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Differences in Abundances of Total *Vibrio* spp., *V. vulnificus*, and *V. parahaemolyticus* in Clams and Oysters in North Carolina

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ABSTRACT Filter feeding shellfish can concentrate pathogenic bacteria, including *Vibrio vulnificus* and *Vibrio parahaemolyticus*, as much as 100-fold from the overlying water. These shellfish, especially clams and oysters, are often consumed raw, providing a route of entry for concentrated doses of pathogenic bacteria into the human body. The numbers of foodborne infections with these microbes are increasing, and a better understanding of the conditions that might trigger elevated concentrations of these bacteria in seafood is needed. In addition, if bacterial concentrations in water are correlated with those in shellfish, then sampling regimens could be simplified, as water samples can be more rapidly and easily obtained. After sampling of oysters and clams, either simultaneously or separately, for over 2 years, it was concluded that while *Vibrio* concentrations in oysters and water were related, this was not the case for levels in clams and water. When clams and oysters were collected simultaneously from the same site, the clams were found to have lower *Vibrio* levels than the oysters. Furthermore, the environmental parameters that were correlated with levels of *Vibrio* spp. in oysters and water were found to be quite different from those that were correlated with levels of *Vibrio* spp. in clams.

IMPORTANCE This study shows that clams are a potential source of infection in North Carolina, especially for *V. parahaemolyticus*. These findings also highlight the need for clam-specific environmental research to develop accurate *Vibrio* abundance models and to broaden the ecological understanding of clam-*Vibrio* interactions. This is especially relevant as foodborne *Vibrio* infections from clams are being reported.

KEYWORDS clams, ecology, food, oysters, shellfish, *Vibrio*

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An estimated 84,000 people contract foodborne *Vibrio* infections each year in the United States, resulting in 500 hospitalizations and 100 deaths (1, 2). Unlike most other major foodborne bacterial pathogens, the number of cases caused by *Vibrio* spp. is increasing and currently is the highest since national reporting began (2, 3). While at least 12 *Vibrio* spp. are potentially pathogenic to humans, the two foodborne *Vibrio* spp. that cause the most infections and the most deaths in the United States are *Vibrio parahaemolyticus* and *Vibrio vulnificus*, respectively (2, 4).

V. vulnificus is the single most fatal foodborne pathogen in the United States, and perhaps the world (4), accounting for 95% of all U.S. seafood-related deaths, with a fatality rate approaching 50% (5). Infections resulting from ingestion typically produce symptoms such as fever, chills, nausea, abdominal pain, hypotension, and the development of secondary lesions on the extremities (5, 6). *V. parahaemolyticus* infections are

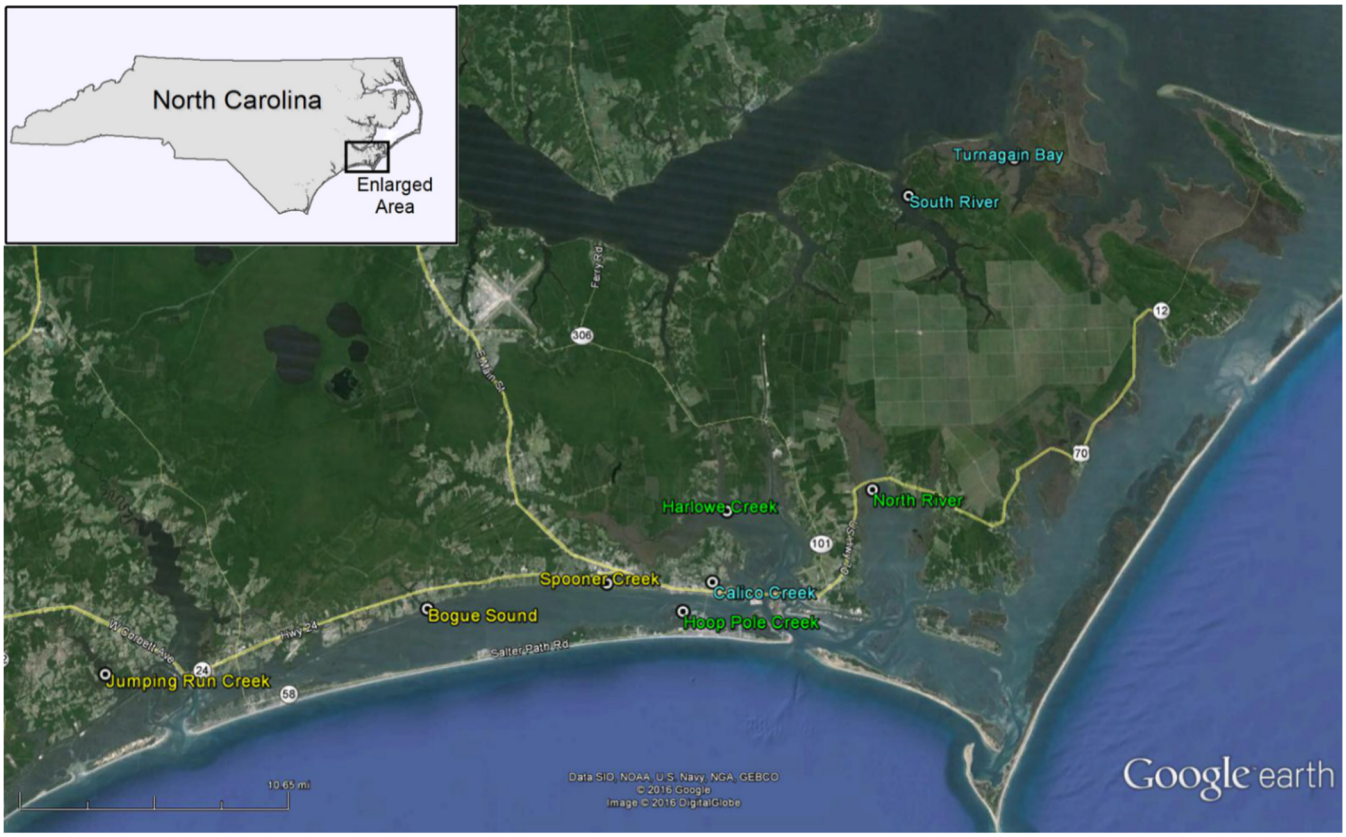

FIG 1 Locations of shellfish harvest sites. The colors of the site names indicate the type of shellfish harvested. Yellow, clams; blue, oysters; green, both clams and oysters. The main map is reprinted from Google Earth; the inset map is reprinted from reference 33.

far more common but are less severe and generally self-limiting. Infection with this species produces a variety of outcomes, with gastroenteritis representing approximately 60 to 80% of cases. Symptoms include diarrhea with cramping, nausea and vomiting, headache, chills, and low-grade fever (7, 8).

Both of these bacterial pathogens occur naturally in estuarine waters worldwide. Molluscan shellfish, such as oysters and clams, concentrate cells from the surrounding water, and efficient filter feeding ability can lead to levels of 10^5 CFU/g of tissue or more (9, 10). Because these concentrations are up to 100 times those of the water column, the consumption of raw or undercooked shellfish represents the primary route for contracting foodborne *Vibrio* infections (9, 11).

Although there are protective regulations in place in the United States to limit the risk of infection from these pathogens, the numbers of cases of vibriosis are still increasing (3, 12). Infections follow a seasonal trend, and the numbers peak in the months of late spring and early summer, corresponding to warm waters and increased abundance of vibrios (10, 13–16). However, temperature differences only partially explain the variations in concentrations seen in shellfish (10, 15, 17, 18). To reduce the number of infections, there needs to be a broader understanding of the environmental conditions that contribute to pathogenic *Vibrio* abundance in shellfish. Moreover, while a growing oyster-environment-*Vibrio* data set is being produced by the scientific community, there have been only a few investigations on the occurrence of these pathogens in clams. This is especially relevant because foodborne *Vibrio* infections from clams are being reported (19–21).

In this work, oysters and clams were collected from various sites in North Carolina (Fig. 1); several locations were habitats for both oysters and clams. It was hypothesized that, because both oysters and clams are filter feeders, the concentrations of *Vibrio* spp. within the shellfish would correlate with the concentrations in the water from which

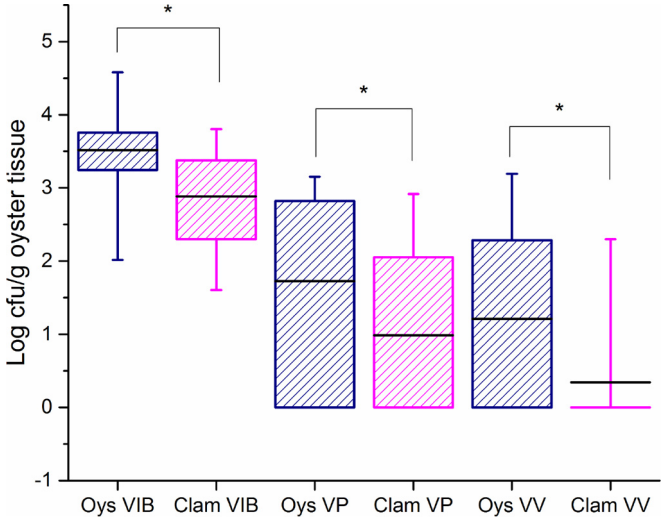

FIG 2 Box plots of levels of total *Vibrio* spp. (VIB), *V. parahaemolyticus* (VP), and *V. vulnificus* (VV) in oysters (Oys) and clams. Horizontal black lines, means; boxes, 25th to 75th percentile values; whiskers, minimum and maximum values. *, significant difference in means.

they were harvested. Additionally, it was hypothesized that, when oysters and clams were harvested from the same site, they would have correlating *Vibrio* abundances. Finally, it was hypothesized that the environmental factors that influenced *Vibrio* loads in oysters and clams would be similar. Thus, our investigations were designed to characterize the presence and levels of *V. vulnificus* and *V. parahaemolyticus* in water, oysters, and clams and to attempt to correlate these levels with several environmental parameters.

RESULTS

Comparing clams and oysters harvested from the same sites. The concentrations of *Vibrio* spp., *V. parahaemolyticus*, and *V. vulnificus* in clams were significantly lower than those in oysters when the shellfish were harvested simultaneously from the same site ($P = 0.0002$, $P = 0.0336$, and $P = 0.0093$, respectively, by paired *t* test). The combined results are shown in Fig. 2. Each paired sampling event was also examined individually. For *Vibrio* spp., all except three samples showed lower concentrations in clams than in oysters (see Fig. S1 in the supplemental material). For *V. parahaemolyticus*, generally two patterns were observed (Fig. S2). In pattern 1, oysters contained *V. parahaemolyticus* while levels in clams were nondetectable. In pattern 2, if both types of shellfish contained *V. parahaemolyticus* at the same sampling event, then the concentrations in the clams were usually slightly higher than those in the oysters. There was only one paired sampling event in which *V. parahaemolyticus* was present in clams but not oysters. All except three clam samples collected from the same sites as oysters had nondetectable concentrations of *V. vulnificus* (Fig. S3), and in only one sample set did the clams have more *V. vulnificus* than the oysters.

Harvest season and *Vibrio* risk. Oyster season in North Carolina is from 15 October through 31 March. Figure 3A and B show the concentrations of *V. vulnificus* and *V. parahaemolyticus*, respectively, in oysters harvested both in and out of oyster season (there is no set clam season in North Carolina). Figure 3C and D show the concentrations of *V. vulnificus* and *V. parahaemolyticus*, respectively, in clams. For both *V. vulnificus* and *V. parahaemolyticus*, the U.S. Food and Drug Administration (FDA) has determined that *Vibrio* concentrations of less than 30 CFU/g are sufficient for post-harvest-processed shellfish or for shellfish that are claimed to have nondetectable levels (22).

Environmental and biological factors correlated with *Vibrio* abundance. *Vibrio* levels in oysters were correlated with the *Vibrio* abundance in water; however, this was

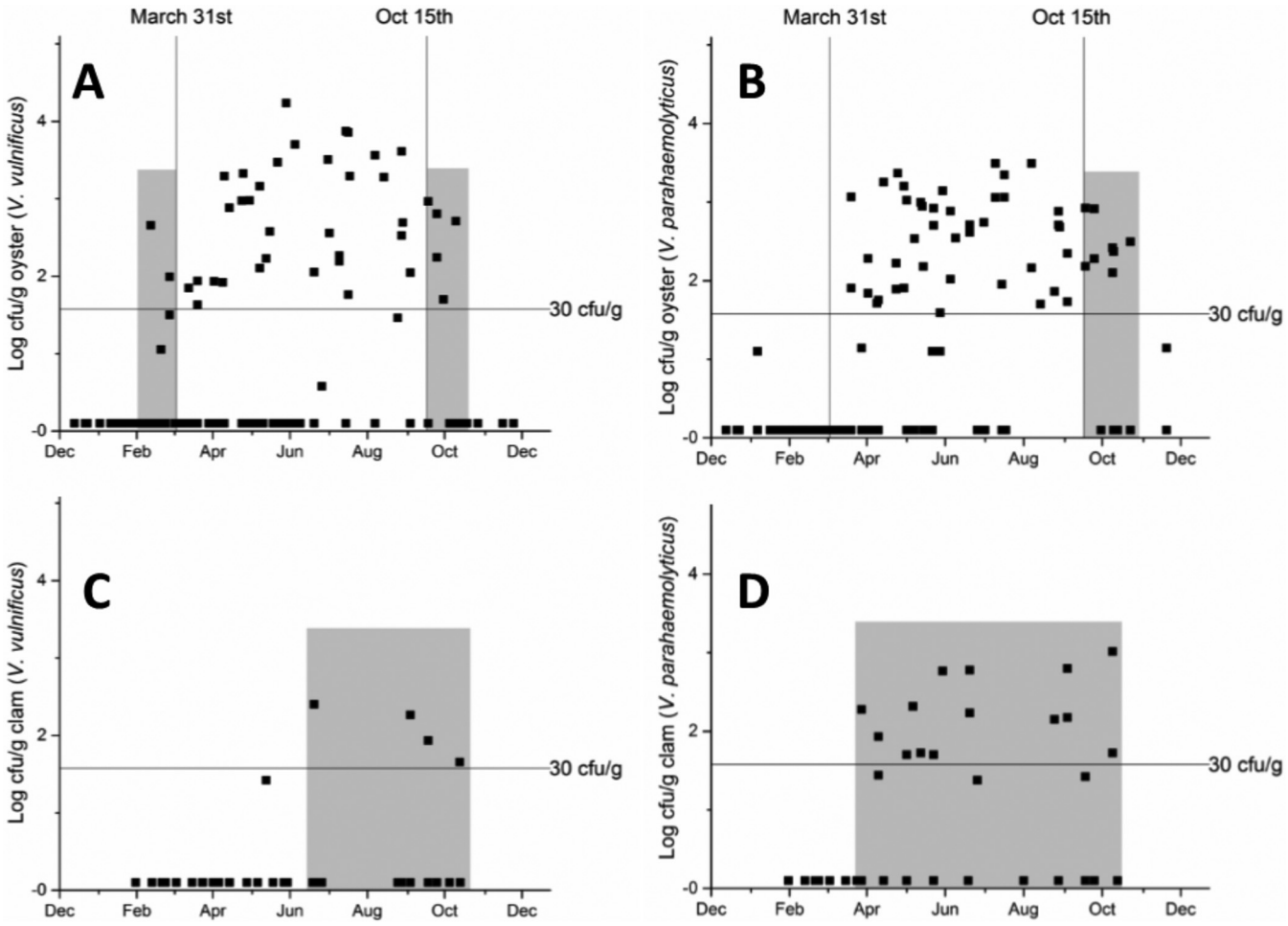

FIG 3 Concentrations of *V. vulnificus* in oysters (A) and clams (C) and concentrations of *V. parahaemolyticus* in oysters (B) and clams (D), according to the time of year of sample collection. Vertical lines in panels A and B, the dates of the opening (15 October) and closing (31 March) of the oyster season in North Carolina. Horizontal lines, concentration of bacteria that the FDA considers nondetectable in oysters. Gray boxes, times of the year when the shellfish are in season and there are *Vibrio* concentrations greater than the FDA nondetectable level. The actual limit of detection was log 1 CFU/g. Values below the limit of detection were assigned a value of log 0 CFU/g.

not true for *Vibrio* levels in clams (Fig. 4). Oyster *Vibrio* levels and water *Vibrio* levels shared all of the same environmental correlations with the exception of 3-day antecedent rainfall, with water temperature and total suspended solid (TSS) levels having some of the highest coefficients. This similarity in response to environmental conditions is demonstrated in Fig. 4 by the temperature-TSS level-oyster-water vertices clustering together. The concentrations of *Vibrio* in clams and oysters were significantly correlated. Water temperature was less strong in determining clam *Vibrio* levels than in determining oyster or water levels. Figure S4 shows that, while water temperature did appear to influence clam *Vibrio* levels, the levels had more variation than water or oyster levels. Nevertheless, heating degree days, which are a measure of cumulative cold days, had a tighter negative correlation with clam *Vibrio* levels than did the instantaneous water temperature (Fig. 4). Clam *Vibrio* levels were apparently affected by salinity, a factor not connected with oyster or water *Vibrio* levels in thus study. Further evidence of this difference is shown in Fig. S5 in the supplemental material, with increases in clam *Vibrio* levels being seen above 25% salinity.

For *V. vulnificus*, oyster and water levels again shared nearly all correlating factors, with water depth having a small but significant correlation with *V. vulnificus* levels in oysters (Fig. 5). The factors included temperature and TSS levels, similar to the *Vibrio* cluster. Salinity was negatively correlated with *V. vulnificus* concentrations in water and oysters but not clams. In both water and oysters, *V. vulnificus* levels increased up to

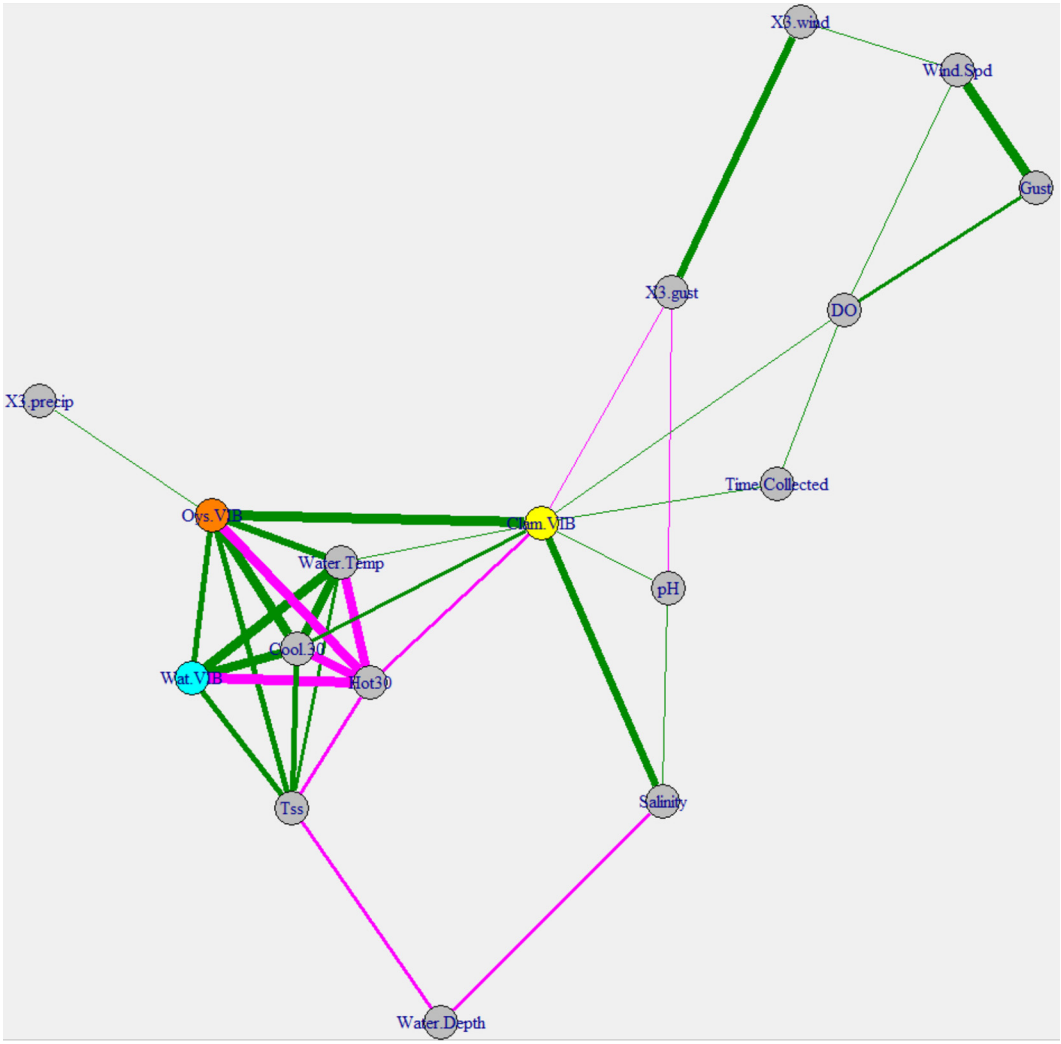

FIG 4 Correlation network map for total *Vibrio* spp. The network is based on Spearman's rank correlation coefficients. Vertices are connected by edges only if the correlation coefficient is greater than 0.25. Edges are colored based on positive (green) or negative (magenta) correlations. The thickness of the edges represents the correlation strength. Vertices were arranged using the Fruchterman-Reingold method, which clusters similar vertices together. Three-day antecedent precipitation values (X3.precip) were summed, while 3-day antecedent wind speed (X3.wind) and wind gust (X3.gust) values were averaged. The values for heating degree days (Hot 30) and cooling degree days (Cool 30) were obtained by summing the degree days 30 days before (and including) the collection date. Vertices representing the total *Vibrio* concentrations in clams (Clam.VIB), oysters (Oys.VIB), and water (Wat.VIB) are colored only for ease of viewing.

~17% salinity and then decreased above that, while clam concentrations changed very little along the salinity gradient (Fig. S6). Dissolved oxygen (DO) levels and pH were correlated with clam *V. vulnificus* abundance but not water or oyster levels (Fig. 5).

Once again, the clam *V. parahaemolyticus* vertex did not cluster with those for oysters and water (Fig. 6). As with *V. vulnificus*, temperature and TSS levels were the most critical correlating environmental factors for oyster and water levels. Water temperature and wind velocity were weakly correlated with *V. parahaemolyticus* levels in clams.

DISCUSSION

There are several approaches to reducing the number of human infections caused by the seafood-borne bacteria *V. vulnificus* and *V. parahaemolyticus*. One approach is the development of easy-to-use predictive models that can provide shellfish consumers or producers with early warnings when shellfish harvested from particular sites might contain dangerous concentrations of either bacterium. To develop such models, an

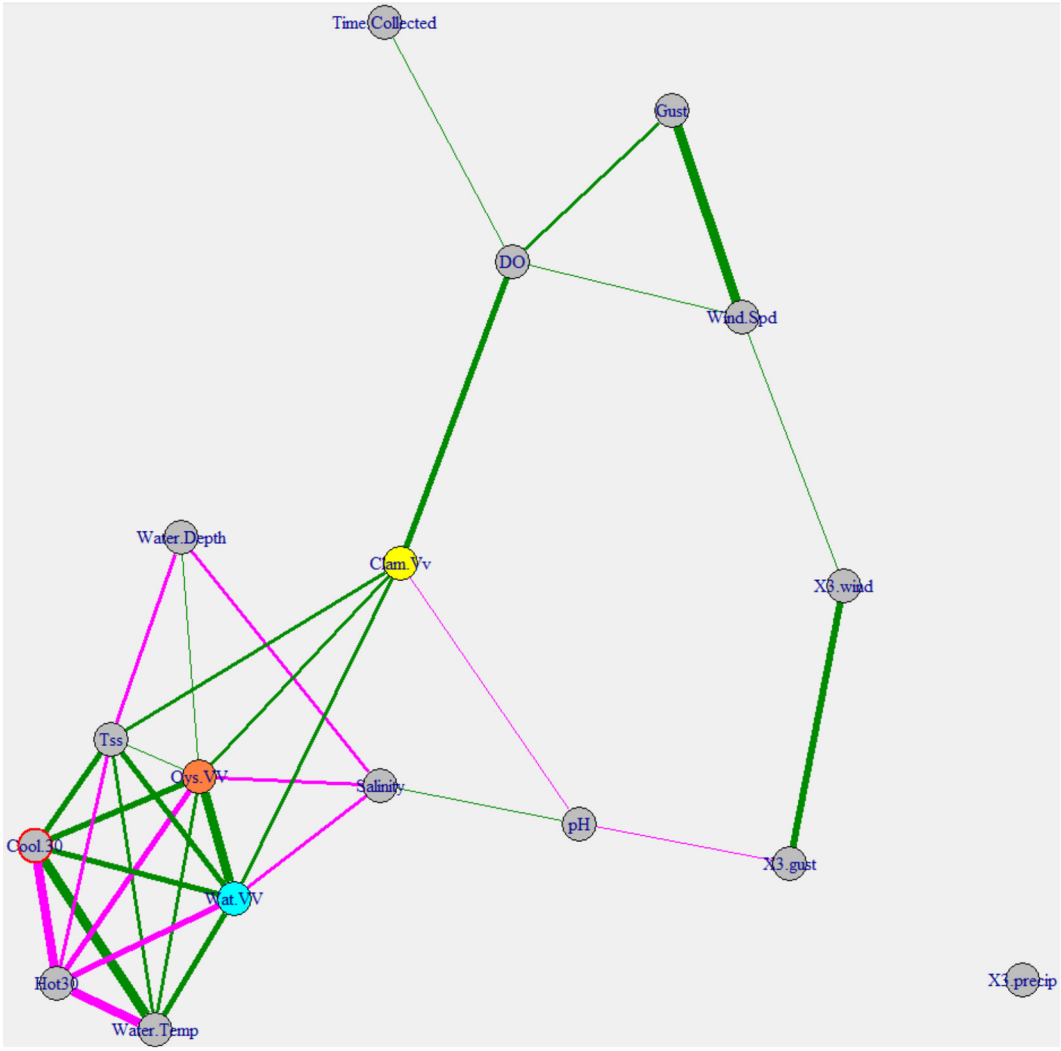

FIG 5 Correlation network map for *V. vulnificus*. The network is based on Spearman's rank correlation coefficients. Vertices are connected by edges only if the correlation coefficient is greater than 0.25. Edges are colored based on positive (green) or negative (magenta) correlations. The thickness of the edges represents the correlation strength. Vertices were arranged using the Fruchterman-Reingold method, which clusters similar vertices together. Three-day antecedent precipitation values (X3.precip) were summed, while 3-day antecedent wind speed (X3.wind) and wind gust (X3.gust) values were averaged. The values for heating degree days (Hot 30) and cooling degree days (Cool 30) were obtained by summing the degree days 30 days before (and including) the collection date. Vertices representing the *V. vulnificus* concentrations in clams (Clam.Vv), oysters (Oys.VV), and water (Wat.VV) are colored only for ease of viewing.

understanding of the biological and ecological parameters that influence bacterial abundance in shellfish is required. To that end, numerous recent studies have compared environmental conditions and *Vibrio* concentrations in oysters. A recent review of the topic presents data from these reports and highlights the site-to-site differences observed in these types of studies (12). Infections with these bacteria are most commonly acquired by eating raw oysters, but a significant number of infections are caused by consuming raw or undercooked clams (19–21). There are strikingly few reports on the environmental levels of these pathogens in hard clams (*Mercenaria mercenaria*), and such data are needed for the development of predictive models. Initially, it might be assumed that, because they are both filter feeding shellfish, clams and oysters would have correlated (if not similar) concentrations of *Vibrio* spp., especially when the shellfish are harvested from the same sites and growing areas. After collecting samples from three sites in eastern North Carolina where clams and oysters could be harvested simultaneously, we found that clams had significantly lower levels of *V. parahaemolyticus*, *V. vulnificus*, and *Vibrio* spp. than did oysters. A study by Jones

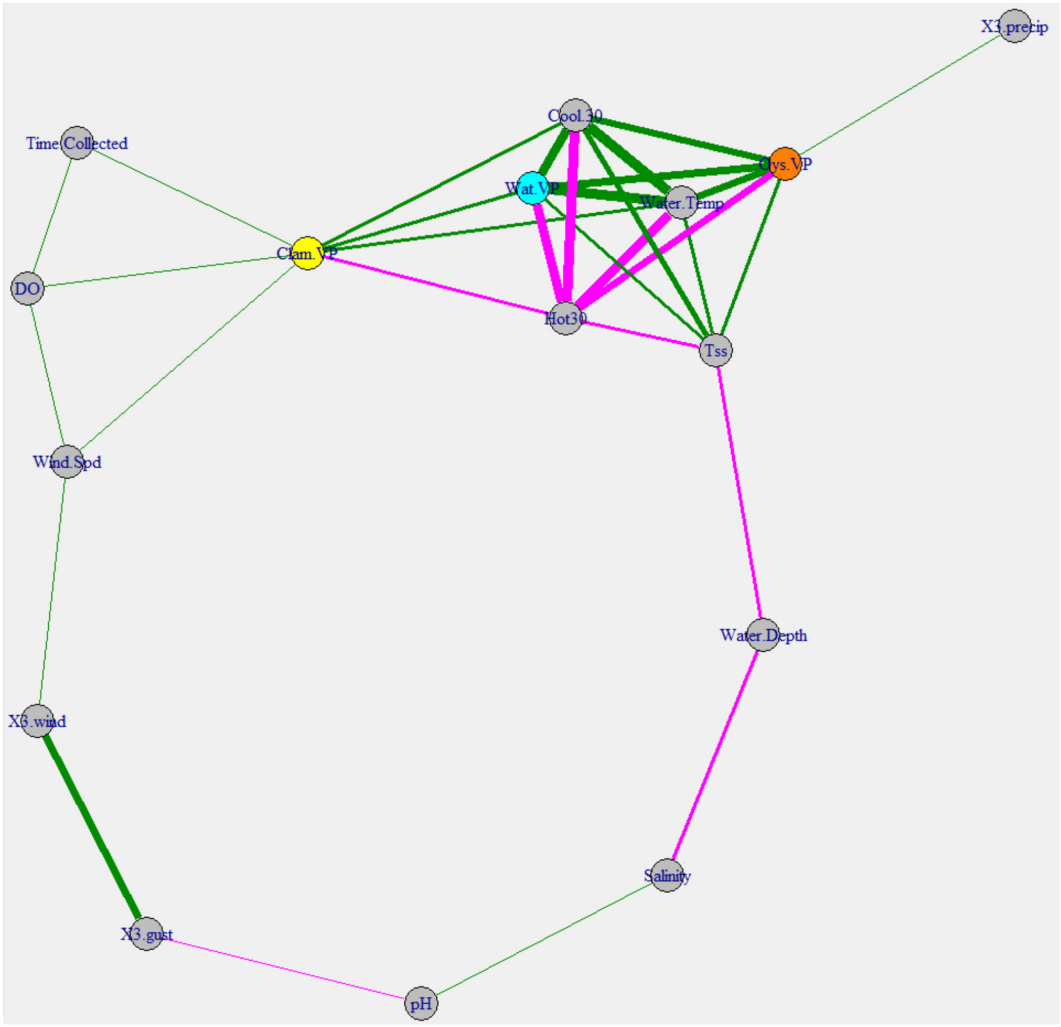

FIG 6 Correlation network map for *V. parahaemolyticus*. The network is based on Spearman's rank correlation coefficients. Vertices are connected by edges only if the correlation coefficient is greater than 0.25. Edges are colored based on positive (green) or negative (magenta) correlations. The thickness of the edges represents the correlation strength. Vertices were arranged using the Fruchterman-Reingold method, which clusters similar vertices together. Three-day antecedent precipitation values (X3.precip) were summed, while 3-day antecedent wind speed (X3.wind) and wind gust (X3.gust) values were averaged. The values for heating degree days (Hot 30) and cooling degree days (Cool 30) were obtained by summing the degree days 30 days before (and including) the collection date. Vertices representing the *V. parahaemolyticus* concentrations in clams (Clam.VP), oysters (Oys.VP), and water (Wat.VP) are colored only for ease of viewing.

et al. (23) that was conducted in Long Island Sound also found that the levels of these human pathogens, as well as *Vibrio cholerae*, were lower in clams than in oysters.

The finding of lower concentrations of these pathogens in clams is welcome news, suggesting that clams are less likely to result in human infections. The oyster season in North Carolina runs from 15 October to 31 March. The timing of the oyster season was established when, historically, oysters were eaten mostly in the winter months to avoid the spawning period during the summer months. The timing of the oyster season exists today because of the scarcity of oysters (S. Jenkins, North Carolina Division of Marine Fisheries, personal communication). Still, this timing likely has a strong effect in keeping the number of oyster-based *Vibrio* infections low. For *V. vulnificus*, the out-of-season period corresponds to the period when the most oysters with levels of >30 CFU/g are found in North Carolina. For *V. vulnificus* and *V. parahaemolyticus*, 30 CFU per gram of oyster is a value that the FDA considers to be below the limit of detection and therefore safe (22). It should be noted that concentrations above 30 CFU/g are not labeled unsafe but do carry increased risk of infection. In this study, only seven in-season oyster

samples, which were collected in February and October/November, had levels above this limit for *V. vulnificus*. *V. parahaemolyticus* concentrations above 30 CFU/g occurred in season only in October and November, again showing the effectiveness of the oyster season regulations for reducing potential infections. It is important to note that private cultured oyster harvests are permitted in the summer off season; these shellfish pose a potential infection risk, and research is under way to understand the risk differences for farmed versus wild shellfish.

While the concentrations of *Vibrio* spp. were found to be lower in clams, there is no clam season in North Carolina, as clams are more abundant than oysters. While there is no FDA limit for *Vibrio* in clams, if the same value of 30 CFU/g that is used as a nondetectable threshold in oysters is applied, then the potential for infection appears greater. Interestingly, there were only five clam samples in this study that contained detectable *V. vulnificus* and the level in one of those samples was below the threshold of 30 CFU/g. Samples with levels above the threshold were found between mid-June and mid-October. The small number of *V. vulnificus* isolates recovered from clams could be due in part to the relatively higher salinity (>20%) of clam growing sites, considering that *V. vulnificus* is sensitive to high salinity. When clam and oyster samples that were collected simultaneously from the same sites were examined, however, it was evident that oysters more often contained these pathogens. Thus, the higher salinity is certainly not the sole cause, and clam biology must play a role. Nevertheless, these data suggest that *V. vulnificus* infections arising from clam consumption will be rare in North Carolina. The *V. parahaemolyticus* data tell a different story; many of the clam samples collected between mid-March and mid-October had concentrations well above 30 CFU/g, and these represent a potential health risk.

Unsurprisingly, water temperature and temperature-related factors, such as heating and cooling days, had strong significant effects on *Vibrio* levels in oysters and water but less so for levels in clams. For both oysters and water, *Vibrio* levels increased as the temperature increased, although the increases slowed at ~22°C. *Vibrio* levels in clams did increase with the temperature but the data were highly varied, making the relationship less obvious. Interestingly, salinity was positively correlated with *Vibrio* levels in clams but not in water or oysters. This is striking, because a previous study conducted in a different body of water in North Carolina also showed a correlation between salinity and *Vibrio* levels in water (24). Furthermore, salinity is one of the variables most often correlated with *Vibrio* levels in water in other studies (25–30). The concentrations of *Vibrio* spp. in oysters remained mostly flat along the salinity gradient, with salt-tolerant *Vibrio* spp. and other bacteria likely occupying oyster matrices vacated by less-salt-tolerant species such as *V. vulnificus* (31, 32). In clams, *Vibrio* levels began increasing at ~25% salinity; currently, we have no explanation for this observation. The temperature factor of heating degree days had a closer relationship with clam *Vibrio* levels than did water temperature at the time of collection. Heating degree days is a measure of how long (in days) the air temperature is below a base temperature; cooling degree days is the opposite. These values can be quickly calculated and represent simple cumulative measurements of hot or cold days. The data in this study provide evidence that lengthy series of cold days are more important (negative correlation) in determining clam *Vibrio* levels than are daily water temperatures.

Like *Vibrio* findings, clam *V. vulnificus* findings clustered more distantly than findings for oysters and water in the Fruchterman-Reingold layout of the correlation network map, indicating that they were less related than the others. Water temperatures and TSS levels were positively correlated with oyster and water *V. vulnificus* levels, while salinity was negatively correlated. For both oysters and water, the levels of *V. vulnificus* appeared to increase to ~17% salinity and then began to decrease, as commonly reported (10, 31–34). Clam concentrations remained mostly stable as salinity increased. Again, the water temperature did not appear to have a significant effect on clam *V. vulnificus* concentrations in this study. Also, surprisingly, salinity was not related to clam bacterial concentrations. These conclusions must be considered carefully, however, as there were few clam samples with detectable *V. vulnificus* levels.

There were fewer *V. parahaemolyticus* isolates in clams than in oysters, but the clam samples with confirmed *V. parahaemolyticus* isolates vastly outnumbered the clam samples with confirmed *V. vulnificus* isolates. Again, temperatures were not as closely related to bacterial concentrations in clams as they were to those in water and oysters. TSS levels were related to oyster and water but not clam *V. parahaemolyticus* levels. Finally, wind-related factors were unique in being related to clam levels, perhaps suggesting that wind-driven mixing or resuspension plays a larger role in the uptake of *Vibrio* by clams than in that by oysters.

This study confirms and expands findings by Jones et al. (23) that clams and oysters harvested simultaneously from the same locations had different, and often uncorrelated, concentrations of *Vibrio* spp., including human pathogens. The concentrations of *Vibrio* spp., *V. vulnificus*, and *V. parahaemolyticus* in oysters and water were significantly correlated and had most of the same environmental determinates. Clam *Vibrio* concentrations, on the other hand, were either unrelated or only weakly related to water column *Vibrio* numbers. Furthermore, clam *Vibrio* concentrations always had influential environmental parameters that were unique (i.e., not shared by levels in oysters or water). Similarly, oyster and water levels had common correlates that clam levels did not have. These findings appear to indicate that oysters contain greater numbers of transient *Vibrio* organisms, which is why the levels are correlated with the *Vibrio* levels in the surrounding water. Clams, however, with weaker correlations with water *Vibrio* levels, might have a more stable *Vibrio* population. This stable population is further evidenced by the resistance to changes in daily water temperatures. With so many unique and unshared environmental variables contributing to oyster and clam bacterial concentrations, we hypothesize that clam biology plays a larger factor in internal *Vibrio* abundance than originally thought. More research is clearly needed, specifically regarding clam *Vibrio* uptake and depuration, for a better understanding of this interesting relationship.

MATERIALS AND METHODS

Sampling sites. Oysters (*Crassostrea virginica*), clams (*Mercenaria mercenaria*), and water samples were collected from nine sites along the eastern North Carolina coast (Fig. 1). At Calico Creek, Turnagain Bay, and South River, only oysters were harvested. Only clams were collected at Jumping Run Creek, Bogue Sound, and Spooner Creek. Oysters and clams were collected simultaneously from Hoop Pole Creek, Harlowe Creek, and North River. Water samples were collected from all sites at each sampling event. Sites were chosen to represent a range of salinity minima and maxima, salinity fluctuations, and suitability for growth for oysters and/or clams.

Shellfish collection and processing. Oyster samples were collected between 4 February 2013 and 11 November 2015, and clam samples were collected between 25 September 2013 and 16 October 2015; this resulted in 112 sampling events for oysters, 36 for clams, and 125 for water. At each sampling event, 5 oysters, 5 clams, or 5 oysters and 5 clams were collected, transported on ice to the laboratory, and processed within 5 h. Shellfish were shucked aseptically, and the hemolymph was drained. Meats were pooled, weighed, and diluted with sterile phosphate-buffered saline (PBS) at a 1:1 (wt/vol) ratio. Shellfish meats were blended for 10 min in a paddle blender (Fisher Scientific, Waltham, MA) at 280 rpm. Homogenates were diluted 1:10 in PBS, and 100-μl aliquots of both diluted and undiluted homogenates were plated on medium as described below.

Water collection and processing. At each site and sampling event, water was collected simultaneously with shellfish. Water was collected in sterile 1-liter bottles, which were rinsed three times with water immediately above the shellfish. As with shellfish, water samples were placed on ice in coolers that were transported to the laboratory. A Hanna HI96822 digital refractometer (Hanna Instruments, Carrollton, TX) was used to measure salinity. Water samples of 1 to 10 ml were passed through a mixed cellulose ester filter (0.45-μm pore size; Pall, Port Washington, NY) and placed on media as described below. To determine TSS levels, water samples were vacuum filtered through predried, preweighed, 25-mm, glass microfiber filters (GE Life Sciences, Pittsburgh, PA), using a minimum of 100 ml of water or continuing until the filter was visibly discolored. Filters were placed in a drying oven until the water was evaporated, and then they were reweighed.

Measurement of environmental parameters. Water temperature and shellfish depth were measured by hand at the time of each collection. DO levels were measured using an Orion 5 Star handheld probe (Thermo Scientific, Waltham, MA). Measurements of pH were made with a Denver Instrument UB-5 pH meter (Denver Instrument, Bohemia, NY). Data on wind velocity or gusts, precipitation, air temperature, and heating and cooling degree days were collected from individual weather stations near each sampling site. A list of the weather stations is included in Table S1 in the supplemental material. Heating and cooling degree days were determined relative to a base temperature of 18°C. Heating degree days for a particular day were calculated by determining the mean of the maximum and minimum temperatures for that day and subtracting that from the base temperature. Cooling days were calculated by

subtracting the base temperature from the mean of the daily temperature. Then, the calculated degree days for individual days were summed for a particular period of time (in this study, 30 days) to determine a cumulative degree day value, representing how far and for how long the daily temperature was above or below a base temperature.

Media and growth conditions. For total presumed *Vibrio* enumeration, thiosulfate-citrate-bile salts-sucrose (TCBS) agar, prepared according to the manufacturer's instructions (Becton, Dickinson, Franklin Lakes, NJ), was used. Green and yellow colonies were counted, and values were summed to determine total *Vibrio* abundance. CHROMagar *Vibrio* plates (CHROMagar, Paris, France), prepared as instructed, were used to isolate presumptive *V. vulnificus* (dark blue) and *V. parahaemolyticus* (dark purple) colonies. To grow pure cultures of each isolate, heart infusion (HI) agar plates were used (Becton, Dickinson). All media were incubated at 37°C for 24 h. After incubation, colonies on plates were counted and the data were converted to CFU per gram of shellfish or CFU per milliliter of water. This number was then multiplied by the fraction of isolates that were confirmed, using the molecular methods detailed below, to be either *V. parahaemolyticus* or *V. vulnificus*; This resulted in a presumptive value of confirmed bacterial abundance for each sample.

Molecular confirmation of isolates. Between 5 and 25 colonies (or all colonies, if fewer than 5 were present) were isolated from each type of sample (water or shellfish) from each sampling event. Isolates were grown overnight in HI broth as pure cultures and then were boiled for 10 min. Centrifugation at 10,000 $\times$ *g* for 10 min separated the aqueous DNA from cellular material. Supernatants, to be used as PCR templates, were stored at −20°C until they were examined. Using the primers described by Tarr et al. (35), *V. parahaemolyticus* was identified on the basis of the *flaE* gene. Confirmation of *V. vulnificus* utilized the *vvhA* gene, with the primers described by Warner and Oliver (36).

Statistical analyses. All data were combined, regardless of the harvest site, and Spearman correlations were determined for all factors. To simplify analysis, the correlation tables were converted into correlation matrices using the igraph package for R (37, 38). Only correlations with absolute correlation coefficients of at least 0.25 were linked with edges in the figures. Vertices were arranged using the Fruchterman-Reingold method, to ensure that related vertices were clustered together (39). The correlation coefficients and *P* values can be found in Tables S2 to S4.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.02265-16.

TEXT S1, PDF file, 0.8 MB.

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