THE ECOLOGY OF C-GENOTYPE AND E-GENOTYPE STRAINS OF THE
BACTERIUM *VIBRIO VULNIFICUS* AND THEIR INTERACTIONS WITH THE
AMERICAN OYSTER *CRASSOSTREA VIRGINICA*

by

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ABSTRACT

BRETT AUSTIN FROELICH. The ecology of C-genotype and E-genotype *Vibrio vulnificus* strains and their interactions with the American oyster *Crassostrea virginica*. (Under the direction of DR. JAMES OLIVER)

Vibrio vulnificus is a pathogenic bacterium, routinely found in waters of estuarine environments as part of the normal microflora. This organism can be divided into two genotypes, a C-type associated with clinical isolation, and an E-type associated with environmental isolation. While it was previously known that C- and E-type cells were genetically distinct, a further distinction was found among C-type strains that has the potential to predict pathogenicity using simple PCR. It was also found that C- types cells are more rapidly taken up by oyster hosts than E-type cells in some cases, but that depuration was just as rapid for both types. These studies revealed that addition of laboratory grown bacterial strains can cause endogenous bacteria in oyster to resuscitate from the viable-but-non-culturable state. Inefficiencies in bacterial uptake in oysters led to a study that concludes that E-type strains are more readily integrated into marine aggregates that C-type strains. Most notably, *V. vulnificus* was observed to decline in North Carolina estuaries in response to a prolonged and severe drought.

DEDICATION

I dedicate this dissertation to Danny Gallegos, for without his unwavering stability, generous support, and constant encouragement this document would surely not exist. I further dedicate this work to my parents Debbie and Harold Froelich who never let me believe, even for a moment, that I was incapable of achieving anything I wanted. Thank you.

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CHAPTER 1: INTRODUCTION

Vibrio vulnificus

Vibrio vulnificus is a gram negative, halophilic, bacterium capable of causing gastroenteritis, wound infections, and fatal septicemia in humans (48, 92, 97). This organism is routinely found in waters of estuarine environments as part of the normal microflora, as well as in oysters and other shellfish inhabiting those estuaries (97). *V. vulnificus* infection is the leading cause of seafood-borne deaths in the United States, usually resulting from the consumption of raw or undercooked oysters (97). Infections caused by ingesting oysters contaminated with *V. vulnificus* commonly result in primary septicemia, almost always require hospitalization, and have a fatality rate greater than 50% (73, 97).   Wound infections are usually the result of exposure of an open wound to sea water containing the bacterium, and can progress to necrotizing fasciitis (49, 96). While causing rapid and highly fatal infections, most often *V. vulnificus* opportunistically targets those individuals with underlying diseases that make them more susceptible to this organism. These can include liver diseases (such as cirrhosis) or immune dysfunction (*e.g.* diabetes).

Vibrio vulnificus exhibits a great deal of genotypic and phenotypic variation (97). The species is divided into three biotypes, all of which are able to cause human infection, but biotype 1 is of greatest importance to oyster producers and consumers (7, 92). Biotype 2 strains routinely infect eels, especially those grown in aquaculture, while

biotype 3 strains have been isolated only in Israel in association with tilapia (3, 7, 49, 137).

Biotype 1 stains of *V. vulnificus* have been further divided into two genotypes, a difference discovered by RAPD-PCR analysis of strains from both clinical and environmental sources (148).   In this classification system, a gene identified as *vcg* (virulence correlated gene) was found to have two variations (117). One variation correlates with strains obtained from clinical sources, while the other variation is correlated with environmentally isolated strains (117, 148). The gene thus has two alleles, designated *vcgC* and *vcgE,* representing clinical and environmental strains, respectively (117). Although a search using the Basic Local Alignment Search Tool (BLAST) failed to identify a specific protein the *vcg* gene encodes, these studies demonstrated that not all biotype 1 strains of *V. vulnificus* are the same. In fact, there appears to be a type (the C-type) that is more pathogenic, a type that might not have been noticed if not for selection by human physiology. From this knowledge, a simple PCR test was devised to rapidly determine the genotype of strains of *V. vulnificus* (117, 147). This test is commonly used to predict a strain's pathogenic potential even though the genes examined in the PCR have yet to be connected to virulence. More recently, it has become understood that genetic differences that C- and E-type strains exhibit in *vcg* are shared genome-wide (116). Thus, we can infer that a genotypic difference at a specific locus is merely an indicator of greater variation throughout the chromosome between strains of the C- and E-genotypes. Separation of clinical and environmental strains has also been performed by comparison of 16s rRNA sequences. This technique classifies environmental isolates as "A-type" and clinical isolates as "B-type" (86). The

vcg and rRNA methods return the same result for most strains, though strains exist that have conflicting classifications.

Vibrio vulnificus infections

A National Health Interview Survey estimated the number of Americans with the underlying health problems that put them at risk for *V. vulnificus* infection is between 12 and 30 million (109), but only 900 infections were reported to the Centers for Disease Control and Prevention between 1998 and 2006, (13). These at-risk individuals include those that are immunocompromised, diabetic, or have elevated serum iron levels, such as those with liver cirrhosis (95). Hospitalization was required for 93% of these cases and mortality was 36%, distinguishing *V. vulnificus* as having the highest case fatality rate of any foodborne pathogen (12, 95).

With this many high-risk people, one would expect to see far more than the ca. 100 total cases that are reported each year (95). The lack of reported cases can be partially explained by the existence of the two *V. vulnificus* genotypes. As previously mentioned, *V. vulnificus* cells possessing the C-type DNA sequence are likely to be more virulent when compared to the *V. vulnificus* population as a whole (95). Thus, infections are lower than expected because some strains of the bacterium appear less able to cause disease. These data, however, can account for only a fraction of the disproportionate number of susceptible individuals versus number of actual cases, suggesting there exists other factors influencing the low infection rate.

The relationship between Crassostrea virginica and Vibrio vulnificus

Oysters, *C. virginica*, are economically important as an a food product, but also ecologically important because they are a keystone species serving to modulate water

quality, as a habitats for other organisms, can serve as bioaccumulators of toxins, and act as vectors for several bacterial pathogens, including *V. vulnificus* (45). *Vibrio vulnificus* is quite prevalent in oysters meant for human consumption, with 67% of raw and 25% of cooked oysters (collected from Louisiana restaurants) found to be harboring the microbe (67). Over 95% of infections resulting in septicemia caused by *V. vulnificus* involved the consumption of raw oysters, with the remainder arising from ingestion of steamed oysters and clams (97).

Studies comparing the population dynamics of the two *V. vulnificus* genotypes have revealed an interesting phenomenon. While there was a nearly even ratio of C-type strains to E-type strains found in North Carolina seawater (before a severe drought altered its presence in 2007), strains isolated from oysters living in that same water were predominately (>84%) E-type (59, 145). This finding helps explain the disparity between the high number of individuals at risk for infection, and the low number of yearly cases. Presently, the reason for this incongruity has not been determined.

Crassostrea virginica pumps water through its gills, straining food particles from the flow (83). The gills create the water flow, filter food and other particles from the water, and also are the main site of respiration (20). This would be the first organ to encounter any *V. vulnificus* cells to ultimately be ingested by oysters. Cells trapped by the gills are shunted towards the oyster mouth, and moved by the esophagus to the stomach (83). Incredibly, *C. virginica* is able to filter water at a rate of $10 \text{ L h}^{-1}\text{g}^{-1}$ dry tissue weight (83). With such a rapid rate of water clearance, one would naturally expect the oyster's internal composition of *V. vulnificus* to mimic that of the surrounding water. There are several potential explanations for this anomaly, including

the possibility that oysters selectively take up and retain E-type strains over C-type strains.

An oyster is able to select the particles of food it eats based on size. The gills act as a sieve, catching particles of optimum size and moving toward the mouth, while particles that are too large are stopped and passed from the oyster as pseudo-feces. Particles smaller than the optimum size pass through the gills without capture. For *Crassostrea virginica,* the optimum particle size is 5-7µm in diameter (143). At this size, particles are retained with 90% efficiency (143). Particle retention rates drop to 50% when the diameter is only 1.8µm, and when particles are the size of a single *V. vulnificus* bacterium (ca. 1µm), oysters only retain about 16% of what is passed through the gills (143). This size selection could limit the effectiveness of a bacterial uptake experiment where bacteria are simply added to oyster tanks.

Genetic variation between strains of *Vibrio vulnificus* is explored in a paper published from my dissertation studies entitled "Orientation of mannitol related genes can further differentiate strains of *Vibrio vulnificus* possessing the *vcgC* allele" (26). This paper describes how a PCR method, based on the *vcg* locus, can further separate C-type strains can into two subtypes. The study suggests that not all C-type strains are virulent, and only a genetically identifiable subpopulation has been actually isolated from clinical cases.

The genetic variations of *V. vulnificus* are examined further, and different genotypes are compared in their ability to be taken up by filter-feeding oysters in a second published manuscript titled "Uptake and depuration of the C- and E-genotypes of *Vibrio vulnificus* by the Eastern Oyster (*Crassostrea virginica*") (27). The paper

offers a possible explanation as to why these two genotypes are found in a nearly even ratio in the aquatic environment, but oysters harvested from those very environments show a *V. vulnificus* genotype ratio disparity, with most being the E-genotype. This study provides evidence that there is significantly greater uptake, but equally effective depuration, of C-type *V. vulnificus* in oyster gill tissues, mantle tissues, and whole oyster homogenates. Because uptake of the C-genotype was generally greater than the E-genotype, it appears unlikely that simple selective uptake is the cause of the predominance of the E-type *V. vulnificus* in oysters.

The experiments that generated the data for the above paper uncovered an unusual phenomenon whereby dormant vibrios resident in oyster tissues responded to laboratory grown strains of *V. vulnificus* added exogenously. The paper, "Increases in oysters of a *Vibrio* sp. upon addition of exogenous bacteria", describes how laboratory grown *V. vulnificus* cultures were added to oysters and periodically sampled. The evidence suggests that the laboratory grown strains were taken up by the oysters, but were rapidly depurated to nearly non-detectable levels. Most interestingly, one of the conclusions of the article is that the number of naturally occurring bacteria within the oysters increased in response to the exogenous bacteria and resisted depuration.

From these oyster studies, and based on previous publications indicating size selection by oysters, it was hypothesized that a more efficient method of bacterial uptake could be obtained by combining the bacteria with larger particles that are more similar to the preferred feeding size of oysters. The work in this area is presented in a paper, currently under review, titled "*Vibrio vulnificus* integration in marine aggregates and subsequent uptake by the oyster *Crassostrea virginica*". Therein are described

marine aggregates which are naturally forming conglomerations of larvacean houses, phytoplankton, microbes, and inorganics adhered together by extracellular polymers. In this paper, the data indicate that, *in vitro, V. vulnificus* can be concentrated into laboratory generated aggregates from surrounding water. It further shows that the environmental (E-type) strains exhibit significantly more integration into these aggregates than clinical (C-type) strains. Experiments where marine aggregates, with attached *V. vulnificus* cells, were fed to oysters (*Crassostrea virginica*) resulted in greater uptake of both C- and E-types than non-aggregated controls. While the reason for the greater attachment of environmental strains to marine aggregates is not yet understood, the fact that *C. virginica* has an uptake efficiency of only ~16% for particles the size of bacteria and ~98% for particles of 7 microns, pre-attachment to marine aggregates could be more effective in bacterial uptake studies in oysters.

The body of knowledge regarding the ecology between bacterial pathogen and bivalve reservoir is then reviewed in the submitted manuscript, "Review: The interactions of *Vibrio vulnificus* and the oyster *Crassostrea virginica* under environmental conditions". This review describes how *Vibrio vulnificus* is concentrated by filter-feeding molluscan shellfish, especially oysters, and summarizes the current knowledge of the environmental interactions between these two organisms. This includes discussion on the effects of salinity and temperature on colonization, uptake and depuration rates of various phenotypes and genotypes of the bacterium, and host-microbe immunological interactions.

Finally, a multi-year study is included titled "Rarity of *Vibrio vulnificus* in North Carolina oysters coincides with drought and increased salinity". This manuscript

describes how *V. vulnificus* is commonly isolated from estuaries using selective media, yet in 2007 it was extremely difficult to culture *V. vulnificus* from North Carolina estuary and oyster samples. After employing culture and non-culture based methods for detection of *V. vulnificus* with negative results, it was concluded that this pathogen had become rare in the North Carolina estuarine ecosystems. The validity of the techniques employed was insured by spiking oysters from North Carolina with *V. vulnificus* and performing the same tests as had been conducted on unadulterated oysters. In spiked oysters, *V. vulnificus* was readily detected using all methods. Furthermore, oysters were obtained from the Gulf of Mexico and *V. vulnificus* was easily isolated, confirming that the oysters and waters of North Carolina were lacking the *V. vulnificus* population our lab has studied for decades. Strikingly, it was discovered that the disappearance of *V. vulnificus* coincided with the most severe drought in the history of North Carolina. The drought continued until the end of 2009, with North Carolina estuaries experiencing elevated salinities throughout this period. When the drought abated in 2010 and salinities returned to normal, *V. vulnificus* was again isolated from the water column, though still could not be cultured from oysters. The study suggests the oysters were colonized with a more salt-tolerant bacterium during the drought, which had displaced *V. vulnificus* and may be preventing its recolonization.

CHAPTER 2: ORIENTATION OF MANNITOL RELATED GENES CAN FURTHER DIFFERENTIATE STRAINS OF *VIBRIO VULNIFICUS* POSSESSING THE vcgC ALLELE

Citation

Froelich, B., and Oliver, J. (2011) Orientation of mannitol related genes can further differentiate strains of *Vibrio vulnificus* possessing the *vcgC* allele. *Adv Stud Biol* **3**: 151-160.

Abstract

The bacterium *Vibrio vulnificus* exhibits a high level of strain to strain genetic variation, and can be divided into three biotypes. The main cause of human infections, biotype 1, has been separated into clinical and environmental strains based on either DNA PCR, 16s rRNA sequence, or intergenic spacer regions. The DNA PCR method is based on the *vcg* (virulence correlated gene) locus, with a C-genotype corresponding to clinical isolates and an E-genotype corresponding to environmental isolation. Here we show that C-type strains can be further divided into two subtypes. Strains of the one subtype have been isolated from environmental sources and human cases, while the other subtype has heretofore only been recovered from the environment

Introduction

The Gram-negative halophilic bacterium, *Vibrio vulnificus,* is able to cause grievous wound infections and potentially fatal septicemia in humans (49). Infections typically result from the consumption and handling of raw or undercooked

oysters, with over 85% of infections occurring in males (49). Infections are primarily associated with individuals that are immunocompromised, diabetic, or have elevated serum iron levels, when compared to the *V. vulnificus* population as a whole (95). Thus, infections may be lower than expected as only some strains of the bacterium are able to cause disease.

Interestingly, it was found that while estuarine water samples contain a mix of almost equal percentages of C-type and E-type cells, the ratio in oysters is 13% C-type to 87% E-type (145). This finding becomes significant when one considers that most *V. vulnificus* infections originate from the consumption of raw or undercooked oysters (95). This suggests that the incidence of infection is further decreased because oysters contain fewer of the more virulent C-type strains.

It has been shown that mannitol fermentation is common among C-type strains, but variable in E-types, a phenomenon that was also found to be true with 16S rRNA typing (18, 29). Examination of the complete sequenced genome of *V. vulnificus* strain CMCP6 (GenBank AE016795) revealed an operon (Fig. 1) used in conversion of mannitol to fructose (56). The genes encoding the IIA domain of the mannitol phosphotransferase system (PTS), mannitol dehydrogenase (*mtlD*), and mannitol operon repressor (*mtlR*) are similar in function and orientation to many species of bacteria that exhibit specific hexitol transport and fermentation (16, 56, 75). Also located in this region, upstream of the mannitol fermentation genes, are two additional genes. These (Fig. 1) code for a putative hemolysin and a TRAP type mannitol transport system (56). The published genome of *V. vulnificus* strain YJ016 shows a similar genetic arrangement (16), albeit with a 53 amino acid hypothetical protein between the

hemolysin and TRAP transporter. As mannitol fermentation has been shown to be correlated with virulence in *V. vulnificus* (18), we decided to study *vcgC* and *vcgE* genotype strains of *V. vulnificus* for the presence of the conserved mannitol operon found in the published clinical isolate genomes (16, 56), to further refine the *vcg* based PCR method of genotyping.

Materials and methods

Bacterial strains and culture conditions

Fifty-eight *V. vulnificus* strains were used in this study (Table 1), including clinical and environmental isolates and C- and E-genotypes. Strains were grown from freezer stocks overnight in Bacto™ Heart Infusion (HI) broth (BD, New Jersey) or on HI agar plates at 30°C.

DNA extraction for PCR analysis

Bacterial cells were grown overnight in HI broth at 30°C. Cells were centrifuged for five minutes at 16,000 x *g* and the pellet resuspended in phosphate buffered saline (PBS). Cells were lysed by boiling for five minutes and centrifuged again at 16,000 x *g* for five minutes. The supernatants containing the DNA template were used in the PCR reactions.

Mannitol fermentation assay

*Vibrio vulnificus s*trains were streaked onto HI agar plates from freezer stocks and incubated at 30°C for 24 hours. An inoculating needle was used to stab a single colony that was then inoculated into 5 ml of mannitol fermentation broth (16 g of BBL™ Phenol Red broth base [BD] and 1.0% D-mannitol [Sigma Cat. No. M-4124] were added per liter of deionized water then autoclaved at 121° C for 5 minutes). These

cultures were incubated at 37° C and examined for mannitol fermentation at 24 and 48 hours.

<div align="center">Strain typing via PCR.</div>

Using the methods developed by Rosche *et al.* (117) and Warner and Oliver (147), each strain was subjected to a multiplex PCR reaction that simultaneously confirmed the isolate as being *V. vulnificus* and identified the genotype as *vcgC* (C-type) or *vcgE* (E-type). Reactions were performed using GoTaq polymerase (Promega) in a Techne Genius thermal cycler using the parameters suggested by the manufacturer for GoTaq (Promega), but with an annealing temperature of 53°C. PCR products were visualized by gel electrophoresis on 1% agarose gels stained with ethidium bromide.

Arrangement of mannitol fermentation genes

After strain typing, all strains were examined for the presence of the three genes that make up the mannitol fermentation operon (the enzyme II of the phosphotransferase system for mannitol, mannitol specific dehydrogenase, and mannitol operon repressor; Fig. 1). All isolates were also examined, via PCR, for the two genes that are immediately upstream of the mannitol fermentation operon in the previously sequenced, clinically isolated CMCP6 strain. These are a putative hemolysin gene as well as a TRAP-type mannitol transport (56). The strains were further examined for a fragment of DNA spanning those two genes (Fig. 1, Table 2). Positive PCR results from primers that were located within a gene indicated the presence of the gene, whereas a positive PCR result from primers than spanned two genes indicated that the two genes were adjacent. Primers were designed for each of the lettered areas (A-J) indicated in Fig. 1. PCR was performed as described for strain typing with the

annealing temperature and extension time modifications listed with the primer pairs in Table 2.

Statistics

Statistical analyses were performed on the data in Table 1, using Chi-square analysis or Fisher Exact test where appropriate. Analyses were performed using SigmaStat statistical analysis software.

Results

All of the 38 of the C-genotype strains were able to ferment mannitol while only eight out of 20 E-genotype strains exhibited this ability. All strains, regardless of genotype, which were able to ferment mannitol also produced PCR bands representing the three genes of the mannitol fermentation operon (Fig. 1, Table 1).

All 58 tested strains of *V. vulnificus*, regardless of genotype (C or E), had the putative hemolysin gene and the TRAP-type transport gene (Table 4). PCR analysis was subsequently used to clarify the arrangement of the genes under investigation. When PCR reactions performed with primers designed to span these two genes were used, the results varied depending on the genotype and isolation source. While possessing both the TRAP and putative hemolysin genes, none of the 20 E-genotype strains examined were found to have these two genes located adjacent to each other ("published arrangement"; Table 4). In contrast, all but one of the 22 C-type strains that had been isolated from human infections were positive for the gene-spanning fragment. Thus, the hemolysin and TRAP genes were adjacent in these strains, similar to the published genome (56) of the clinically isolated C-type strain (Fig. 1, Table 4). When

the environmentally isolated (oyster or water) C-type strains were examined by the same method, only seven of 16 had these genes adjacent to each other (Table 4).

<div align="center">Discussion</div>

More than 90% of *V. vulnificus* strains recovered from patients are of the C-genotype (117), but this does not necessarily mean that all C-type strains found in the environment are similar or even able to cause disease. Our examination of both clinically- and environmentally-derived C-type strains suggests that the two published *V. vulnificus* genomes [(16, 56); GenBank AE016795 and GenBank BA000037] may not accurately represent all of the C-type strains, especially those isolated from a water or oyster source (the only published genomes of C-types strains have been from cells isolated from clinical sources). Although all strains (regardless of genotype) were found via PCR to possess both the putative hemolysin and TRAP-type transport genes, many did not share the same arrangement of those genes as seen in the two published *V. vulnificus* genomes (Fig. 1). Not surprisingly, none of the E-type strains were homologous to the available genomic sequence data. Most notably, when testing C-type strains from clinical sources, nearly all were in accord with the published genomic sequences; however many C-type strains of environmental origin have a gene arrangement that is yet unknown.

The human body appears to select for those C-type strains that exist in the environment only when they have genomes similar to the two published *V. vulnificus* genomes. This suggests that the number of *V. vulnificus* strains that are highly virulent is even lower than indicated by possession of the *vcgC* allele. This is somewhat analogous to the situation with *V. parahaemolyticus*, where only a very small

percentage of strains in the environment possess the hemolysin genes required to initiate infection, with the latter being found almost exclusively in clinical samples.

A similar phenomenon was uncovered recently by Roig and coworkers (115), who looked at seven environmentally isolated C-type strains of *V. vulnificus*. They found four of these strains to be potential pathogens (having resistance to human serum), while three of the strains were likely non-pathogenic (inhibited by human serum). Coupled with the results of the present study, this suggests that C-type *V. vulnificus* strains may vary enough to warrant a further classification, and can be differentiated using simple PCR.

Acknowledgements

CHAPTER 3: UPTAKE AND DEPURATION OF THE C- AND E-GENOTYPES OF VIBRIO VULNIFICUS BY THE EASTERN OYSTER (CRASSOSTREA VIRGINICA)

Citation

Abstract

The human pathogen *Vibrio vulnificus* is a gram-negative estuarine bacterium that infects via wounds and ingestion, and is the leading cause of seafood-borne death in the United States. *V. vulnificus* is part of the naturally occurring flora of both estuaries and estuarine mollusks (especially oysters). *V. vulnificus* is divided into two genotypes, including a clinically associated C-type, and an environmentally associated E-type that is more rarely involved in septicemia. These two genotypes are found in a nearly even ratio in the aquatic environment, but oysters harvested from those very environments show a *V. vulnificus* genotype ratio disparity, with 87% of the species being of the E-genotype. To determine if oysters selectively incorporate E-types over C-types, we placed oysters in water inoculated with either C- or E-type *V. vulnificus* strains that were phenotypically different from the normal flora and measured the uptake and depuration over a course of 6 days. We found significantly greater uptake, but equally

effective depuration, of C-type *V. vulnificus* in oyster gill tissues, mantle tissues, and whole oyster homogenates. Because uptake of the C-genotype was generally greater than the E-genotype, it appears unlikely that simple selective uptake is the cause of E-type *V. vulnificus* predominating in oysters.

Introduction

Vibrio vulnificus is a gram negative, halophilic bacterium capable of causing gastroenteritis, wound infections, and fatal septicemia in humans (48, 92, 97). This organism is routinely found in oysters and waters of estuarine environments as part of the normal flora (97). *V. vulnificus* is the leading cause of seafood-borne deaths in the United States, usually resulting from the consumption of raw or undercooked oysters (97).

Vibrio vulnificus can be divided into three biotypes (1-3), all of which are able to cause human infection, but biotype 1 is of greatest import to oyster producers and consumers (7, 92, 137). Biotype 1 strains of *V. vulnificus* can be further divided into a C- and an E-genotype, with the former genotype most frequently isolated from clinical sources and the latter predominately occurring in environmental samples (117). Oysters feed by filtering out and sorting particles, including bacteria, suspended in the water column using gills and labial palps (61, 83). Oddly, while the ratio of C- to E-genotype strains in the aquatic environment is nearly even, population studies have shown that in oysters the E-genotype strains are overwhelmingly dominant, averaging 87% of the total *V. vulnificus* population (145).

The purpose of the study was to compare the uptake and depuration rates of the two genotypes of *V. vulnificus* biotype 1 in the oysters, *Crassostrea virginica*. It was

hypothesized that the disparity between the genotype ratios in water vs. oysters might be due to selective uptake of E-type strains and/or increased elimination/depuration of C-type strains. For these studies, genetically or phenotypically marked strains were utilized to distinguish the added bacterial cells from the background *V. vulnificus* population already present in oysters. In addition to testing the whole-organism uptake and depuration of *V. vulnificus* in oysters, we also determined distribution of the introduced bacterial strains in different tissues of oysters including the gills (which serve not only as a gas exchange site, but also as an organ for acquiring food), the digestive gland, and the mantle tissue, which covers the internal organs of oysters and secretes shell materials (10, 20, 61, 83), in order to test for the potential tissue-specific differences in uptake and/or depuration rates of these bacteria. .

Materials and methods

Bacterial strains and culture conditions

Vibrio vulnificus CVD713 is a C-genotype strain possessing a TnphoA transposon that confers kanamycin resistance and alkaline phosphatase activity and is stable for at least 10 days when the bacteria are maintained above 17°C (76, 80, 155). This strain forms blue colonies when grown on Tn Agar, consisting of Luria Agar with the addition of 0.2 g L^{-1} kanamycin, 2 g L^{-1} glucose, and 0.04 g L^{-1} 5-bromo-4-chloro-3-indolyl phosphate (BCIP). Tn Agar selects for the TnphoA-possessing strain via its kanamycin resistance and is differential by means of the BCIP (155). *V. vulnificus* strain VVL1 is a naturally occurring E-genotype strain that is bioluminescent on normal solid media (101). Studies have shown that this organism does not differ phenotypically from others strains in the species except for its luminescence (101).

Vibrio vulnificus strain pGTR-Env1 is an E-type strain that contains the stable pGTR plasmid which confers kanamycin resistance when grown on Luria Agar containing 10 g L^{-1} L-arabinose and 0.3 g L^{-1} kanamycin (90). Strains were grown overnight in Bacto™ Heart Infusion (HI) broth (BD, New Jersey) at 30°C with vigorous shaking.

Oyster maintenance

Oysters (*Crassostrea virginica*) from the coast of North Carolina were collected by hand in the intertidal zone, rinsed, and placed into holding aquarium tanks to acclimate to laboratory conditions. The tanks contained a 1:1 mixture of artificial seawater (ASW, Instant Ocean, Aquarium Systems, Mentor , OH) and natural seawater (NSW, collected from North Carolina Coast), passed through a 0.45μm filter (Millipore, Bedford, MA) and adjusted to 20 ‰ salinity. Tank water was kept at 23°C. Oysters were fed an algal mixture of *Skeletonema, Rhodomonas,* and *Isochrysis* species daily, at 5ml of culture per oyster. The algal cultures were grown at room temperature in vented, 1 liter flasks containing F/2 medium and were provided with constant fluorescent light (46, 74).

Oyster Infection and depuration

For each experiment, oysters were fed 24 h prior to being removed from maintenance tanks and placed in experimental tanks with 20 ‰ salinity ASW at 23°C. Twenty-five oysters were placed into each tank and five oysters were sampled at each time. Before infection, five oysters were removed from the tanks for sampling to establish a background population count of *V. vulnificus* (described below). *V. vulnificus* cells grown to a concentration of 10^{8-9} CFU per ml were added to the

experimental tanks, at a ratio of 0.075 ml of liquid culture per liter of ASW. Oysters

were allowed to bathe in the *V. vulnificus*-infected water for 24 h.

After the initial 24h exposure, and every 48 hours thereafter, the oysters were

removed from the tanks and the aquaria were cleaned, sanitized, and refilled with fresh

ASW (20 ‰ salinity). The oysters were then placed back into the clean tanks before

selecting five oysters that were removed for sampling. All studies were conducted in

duplicate.

Oyster dissection and homogenization

Oysters, once removed from tanks, were rinsed with ethanol and patted dry with

paper towels. The oysters were shucked with a flame-sterilized oyster knife, and the

meat washed with sterile ASW of 20‰ salinity. Using flame-sterilized instruments,

pieces of the oyster gill, mantle tissue, and digestive gland were removed and placed

into sterile tubes. The remaining oyster tissues were separated from the shell and

placed in a sterile test tube.

One ml of ASW (20 ‰) was added to each sample of gill, mantle and digestive

gland tissues, and the samples were homogenized using an ethanol and flame-sterilized

Tissue Tearor (Biospec Products, Inc., Bartlesville, OK) until all tissue was liquefied.

The remaining oyster body was homogenized in 20 ‰ ASW at 1:1 w:v ratio (minimum

3 ml ASW) using sterile blender cups (Warring, Torrington, CT) and 3 bursts of 15 s

each, with a 5 s pause between the bursts.

Sampling methods

After homogenization, samples were serially diluted in sterile phosphate

buffered saline (PBS) and spread onto non-selective agar (1 L of 20 ‰ ASW combined

with 10 g Bacto Peptone [BD, Sparks, MD], 3 g Bacto Yeast Extract [BD, Sparks, MD], and 15 g Agar [Sigma, St. Louis, MO] autoclaved at 121°C for 15 minutes), and on CPC+ plates, a medium selective for *V. vulnificus* (146). Aliquots of tissue samples were also plated onto the appropriate medium for the inoculated strain of *V. vulnificus*, as previously described. Total colony forming units (CFU) per gram of oyster tissue were calculated.

Statistics

Data were compared using a two-way analysis of variance followed by post-hoc tests with Bonferroni corrections for multiple comparisons (124). Data were analyzed using SigmaStat statistical software (Version 2.0, Access Softek Inc).

Results

Uptake and depuration of the C- and E-genotypes of V. vulnificus

In all tissues examined, including the whole oyster homogenates, there was significant (P<0.001) uptake of the marked strains of *V. vulnificus* into the oysters after 24 h of incubation (Figure 2). After allowing the oysters to depurate for 48 h in clean water, all tissue types showed a significant drop in the CFU/g of the marked strains (p<0.001) to a level that was not significantly different than the non-detectable levels present in the non-inoculated (zero time point) oysters (p>0.05, Figure 2).

Examination of the gill tissues of oysters revealed that 24 h after inoculation with *V. vulnificus,* the C-type strain had significantly greater uptake than the E-type strains (p<0.001). After 48 h in clean water, the proportion of C- and E-types in the oyster tissue was even (p>0.05), and six days after inoculation, both genotypes were almost undetectable in the oyster gills (Figure 2).

The mantle tissue from artificially infected oysters also showed a significantly greater uptake of C-genotype *V. vulnificus* cells than strains of the E-genotypes (p<0.001). This difference disappeared after the oysters were allowed to depurate in clean water (Figure 2), with no significant differences at the three and six-day time points.

In digestive gland tissues, there were no significant differences in uptake or depuration rates of the C- and E-genotypes at any time point (p>0.05, Figure 2). The C-genotype strain of *V. vulnificus* showed significantly greater uptake 24h after inoculation than the E-genotypes (p=0.036) in whole-body tissue, and was the only sample time to also show significant difference between the genotypes at six days after inoculation (p=0.029, Figure 2).

Discussion

It is quite noteworthy that the clinically associated C-genotype and the environmentally associated E-genotype of *V. vulnificus* are found in a nearly equal ratio in the estuarine waters of North Carolina, but oysters harvested from those same waters contain a highly disproportionate (approximately 87%) number of E-type strains (15, 145). We hypothesized that a possible cause of this disparity was an increased selective uptake of E-genotype cells facilitated by the oyster, or an increased ability of the E-type cells to colonize oyster tissues over their C-type relatives. By using strains of *V. vulnificus* that are distinguishable from the background *V. vulnificus* flora of oysters, we were able to estimate the uptake and depuration rates of both the C- and E-genotypes.

Contrary to our hypothesis, the E-type strains did not have increased uptake in oysters as a whole or in certain oyster tissues after 24 h of incubation in *V. vulnificus-*

infected water. In fact, in gill and mantle tissues, as well as whole oyster-tissue homogenates, the opposite was true, with C-type cells showing significantly greater uptake (Figure 2). With this knowledge gained, it seems unlikely that simple selective uptake and/or colonization generates the high internal levels of E-type strains in oysters while the external, environmental levels retain a nearly equal ratio of C- and E-type.

Our experiments also suggest that neither genotype experiences greater (or less) depuration than the other (Figure 2). In all cases, 48 hours of depuration in clean ASW was able to reduce the number of marked cells in the artificially infected oysters to the levels close to those of the non-inoculated control oysters. This indicates that recently acquired C- and E-genotype cells are quickly purged from oysters and their tissues (Figure 2) and simple differential depuration is unlikely to create an overabundance of E-types within the oyster.

CHAPTER 4: INCREASES IN OYSTERS OF A *VIBRIO* SP. UPON ADDITION OF
EXOGENOUS BACTERIA

Citation

Froelich, B., and Oliver, J. (2012) Increases in oysters of a *Vibrio* sp. upon addition of

exogenous bacteria. *In prep*.

Abstract

CPC+ is a medium used to select for and isolate *Vibrio vulnificus* from the

environment. In this study, oysters were collected from the coast of North Carolina

during winter months, homogenized, and plated onto CPC+. The number of

presumptive *V. vulnificus* colonies was minimal, an expected result as the concentration

of culturable vibrios decreases seasonally as the temperature declines. These oysters

were kept in aquaria under laboratory conditions for up to 5 month with no significant

increase in the number of presumptive *V. vulnificus* colonies obtained on CPC+. These

oysters were also used in bacterial uptake experiments in which laboratory grown *V.

vulnificus* cultures were added to the aquaria and oysters periodically sampled. We

found that the laboratory grown strains were taken up by the oysters, but were rapidly

depurated to nearly non-detectable levels, as previously reported by our lab as well as

several others. Most interestingly, the number of naturally occurring bacteria increased

and resisted oyster depuration.

Introduction

The Gram-negative bacterium, *Vibrio vulnificus*, is an opportunistic pathogen capable of causing gastroenteritis, wound infections, and fatal septicemia in humans (48, 92, 97). Routinely found in waters of estuarine environments as part of the normal microflora, as well as in oysters and other shellfish inhabiting those estuaries, this organism is remarkable as it has the potential for infection through preexisting wounds (e.g. from seawater to lesion) or though ingestion (primarily oyster and other shellfish meats) (97). *V. vulnificus* is present in the majority of oysters meant for human consumption, with 67% of raw and 25% of cooked oysters, collected from Louisiana restaurants, found to be harboring the microbe (67). Infections caused by *V. vulnificus* are the leading cause of seafood-borne deaths in the United States, with most of these infections resulting from the consumption of raw or undercooked oysters (97). Infections caused by ingesting oysters containing *V. vulnificus* commonly result in primary septicemia, almost always require hospitalization, and have a fatality rate greater than 50%, distinguishing *V. vulnificus* as having the highest case fatality rate of any foodborne pathogen (12, 73, 95, 97).

Many aquatic bacteria and most vibrios (including *V. vulnificus)*, are affected by seasonal environmental shifts, with increases in bacterial isolations correlating with warmer temperatures (100). Conversely, a sensitivity to low temperature causes a decrease in the culturable populations of *V. vulnificus* (51, 54, 78, 108, 110). While some of these decreases can be attributed to decreased survival at the colder temperatures, at least part of our reduced ability to isolate aquatic bacteria in the winter is due to a phenomenon known as the viable but nonculturable (VBNC) state (93). In

this state, cells are viable (as confirmed via detection of RNA transcription, intact

membranes, and other methods) but cannot be cultured on the routine media normally

employed for their culture (94). Thus the cells enter a type of dormancy, a result of

some form of environmental stress (e.g. cold temperature), in which it is hypothesized

that they are able to better survive other unrelated stresses, a phenomenon referred to as

cross-protection (93, 94). When the initial stress is alleviated, the bacteria can

potentially emerge from the VBNC state in a process known as resuscitation (85, 94,

99). In *V. vulnificus*, the VBNC state can be induced *in vitro* by a temperature

downshift, and resuscitation by a simple temperature upshift (85, 99, 152).

In the present study, we show the possible existence of a VBNC population of vibrios in

oysters harvested from the coast of North Carolina. After adding either *V. vulnificus* or

Escherichia coli to such oysters held in laboratory aquaria, this VBNC population

appears to resuscitate in rapid response to the added bacteria.

Materials and methods

Bacterial strains and isolation media

Vibrio vulnificus strain CVD713 possesses a Tn*phoA* transposon that confers

kanamycin resistance and alkaline phosphatase activity and is stable for at least 10 days

when the bacteria are maintained above 17°C (76, 80, 155). This strain forms blue

colonies when grown on Tn Agar, consisting of Luria Agar with the addition of

0.2 g L^{-1} kanamycin, 2 g L^{-1} glucose, and 0.04 g L^{-1} 5-bromo-4-chloro-3-indolyl

phosphate (BCIP). Tn Agar selects for the Tn*phoA*-possessing strain via its kanamycin

resistance and is differential by means of the BCIP (155). Liquid cultures of this strain

were grown in the same medium without the addition of BCIP and agar. *Vibrio*

vulnificus strain VVL1 is a naturally occurring E-genotype strain that is bioluminescent on normal media (101). Studies have shown that this organism does not differ phenotypically from other strains in the species except for its luminescence (101). This strain was grown in Bacto™ Heart Infusion (HI) broth (BD, New Jersey) for liquid cultures or with the addition of 1.5% agar for solid medium. *V. vulnificus* strain pGTR-Env1 contains the stable pGTR plasmid which confers kanamycin resistance when grown on Luria Agar containing 10 g L^{-1} L-arabinose and 0.3 g L^{-1} kanamycin (90), or in liquid without the addition of agar. *Escherichia coli* strain K12 was grown in LB broth or on MacConkey Agar (Sigma-Aldrich, St. Louis, MO). All strains were grown at 30°C (with vigorous shaking for liquid cultures). CPC+ agar was employed to isolate *V. vulnificus* and other vibrios from oyster meat. CPC+ (a derivative of CPC) is both selective and differential for *V. vulnificus* (71, 146), using the antibiotics colistin and polymyxin B to inhibit most bacterial growth, and the fermentation of cellobiose to differentiate colonies of this species from other bacteria and vibrios (71, 146). *V. vulnificus* colonies growing on CPC+ produce yellow colonies with a yellow zone surrounding them, while *V. cholerae* produces purple colonies with a blue zone, thus allowing easy distinction between the two species (71, 146).

<div align="center">Oyster collection and maintenance</div>

Oysters (*Crassostrea virginica*) from the coast of North Carolina were collected by hand in the intertidal zone, rinsed, and placed into holding aquaria to acclimate to laboratory conditions. The tanks contained artificial seawater (ASW, Instant Ocean, Aquarium Systems, Mentor, OH) adjusted to 20‰ salinity and kept at 23°C. Oysters were fed an algal mixture of *Skeletonema, Rhodomonas,* and *Isochrysis* grown at room

temperature in vented, 1 liter flasks containing F/2 medium and provided with constant fluorescent light (4, 46).

Oyster Infection and depuration

For each experiment, oysters were fed 24 h prior to being removed from maintenance aquaria and placed in experimental aquaria as described above. Twenty-five oysters were placed into each tank and five oysters were sampled at each time point. Before addition of exogenous bacteria, five oysters were removed from the tanks and sampled to establish a background population count of vibrios and of the marked strains (see below). Cells used for addition to the oysters were grown to a concentration of 10^{8-9} CFU per ml, then added to the experimental tanks at a ratio of 0.075 ml of liquid culture per liter of ASW. Cells grown in the presence of antibiotics were washed 3 times with phosphate buffered saline (PBS) prior to introduction to the aquaria. Oysters were exposed to the bacteria in the inoculated water for 24 h.

After the initial 24h exposure, and at days 3 and 6 after bacterial cell addition, the oysters were removed from the tanks and the aquaria were cleaned, sanitized, and refilled with fresh ASW (20‰ salinity). The oysters were then placed back into the clean tanks before selecting five oysters that were removed for sampling.

Oyster shucking and homogenization

Oysters, once removed from tanks, were rinsed with ethanol and patted dry with paper towels. The oysters were shucked with a flame-sterilized oyster knife, and the tissues washed with sterile ASW (20‰ salinity). The oyster tissues were homogenized in 20‰ ASW at a 1:1 w:v ratio (minimum 5 ml ASW) using sterile blender cups

(Warring, Torrington, CT) using 3 bursts of 15 seconds each, with a 5 second pause between the bursts.

Bacterial sampling methods

After homogenization, samples were serially diluted in sterile PBS and spread onto CPC+ plates and the medium used to specifically select for each genetically marked *V. vulnificus* or *E. coli* strain (see above). Total colony forming units (CFU) per gram of oyster tissue were calculated.

Sampling methods

Presumptive *V. vulnificus* (yellow colonies from CPC+) were picked to HI agar and allowed to grow overnight at 30°C. Using the methods described by Rosche *et al.* (117) and Warner and Oliver (147), each strain was then subjected to a PCR reaction that confirmed the isolate as *V. vulnificus*. Reactions were performed using GoTaq polymerase (Promega, San Luis Obispo, CA) in a Techne Genius thermal cycler using the parameters described by Warner and Oliver (147). PCR products were visualized by gel electrophoresis on 1% agarose gels stained with ethidium bromide.

Statistics

Statistical analyses were performed using a two way ANOVA, with Bonferroni post-tests. The variables included time and bacterial treatment.

Results and discussion

We examined the uptake and depuration of *V. vulnificus* in the Eastern oyster, *Crassostrea virginica,* by placing oysters collected from the estuaries of North Carolina (NC) into aquaria containing lab-grown strains of the bacterium. Such an experiment has been performed numerous times by several laboratories including ours (14, 27, 37,

55, 80, 105, 126). For these experiments, however, we used "marked" strains of *V. vulnificus* that could be distinguished from background bacterial populations. For our first experiment strain CVD713 (80) was used, which contains a stable transposon, Tn*phoA,* providing resistance to kanamycin along with alkaline phosphatase activity.

We sampled oysters harvested from estuaries during cooler months (November – March; temperatures of 6.0 - 11.2°C) and plated homogenized meat onto Tn agar, which is selective for CVD713 (80). There were no colonies recovered on this medium, as expected (Figure 3, time zero). After incubation in the inoculated water, but before being placed into clean water to allow depuration, we again sampled oysters for the marked strain and recovered ca. 1100 CFU/gram of oyster tissue (Figure 3, t=1 day). We sampled again at 3 and 6 days as the oysters were transferred to clean water. During this time, the number of CFU of the marked strain recovered dropped to less than 10. This observation, that oysters concentrate the bacteria from the surrounding waters and then quickly clear the added cells upon the removal of the inoculum, is consistent with previous reports that utilized protocols which included a method for distinction of bacterial inoculum from naturally occurring bacterial populations (27, 37, 55, 105, 126).

While performing this study, we simultaneously plated the oyster homogenates onto CPC+ medium to observe changes in total *V. vulnificus* populations; these results are also shown in Figure 3. Prior to incubation in inoculated water, we counted less than 10 CFU *V. vulnificus*/gram of oyster tissue. After uptake of the marked *V. vulnificus* strain, the oysters contained an average of 10^4 CFU *V. vulnificus*/gram when plated onto CPC+ agar, an increase of 3 logs from the initial values. This increase initially appeared to be a result of the uptake of the marked strain of *V. vulnificus* added

to the aquaria, but upon closer inspection it was evident that the number of cells detected on CPC+ at 1d exceeded the number of marked strain counts by nearly an order of magnitude (Figure 3). Furthermore, while the marked strain was not detectable by day 3, the number of cells observed on the CPC+ plates, while declining slightly, were still far greater at day 6 than the number recorded prior to uptake of the lab-grown cells. Although the authors did not comment on the finding, a similar phenomenon was recorded by Groubert and Oliver (37), where the uptake of an exogenous *V. vulnificus* strain caused a greater increase in total vibrios than could be accounted for by the added strain alone (Figure 4).

The transposon used in CVD713 has been shown to be stable for at least 10 days in artificial sea water (80). However, while our experiments only lasted 6 days, there are no published data showing the stability of this transposon in oysters. To ensure we were seeing an increase in total vibrios, even after the depuration of the marked *V. vulnificus* strain, and not simply cells of the marked strain which had lost the transposon, we employed two additional strains of *V. vulnificus* and performed the same study. One of these strains, pGTR Env1, exhibits kanamycin resistance via a plasmid, pGTR (90). This strain, once taken up by oysters, caused an increase in CFU on CPC+ that was over 1 log greater than the number of marked cells taken up by the oysters (Figure 5). The third strain used for uptake and depuration experiments was VVL1, which is a naturally occurring bioluminescent strain (101), and which could be differentiated from the background bacterial population by viewing and counting the cells in the dark. This strain thus relies neither on genetic manipulation nor on antibiotic resistance. Similar to the results observed with both CVD713 and pGTR Env1, this

strain was taken up and concentrated by the oysters and was completely non-detectable once placed into clean water (Figure 6). Despite this, we continued to observe colonies on CPC+ at days 3 and 6 at levels that were greater than those present in oysters before the marked strain was introduced. This strain provided the best evidence that the phenomenon we were observing was not due to the loss of antibiotic resistance genes on the transposon in CVD713 nor of the plasmid present in pGTR Env1, as the luminescence of this bacterium occurs naturally and is not on a mobile genetic element. These results of these three independent studies were pooled, log transformed, and the data are shown in Figure 7.

The presumptive colonies recovered from CPC+ were subsequently isolated and subjected to PCR analysis to confirm they were *V. vulnificus*. Only four of 144 colonies tested (2.8%) were found to be *V. vulnificus*. This was surprising, given the selective nature of CPC+, but provided strong evidence that the cells appearing on CPC+ were not the marked *V. vulnificus* cells we had added to the oyster aquaria. Thus, it was evident that the addition of exogenous *V. vulnificus* cells to oysters was causing an increase in a bacterial population of a different *Vibrio* species. Further evidence that a totally different species was being induced in these studies came when we performed the same study using *E. coli* as the added bacterium. The *E. coli* K12 we used does not grow on CPC+, but can be detected using MacConkey agar. When oysters were incubated with *E. coli* we observed results similar to those seen when the marked *V. vulnificus* strains were added, with an increase of colonies recovered from CPC+ from 2.9 log initially to 3.8 log after 24 hours, even though no vibrios were added to the

oysters (Figure 8). This increase was highly significant (p=0.02) compared to control oysters that were not inoculated with *E. coli* (Figure 8).

As numerous studies have shown, while adult oysters are able to concentrate bacteria from the surrounding water, this results in a transient population that is rapidly lost when the oysters are removed from the externally added bacteria (27, 37, 55, 77, 105, 126). This is in contrast to the normal gut microflora, which potentially develops during the larval stage of oyster development (Doyle and Oliver, submitted), and which does not appreciably depurate (14, 37, 105).It thus appears that the established population of bacteria in oysters prevents exogenous bacteria from permanently colonizing oyster tissues, possibly through competition of adhesion sites on gut tissues or other surfaces, an effect that provides the basis for the probiotic prophylaxis against *Vibrio* species (32, 60, 104, 140). However, this does not explain how oysters that appeared to initially contain very low numbers of vibrios suddenly contained several logs more of such cells after exposure to a different bacterial genus or species. Such rapid development of this population could be due to the presence of a natural microflora population present in a viable but nonculturable state during the winter months (84, 93, 94, 99). It is conceivable that the established microbiota enters this state to survive the reduced temperatures, and thus oysters sampled during the winter months have fewer culturable vibrios. Certainly, numerous studies have documented the apparent lack of vibrios in oysters during cold water months, and their "reappearance" when the waters become warmer (51, 54, 64, 78, 84, 89, 108, 110, 145, 152, 154). We propose that, in response to the addition of a substantial population of exogenous, actively metabolizing bacteria, these VBNC cells resuscitate (6, 85, 99,

150). Perhaps this is a signal to the VBNC cells that the environment is better able to

support their existence without the protective effects of the non-culturable state.   This

seems likely in contrast to rapid multiplication of only a few actively metabolizing cells,

which would not have the time to generate the numbers of cells that so rapidly appeared

in these studies. This is further suggested by a study wherein we collected oysters from

cold waters, transported them to the laboratory and kept them in aquaria at warm (27°C)

conditions for several months. These oysters, and their bacterial populations, were no

longer at inhibitory temperatures, yet when sampled were still low in culturable *Vibrio*

populations for 5 months (data not shown). These were the same oysters used in the

experiments described above and which, upon the addition of *V. vulnificus*, developed

the large and stable population of non-*V. vulnificus* vibrios. Therefore these bacteria

must have been present in the oysters from the outset, but resuscitated not in response to

a temperature increase, but to a as yet unknown factor associated with the exogenously

added, culturable bacteria.

<div align="center">Acknowledgements</div>

CHAPTER 5: *VIBRIO VULNIFICUS* INTEGRATION IN MARINE AGGREGATES AND SUBSEQUENT UPTAKE BY THE OYSTER *CRASSOSTREA VIRGINICA*

Citation

Abstract

Marine aggregates are naturally forming conglomerations of larvacean houses, phytoplankton, microbes, and inorganics adhered together by exocellular polymers. In this study, we show *in vitro* that the bacterial pathogen *Vibrio vulnificus* can be concentrated into laboratory generated aggregates from surrounding water. We further show that the environmental (E-type) strains exhibit significantly more integration into these aggregates than clinical (C-type) strains. Experiments where marine aggregates, with attached *V. vulnificus* cells, were fed to oysters (*Crassostrea virginica*) resulted in greater uptake of both C- and E-types, than non-aggregated controls. While the reason for the greater attachment of environmental strains to marine aggregates is not yet understood, because *C. virginica* has an uptake efficiency of only ~16% for particles the size of bacteria and ~98% for particles of 7 microns, pre-attachment to marine aggregates could be more effective in bacterial uptake studies in oysters.

Introduction

Vibrio vulnificus is a gram negative, halophilic, bacterium capable of causing gastroenteritis, wound infections, and fatal septicemia in humans (48, 92, 97). This organism is routinely found in waters of estuarine environments as part of the normal microflora, as well as in oysters and other shellfish inhabiting those estuaries (97). *V. vulnificus* infection is the leading cause of seafood-borne deaths in the United States, usually resulting from the consumption of raw or undercooked oysters (97). Infections caused by ingesting oysters contaminated with *V. vulnificus* commonly result in primary septicemia, almost always require hospitalization, and have a fatality rate greater than 50% (73, 97). Wound infections are usually the result of exposure of an open wound to sea water containing the bacterium, and can progress to necrotizing fasciitis (49, 96). While causing rapid and highly fatal infections, most often *V. vulnificus* opportunistically targets those individuals with underlying diseases that make them more susceptible to this organism. These can include liver diseases (such as cirrhosis) or immune dysfunction (*e.g.* diabetes).

V. vulnificus exhibits a great deal of genotypic and phenotypic variation (97). The species is divided into three biotypes, all of which are able to cause human infection, however biotype 1 is of greatest import to oyster producers and consumers (7, 92). Biotype 2 strains routinely infect eels, especially those grown in aquaculture, while biotype 3 strains have only been isolated in Israel from wound infections in association with the handling of tilapia (3, 7, 49, 137).

Biotype 1 stains of *V. vulnificus* have been further divided into two genotypes, a difference discovered by RAPD-PCR analysis of strains from both clinical and

environmental sources (148).   In this classification system, a gene identified as *vcg* (virulence correlated gene) was found to have two variations (117). One allele (*vcgC)* correlates with strains obtained from clinical isolation, designated the C-genotype, while the other (*vcgE*) is correlated with environmentally isolated strains and is designated the E-genotype (117, 148). Although a search using the Basic Local Alignment Search Tool (BLAST) failed to identify a specific protein the *vcg* gene encodes, it appears that the C-type is more pathogenic for humans. More recently, it has been shown that the genetic differences that C- and E-type strains exhibit in *vcg* are shared genome-wide, with analysis of any gene resulting in a phylogenetic tree splitting strains into C and E types (116). Thus, we can infer that a genotypic difference at a specific locus is merely an indicator of greater variation throughout the chromosome between strains of the C- and E-genotypes. A similar separation of clinical and environmental strains has also been performed by comparison of 16s rRNA sequences. This technique classifies environmental isolates as "A-type" and clinical isolates as "B-type" (86). The *vcg* and rRNA methods agree on most strains, though strains exist that have conflicting classifications (*e.g.* A-type but E-genotype).

Over 95% of infections resulting in septicemia caused by *V. vulnificus* involve the consumption of raw oysters, with the remainder arising from ingestion of steamed oysters and clams (97). Studies comparing the population dynamics of the two *V. vulnificus* genotypes have revealed an interesting phenomenon. While there was a nearly even ratio of C-type strains to E-type strains found in North Carolina seawater (before a severe drought altered the ecosystem in 2007), strains isolated from oysters living in that same water were found to be predominately (>84%) E-type (59, 145). This finding helps

explain the disparity between the high number of individuals at risk for infection and the low number of yearly cases. Presently, the reason for this incongruity has not been determined. It is especially odd considering that oysters are filter feeders. The oyster, *Crassostrea virginica*, pumps water through its gills, straining food particles from the flow (83). Incredibly, *C. virginica* is able to pump water at a rate of 10 L $h^{-1}g^{-1}$ dry tissue weight (83). With such a rapid rate of water clearance, one would naturally expect the oyster's internal composition of *V. vulnificus* to mimic that of the surrounding water. In experiments employing marked *V. vulnificus* strains of the C- or E-genotype to oysters and measuring their entry into and exit out of oysters, it was found that there was no difference between uptake or clearance rates of the two types (27).

An oyster is able to select the particles of food it eats based on size. The gills act as a sieve, catching particles of optimum size and moving them toward the mouth, while particles that are too large are stopped and passed from the oyster as pseudo-feces. In contrast, particles smaller than the optimum size pass through the gills with low capture efficiency. For *Crassostrea virginica,* the optimum particle size is 5-7µm in diameter (143). At this size, particles are retained with 90% efficiency. Particle retention rates drop to 50% when the diameter is only 1.8µm, and when particles are the size of a single *V. vulnificus* bacterium (ca. 1µm), oysters only retain about 16% of what is passed through the gills (143). This size selection could limit the effectiveness of a bacterial uptake experiment where bacteria are simply added to oyster tanks.

Marine aggregates, also as known marine snow, are a natural part of marine waters. These particles consist of fecal pellets, larvacean houses, phytoplankton, microbes, and inorganics brought together by shear forces and Brownian movement.

These particles are stuck together by exocellular polymers and physical/chemical forces (2) and once achieving a critical size, particles sink to the ocean and estuary floor. Visible aggregates are termed "marine snow" after the "long snowfall" of sedimentary material described by Rachel Carson (2, 11, 130).

The purpose of this study was to compare the integration of C- and E-genotype *V. vulnificus* cells into laboratory-produced marine aggregates. These marine snow particles with added *V. vulnificus* were then fed to oysters to measure uptake and depuration rates of this pathogen. We hypothesized that differences in the ability of *V. vulnificus* strains to incorporate into marine aggregates could play a role in the population disparity we have observed within oysters.

Materials and methods

Bacterial strains and growth conditions

Vibrio vulnificus CVD713 is a C-genotype strain possessing a stable (for at least 10 days) TnphoA transposon that confers kanamycin resistance and alkaline phosphatase activity (76, 80, 155). This strain forms blue colonies when grown on Tn Agar, consisting of Luria Agar with the addition of 0.2 g L^{-1} kanamycin, 2 g L^{-1} glucose, and 0.04 g L^{-1} 5-bromo-4-chloro-3-indolyl phosphate (BCIP). Tn Agar selects for this TnphoA-possessing strain via its kanamycin resistance and is differential by means of the BCIP breakdown (155). *Vibrio vulnificus* strain pGTR-JY1305 is an E-type strain that contains the stable pGTR plasmid which confers kanamycin resistance when grown on Luria Agar containing 10 g L^{-1} L-arabinose and 0.3 g L^{-1} kanamycin (90). These genetically marked strains were used in aggregation and oyster uptake experiments to

separate these strains from naturally occurring *Vibrio* spp. and other bacteria found in oysters and seawater.

Oyster maintenance

Oysters (*Crassostrea virginica*) from the coast of North Carolina were collected by hand in the intertidal zone, rinsed, and placed into holding aquarium tanks to acclimate to laboratory conditions. The tanks contained a 1:1 mixture of artificial seawater (ASW, Instant Ocean, Aquarium Systems, Mentor, OH) and natural seawater (NSW, collected from North Carolina coast) which had been passed through a 0.45μm filter (Millipore, Bedford, MA) and finally adjusted to 20‰ salinity. Tank water was kept at 23°C. Oysters were fed an algal mixture of *Skeletonema, Rhodomonas,* and *Isochrysis* species daily. The algal cultures were grown at room temperature in vented, 1 liter flasks containing F/2 medium and were provided with constant fluorescent light (46, 74).

Marine aggregates

Laboratory-created aggregates (marine snow) were generated using the method described by Shanks and Edmondson with modifications suggested by Ward and Kach (121, 142). Briefly, cells and seawater are transferred to 250 ml roller bottles, 10 $\mu g\ l^{-1}$ hyaluronic acid was added to the bottles, and the bottles were placed on a roller table at 15RPM for 24 hours. Static bottles were placed next to the roller table to serve as controls (no aggregates).

Bacterial incorporation into aggregates was measured by allowing the aggregates to settle for 20 minutes and removing a 750μl sample from above the aggregates (or non-aggregated particulate matter in the static controls), disrupting the aggregates via vortexing, and plating onto media specific for the added *V. vulnificus* strain. Control

bottles not rolled were inverted three times, allowed to settle for 20 minutes prior to sampling. Each experiment involved four static control bottles and four rolled bottles per bacterial strain, and each experiment was performed in triplicate. For uptake experiments, roller bottles containing aggregates, and control bottles without aggregates, were inverted three times before being gently poured into oyster aquaria.

Oyster uptake and depuration

For each experiment, oysters were fed 24 h prior to being removed from maintenance tanks and placed in experimental tanks with 20‰ salinity ASW at 23°C. Twenty-five oysters were placed into each tank and five oysters were sampled at each time point. Five oysters were also removed from the tanks for sampling to establish a background population count of *V. vulnificus* (sampling methods described below). *Vibrio vulnificus* cells grown to a concentration of 10^{8-9} CFU per ml were added to the experimental tanks, at a concentration of 7.5×10^{4} CFU/ml of tank water. Oysters were incubated in the *V. vulnificus*-infected water for 24 h.

After the initial 24h exposure, and every 48 hours thereafter, the oysters were removed from the tanks and the aquaria cleaned, sanitized, and refilled with fresh ASW (20‰ salinity). The oysters were then placed back into the clean tanks before selecting five oysters that were removed for sampling, allowing the measurement of bacterial uptake and elimination rates. All studies were conducted in triplicate.

Oyster dissection and homogenization

Oysters, once removed from experimental tanks, were rinsed with ethanol and patted dry with paper towels. The oysters were shucked with a flame-sterilized oyster

knife, and the meat washed with sterile ASW of 20‰ salinity. The oyster tissue was separated from the shell and placed in a sterile test tube.

The whole oyster tissues were homogenized in 20‰ ASW at 1:1 w:v ratio (minimum 5 ml ASW) using sterile blender cups (Warring, Torrington, CT) and a blending pattern of 3 bursts of 15s each, with a 5s pause between the bursts. Sampling methods for marked strains

After homogenization, samples were serially diluted in sterile phosphate-buffered saline (PBS) and plated onto the appropriate medium for the inoculated strain of *V. vulnificus*, as previously described. Total colony forming units (CFU) per gram of oyster tissue were calculated.

Statistics

Data were compared using a two-way analysis of variance followed by post-hoc tests with Bonferroni corrections for multiple comparisons (124). Variable included time and treatment. Data were analyzed using SigmaStat statistical software (Version 2.0, Access Softek Inc).

Results and discussion

For decades, experiments looking at the uptake of *V. vulnificus* by oysters have been conducted by simply adding planktonic bacteria to tanks containing the oysters or, occasionally, by adding the bacteria to algae and feeding the algae to oysters. While significant uptake has been observed, it may actually underestimate the potential uptake. Using the method modified from Ward and Kach (142), we formed aggregates of particles suspended in natural sea water by rotating the water on a roller table. By adding

C- or E- strains of *V. vulnificus* to these aggregates as they formed, we were able to measure the incorporation of the bacteria into these particulate conglomerations.

Macroscopic aggregates were always observed in the bottles placed onto the roller table, whereas the static control bottles never formed visible aggregates. The concentration of *V. vulnificus* cells was significantly greater in the samples with aggregated marine snow than in the samples where aggregates did not form (p<.001), regardless of genotype (Figure 9). In the environment, *V. vulnificus* and other vibrios have also been reported to be in higher concentrations in natural marine aggregates compared to the surrounding water (68, 69). Persistence in marine aggregates can increase bacterial survival when moving from host to host (68). Furthermore, direct access to organic substrates and protection from chemical or physical stress can be gained by association with aggregates (69, 135). Thus it is beneficial for the cells to concentrate in marine snow in a short time.

E-type cells showed significantly more integration into marine aggregates than C-type cells (p<.001) while the non-aggregated *V. vulnificus* concentrations between the two genotypes was not statically different (p=.084, Figure 9). The cause of the increased affinity for the E-genotype strain to demonstrate increased snow affinity is not known, but because aggregate affinity and assimilation involves cell-cell interactions, motility, chemokinetics, and exoenzyme production, we assume that one of these or other factors are different between the two genotypes (17, 57, 68, 135).

Aggregates, with integrated *V. vulnificus* cells, were added to oyster tanks, and the uptake and depuration of the bacteria was recorded. Oysters sampled after one day of incubation with aggregated *V. vulnificus* treatments were found to have significantly

more E-genotype cells or more C-genotypes cells than the non-aggregated control treatments (p=.025 and p=.002, respectively; Figure 10). The aggregated C-type cells were also significantly higher than controls at 3 days after incubation (p=.03) but by day 6 were undetectable (Figure 10B). We could still detect E-type cells at day 6, but detection of pre-aggregated cells was not different from controls, and were very low in number (p>.05, Figure 10A).

When the uptake and depuration rates of C- and E-genotype cells were compared, we observed no differences at any time point (p>.05, Figure 11). This apparent lack of uptake difference between the two genotypes, despite their differences in aggregate integration, might be explained by the fact that all of the bacterial cells from each microcosm, whether aggregated or free-living, were added to the oyster tanks. We have previously reported that free-living C-genotype cells are more rapidly taken up by oysters than E-type cells (6), and thus the effects of the aggregation could be masked, resulting in equal uptake (27). Future experiments will involve the addition of only the aggregated portion of the bacterial preparations, and not the entire assemblage of aggregated and free-living cells. Additionally, C- and E-genotype cells will be added to the same aggregate generation bottles, allowed to co-incubate, and then fed to bacteria in a competition study that will allow the cell-cell interactions and communications.

CHAPTER 6: THE INTERACTIONS OF *VIBRIO VULNIFICUS* AND THE OYSTER
CRASSOSTREA VIRGINICA UNDER ENVIRONMENTAL CONDITIONS

Citation

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Submitted

Abstract

The human bacterial pathogen, *Vibrio vulnificus*, is found in brackish waters and

is concentrated by filter-feeding molluscan shellfish, especially oysters, which inhabit

those waters. Ingestion of raw or undercooked oysters containing virulent strains of *V.

vulnificus* can result in rapid septicemia and death in 50% of victims. This review

summarizes the current knowledge of the environmental interactions between these two

organisms, including the effects of salinity and temperature on colonization, uptake and

depuration rates of various phenotypes and genotypes of the bacterium, and host-

microbe immunological interactions.

Introduction

Vibrio vulnificus is a gram negative, halophilic bacterium capable of causing

gastroenteritis, wound infections, and fatal septicemia in humans (48, 92, 97). This

organism is routinely found in waters of estuarine environments as part of the normal

microflora, as well as in oysters and other shellfish inhabiting those estuaries (97). *V.

vulnificus* infection is the leading cause of seafood-borne deaths in the United States,

usually resulting from the consumption of raw or undercooked oysters (97). Infections caused by ingesting oysters harboring *V. vulnificus* commonly result in primary septicemia, almost always require hospitalization, and have a fatality rate greater than 50% (73, 97). Wound infections are usually the result of exposure of an open wound to sea water containing the bacterium, and can progress to necrotizing fasciitis (49, 96). While causing rapid and highly fatal infections, most often *V. vulnificus* opportunistically targets those individuals with underlying diseases that make them more susceptible to this organism. These can include liver diseases (such as cirrhosis) or immune dysfunction (*e.g.* diabetes). The pathogenesis of this bacterium has been recently reviewed (49).

Vibrio vulnificus exhibits a great deal of genotypic and phenotypic variation (97). The species is divided into three biotypes, all of which are able to cause human infection, but biotype 1 is of greatest import to oyster producers and consumers (7, 92). Biotype 2 strains routinely infect eels, especially those grown in aquaculture, while biotype 3 strains have only been isolated in Israel in association with handling of tilapia (3, 7, 49, 137).

Biotype 1 stains of *V. vulnificus* have been further divided into two genotypes, a difference discovered by RAPD-PCR analysis of strains from both clinical and environmental sources (148). In this classification system, a gene identified as *vcg* (virulence correlated gene) was found to have two variations (117). One variation correlates with strains obtained from clinical isolation, while the other variation is correlated with environmentally isolated strains (117, 148). The gene was has two alleles, *vcgC* and *vcgE,* representing clinical and environmental strains, respectively.

Separation of clinical and environmental strains has also been performed by comparison of 16s rDNA sequences. This technique classifies environmental isolates as "A-type" and clinical isolates as "B-type" (86). The *vcg* and rRNA methods agree on most strains, though strains exist that have conflicting classifications.

The American oyster, *Crassostrea virginica*, used as a food source for thousands of years, survives in a wide array of habitat conditions but prefers salinities between 5-40‰ and temperatures from 20 to 30°C (19, 123, 128). This species of oyster is found naturally along the western Atlantic Ocean from the Canadian Maritime Provinces down to the Gulf of Mexico, Panama, and the Caribbean Islands (19). *C. virginica* pumps water through its gills, straining food particles from the flow (83). Incredibly, *C. virginica* is able to pump water at a rate of 10 L $h^{-1}g^{-1}$ dry tissue weight (83). This remarkable filtration rate allows oysters to concentrate *Vibrio* spp., reportedly to levels as great as 6 x 10^4 CFU/g, from surrounding waters containing only 7 CFU/ml (103), making oysters an important reservoir for *V. vulnificus*. The interactions between these two organisms are complex, and still largely unknown.

Populations and seasonality

Vibrio vulnificus population dynamics in oysters

Studies separating the natural *V. vulnificus* populations present in *C. virginica* by genotype, or other similar classification, agree that environmental strains of the species outnumber the clinical strains (38, 86, 127, 141, 144). Environmental (E-type) strains can range from 50% to almost 85% of the total *V. vulnificus* population in oysters, depending on the season (38, 63, 144). A similar disparity between the two genotypes has been reported for the Pacific oyster, *Crassostrea gigas* (58). This

unequal distribution could reflect the population differences of the bacteria in the water, or could potentially reflect a selective advantage of environmental type stains as a result of differential uptake by, or survival within, oysters (144). Evidence for such a selective advantage is provided by studies that compared the populations of *V. vulnificus* in oysters to the populations in the waters surrounding those oysters. A report by Warner and Oliver found that the water column had a nearly even mix of C- and E-genotypes while oysters were predominated by E-types, and research by Hoi showed the same phenomenon, albeit in mussels (44, 144). Such studies suggest that oyster bacterial populations are not directly dependent on the levels present in the surrounding waters. While there are individual oysters that can contain a greater proportion of clinical types to environmental types, these oysters are rare and do not appear reflect to the population (38, 127, 144).

<div align="center">Seasonality of V. vulnificus in oysters</div>

It is generally accepted that *V. vulnificus* populations fluctuate seasonally, regardless of their environment (e.g. shellfish or estuarine waters). Multiple studies have shown that oysters harvested from the summer months have a greater likelihood of containing *V. vulnificus* cells, and at higher concentrations, than oysters from the winter months (19, 38, 47, 63, 64, 106, 132, 136, 144). Studies that have separated *V. vulnificus* into the clinical and environmental subtypes concur that the clinical type strains show a greater seasonal shift than the environmental type strains (38, 64, 144). This appears to be true regardless of the method used to determine strain type (38, 64, 144). One study designed to characterize *V. vulnificus* diversity in oysters found that during summer months there was a major shift in structure and intraspecific diversity in

the *V. vulnificus* populations (63). Such studies suggest there is a population of *V. vulnificus* that is more suited for oyster colonization, but that the summer season can reduce this advantage and allow the less capable populations to proliferate. Thus the question arises, "are increased *V. vulnificus*-related diseases in the summer months (43, 122) due to increased concentrations of *V. vulnificus* in oyster meats or an increased percentage of infectious strains"?

<div align="center">Temperature and salinity</div>

<div align="center">The effects of temperature on intra-oyster *V. vulnificus* populations</div>

That warmer water temperatures are associated with increased *V. vulnificus* population in oysters has been well established, and may account for as high as 50% of *V. vulnificus* density variability (30, 44, 47, 51, 63, 78, 108, 110, 132, 136, 139, 144). Most researchers agree that the lowest range of temperatures for finding culturable *V. vulnificus* in oysters ranges from 12° to 17°C and the density of the population increases as temperatures increase, with no natural maximum temperature being observed in an estuary (30, 78, 89, 110, 132, 136). There have been studies where no correlation with temperature was found (106, 112), but these observations occurred in tropical climates where seasonal temperature changes are not as dramatic as they are in temperate climes (106). In fact, the effect of temperature may not be visible in these tropical waters if temperatures are consistently above 26°C, as reported in India by Parvathi and colleagues (106). Similarly, Motes *et al.* (78) found that *V. vulnificus* populations remained unchanged above this temperature. The lower temperature limit for *V. vulnificus* varies considerably from report to report. Tamplin *et al.* (132), as well as Tilton and Ryan (136), found no *V. vulnificus* below 17°C while Fukushima and Seki

(30) and O'Neill *et al.* (89) suggest 15°C as the minimum temperature. An extensive study by Randa *et al.* (110) found, *in vivo,* that the lowest temperature of *V. vulnificus* recovery was 12°C, while the lowest *in vitro* temperature was 13°C. In contrast, Wright *et al.* (154) reported relatively high numbers of *V. vulnificus* in oysters harvested from the Chesapeake Bay area when water temperatures were as low as 7.6°C, and suggested that the bacteria were adapting to the colder climate of this area. However, studies from regions farther north, such as New Jersey, New York, New Hampshire, and Maine, do not support this explanation, and Wright's findings may be due to other factors (89, 110, 136).

Temperature is not only a major factor in the distribution of *V. vulnificus* in the environment, but appears to affect the rate at which *V. vulnificus* is depurated from oysters. At cold temperatures the bacteria may have sharply stunted replication rates, yet depuration has little effect on the density of *V. vulnificus* as oysters slow their pumping activity (14, 66). Kelly and Dinuzzo (14) reported that cool temperatures allow oysters to increase pump rates, causing a net decrease in the density of *V. vulnificus* contained within their tissues, but this was not seen by Lewis *et al* (62). Warm temperatures usually show no change in *V. vulnificus* numbers as replication and depuration rate become balanced, and significantly increased temperatures can actually bring about an increase in the number of *V. vulnificus* cells within the oyster, with as many as 10^5 *V. vulnificus* cells being released per hour per oyster (14, 41, 133).

The effects of salinity on intra-oyster *V. vulnificus* populations

Vibrio vulnificus is an obligate halophile which has only been recovered from water with a salinity of at least 5‰ but never from the open ocean (97). Conclusions on

the role of salinity on the density of *V. vulnificus* population within oysters are mixed, ranging from a positive correlation, negative correlation, or no correlation (47, 63, 106, 110, 112, 132, 154). Zimmerman *et al.* (156) stated that a non-linear relationship between cell density and salinity would not be identified if the variation in salinity lies only on one side of the optimum salinity level for *Vibrio parahaemolyticus*, and the same may apply for *V. vulnificus*. If studies were conducted, or samples collected, in a temperature range that was too narrow, it would likely disguise such correlations (47, 156). Parvathi *et al.* (106) and Johnson *et al.* (47) sampled in salinities ranging from <3‰ to >30‰, and both found a correlation between salinity and *V. vulnificus* density in oysters. On the other hand, Lin *et al.* (63) sampled oysters in salinities ranging from 5-25‰ and found no correlation, though the authors pointed out that this span was completely within the non-limiting range for *V. vulnificus*. Furthermore, two other teams found a correlation in *V. vulnificus* densities in water, but not in the oysters inhabiting those waters, when the salinity in these environments ranged from 0 to >30‰ (47, 132). Such ranges should be wide enough to detect differences in *V. vulnificus* population changes if they existed, so it is possible that other factors such as temperature interact with salinity to make direct salinity correlations difficult. Evidence of this is discussed in the following section.

While moderate salinity appears to be the most permissive for *V. vulnificus* populations in *C. virginica*, some interesting phenomena occur at the extreme ends of the range. Reports of high numbers of recovered *V. vulnificus* at low salinities were detailed by Fukushima and Seki as well as Parvathi *et al.* (30, 106). Both groups found *V. vulnificus* in oysters from brackish water (~6‰) as well as a very low salinity

(<2.6‰), with the study by Parvathi *et al.* reporting the maximum density of cells, during a monsoon season in India, being found at salinities close to 2‰ (30, 106).

Data regarding high salinities (~25‰ or greater) all point in to the same conclusions. An anomaly in the number of *V. vulnificus* isolates obtained in yearly studies conducted by Motes and his coworkers was recorded when they obtained much lower concentrations one year, coinciding with unusually high salinity (78). Parvathi found that at 25‰ *V. vulnificus* numbers decreased and disappeared completely above 30‰ (106). When oysters harvested from one location were relayed to another, ranging from 32-35.3‰ salinity, *V. vulnificus* counts were reduced from as high as 14000/g to less than 10/g (77). Such a reduction is far better than is traditionally achieved by conventional depuration in moderate salinity waters (more detail below), and appears to significantly reduce the natural populations of *V. vulnificus* which are notoriously difficult to depurate. We found that oysters experiencing long durations of elevated salinity, even at levels less than the non-permissive limit of 25‰, show dramatically reduced *V. vulnificus* levels, even many months after the salinity of the surrounding waters returned to a more habitable level for this pathogen (28). It appears that extreme salinity events near the upper regions of the limit actually cause death or perhaps depuration of *V. vulnificus* from *C. virginica* and not simply a retardation of growth.

Interactions of salinity and temperature

While there is some agreement that temperature can affect how *V. vulnificus* responds to different salinities, there is no consensus on what that effect is. Randa *et al.* published that *V. vulnificus* was more dense in salinities above 15‰ when the temperature was greater than 22°C, and this was even more pronounced at or above

30°C (110). The opposite effect was claimed by Fukushima and Seki, who state that lower temperatures increase the tolerance to higher salinities (30). Both of these publications cite as evidence an earlier paper by Kaspar and Tamplin (51), which reported that when temperatures were above 22°C, *V. vulnificus* levels remained unchanged or only dropped slightly, while at 14°C *V. vulnificus* populations encountering higher salinities exhibited sparser densities, supporting Randa *et al.* (51, 110). However, Kaspar and Tamplin (51) also showed that *V. vulnificus* numbers were higher at lower temperatures over a broader range of salinities, giving support to Fukushima and Seki (30, 51). The extent to which temperature affects the ability of *V. vulnificus* to survive at various salinities remains unclear, but is likely influenced by other factors often not documented, or even spatial or temporal differences between the studies.

<div align="center">Uptake and elimination of *V. vulnificus* in *C. virginica*</div>

As a filter feeder, *Crassostrea virginica*, pumps water through its gills, straining food particles from the flow (83). Incredibly, *C. virginica* is able to pump water at a rate of 10 L $h^{-1}g^{-1}$ dry tissue weight (83). Depuration is the process where filter feeders are placed into fresh water to purge bacteria over time (14, 37). Experiments in which oysters were placed into water inoculated with cultured *V. vulnificus* bacteria were usually found to take up the cells quite rapidly, usually within a few hours. However, such laboratory grown bacteria were quickly eliminated from the oysters once they were placed into clean water, often in less than 72 hours (27, 37, 113, 126). Two studies indicated that elimination of laboratory grown bacteria took longer, with one reporting 6 days and another 2 weeks (55, 62). Those studies were different from others of this

type as these researchers exposed the oysters to strains of *V. vulnificus* that were indistinguishable from the background microflora whereas most studies employ "marked" strains of *V. vulnificus* in uptake studies so that uptake rates of only the bacteria added by the investigators are measured. Such bacteria contain (*e.g.*) antibiotic resistance, alkaline phosphatase activity, or natural luminescence to distinguish them from the background natural flora (76, 80, 101, 155). In the two studies that did not use marked laboratory strains, the oysters they sampled prior to inoculation may have appeared to be free of *V. vulnificus*, but it is conceivable that the bacteria were in the viable but non-culturable (VBNC) state (93).   Cells in the VBNC state cannot be grown on their routine media but still retain viability (97). *V. vulnificus* cells that enter into this state can return to a culturable, actively metabolizing, state in a process termed "resuscitation" (97). Thus cells existing in the VBNC state would not be counted at the beginning of these experiments but if these endogenous cells resuscitated upon the addition of exogenous bacteria (described later) the researchers may have actually been enumerating reemerging natural flora rather than the added strains.

It has been suggested that laboratory acquired vibrios are purged quickly because they become trapped in the feces of the oyster and are rapidly passed through the digestive tract (111). Regardless, close associations of vibrios with the oyster's hepatopancreas cells could allow colonization within those cells, creating "persistently infected shellfish" (9, 22, 35, 111). The constant presence of *V. vulnificus* in surrounding waters could allow these closer associations with the intestinal tissues, but these processes could occur quite slowly and would not be observed in laboratory experiments that only allow uptake of the *V. vulnificus* strains for a short time. This

could help explain why oysters eliminate laboratory-introduced *V. vulnificus* cells more easily than naturally-acquired cells (111). Conversely, neither incidence nor loads of *V. vulnificus* were shown by Sokolova *et al.* to increase with oyster age, suggesting there are yet undiscovered factors involved in the depuration process (125).

<div align="center">Uptake of laboratory strains of V. vulnificus in oysters</div>

While the *in vitro* uptake of laboratory grown cultures of *V. vulnificus* is very different from the *in situ* uptake of natural bacterial populations, several factors that affect uptake or depuration rates in the lab may also affect populations in the environment. The role of bacterial pili in oyster uptake has been examined primarily by two laboratories and conflicting reports have been reported. Paranjpye *et al.* used *V. vulnificus* strains that were pili deficient (by mutations in either the *pilA* or *pilD* genes) and found that these strains were taken up by oysters with the same efficiency as the wild type strain (105). In contrast, when Srivastava *et al.* performed a similar experiment using the *pilA* mutant, they reported a reduction in uptake in whole oyster and hemolymph samples when compared to the wild type strain (126). They did not see this difference in gill or digestive tissue, and they concluded that *pilA* is important for oyster uptake but not for dissemination to the tissues (126). This same study examined both non-motile and rugose strains, the latter being copious biofilm producers and also non-motile, and found that while motility was not involved in uptake, rugose *V. vulnificus* cells did show less uptake in whole oysters, gills, and hemolymph but not digestive tissue (34, 126).

<div align="center">The response of natural bacterial populations to exogenous bacteria in oyster uptake experiments.</div>

We recently observed that when oysters with low numbers of culturable vibrios were exposed to with *V. vulnificus*, a greater number of culturable cells than were added would emerge from the oysters (submitted). These vibrios apparently had been present in a nonculturable/dormant state, but resuscitated and multiplied in response to the exogenous addition of a different species. This phenomenon occurred whether *V. vulnificus* or *Escherichia coli* was added to the oysters. A similar phenomenon has been documented by other researchers as well. Groubert *et al.* reported almost exactly the same results, using strains similar to those we employed (37). Murphree and Tamplin (79) saw an increase of *V. vulnificus* cells in oysters that were inoculated with *Vibrio cholerae*, and Srivastava *et al.*(126) described an effect whereby the addition of *V. vulnificus* caused an increase in total bacteria in the oysters. This effect was not seen by Paranjpye *et al.*, but their study examined total aerobic bacteria, and not *V. vulnificus* specifically (105).

<div align="center">Depuration of *V. vulnificus* by *C. virginica*</div>

Many studies have been conducted to determine what affects the rate of depuration of *V. vulnificus* by *C. virginica*. Most agree that laboratory-grown bacterial strains exhibit rapid depuration from oysters yet there is a persistent, depuration resistant, natural microflora (27, 37, 113). A unique study reported by Lewis *et al.* used a flow-through depuration system, and they concluded that this type of depuration employing rapid (60L/min.) flow was effective at reducing *V. vulnificus* numbers in oysters. However, this experiment employed water that was at >30‰ salinity, and a similar experiment using a salinity within the preferred range of *V. vulnificus* actually showed an increase of natural *V. vulnificus* cells (62). Depurating oysters in water near

or above 30‰ has been shown to be effective in reducing the persistent, naturally present *V. vulnificus* cells (28, 77)

Other studies moved beyond basic depuration and often took advantage of genetically modified or phenotypically unusual strains in the experimental process. Temperature is one factor that appears to affect the rate of depuration, and we refer the reader to the previous section of this review that discusses temperature for details. Our laboratory found that "pre-depuration", allowing oysters to depurate before the addition of laboratory grown strains, had no effect on subsequent depuration rates of the introduced bacteria (37).

Two studies have concluded that the clinical variant of *V. vulnificus* is depurated at the same rate as the environmental variant, although Lewis *et al.* concluded this even after finding more of the environmental Type-A strains before depuration and more of the clinically associated Type-B strains after depuration in their experiments (27, 62).

Despite the benefits of an antiphagocytic capsule, some strains of *V. vulnificus* exhibit a reduction in capsule expression (49). Those strains will full capsule production are termed "opaque" and those without or with reduced capsule are referred to as "translucent" (49). Opacity is phase-variable and strains can revert to either morphology (49). Groubert *et al.* and Srivastava *et al.* both found that opaque and translucent strains have similar uptake and depuration rates (37, 126). The latter study also examined a translucent strain that has lost the ability to revert to the opaque phenotype, and reported significantly lower concentrations of this phenotype from oysters that were inoculated exogenously. Whereas oysters infected with the opaque strain retained 3.2 log CFU/g of oyster tissue, the non-encapsulated form was reduced

to 2.8 log CFU/g oyster tissue (126). The role of pilus-based attachment was

determined to be important in oyster colonization by Paranjpye *et al.*(105). In contrast

with the effects on uptake, pilus mutants (*pilA* and *pilD*) were depurated more rapidly

than the wildtype, a phenomenon that was confirmed by Srivastava *et al.* (126) when

they repeated the experiment. The latter authors additionally reported that the rugose

phenotype of *V. vulnificus* underwent a significant increase in depuration (105, 126). It

was proposed by Paranjype *et al.* that *V. vulnificus* pili may bind specifically to

carbohydrate-containing receptors on oyster cells (105).

<div align="center">Fate of bacteria inside the oyster</div>

It has been shown that *V. vulnificus* does not exist only on oyster surfaces, but

within tissues as well (125, 129, 133). Sun and Oliver found that over 95% of oyster-

associated *V. vulnificus* cells are within tissues rather than on meat surfaces (129).

Environmental *V. vulnificus* cells that have been taken up by *C. virginica* appear to

concentrate primarily in the digestive gland, followed by the adductor muscle, then the

mantle and gills (9, 27, 111, 126, 133). A study by Aldrich *et al.* that utilized

immunoelectron microscopy, however, asserts that free-living bacteria are not found in

the adductor muscle, but are contained specifically in hemocytes within the adductor

muscle (1). Interestingly, as oyster size increases, the concentration of *V. vulnificus*

inside the oyster decreases, possibly due to a decrease in tissue surface area as

compared to volume (125).

Opaque and translucent strains of *V. vulnificus* have been introduced into

oysters, as mentioned previously. Phase variation from the translucent phenotype to the

opaque phenotype was not observed by Groubert and Oliver (37) but was seen by

Srivastava *et al*. (126). The rate at which opaque cells phase shift to the translucent phenotype was reported to be unaffected by oyster passage (37).

The ratio of clinical type strains to environmental type strains does not shift in natural oysters according to one study (127), nor is virulence affected by oyster passage (37). However, it should be noted that virulence was only examined in laboratory-grown bacteria; natural populations have not been investigated.

<div align="center">Oyster immune system and V. vulnificus</div>

Immunoelectron microscopy has shown that oysters remove bacteria from the environment using phagocytic cells, known as hemocytes, present in the hemolymph (1). Schematically, this process begins when bacteria are phagocytized by hemocytes in the hepatopancreas/digestive gland, digested in the lysosome of the hemocyte, with residual material being discharged into the periphery of gills, muscle, and mantle tissues (1, 23). Fisher termed this process "dispedesis" (23).

Oyster hemocytes are multifunctional, serving in defense, excretion, repair, and digestive roles (23, 33). Agglutinins traditionally increase phagocytosis by aggregating bacteria but this process was not found to occur with *V. vulnificus* in oyster hemolymph (33, 134). The oyster hemocytes ingest the *V. vulnificus* cells independently of bacterial contact with humoral factors (41, 138). Multiple studies have shown that the encapsulated phenotype of *V. vulnificus* is more resistant to phagocytosis than the less or non-encapsulated forms, and it is suggested that encapsulation also allows for resistance to degradation and the ability for this pathogen to survive with the hemocyte (33, 40, 41). No differences in the amount of hemocyte lysozymal or acid phosphatase

activities were seen following ingestion of encapsulated vs. non-encapsulated strains (40).

Another explanation for *V. vulnificus* persistence inside these phagocytic cells is the inability of some hemocytes to kill *V. vulnificus*. While granular hemocytes are quite lethal to *V. vulnificus*, agranular hemocytes are not, possibly due to the lack of the lysosomal enzymes responsible for bacterial degradation (24, 40, 114). In the summer, the percentage of agranular hemocytes in oysters is greater than granular hemocytes, possibly contributing to the increase of *V. vulnificus* seen in the summer months (40, 72). However, this seems in contrast to the observation that increased temperatures encourage increased phagocytosis (41, 114).

Oliver found that *V. vulnificus* populations incubated in the presence of oyster broth exhibited a rapid decline at low temperatures, and suggested the presence of antimicrobials within the oyster tissues (91). Pelon *et al.* showed that an oyster extract component was lethal to *V. vulnificus* cells (107) and Seo *et al.* extracted a protein from *C. virginica* that was able to inhibit *V. vulnificus* (120). Because these studies used similar extraction techniques, it is likely that both teams were reporting on the same antimicrobial polypeptide, a protein that Seo and team have named American oyster defensin. They suggested this might be the basis of the mechanism for the anti-*V.vulnificus* properties of oyster hemocytes (107, 118, 120). In a very recent study, more proteins with *V. vulnificus* inhibitory effects have been discovered. Seo *et al.* described histone 2B proteins with strong activity on *V. vulnificus*, and speculated that the significant concentrations of these proteins inside oyster tissues might be involved in regulating the prevalence of *V. vulnificus* in oysters (119).

Pollution and *V. vulnificus* in oysters

It has been reported that the numbers of *V. vulnificus* in oysters do not correlate to contamination or pollution (62, 153). A recent study, however, reported that oysters exposed to a cadmium-hypoxia double challenge showed increased *V. vulnificus* concentrations when compared to controls (45). Vibrio species are known to be tolerant to heavy metals, including cadmium, in comparison to other bacteria (5, 21, 31, 53, 131).

Conclusion

The interactions between oysters and *V. vulnificus* are sufficiently complex that while we have a general understanding of such interactions, there remain numerous unanswered questions. Because oysters harbor natural *V. vulnificus* populations, laboratory exposure experiments can be problematic, but the trend of moving towards molecular, rather than culture based, techniques is likely to prove useful in filling in these knowledge gaps. As we continue to discover the biological workings that govern bacterial colonization, oyster immunology, and even location specific interactions between oysters and their natural microflora, we can hopefully keep seafood safer for human consumption and gain greater insight into *V. vulnificus* physiology in the process.

Acknowledgements

recommendations expressed in this publication are those of the authors and do not

necessarily reflect the views of the U.S. Department of Agriculture.

CHAPTER 7: APPARENT LOSS OF *VIBRIO VULNIFICUS* IN NORTH CAROLINA OYSTERS COINCIDES WITH DROUGHT-INDUCED INCREASE IN SALINITY

Citation

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Abstract

Despite years of successful isolation of *Vibrio vulnificus* from estuarine waters, beginning in 2007 we found it extremely difficult to culture *V. vulnificus* from either North Carolina estuary or oyster samples. After employing culture and non-culture based methods for detection of *V. vulnificus*, always with negative results, we concluded that this pathogen had become nearly undetectable in North Carolina estuarine ecosystems. We ensured our techniques were sound by spiking North Carolina oysters with *V. vulnificus* and performing the same tests as previously conducted on unadulterated oysters. We were able to readily detect *V. vulnificus* in the spiked oysters using all methods. Furthermore, we obtained oysters from the Gulf of Mexico and easily isolated *V. vulnificus*, confirming that our methodology was sound but that the oysters and waters of North Carolina were lacking the *V. vulnificus* population our lab has studied for decades. Strikingly, we discovered that the apparent disappearance of *V. vulnificus* coincided with the most severe drought in the history of

North Carolina. The drought continued until the end of 2009, with elevated salinity experienced throughout this period, and with *V. vulnificus* nearly non-existent. When salinities returned to normal after the drought abated in 2010, we were again able to routinely isolate *V. vulnificus* from the water column, though still unable to culture it from oysters. We suggest the oysters were colonized with a more salt-tolerant bacterium during the drought, which displaced *V. vulnificus* and may be preventing its recolonization.

Introduction

Vibrio vulnificus is a gram negative, halophilic, bacterium capable of causing gastroenteritis, wound infections, and fatal septicemia in humans (48, 92, 97). This organism is routinely found in waters of estuarine environments as part of the normal microflora, as well as in oysters and other shellfish inhabiting those estuaries (97). *V. vulnificus* is present in virtually all oysters meant for human consumption, with 67% of raw and 25% of cooked oysters collected from Louisiana restaurants found to be harboring the microbe (67).

Infections caused by *V. vulnificus* are the leading cause of seafood-borne deaths in the United States. Infections caused by ingesting oysters contaminated with *V. vulnificus* commonly result in primary septicemia, almost always require hospitalization, and have a fatality rate greater than 50%. This distinguishes *V. vulnificus* as having the highest case fatality rate of any foodborne pathogen (12, 73, 95, 97). This pathogen is credited with causing over 900 infections from 1998 to 2006 (97).

Vibrio vulnificus exhibits a great deal of genotypic and phenotypic variation (36, 97). The species is divided into three biotypes, all of which are able to cause human

infection, but biotype 1 is of greatest importance to oyster producers and consumers (7, 92). Biotype 2 strains routinely infect eels, especially those grown in aquaculture, while biotype 3 strains have only been isolated in Israel in association with tilapia (3, 7, 49, 137).

Biotype 1 stains of *V. vulnificus* can be further divided into two genotypes based on genetic polymorphisms originally identified using RAPD-PCR analysis (148).   In this classification system, a gene identified as *vcg* (virulence correlated gene) was found to have two variations (117). One variation occurs predominantly in strains obtained from clinical sources, while the other variation is seen primarily in environmentally isolated strains (117, 148). The gene has two alleles, *vcgC* and *vcgE,* representing clinical and environmental strains, respectively (117)

The two most important factors affecting the number of *V. vulnificus* cells populating harvested oysters are temperature and salinity. The temperature effect is easily seen through seasonal and experimental data (with temperatures ranging between 13 and 22°C the most permissive to *V. vulnificus* survival) but salinity is also an important , though less obvious survival factor (51, 54, 77, 78, 99, 103, 108).

Historically, *V. vulnificus* has been easily isolated from North Carolina and Gulf Coast estuaries and oysters. Collecting and homogenizing oyster meats and plating them onto selective media has been a standard procedure for the isolation of strains of this organism. These *V. vulnificus*-specific media are used for primary isolation, but a confirmatory step using immunological or molecular methods is common and often required to eliminate incomplete sensitivity or specificity (42). CPC (colistin-polymyxin B-cellobiose) based media have been used since 1992 by our lab and others

to isolate *V. vulnificus* with great success (98, 108, 145, 151). However, beginning in

the spring of 2007, colonies presumptively identified as *V. vulnificus* on this and other

selective media could not be confirmed as this species using molecular methods.

Coincidently, in 2007 North Carolina entered into what would become the worst

drought in the state since recordkeeping began in 1895 (88). This drought, which

significantly elevated the salinity of estuaries in North Carolina, lasted from May 2007

until the winter of 2009 (82). In the six-year study we report here, we elaborate on the

coincidence between extended extreme environmental changes and *V. vulnificus* oyster

colonization, and present a possible explanation for continued lack of *V. vulnificus*

isolation from NC oysters, despite a return of this species to NC estuarine waters.

<div style="text-align:center">Materials and methods</div>

<div style="text-align:center">Media</div>

Two media were used to isolate *V. vulnificus* from environmental samples.

CPC+ (a derivative of CPC) is both selective and differential for *V. vulnificus* (71, 146).

It uses the fermentation of cellobiose to presumptively identify *V. vulnificus* colonies

from other bacteria and vibrios (71, 146). *V. vulnificus* colonies growing on CPC and

CPC+ produce yellow colonies with a yellow zone surrounding them, while *V. cholerae*

produces purple colonies with a blue zone, thus allowing easy distinction between the

two species (71, 146). Presumptive *V. vulnificus* colonies grown on these media can be

examined using PCR amplification to confirm their identity (147). CHROMagar Vibrio

(CHROMagar, Paris, France) is a chromogenic medium that can distinguish four types

of vibrios, including *V. parahaemolyticus, V. vulnificus, V. cholerae,* and *V.

alginolyticus* (39, 81, 151). Colonies of *V. vulnificus* appear dark blue or teal on this

medium, but as with CPC+, this medium can only be used for presumptive detection, and a confirmation step must be included for complete accuracy.

Oyster collection and maintenance

More than 650 oysters (*Crassostrea virginica*) from the coast of North Carolina were collected by hand from the intertidal zone between 2005 and 2010, at various times during the year, including spring, summer, fall, and winter harvest dates. These were rinsed and placed into holding aquarium tanks to acclimate to laboratory conditions. Oysters from Gulf Coast sites were shipped overnight with ice packs and placed into acclimation tanks once received at the Charlotte laboratory. The tanks contained a 1:1 mixture of artificial seawater (ASW, Instant Ocean, Aquarium Systems, Mentor , OH) and natural seawater (NSW, collected from North Carolina Coast), which was passed through a 0.45 μm filter (Millipore, Bedford, MA) and adjusted to 20‰ salinity. Tank water was kept at 23°C. Oysters were fed daily an algal mixture of *Skeletonema, Rhodomonas,* and *Isochrysis* spp. which had been grown at room temperature in vented, 1 liter flasks containing F/2 medium and provided with constant fluorescent light (4, 46).

Oyster sampling

Oysters were removed from tanks, rinsed with ethanol and patted dry with paper towels. The oysters were shucked with a flame-sterilized oyster knife, and the meat washed with sterile ASW of 20‰ salinity. Oyster tissues were aseptically separated from the shell and placed in sterile test tubes. The tissues were homogenized in 20‰ ASW at 1:1 w:v ratio (minimum 5 ml ASW) using sterile blender cups (Warring, Torrington, CT) and 3 bursts of 15 s each, with a 5 s pause between the bursts. After

homogenization of oysters, the samples were serially diluted in sterile phosphate buffered saline (PBS) and spread onto both CPC+ and CHROMagar Vibrio.

Water sampling

To improve sensitivity of detection of cells in estuarine waters, 10-ml water samples were filtered by vacuum suction onto 0.22μm filters which were then aseptically placed onto the solid media plates, either CPC+ or CHROMagar Vibrio.

Sampling methods

Presumptive colonies were picked from these selective media to heart infusion (HI) agar and allowed to grow overnight at 30^oC. Using the methods developed by Rosche *et al.* (117) and Warner and Oliver (147), each strain was subjected to a PCR reaction that confirmed the isolate as *V. vulnificus*. Reactions were performed using GoTaq polymerase (Promega, San Luis Obispo, CA) in a Techne Genius thermal cycler using the parameters suggested by Warner and Oliver (147). PCR products were visualized by gel electrophoresis on 1% agarose gels stained with ethidium bromide.

Spiking oysters with *V. vulnificus*

Oysters were fed 24 h prior to being removed from maintenance tanks and placed into two separate tanks with 20‰ salinity ASW at 23°C. *V. vulnificus* cells (of the E-genotype) were added to one tank at a final concentration of ~10^4 CFU per ml of ASW. The other tank served as a control without a *V. vulnificus* inoculum. Oysters were allowed to take up *V. vulnificus* cells for 24 h. Oysters from both tanks were then removed, shucked and homogenized as described above.

PCR and Q-PCR analysis of oyster homogenate

Diluted oyster homogenate (1 ml) from each oyster (four control oysters, and one artificially infected oyster) was treated with the Wizard Genomic DNA Purification System (Promega), and isolated DNA subjected to both standard PCR (described previously) and quantitative PCR.

Q-PCR was performed using the QuantiTect SYBR Green PCR kit (Qiagen, Valencia, CA) using a Lightcycler 2.0 (Roche, Basel, Switzerland), employing the E and C genotype-specific primers described by Warner and Oliver (147).

Microbial identification

Colonies that presumptively appeared positive as *V. vulnificus* on CPC+ and/or CHROMagar Vibrio, but which subsequently were not confirmed to be this species, were subjected to genetic identification by sequencing of the 16s rRNA gene, provided by Accugenix (Newark, DE).

Results

Sampling of North Carolina water and oysters

Oyster samples plated onto CPC+ resulted in 3990 presumptive *V. vulnificus* isolates which were subjected to PCR confirmation. In both 2005 and 2006, 40.7% of the presumptive isolates were positively confirmed as this species, whereas in the period 2007-2010, confirmation dropped to 0.7% or less (Table 5). To ensure our lack of *V. vulnificus* recovery was not due to deficiencies in CPC+, in 2010 we also began utilizing an additional medium, CHROMagar Vibrio, to collect bacteria from oyster samples. We confirmed only 4% of 456 colonies isolated on this medium as *V. vulnificus* (Table 5). There was a highly significant difference between the number of presumptive isolates confirmed in drought years versus the number confirmed from pre-

drought years (p<0.001 using Chi-square analysis with Yates correction for continuity).

In addition, between 2006 and 2010, water samples were taken from the same North

Carolina estuaries from which the oysters were harvested, and plated onto CPC+. These

samples generated 2404 total presumptive *V. vulnificus* isolates. In 2006, before the

drought began, 45.7% of bacterial samples isolated using CPC+ from North Carolina

waters were determined to actually be *V. vulnificus*. In 2007 (during the drought), none

of the samples were positively confirmed (although the sample size was small) and in

2008, only 2.4% of these isolates were determined to be *V. vulnificus*. In the last part of

2009 and in 2010, after the drought period, the confirmed percentages were 38.1% and

42.4%, respectively (Table 6), returning to pre-drought percentages.

   In 2007, North Carolina entered the driest year of its history and continued to

experience drought conditions until the winter of 2009. Estuarine salinity levels, which

typically vary between 10 and 20‰, were consistently above normal (ca. 20 to >25‰)

during this period (Figure 5). Thus, before the drought of 2007, we were able to

positively confirm over 40% of presumptive colonies from CPC+ from North Carolina

oysters as *V. vulnificus*. After the drought began in the late spring of 2007, this figure

dropped to less than 1% (Table 5).

   To confirm that our methodology was not causing this sudden and dramatic

change, we spiked oysters from the Neuse River estuary in North Carolina with *V.

vulnificus* cells. When these oysters were homogenized and plated onto CPC+, 79 of 80

(99%) presumptive isolates were confirmed as *V. vulnificus* using our standard PCR

protocol. To further document that it was North Carolina oysters which were exhibiting

a lack of indigenous *V. vulnificus* cells, oysters harvested from Gulf Coast waters

between 2008 and 2010 were plated onto CPC+ agar. These generated 131 presumptive

V. vulnificus isolates with an additional 10 isolates obtained from CHROMagar Vibrio.

During this period, when North Carolina oysters were subjected to the drought

conditions and contained <1% *V. vulnificus*, PCR confirmation of the Gulf Coast

isolates yielded > 96% positive confirmation of *V. vulnificus* (Table 7). Relatively few

Gulf Coast isolates were sampled, compared to North Carolina isolates, as *V. vulnificus*

was readily identified from Gulf Coast oysters, and further confirmation was not

necessary.

PCR and Q-PCR detection of *V. vulnificus* in oyster homogenates

To support our culture-based *V. vulnificus* detection methods, we supplemented

our data with non-culture based identification techniques. Oysters (both natural and

those spiked with *V. vulnificus* cells) were homogenized and total DNA extracted. PCR

analysis of control oysters revealed no indication of the presence of *V. vulnificus* cells,

while the spiked oyster homogenates produced PCR amplicons matching the expected

size for the *V. vulnificus*-specific *vvhA* (hemolysin) and the *vcgE* (virulence correlated)

genes (data not shown).

These same DNA extracts were also subjected to quantitative PCR analysis with

primers specific for the E- and C-genotypes of *V. vulnificus* (147). The number of

copies of the *V. vulnificus* C-genotype specific gene (*vcgC*) were below the limit of

detection in all tested oysters (control and spiked). Only the oyster spiked with E-

genotype *V. vulnificus* cells contained enough *V. vulnificus* DNA to be detected by E-

type specific probes, yielding 2.5×10^4 copies in 2ul of concentrated sample (data not

shown).

Sequence-based identification

Two false positive isolates on CPC+ (using PCR to confirm they were not *V. vulnificus*) were identified to the genus level based on 16S rRNA sequencing. Neither of these were identified as *V. vulnificus* (>7% sequence mismatch). Differing by <2% sequence alignment, the top matches were *V. coralliiyticus, V. mediterranei, V. nereis, V. tubiashii, and V. sinaloensis* (Table 8).

Discussion

Isolation of *V. vulnificus* from the oysters and water of North Carolina estuaries has been routinely accomplished by our lab and others (8, 25, 78, 98, 102, 108, 148, 149), especially during the summer months. Historically, using CPC+ agar designed for the isolation of *V. vulnificus* from environmental samples (146), over 40% of presumptively identified *V. vulnificus* were positively confirmed as this species (Table 5). In 2007, this statistic dropped to less than 1% (Table 5), even though the number of samples tested was increased by 500% (Table 5). Concerned about a possible deficiency in the quality and function of our isolation medium, we added CHROMagar Vibrio to presumptively detect *V. vulnificus*, but this medium fared only slightly better. The phenomenon of *V. vulnificus*-specific media losing specificity when samples contain a large number of competing *Vibrio* spp. has been reported previously by Macian *et al.* (70), thus offering a possible explanation for the presence of false positive *V. vulnificus* colonies on these typically reliable selective and differential media.

Puzzled by our inability to isolate *V. vulnificus* we employed non-culture based methods of detection, including PCR and Q-PCR, on DNA extracted from oyster tissue. No *V. vulnificus* could be detected by either assay method (data not shown).

To confirm that our isolation and confirmation techniques were sound, we spiked oysters with *V. vulnificus* cells. These oysters yielded positive confirmation of *V. vulnificus* with culture and Q-PCR detection methods, providing evidence that our media and techniques were working correctly. Further verifying our methodology, we collected Gulf Coast oysters and processed them in the same fashion as the North Carolina oysters and recovered confirmed *V. vulnificus* cells with ease (Table 7).

Since it was established that we were indeed observing North Carolina oysters with extremely reduced numbers of *V. vulnificus* cells we began to investigate potential conditions or events that might have caused such a sudden and significant loss. The drought (Figure 12) that occurred during our study period was the most severe ever recorded since record keeping began in 1895 (88). These conditions, which began in 2007 and persisted until the end of 2009 (82), resulted in a long-term increase in average salinity of the estuary (Figure 12). While previous increases of salinity to this level had occurred (Figure 12), these were short-term and unlike the extended interval (>2 ½ years) of elevated salinity we document in this study (87).

Prior reports have indicated that *V. vulnificus* is sensitive to salinity shifts. Kaspar and Tamplin (51) determined experimentally that in seawater with salinity greater than 25‰, *V. vulnificus* survival decreased. These findings were bolstered with *in situ* data by Motes *et al.* (78) who showed increases in salinity in Apalachicola Bay were linked with declines in *V. vulnificus* recovered from oysters and by Noble *et al.* (149) who found that salinity-lowering storm events resulted in increased recovery of *V. vulnificus* (finding it in 80% of their samples). Consistent with such observations, Jones (50) found that oysters moved to elevated (25‰) salinity waters were cleared of *V.*

vulnificus, and Motes and DePaola (77) reported that relaying oysters from estuarine to offshore sites with salinity of at least 32‰ similarly reduced the numbers of *V. vulnificus* in those oysters.   Past studies, however, have not examined the presence of *V. vulnificus* in high salinity waters for periods lasting longer than 21 days (8, 44, 50, 51, 54, 65, 77, 78, 108, 110, 132, 139, 154), while our study suggests that long-term elevated salinity (even those less than 25‰, considered to be the upper limit of *V. vulnificus* salinity preference) could negatively impact oyster colonization by *V. vulnificus*.

It is important to note that during this prolonged drought, several other environmental parameters may have shifted as well (which were unaccounted for in this study). Thus, it is possible that other environmental changes could have contributed to the loss of *V. vulnificus*. That considered, it is quite conceivable that the lengthy drought and resulting shift in estuarine salinity either induced *V. vulnificus* to be purged from the oyster habitat or else had outright bactericidal effects. Either possibility would lead to a loss of *V. vulnificus* in oysters, leaving an empty niche ready to be occupied by an organism with similar physiological characteristics but one which could tolerate the elevated salinity caused by the drought. Furthermore, we sampled only oyster and water for *V. vulnificus,* and these bacteria could have been residing in the sediment during the unprecedented drought (78, 110, 139).

After the drought eased at the end of 2009, the salinity of the NC estuary returned to normal (Figure 12). The number of *V. vulnificus* presumptive isolates obtained from water samples which were positively confirmed as this species quickly increased to pre-drought levels (Table 6), indicating that *V. vulnificus* had returned to

the NC estuary after cessation of the drought. Nevertheless, oysters harvested from the same waters in 2009 and 2010 still contained extremely low numbers of *V. vulnificus* cells (Table 6).

Although it is not known why *V. vulnificus* has returned to the estuary while the oysters which are filtering water from these estuaries continue to have very low levels of detectable *V. vulnificus*, we speculate that the answer may lie in results obtained by our lab and many others over the last 30 years. A number of researchers have examined uptake and depuration of *V. vulnificus* from oysters by placing them into water inoculated with this species. Such experiments have all provided similar data, indicating that *V. vulnificus* cells are rapidly taken up by the oysters, but are not retained and are quickly eliminated (14, 27, 37, 52, 55, 105, 126). It seems possible that if the oyster microflora is firmly established during the early stages of oyster development, transient bacterial cells acquired through gill filtration would be unable to establish residency in the oyster gut. Such an "original" population would likely only be displaced by extreme events, such as large and acute shifts in salinity which occur when oysters are relayed to much higher salinity waters (77, 78), or as in the moderate yet chronic salinity increases described in our study. If this is correct, then the re-emergence of a significant *V. vulnificus* population in adult North Carolina oysters may only be observed when oyster larvae produced after the drought conditions (*i.e.* >2009) develop into harvestable adults, a period of ca. 2 years. This is a very testable hypothesis which we intend to pursue over the next several years.

In summary, we have observed a sudden and dramatic decline in the *V. vulnificus* population of North Carolina estuaries and the oysters in those estuaries.

Before 2007, *V. vulnificus* could be isolated from North Carolina coastal waters and oysters with ease, but isolation of this bacterium from oysters has now become rare. This major disruption of the ecosystem appears to have been caused by a severe and enduring drought, the worst in the history of the state, which resulted in an up-shift in the salinity of North Carolina coastal waters to unusually high levels. What made these conditions unique was the duration of the elevated salinity conditions, from 2007 through 2009. While the salinity did not reach the upper limit of *V. vulnificus* salt preference (25‰), the long-term effect appears to have been the loss of the organism from the environment. The most striking findings of our report, however, may be those that occurred after the cessation of the drought conditions when the salinity of the estuary returned to normal and *V. vulnificus* re-emerged as a commonly isolated bacterium in water samples. Despite repopulation of the water, oysters that are actively filtering this water have yet to regain their pre-drought levels of *V. vulnificus*. It is worth considering the possibility that the significant loss of *V. vulnificus* from oysters left a niche that has been filled by a more salt tolerant bacterium, such as the *V. mediterranei* we identified growing on CPC+ agar. This bacterium may in fact be preventing *V. vulnificus* from re-colonizing oysters. We will continue to monitor North Carolina oysters for the presence of this "new" *Vibrio*, and attempt to determine if a species shift has taken place by comparing future non-*V. vulnificus* isolates with the sequenced strains.

Acknowledgements

Table 1: Number, genotype, phenotype, and isolation source of strains used in this
study.

Type	No. Strains	Genotype	Isolation Source	Mannitol Fermentation	Gene order same as Fig 1
I	21	C-type	Clinical	Yes	Yes
II	7	C-type	Environmental	Yes	Yes
III	9	C-type	Environmental	Yes	No
IV	1	C-type	Clinical	Yes	No
V	1	E-type	Clinical	Yes	No
VI	6	E-type	Clinical	No	No
VII	7	E-type	Environmental	Yes	No
VIII	6	E-type	Environmental	No	No

Table 2. Product predicted by primer pairs employed in this study, with annealing temperatures and extensions times that differ from manufacturer's instructions.

PCR Product (from Fig. 1)	Annealing Temp. (°C)	Extension Time (s)
A-B	56.1	30
A-D	54.5	90
C-D	54.5	30
E-F	60.8	30
G-H	60.4	30
I-J	54.2	30

Table 3. Primers used in Chapter 2

Primer	Sequence (5' - 3')	Corresponding Location in Fig. 1
Mann Hemo F	ACATTTGACGGATGAGCAG	A
Mann Hemo R	TCCCAGACAAACAGATGATG	B
Mann TRAP F	CGCTGAAGAAATGTCAAACG	C
Mann TRAP R	ACGCATTTTCAACCCTTT	D
Man IIA F	GATGTTGGTGAACAACTTCTCTGC	E
Man IIA R	TCTGAAGCCTGTTGGATGCC	F
Man Dehydro F	CAAAACGCTTTGCCGCTG	G
Man Dehydro R	CAATGGATGGCACTTCGC	H
Man Rep F	CGTCGATGGCTTGGTACAA	I
Man Rep R	TCGGTAAACTCGTATTCTGTG	J

Table 4. PCR results of tested strains. Values represent number of isolates possessing the listed gene (generating a PCR product of the correct size) or gene arrangement (primers overlapping two genes producing a product of the expected size) over the number of strains tested. The "Published arrangement" heading refers to those strains that have the putative hemolysin gene directly upstream of the TRAP-type transport gene, as shown in Fig. 1.

V. vulnificus Isolate	Putative hemolysin	TRAP type transport	Published arrangement
Clinical C-type	22/22	22/22	21/22
Environmental C-type	16/16	16/16	7/16
E-type	20/20	20/20	0/20

Table 5: Presumptive *V. vulnificus* isolates, obtained from North Carolina oysters using either CPC+ or CHROMagar Vibrio, confirmed as *V. vulnificus* following PCR analysis.

Presumptive *V. vulnificus* from North Carolina oysters							Isolated using CHROMagar Vibrio
Isolated using CPC+							
Year	2005	2006	2007	2008	2009	2010	2010
No. Tested	166	201	1041	1428	404	750	456
% Confirmed	40.7%	40.7%	0.6%	0.6%	0.7%	0.7%	4%

Table 6: Presumptive *V. vulnificus* isolates, obtained from North Carolina estuarine waters using CPC+ and confirmed as *V. vulnificus* following PCR analysis.

Presumptive *V. vulnificus* from North Carolina estuarine water					
Year	2006	2007	2008	2009 (Sept. – Dec.)	2010
No. Tested	138	45	245	425	1551
% Confirmed	45.7%	0%	2.4%	38.1%	42.4%

Table 7: Presumptive *V. vulnificus* isolates, obtained from Gulf Coast oysters using either CPC+ or CHROMagar Vibrio and confirmed as *V. vulnificus* following PCR analysis

Presumptive *V. vulnificus* from Gulf Coast oysters				
Isolated using CPC+				Isolated using CHROMagar Vibrio
Year	2008	2009	2010	2010
No. Tested	80	31	20	10
% Confirmed	96%	98%	100%	100%

Table 8: Molecular identification of false positive isolates from CPC+ and CHROMagar Vibrio using 16s rRNA gene sequencing. Species listed are hits from the sequence library that aligned to our unknown sequences with less than 2% difference.

Colony type	Presumptively positive on CPC+	Presumptively Positive on CPC+ and CHROMagar Vibrio
Sequence match (alignment difference)	*V. coralliilyticus* (1.3%) *V. nereis* (1.4%) *V. tubiashii* (1.7%) *V. sinaloensis* (1.8%)	*V. mediterranei* (1.0%)

Figure 1: Configuration of genes (from *V. vulnificus* CMCP6) examined in this study. Letters A-J represent binding sites for primers listed in Table 3
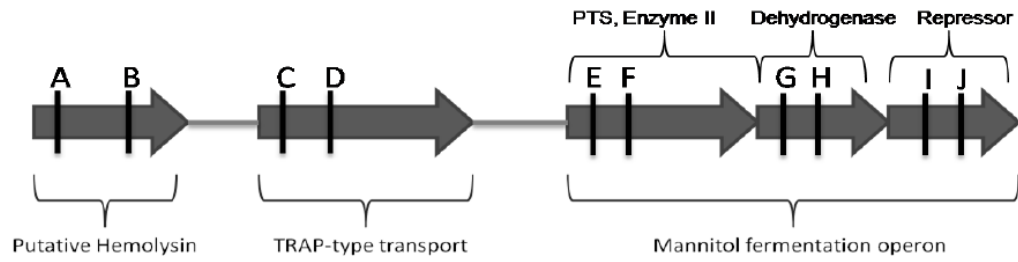
Figure 2: Geometric means of recovered C-type (closed squares) and E-type (open squares) strains of *V. vulnificus* from dissected oyster tissues and whole oyster homogenates. Asterisks indicate significant differences between C- and E-type values. Bars represent standard error of the mean.
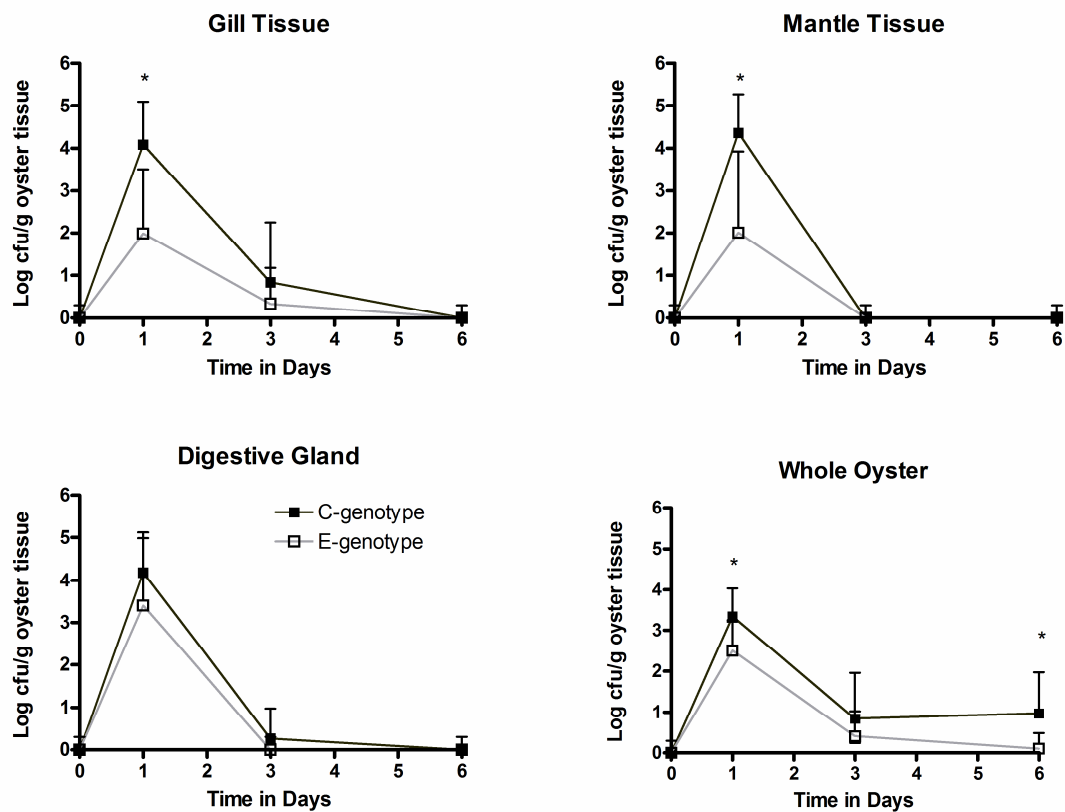
Figure 3. Recovery of vibrios and *V. vulnificus* from oysters exposed to exogenous *V. vulnificus* (strain CVD713)
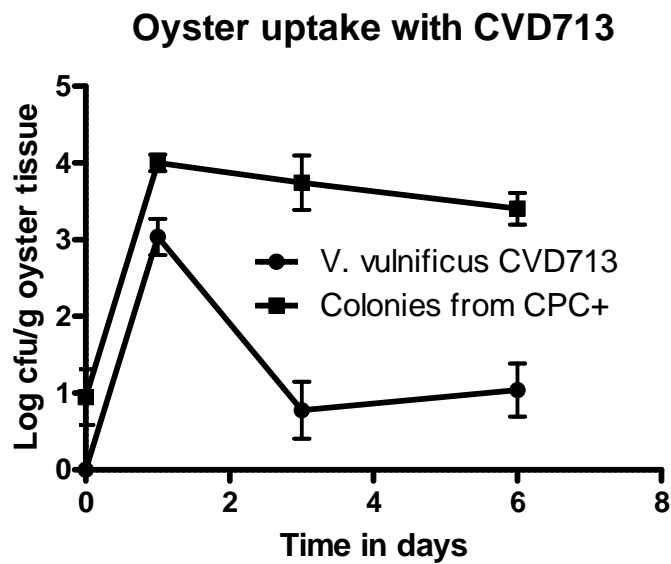

Oyster uptake with CVD713

Figure 4. Recovery of vibrios and *V. vulnificus* from oysters exposed to exogenous *V. vulnificus* (strain CVD713), Adapted from Groubert and Oliver, 1994.
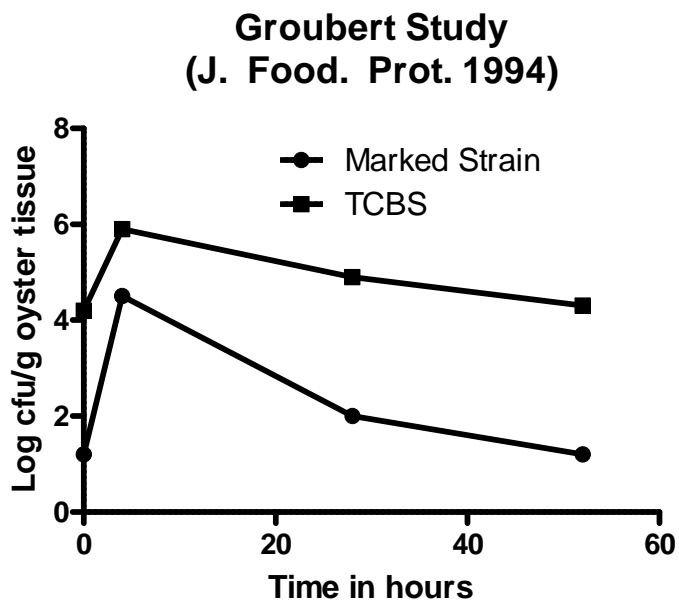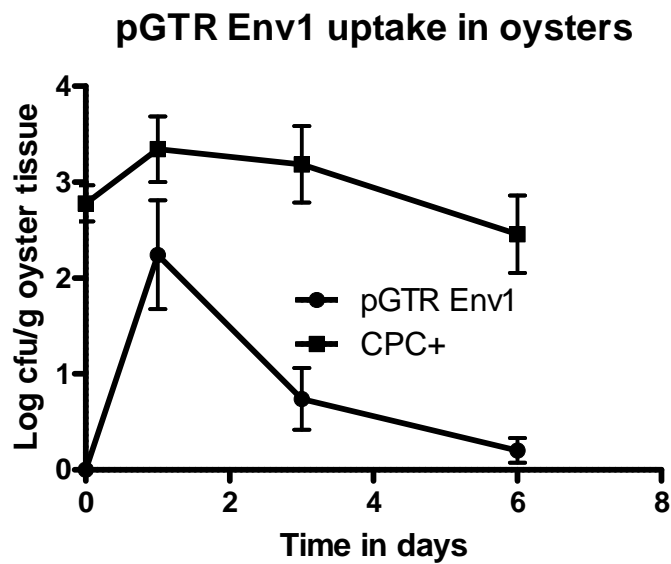

Groubert Study
(J. Food. Prot. 1994)

Figure 5. Recovery of vibrios and *V. vulnificus* from oysters exposed to exogenous *V. vulnificus* (strain pGTR Env1)

Figure 6. Recovery of vibrios and *V. vulnificus* from oysters exposed to exogenous *V. vulnificus* (strain VVL1)
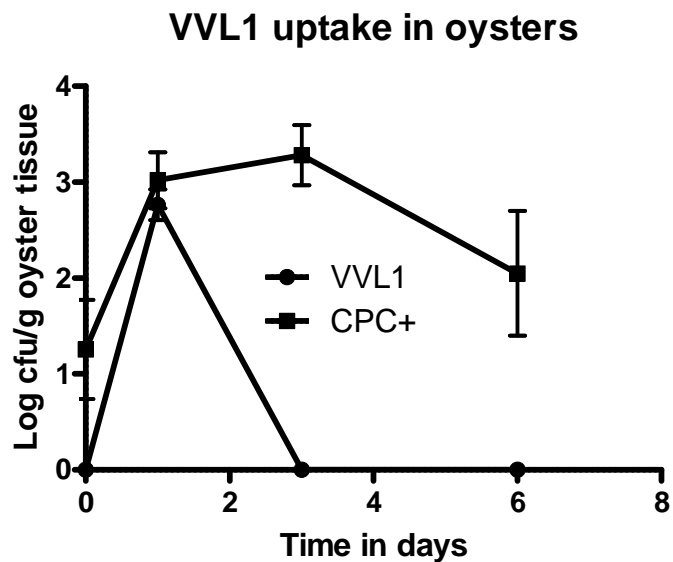

VVL1 uptake in oysters

Figure 7. Combined recovery of vibrios and *V. vulnificus* from oysters exposed to marked strains of *V. vulnificus*
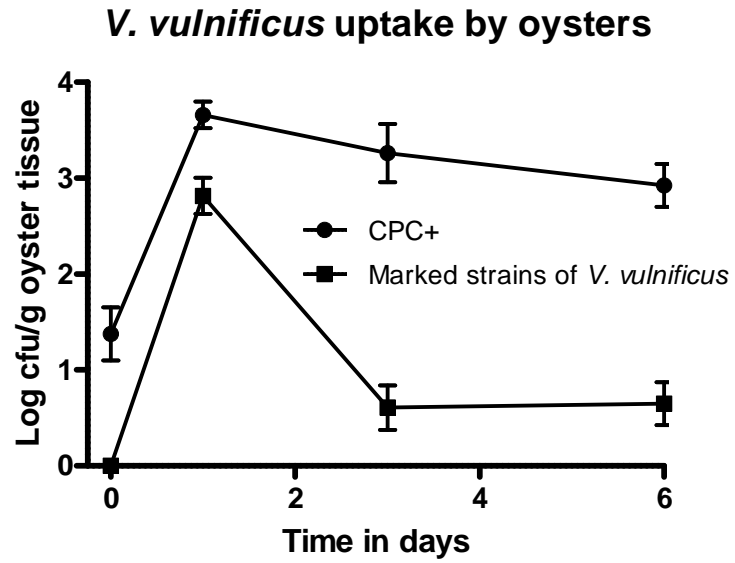
Figure 8. Recovery of vibrios on CPC+ and *E. coli* on MacConkey agar from oysters
exposed to *E. coli* K12. Control data included vibrios growing on CPC+ from non-
inoculated oysters. One day after inoculation with *E. coli,* the number of vibrios
recovered from inoculated oysters was significantly greater than non-inoculated control
oysters (p=.02).
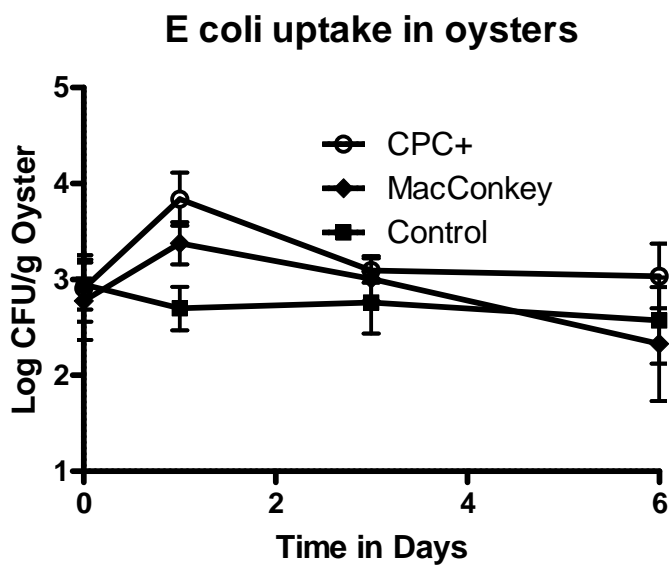

E coli uptake in oysters

Figure 9. Concentrations of *V. vulnificus* cells by genotype recovered from microcosms allowed to form aggregates by rolling, or from static control samples. Bars with different letters are significantly different from each other as determined by 2-way ANOVA with Bonferroni posttest comparisons.
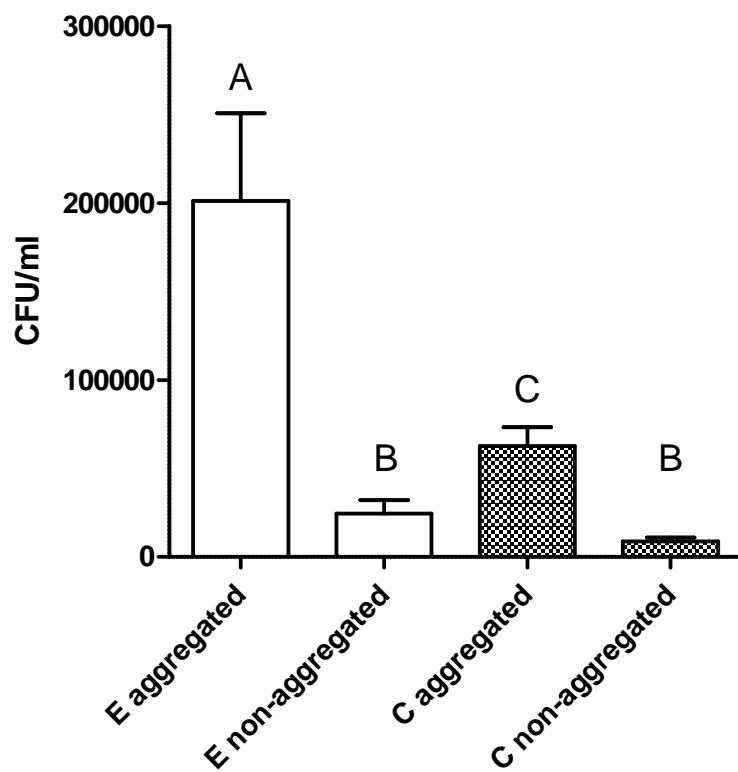
Figure 10A. Uptake and elimination of E-genotype cells in oysters exposed to aggregated and non-aggregated (static) bacterial cultures. Asterisk indicates time point at which experimental group was significantly different from control.
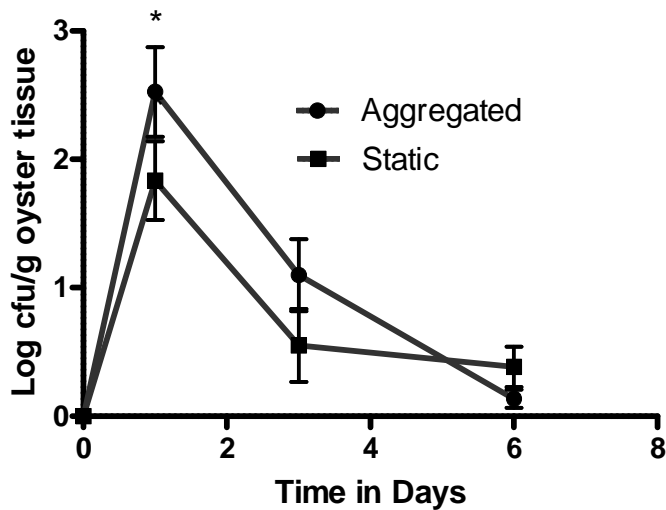

Figure 10B. Uptake and elimination of C-genotype cells in oysters exposed to aggregated and non-aggregated (static) bacterial cultures. Asterisks indicate time points at which experimental group was significantly different from control.
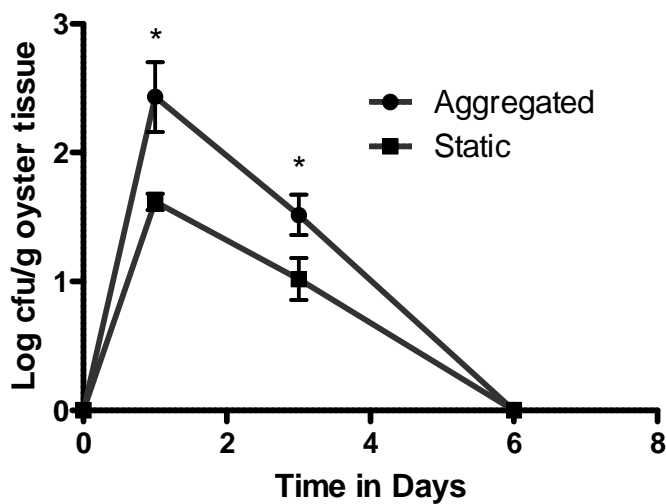
Figure 11. Comparison of uptake and elimination of aggregated C- or E-type cells fed to oysters. Graphs are not statistically different at any time point.
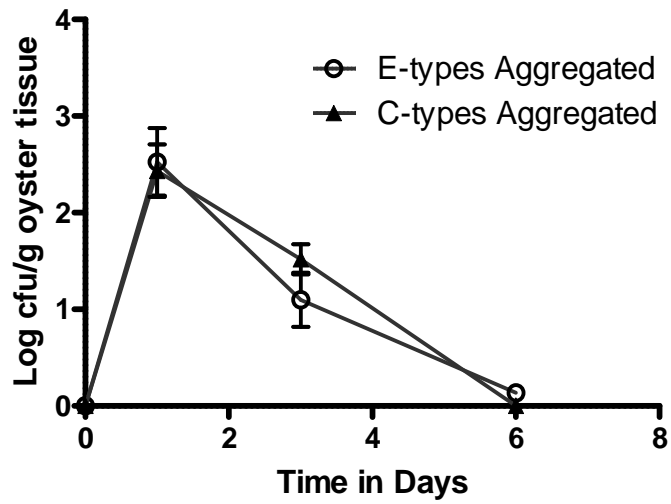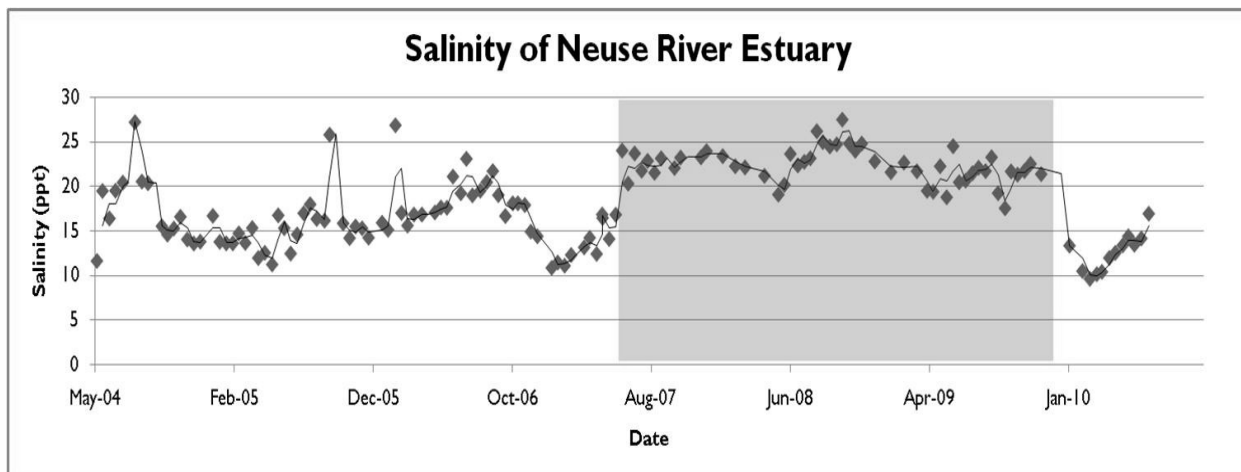
Figure 12: Biweekly salinity data from the Neuse River Estuary in NC. Black line represents the monthly moving salinity average; shaded area indicates the drought period.

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