Title: Improving *V. parahaemolyticus* predictive models via sampling during anomalous events

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Abstract: Understanding the biotic, atmospheric, and environmental conditions that drive total and pathogenic *Vibrio parahaemolyticus* (VP) abundance is critical to reducing infections or infection risk from these bacteria. Unfortunately, at this time, our capability to predict the presence of virulent forms of VP is limited. Furthermore, as a research community we well understand that predicting total populations of *Vibrio* bacteria is an inaccurate mode of public health protection. Knowing the factors that contribute to increasing concentrations of VP is the first step to developing more robust predictive models, identifying hotspots/ high risk growing areas, developing technology, and targeting future research to reduce VP loads. The current FDA model for VP relies solely on temperature, which is likely the largest driver of VP concentrations, but this dependence on a single factor leaves vast room for improvement. Predictive models for VP that use more than temperature have been tricky to develop, and findings have sometimes been contradictory. Models of *V. vulnificus* concentration, in contrast, have been much easier to create. Attempts at using the same data to predict VP loads demonstrates the relatively low accuracy of such a simplistic modeling attempt. In fact, a review of the literature reveals that many environmental variables measured have widely different effects on the abundance of VP depending on the study. This study used data on VP sampled from water and oysters from waters in eastern North Carolina (NC). Routine sampling was combined with targeted sampling. Targeted sampling during extreme events including periods of high salinity and immediately before and after a hurricane.

Methods utilized

Site selection: Samples were collected from the Newport River Estuary in NC during low tide. This site was selected because of moderate salinity and because this site had been sampled extensively in previous projects. Data was collected from February 2013 to November 2018.

Oyster sampling and processing: During each of the 42 sampling events, 12 oysters of at least two inches in length were collected. The oysters were stored on top of ice during transport to the laboratory and were processed within three hours of collection. Oysters were shucked using sterilized equipment

and cleaned of excess internal fluids, sediment, and pseudo-feces. The oyster soft tissues were placed together in a blender bag then weighed and diluted with sterile phosphate-buffered saline (PBS) in a 1:1 w:v ratio. The post-diluted weight was measured and the oysters were homogenized in a paddle blender (Fisher Scientific, Waltham, MA) for ten minutes at a rate of 280 rpm. Undiluted oyster homogenate as well as homogenate that was diluted with PBS at a 1:10 ratio was portioned into 100 µL aliquots then plated in the fashion detailed below.

Water sampling and processing: Water samples were collected congruently with oyster samples. Water was collected in 1-liter sterilized bottles that were rinsed thrice *in situ* directly adjacent to where the oyster samples were collected. Water samples were transported on ice, with oysters, and processed within three hours of collection. A digital seawater refractometer was used to determine salinity. The content of total suspended solids (TSS) was measured by vacuum filtration of at least 150 mL of sample water through pre-weighed, pre-dried 25 mm wide fiberglass filters. These filters were oven dried for at least one week at 55ºC then re-weighed. Concentrations of *Enterococcus* and *E. coli* were measured using the methods established by IDEXX for their proprietary IDEXX Quanti® -Tray 2000 system (IDEXX Laboratories, Westbrook, ME). 10 mL of sample water was diluted with deionized water in a 1:10 ratio in sterilized 100 mL sealable bottles and media selective for either *Enterococcus* or *E. coli* was added to the solution. The solution and media were thoroughly mixed and poured into the Quanti® -Tray 2000 which is then sealed and incubated for either 18 or 24 hours for *E. coli* and *Enterococcus* respectively. One tray of each *E. coli* and *Enterococcus* was completed for each sampling date and bacterial concentrations were determined using IDEXX's proprietary most probable number algorithm. Meteorological data for the sampling site, including air temperature, rainfall, and wind speed, was gathered from Wunderground (Weather Underground, 2018).

Bacterial analysis: To estimate total *Vibrio spp.* concentrations, oyster homogenate was plated on thiosulfate-citrate-bile salts-sucrose (TCBS) agar. Green and yellow colonies were counted, with the combined count representing total *Vibrio spp.* concentration. CHROMagar proprietary plates were used to assess presumptive counts of *V. parahaemolyticus* and *V. vulnificus* (CHROMagar, Paris, France). Colonies that grew into a rich blue color were presumed to be *V. vulnificus* and colonies that produced a dark purple color were presumed to be *V. parahaemolyticus.* The presumption of each species was due to the possibility of the CAV plates producing false positives and so the colonies were later subjected to molecular identification. Quantification of V. vulnificus and V. parahaemolyticus was performed as described by Froelich et al. (2015) where concentrations in CFU/g obtained from culture data were

multiplied by the percentage of vvhA-positive and toxR-positive (respectively) isolates. The same process was used in quantifying abundance and percent potentially pathogenic V. vulnificus (vcgC-positive) and V. parahaemolyticus (tdh/trh-positive).

Gene target name	Direction	Sequence (5'-3')	Amplicon Size (bp)	Source
vvhA1	F	TTCCAACTTCAAACCGAACTATGAC	205	Panicker and Bej (11)
	R	ATTCCAGTCGATGCGAATACGTTG		
vvhA2	F	CCGGCGGTACAGGTTGGCGC	519	Hill et al. (12)
	R	CGCCACCCACTTTCGGGCC		
vcgC1	F	AAAACTCATTGARCAGTAACGAAA	146	Warner and Oliver, (13)
	R	AGCTGGATCTAAKCCCAATGC		
toxR3	F	GTCTTCTGACGCAATCGTTG	368	Kim et al. (14)
	R	ATACGAGTGGTTGCTGTCATG		
tdh1	F	GTAAAGGTCTCTGACTTTTGGAC	269	Bej et al. (15)
	R	TGGAATAGAACCTTCATCTTCACC		
trh1	F	TTGGCTTCGATATTTTCAGTATCT	500	Bej et al. (15)
	R	CATAACAAACATATGCCCATTTCCG		

Table 1. Primer sequences. All isolates were typed for toxR and vvhA via both conventional PCR and SYBR® Green-PCR. While toxR used the same primer set for both methods, vvhA analysis used two primer sets, due to difficulties in transitioning the conventional vvhA primer set to SYBR® Green-PCR. Isolates were also typed for three other genes (vcgC, tdh, trh) but were only done so via SYBR® Green-PCR

1 Primers were used for SYBR® Green-PCR only

2 Primers were used for conventional PCR only

3 Primers were used for both PCR methods

DNA extraction: To genetically identify the bacterial colonies isolates were taken from each colony that was presumed to be either *V. parahaemolyticus* or *V. vulnificus.* up to 10 isolates of blue or purple colonies were taken, unless there were fewer than 5, in which all presumptive isolates were captured. A

sterile pipet tip was used to collect the isolate from the agar and this was transferred to microcentrifuge tubes prefilled with 100µl of sterile ultra-ultrapure water. The microcentrifuge tubes were placed into a heat block at ~100°C for 10 minutes and then centrifuged at 10,000 times gravity for 10 minutes. The isolates were stored in a freezer at -20ºC until they were processed.

PCR verification of isolates: Molecular species identification of both *V. vulnificus* (*vvhA*) and *V. parahaemolyticus* (*toxR*) was performed by conventional PCR on the C1000 Touch™ Thermal Cycler (BioRad, Hercules, CA, US) using GoTaq® Green Master Mix (Promega, Madison, WI, US) with subsequent gel (1.5% agarose) electrophoresis in tris-acetate (TAE) buffer at 140 mAmps for 25 minutes. The resultant bands were visualized via ethidium bromide staining and subsequent exposure to UV light Primers are listed in Table 1. Species identification was also

determined using qPCR. Molecular species identification of both V. vulnificus (vvhA) and V. parahaemolyticus (toxR), and subsequent potentialfor pathogenicity (vgcC for V. vulnificus, and tdh/trh for V. parahaemolyticus) was performed via PCR amplification on the BioRad CFX96™ Real-Time System (BioRad) using the PowerUp™ SYBR® Green Master Mix (ThermoFisher Scientific, Pittsburg, PA). Following SYBR® Green-PCR, a melt curve was generated in order to confirm amplification of only the target amplicon and only those peaks that matched the positive control (see below) were considered positive for the corresponding gene. Primers are listed in Table 1.

Two positive controls were used for these studies. For V. parahaemolyticus assays, toxR, tdh, and trh, the positive control was the tdh-positive/trh-positive environmental isolate, F11-3A, which is an ST36 pandemic strain (16, 17). The positive control for vvhA and vcgC assays was the septicemia isolated, vcgC-positive strain, MO6 (18). Both positive controls were inoculated from freezer stocks in heart infusion broth at 37°C overnight, then boiled-lysed for 10 minutes. Extracted controls were stored at -20°C. A non-template control (NTC) was used for all analyses.

Results:

Pre and post hurricane sampling: In September of 2018 Hurricane Florence made landfall in Eastern North Carolina and brought two-meter-high storm surges and 34 inches of rain to the area in less than a

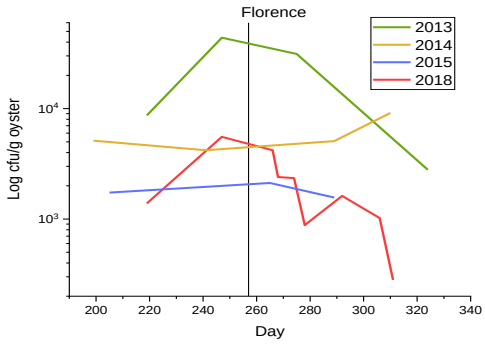

Figure 1: Total *Vibrio* in oysters from Newport River by sample date. Each line represents one year of the study. The vertical line shows the occurrence of Hurricane Florence.

week (Pregizer, 2018). Once safe to do so, The Newport River Estuary Site was sampled eight times in two months post-hurricane. These data were compared to the pre-hurricane and baseline sampling that occurred at the site. In 2018, at the Newport River Estuary Site, The total Vibrio concentrations seen in oysters increased up until the occurrence of Hurricane Florence, and decreased rapidly after (Figure 1). This trend was not observed in 2014 or 2015, but a similar trend was seen in 2013, even though there was no major storm that year (Figure 1).

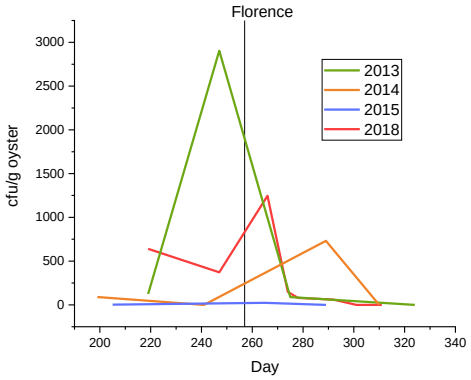

Figure 2: Total *V. vulnificus* in oysters from Newport River by sample date. Each line represents one year of the study. The vertical line shows the occurrence of Hurricane Florence.

There was a spike in the concentrations of V. vulnificus and V. parahaemolyticus after Hurricane Florence (Figures 2 and 3, respectively) that quickly dropped afterwards. In the years of 2014 and 2013 there were also spikes in these species, without the associated storm (Figures 2 and 3).

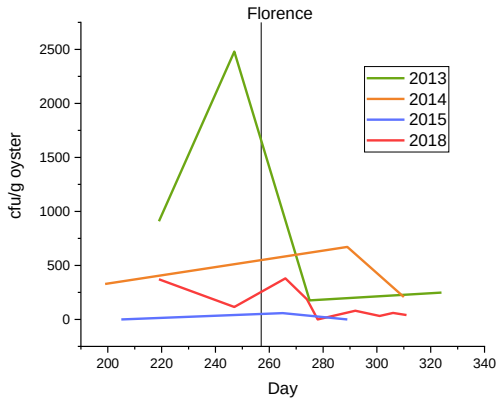

Figure 3: Total *V. parahaemolyticus* in oysters from Newport River by sample date. Each line represents one year of the study. The vertical line shows the occurrence of Hurricane Florence.

Pathogenic V. vulnificus spike just before the storm in 2018, and a similar spike at the same time was seen in 2013, a non-storm year (Figure 4). The concentrations of pathogenic strains of V. vulnificus in years 2018 (storm year) and 2013 (non-storm year) are far greater than in 2014 and 2015 (Figure 4). When the most influential environmental factor in determining Vibrio concentrations, temperature, was included in the analysis, it was observed that while 2018 was trending toward greater average temperatures, this does not explain the spike in pathogenic V. vulnificus, as 2013 had statistically lower temperatures than all other years (Figures 5 and 6).

Average temperature at the site was significantly lowest at the site in 2013, and showed an increasing trend to 2018 (Figure 6). Average wind speed and precipitation also showed increasing trends at the site over the study period (Figures 7 and 8).

Study of pathogenic Vibrio in oysters, farmed vs. wild.

A secondary study that occurred simultaneously, since we were already collecting oysters for this study, was a comparison of the bacteria growing in farmed oysters vs. wild oysters. Commercially, NC oysters can be harvested in two ways; wild-caught or grown as part of aquaculture programs. Farmed oysters are often grown in floating cages, which mean that these oysters experience vastly different conditions than wild oysters. Some of these differences in growth conditions include exposure, UV, temperature, agitation, water column height, handling, etc. The oysters collected with ISSC funding were also included in a multi-year farmed shellfish monitoring program, as well as a short-term, in-depth experiment. Farmed oysters were collected from seven locations in Carteret County, NC). Farms were located in the Newport River, Cedar Island Bay, Jarrett Bay, and Nelson Bay. That data was combined with additional locations with wild oysters which include Calico Creek, Harlowe Creek, Hoop Pole Creek, Newport River, South River, Turnagain Bay, Cedar Island Bay, and Jarrett Bay. . For the short-term experiment, both wild and farmed oysters were collected from Cedar Island Bay, Jarrett Bay, and Newport River. Long-term sampling of farmed oysters occurred from June 5, 2016 to October 20, 2017. Short-term oyster sampling occurred between late July 2018 and September 2018. For short-term sampling, each site contained a wild location and a farm location, and they were within no more than 1000m distance and within 3 ppt salinity difference, except during a single extreme rainfall condition. The farm location and its corresponding wild location from each

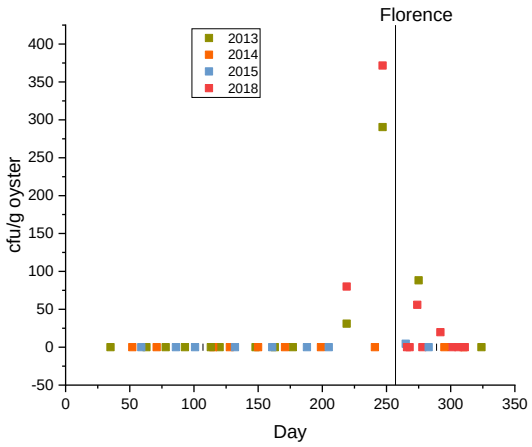

Figure 4: Pathogenic strains of *V. vulnificus* in oysters in the Newport River. Data points are color coded by year and data are presented by date. Vertical line shows the occurrence of Hurricane Florence

Figure 5: Pathogenic strains of *V. vulnificus* in oysters in the Newport River (large squares), and temperature (small squares). Data points are color coded by year and data are presented by date. Vertical line shows the occurrence of Hurricane Florence

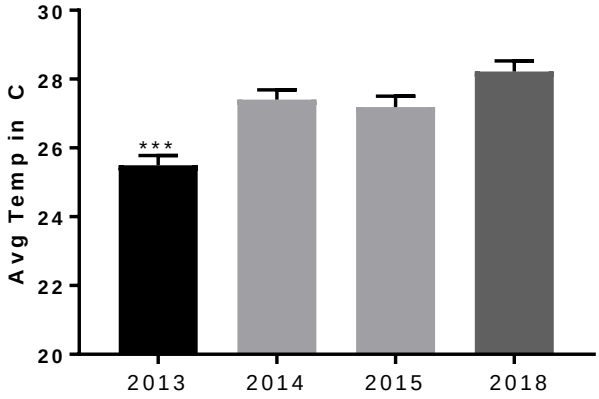

Figure 6: Average daily air temperature at Newport River wild oyster site by year. Asterisks indicate significantly different mean. Error bars are standard error of the mean.

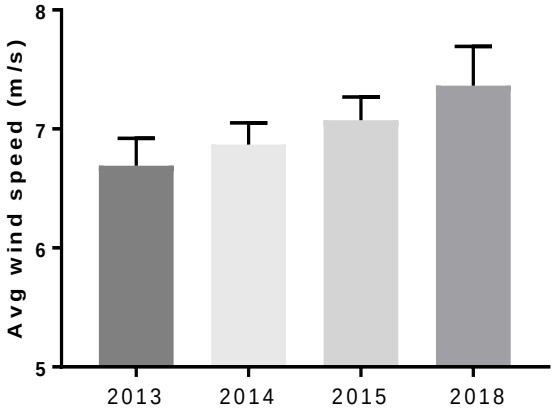

Figure 7: Average daily wind speed at Newport River wild oyster site by year. Error bars are standard error of the mean.

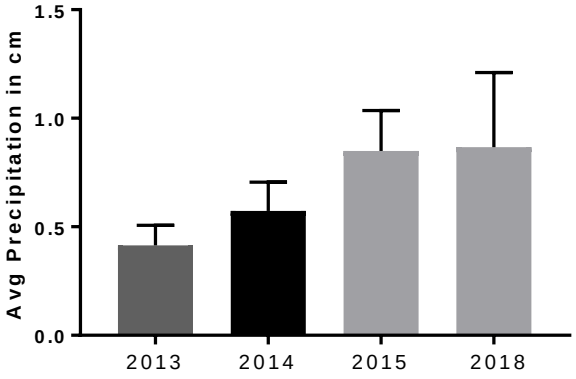

Figure 8: Average daily precipitation at Newport River wild oyster site by year. Error bars are standard error of the mean.

site were sampled on the same day at or within 3 hours of low tide, with oysters harvested typically within an hour of each other. Each site was sampled twice within the two-month period. For long-term sampling, 12 oysters were collected from each site, and divided into two bags of 6. For the short-term sample design, two sites were sampled comparing off-bottom farmed oysters and nearby wild oysters, while the third site was on-bottom farmed oysters and wild oysters, which served as a control. At each sampling day, 48 oysters were collected from the wild site and 48 oysters were collected from the farmed site. Each site was sampled on two separate occasions.

Long-term study of pathogenic Vibrio in oysters, farmed vs. wild

The data of the long-term results were merged and averaged by week of collection. Farmed and wild data were compared as yearly means. Total *Vibrio* was found to be nearly identical in farmed and wild oysters (Figure 9). Total *V. parahaemolyticus* (Figure 10) was found to be significantly greater in farmed

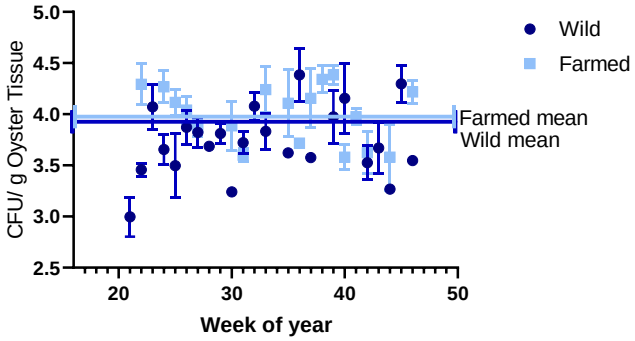

Figure 9: Total *Vibrio* in farmed (light blue) and wild (dark blue) oysters. Samples are averaged by week of year. Error bars are standard error.

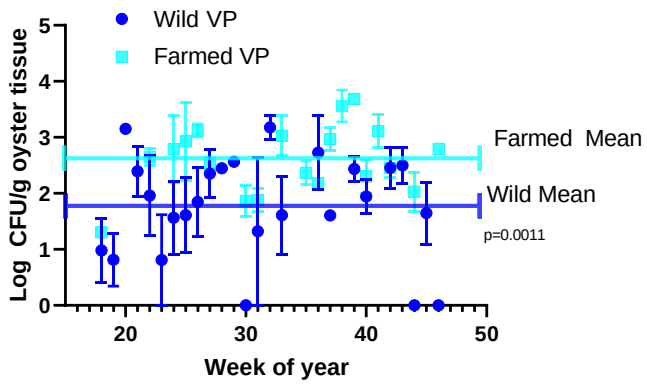

Figure 10: Total *V. parahaemolyticus* in farmed (light blue) and wild (dark blue) oysters. Samples are averaged by week of year. Error bars are standard error.

oysters than in wild oysters (p=0.0011). Pathogenic *V. parahaemolyticus* were found too infrequently to be compared in this study, but this finding is discussed in detail in a later section. Total *V. vulnificus* showed no significant difference between farmed and wild oysters in the long-term results (p=0.17, Figure 10). The percent and total pathogenic *V. vulnificus* were both significantly greater in farmed oysters than in wild (Figure 11, p=0.02 and Figure 12, p=0.004, respectively).

Short-term, in-depth comparison of surface vs. bottom oysters

Total *Vibrio* concentrations did not vary statistically from site to site (Table 2A) nor from sampling date to sampling date (Table 2B). There was no difference in concentration of total *Vibrio* between suspended and on-bottom oysters, nor at the control site with both farmed and wild oysters being grown on bottom (Figure 13). There was also no difference in total *V. parahaemolyticus* in farmed or wild oysters at both the experimental (off vs on bottom) and control (both on bottom) sites (Figure 14). Analysis of pathogenic *V. parahaemolyticus* was not performed due to too few samples containing these bacteria. More information about this finding is discussed later.

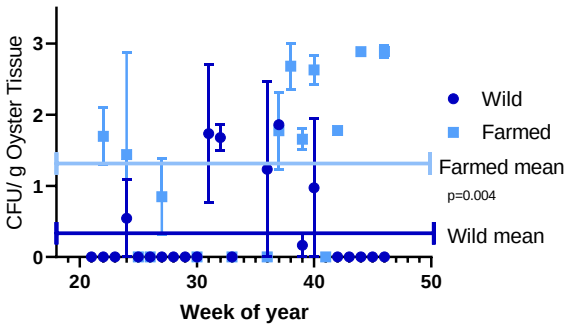

Figure 11: Concentration of pathogenic *V. vulnificus* in farmed (light blue) and wild (dark blue) oysters. Samples are averaged by week of year. Error bars are standard error.

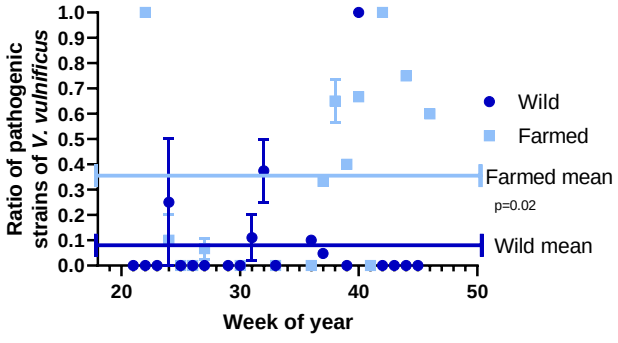

Figure 12: Percent of pathogenic V. vulnificus in farmed (light blue) and wild (dark blue) oysters. Samples are averaged by week of year. Error bars are standard error

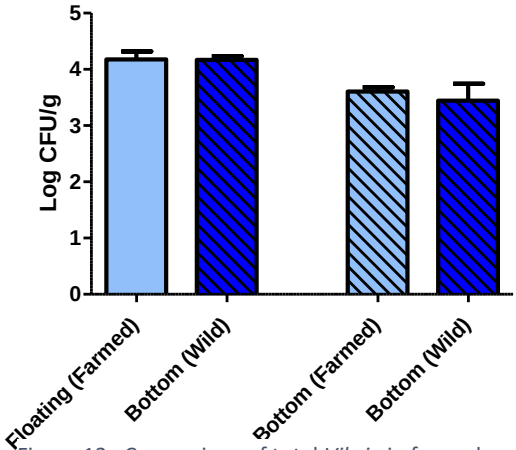

Figure 13. Comparison of total *Vibrio* in farmed and wild oysters. Error bars are standard error of the mean. There were no significant differences.

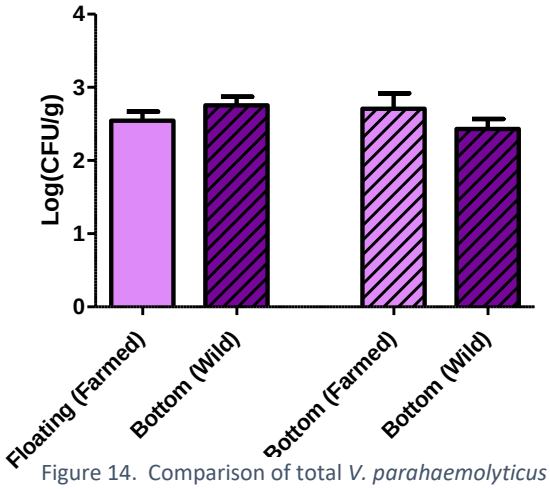

Figure 14. Comparison of total *V. parahaemolyticus* in farmed and wild oysters. Error bars are standard error of the mean. There were no significant differences.

A significant difference was seen in total *V. vulnificus* concentrations, shown in Figure 15, with off-bottom farmed oysters having fewer total *V. vulnificus* than wild oysters (p=0.334). This difference was not mirrored in the control site with both farmed and wild oysters being grown on-bottom (p=0.8379). *V. vulnificus* was found in 87.5% of samples in this study, with 91.7% of bottom grown and 81.3% of off-bottom oyster samples containing the bacteria. Ten samples were devoid of confirmed *V. vulnificus*, four from on-bottom oysters and six from suspended oysters. Half of the suspended oyster samples that were devoid of *V. vulnificus* came from sampling at JB-FS on 8/24/2018, meaning that three of the eight suspended oyster samples from that date did not have any confirmed *V. vulnificus*. The corresponding on-bottom site (JB-WB) had confirmed *V. vulnificus* in ten out of ten oyster samples for that date. Oyster samples taken from waters with salinities lower than 20 ppt all had confirmed *V. vulnificus*. Of the 266 confirmed *V. vulnificus* (*vvhA*-positive) isolates throughout the entire study, 44 contained the virulence correlated gene, *vcgC*, constituting 16.5% of the sample population. When analyzed according to growing approach, i.e. by suspended and on-bottom oysters, however, 20.1% of on-bottom oysters were *vcgC*-positive and only 10.3% of suspended oysters were potentially pathogenic.

Similar to confirmed *V. vulnificus,* potentially pathogenic *V. vulnificus* and *percent* potentially pathogenic *V. vulnificus* were also lower in suspended farmed oysters than on-bottom wild oysters at the experimental sites (p=0.0366 (Figure 16) and 0.0342 (not

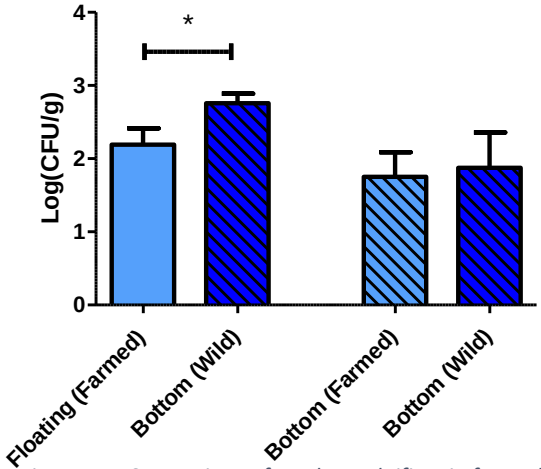

Figure 15. Comparison of total *V. vulnificus* in farmed and wild oysters. Error bars are standard error of the mean. Asterisk indicates significant difference in mean.

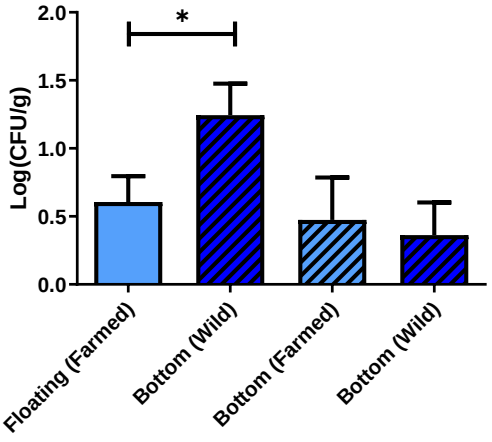

Figure 16. Comparison of pathogenic *V. vulnificus* in farmed and wild oysters. Error bars are standard error of the mean. Asterisk indicates significant difference in mean.

A.

Site	W/F	S/B	Average total *Vibrio* log(CFU/gram)
CIB-FS	F	S	4.3 ± 1.1
CIB-WB	W	B	4.2 ± 0.9
JB-FS	F	S	4.1 ± 0.9
JB-WB	W	B	4.2 ± 1.1
NR-FB	F	B	3.5 ± 1.3
NR-WB	W	B	3.1 ± 1.1

B.

Date of Harvest	Site	W/F	S/B	Average total *Vibrio* log(CFU/gram)
7/22/2018	CIB-FS	F	S	3.9 ± 1.2
	CIB-WB	W	B	4.2 ± 1.3
8/3/2018	JB-FS	F	S	4.1 ± 1.3
	JB-WB	W	B	4.3 ± 1.6
8/7/2018	NR-FB	F	B	3.5 ± 1.3
	NR-WB	W	B	3.1 ± 1.1
8/13/2018	CIB-FS	F	S	4.6 ± 1.6
	CIB-WB	W	B	4.2 ± 1.3
8/24/2018	JB-FS	F	S	4.2 ± 1.4
	JB-WB	W	B	4.0 ± 1.3
9/4/2018	NR-FB	F	B	3.7 ± 1.3
	NR-WB	W	B	3.7 ± 1.3

Table 2. Log total *Vibrio* concentrations in farmed and wild oysters. Total *Vibrio* concentrations were separated by site (A) and by date of harvest (B). Total *Vibrio* concentrations were obtained from culture-based analyses data. “F” indicates farmed oysters and “W” indicates wild oysters

shown), respectively). Again, this was not demonstrated at the control site (p=0.7832 (Figure 16) and 0.8924 (not shown), respectively). Potentially pathogenic *V. vulnificus* was found in 35.0% of the oyster samples in this study: 41.7% of on-bottom oysters contained *vcgC*-positive *V. vulnificus* and 25% of suspended oysters contained *vcgC*-positive *V. vulnificus*. Two samples contained 100% *vcgC*-positive *V. vulnificus*, both from the same sample site and day (NR-FB and NR-WB on 8/7/2018). Salinity was 23 ppt and daily air temperature was 28°C. aily air temperatures during this time period averaged at 27°C, with a range of 24°C-29°C. Throughout this study period, temperature and salinity exhibited very weak correlations with total *Vibrio* concentrations. Low salinities observed early in the study period (August 3, 2018) were due to heavy rainfall in July. In Carteret County, NC, rainfall total for the month of July was between 11.47 inches and 12.95 inches making it the wettest July on record (NOAA, NC Coastal Fed. 2018). July 24, 2018, alone, had 3.51 inches of rain. Heavy rainfall frequently results in shellfish harvest closures due to high concentrations of fecal indicator bacteria in harvest waters as a result of stormwater runoff (19). Shellfish harvesting closures were implemented sporadically between July 8-August 20 in and near the sampling area of this project due to rainfall (North Carolina Division of Marine Fisheries 2018). Although salinity did not correlate with total *Vibrio*, weak correlations were observed between total *Vibrio* and rainfall. Specifically, although total *Vibrio* in wild, on-bottom oysters correlated weakly with 24-hour rainfall (R^2=0.329, n=64, (figure not shown)), total *Vibrio* in suspended oysters had weak, negative

correlations with three-day and seven-day rainfall (R^2=-0.618, R^2=-0.439, respectively, n=32). Prevailing wind direction across coastal NC is along the SW-NE trajectory. Additionally, the shallow estuaries in North-Eastern NC are largely freshwater and wind dominated (tidal influence is dampened by presence of barrier islands) (20). During the study period, winds came predominantly from SW/SSW. Average wind speed was 9.7 mph. On each day that the wind direction was not SW/SSW, the wind speed was below average, except for a WSW wind on August 9 that was just above the average (10.0 mph). The maximum wind speed was 18.2 mph coming from the SW. Daily wind speeds negatively correlated with total *Vibrio* concentrations in surface oysters (R^2 = -0.617, n=32 (figure not shown)). There was no correlation between wind and total *Vibrio* in on-bottom oysters.

All isolates of confirmed *V. parahaemolyticus* collected from this and other studies in eastern North Carolina were tested molecularly to determine if they were potentially pathogenic. A total of 3036 oysters were examined for pathogenic *V. parahaemolyticus* from 2013 to 2018. This included samples from 5 wild sites, 6 farmed sites, and an additional 3 farm/wild combination sites. Only four samples yielded pathogenic *V. parahaemolyticus* (Figure 17).

Discussion

Oysters from a variety of farmed and wild sites were collected over two years and analyzed for human pathogenic *Vibrio* species, including *V. parahaemolyticus* and *V. vulnificus.* Initially, only a passive monitoring program was in place, in which samples were regularly collected throughout the year to determine the concentrations of these pathogens, and of the total *Vibrio* in oysters. Initially the data showed that only *V. parahaemolyticus* concentrations were significantly different between farmed and wild oysters. Interestingly, while the total *V. parahaemolyticus* were elevated in farmed oysters,

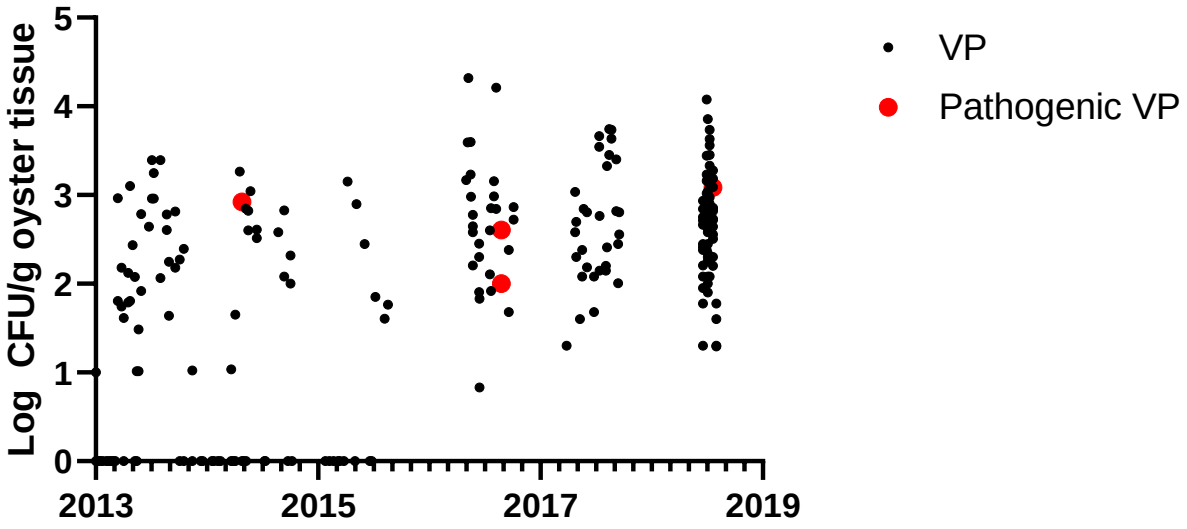

Figure 17: *Vibrio parahaemolyticus* concentrations (black circles) in oysters. Data was obtained from this and previous studies. Red circles indicate samples that were confirmed to contain pathogenic strains of *V. parahaemolyticus*.

pathogenic strains were exceedingly rare in both groups. The average number of *Vibrio* spp. and *V. vulnificus* were nearly identical in farmed and wild oysters. The observation that total *Vibrio* remained the same but *V. parahaemolyticus* was elevated in farmed oysters suggests that farming practices could have species specific effects. Even more striking, was that even though the number of *V. vulnificus* was not different between farmed and wild oysters, the number of pathogenic *V. vulnificus* was significantly different. Again, indicated that farming has targeted effects not only at the species level but at the strain level as well. Because this finding could have strong implications for the aquaculture industry, a second, highly-focused and statistically rigorous experiment was executed to further examine this phenomenon.

This second experiment controlled for confounding factors in the first experiment, including distal collection sites and uneven sampling dates and times between wild and farmed oysters. In this second experiment, three sites were chosen in which wild oysters were found in close proximity to farmed oysters. Additionally, the oysters were harvested together, within a short time frame. The proximity and simultaneous collection ensured that most environmental effects were controlled for. Additionally, a robust sampling scheme was performed, with each site being sampled twice, and 48 oysters being collected at each sampling date, from both the farmed and wild paired locations. The third site, which served as a control, contained farmed oysters that were grown on bottom, while at the other two sites oysters were grown in floating cages, off-bottom. This design allowed us to control for aquaculture methodology.

The intense short-term experiment had results that differed from the longer experiment that included greater seasonality. In this second short-term experiment, once again there was no observable difference in the number of total *Vibrio* in farmed or wild oysters, regardless of aquaculture practice. This appears to indicate that *Vibrio* will fill an oyster to maximum capacity, either by uptake or by replication. Yet, in this study it was wild oysters that contained significantly more *V. vulnificus* including pathogenic forms. This reinforced the finding that oysters growing on the surface vs. on-bottom can contain differing concentrations of specific *Vibrio* species, even when the total number of *Vibrio* is nearly identical. The distinction between farmed and off-bottom oysters is important, as the control site with farmed oysters grown on-bottom showed no differences with wild oysters. Thus, it is less likely that the handling and other aquaculture procedures that occur with farming are influencing the concentration of pathogenic *Vibrio,* but rather the use of floating cages that is the important factor. The differences between the long and short-term studies could possibly be explained by a few factors. The first is that the short-term experiment was performed at a time when temperatures remained fairly constant, thus having little effect on the number of *Vibrio.* The second, as mentioned previously, is that the short-term experiment controlled for several environmental factors by harvesting the wild and farmed oysters from nearby sites and at the same time. These confounding results could be clarified by a longer-term farm/wild hybrid experiment, specifically testing floating cages. Oysters placed in floating cages and in on-bottom cages could be tested simultaneously.

In September of 2018 Hurricane Florence made landfall in Eastern North Carolina and brought two-meter-high storm surges and 34 inches of rain to the area in less than a week (Pregizer, 2018). Continued testing of the wild oyster site in the Newport River after the passing of Hurricane Florence permitted the observation of pathogenic *Vibrio* before and after a major storm. While there was some interesting changes in the concentrations of *Vibrio,* by comparing the oysters from before and after the storm to non-storm years, it was concluded that the storm did not significant affect the pathogenic *Vibrio* concentrations.

Analysis of pathogenic *V. parahaemolyticus* in oysters was confounded by the fact that very few pathogenic strains were detected, during this study and in previous studies. But this is itself an interesting finding. It appears that the waters of Carteret County contain very few pathogenic *V. parahaemolyticus* strains, despite the concentrations of total *V. parahaemolyticus* being quite high. With concentrations reaching as much as $5x10^4$ per gram of oyster tissue. The rarity of these pathogenic strains has been observed as far back as 2013. With 3036 oysters being tested and only four samples containing detectable pathogens. This finding is potentially good news for the oyster industry in NC, as fewer of these bacteria in oysters could mean fewer infections from NC oysters.

Broader Impacts

Funding from ISSC was used in projects that resulted in the training of an undergraduate, a post-baccalaureate, and a Masters student. Results of the ISSC study were presented by the undergraduate as part of the Institute for the Environment Morehead City Field Site Semester. The undergraduate has entered in a science career, and the post-baccalaureate has entered into graduate school at the University of Maine, with a project focused on oysters and oyster aquaculture.

Data generated from the ISSC funded work was presented at the meeting *Aquaculture 2019*, in New Orleans, referenced below

A comparison of human pathogenic *Vibrio* in farmed and wild oysters (*Crassostrea Virginica*); Invited presentation to **Aquaculture 2019**, New Orleans, LA (2019)

Data generated as a result of ISSC funding was used in two publications, which are referenced below.

Brett Froelich and Dayle Daines; In hot water: effects of climate change on *Vibrio*-human interactions; **Environmental Microbiology**; March 2020; **https://doi.org/10.1111/1462-2920.14967**

R Canty, D Blackwood, R Noble and B Froelich; A comparison between farmed oysters using floating cages and oysters grown on-bottom reveals more potentially human pathogenic *Vibrio* in the on-bottom oysters; **Environmental Microbiology**; February 2020; **https://doi.org/10.1111/1462-2920.14948**

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