# A comparison between farmed oysters using floating cages and oysters grown on-bottom reveals more potentially human pathogenic *Vibrio* in the on-bottom oysters

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## Summary

**Eating raw oysters can come with serious health risks, as oysters can potentially contain bacteria of the *Vibrio* genus that cause food-borne infections. *Vibrio* bacteria are concentrated by oysters and, when consumed, infections can result with severe symptoms such as diarrhoea, lesions on the extremities, or even death. *Vibrio* spp. concentrations are strongly affected by season, location, and other factors such as temperature and salinity. Previous research in North Carolina oysters has been conducted on wild and farmed oysters but not at the same time. Farmed, or aquaculture raised, oysters are considerably different from wild oysters and could possibly pose different health risks. Farmed oysters are handled, raised from seed, and often grown using suspended grow-out systems called 'floating cages'. Therefore, farmed oysters can be grown at the surface of the estuary, while wild oysters typically grow at the bottom of the water column. This project compared the concentrations of *Vibrio spp.* in suspended, farm-grown oysters and wild oysters at three sites, using a paired approach with farmed and wild oysters sampled in proximity. An important part of this comparison was identifying pathogenicity of the bacteria isolated from the samples. Distinction was made between off- and on-bottom farming. Interestingly, on-bottom oysters had more pathogenic *V. vulnificus* than off-bottom oysters.**

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## Introduction

There is a $14B+ United States (US) seafood trade deficit (NOAA Fisheries, 2020). To close this gap, a nationwide expansion of shellfish aquaculture has occurred, with the goals of expanding commercial oyster markets and streamlining the permitting process for growers in most coastal states. For example, in 2018, North Carolina prepared a Shellfish Mariculture Plan that provides a roadmap for increasing shellfish aquaculture production in NC by an order of magnitude by 2030. This type of expansion is mirrored by many coastal states, with increases in available shellfish leasing areas, marketing of boutique shellfish products, and improvements in food safety regulations for shellfish production. During this period of rapid mariculture expansion, the health of the shellfish consumer and the reputation of growers must be protected by minimizing the health risks that come from consuming raw or undercooked oysters.

Eating raw oysters comes with serious risks as many oysters contain naturally occurring harmful bacteria, including strains of *Vibrio vulnificus* and *Vibrio parahaemolyticus* that can cause severe illness, or even death, when consumed. Commercially, oysters can be harvested in two ways; wild-caught or grown as part of aquaculture or farming activities. Many states have regulations that limit the size, quantity, area, season, and times of collection for wild oysters in order to promote native shellfish reefs, but these restrictions are not in place for farmed oysters. Most of the expansion in shellfish commercial harvest will be achieved by shifts in oyster farming practices, such as the use of suspended farming system, usually referred to as 'floating cages' for oyster grow-out.

Both *V. vulnificus* and *V. parahaemolyticus* occur naturally in estuarine waters worldwide. The vector to humans is primarily raw/undercooked oysters, which account for 93% of ingestion cases (Oliver, 2013). *Vibrio vulnificus* is the single most fatal foodborne pathogen in the United States and possibly in the world (Baker-Austin and Oliver, 2018). It accounts for 95% of all U.S. seafood-related deaths and has a fatality rate of

ca. 50%, greatly exceeding that of other foodborne pathogens such as *Salmonella* (0.6%), *Escherichia coli* (3%–5%), and *Clostridium botulinum* (<8%), although the number of cases are lower (Mead *et al*., 1999; Jones and Oliver, 2009). Symptoms of a *V. vulnificus* infection include fever, chills, nausea, hypotension, and secondary lesions, which develop on the extremities. The incubation period is very short, and death can occur in 24–72 h after eating a single oyster (Jones and Oliver, 2009). *Vibrio parahaemolyticus* infections, unlike those caused by *V. vulnificus*, are usually not fatal but instead come with symptoms that include diarrhoea with abdominal cramps, nausea, vomiting, headache, chills, and low-grade fever (Yeung and Boor, 2004). While foodborne *Vibrio* infections are sometimes self-limiting and typically last 3 days, fatal cases of septicemia may occur in immunocompromised patients or those with pre-existing medical conditions. In the United States, an estimated 84 000 people contract foodborne *Vibrio* infections each year (Scallan *et al*., 2011). Unfortunately, in contrast to other leading foodborne bacteria, numbers of *Vibrio* cases are increasing (Centers for Disease Control and Prevention, 2007).

There is a critical need for information about pathogenic *Vibrio* in oysters, particularly studies on various farming practices. Farmed oysters have generally not been wellstudied in the context of growing practices and *Vibrio* abundance. Furthermore, there are no studies in NC comparing *Vibrio* concentrations in farmed and wild oysters. When compared to wild oysters, farmed oysters are often grown in floating cages, which mean that these oysters experience vastly different conditions than wild oysters, which grow along the benthos of the estuary. Some of these differences in growth conditions include air exposure, UV irradiation, temperature, agitation, water column height, and/or handling. Because these oysters experience such disparate conditions, it was hypothesized that there could be a difference in the concentration or type of human *Vibrio* pathogens found in suspended farmed versus on-bottom wild oysters, even when these oysters are harvested from matching estuarine locations under with very similar environmental conditions. The objectives of this project were to examine and compare suspended, farm-grown off-bottom oysters and wild on-bottom oysters at three sites, with farmed and wild oysters collected at paired sites in close proximity to one another. Over the course of the study, total *Vibrio*, *V. vulnificus* and *V. parahaemolyticus* concentrations were determined in composite oyster samples using both culture-based methods and molecular confirmation of pathogenicity. Attention was paid to ensure that samples were collected from paired wild and farmed oyster sites under similar environmental conditions, with sampling conducted within 2 h. Molecular confirmation methods were developed over the course of the study to rapidly type isolates selected from culture-based analyses.

## Experimental procedures

### *Oyster collection*

Both wild and farmed oysters (*Crassostrea virginica*) were collected by hand from Cedar Island Bay, Jarrett Bay, and the Newport River Estuary (Fig. 1). Oyster sampling occurred between late July 2018 and September 2018. Each site contained a wild location and a farm location, and they were within no more than 1000 m distance and within 3‰ salinity difference, except during a single extreme rainfall condition (Fig. 1 and Supporting Information Table S1). Cedar Island Bay was sampled on 22 July and 13 August, Jarret Bay was sampled on 3 August and 24 August, and the Newport River Estuary was sampled on 7 August and 4 September. The farm location and its corresponding wild location from each site were sampled on the same day at or within 3 h of low tide, with oysters harvested typically within an hour of each other. Cages were removed from water to retrieve samples. Each site was visited twice within the 2-month period, with 16 samples occurring at each visit (eight from farm and eight from wild). All oysters were transported to the laboratory on ice and processed within 5 h.

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 (Fig. 2). On each sampling date, 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.

### *Oyster processing*

Shellfish were rinsed with cold water, shucked, aseptically, and the hemolymph drained. Any internal sediment was rinsed with phosphate buffered saline (PBS). Meats of six oysters were pooled, weighed, and diluted with PBS at a 1:1 (weight/volume) ratio. Eight samples of six oysters each were collected from each site (farmed and wild) on each sampling day. 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 media as described in the next section.

### *Colony growth and isolation*

For all samples, 100 μl of both undiluted and diluted homogenate were spread plated on thiosulfate-citrate-bile salts-sucrose (TCBS) and CHROMagar *Vibrio* (CAV)

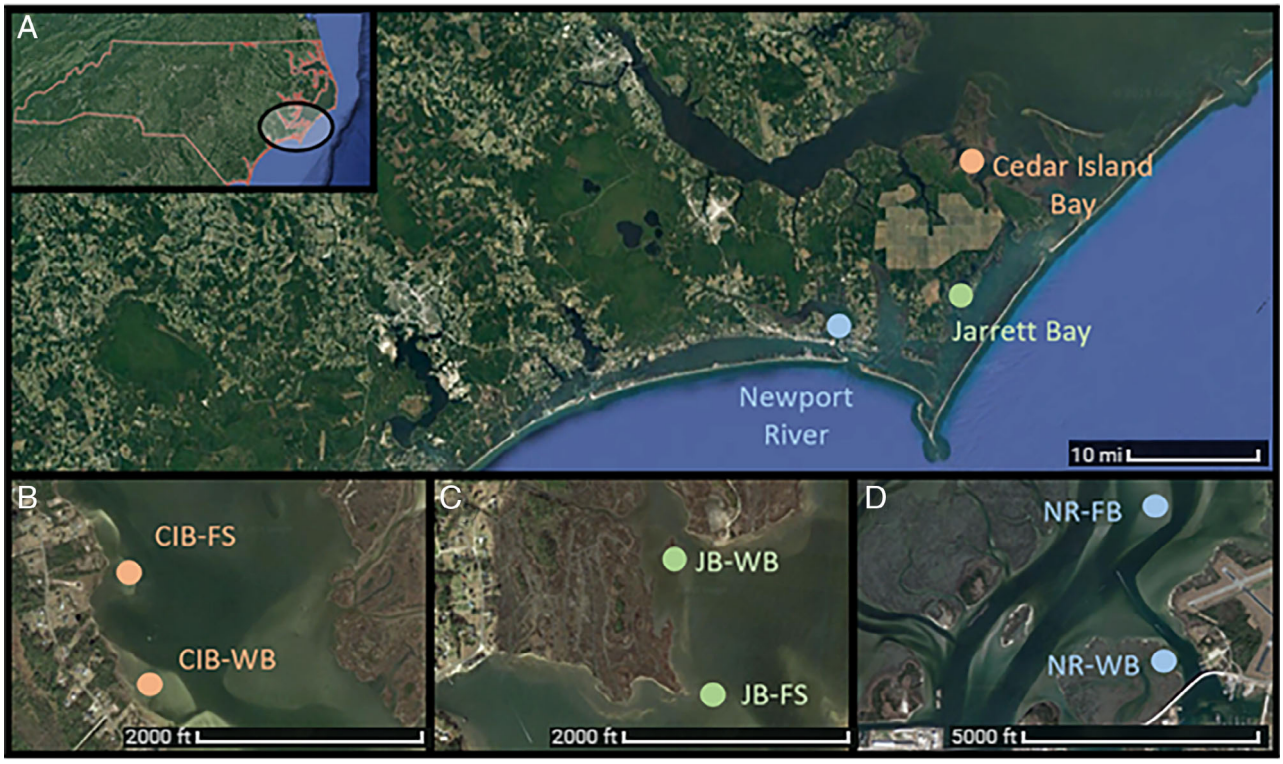


**Fig. 1.** A. Study area in eastern NC. Sampling sites included Cedar Island Bay (CIB), Jarrett Bay (JB), and Newport River (NR).
B and C. Farmed/surface (FS) and wild/bottom sites (WB).
D. Farmed/bottom site (FB) and wild/bottom site (WB).

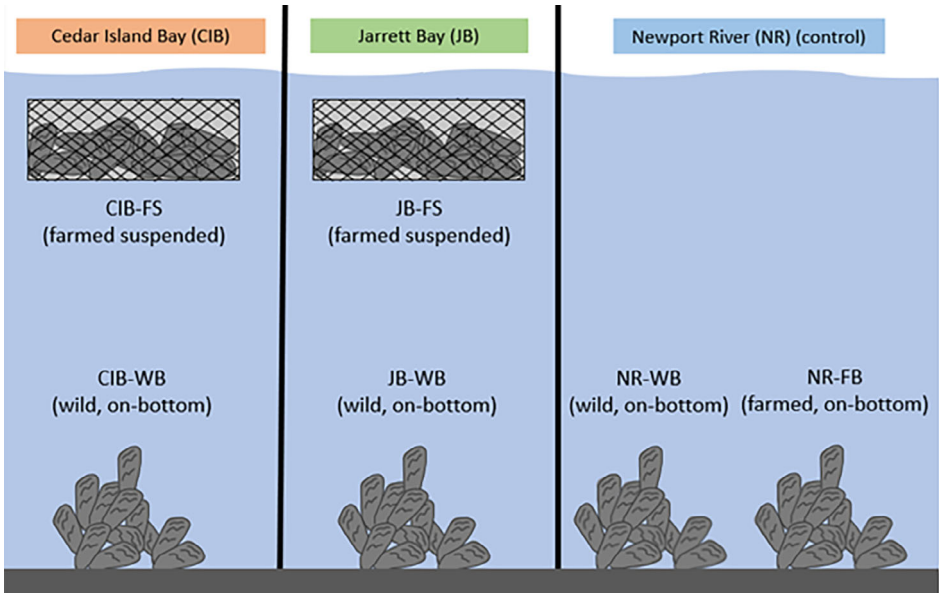


**Fig. 2.** Sample design of short-term experiment. The study had three sites, with the Newport River site acting as a control (with on-bottom farmed oysters).

(CHROMagar, Paris, France). TCBS plates, prepared as instructed (Himedia), were used to enumerate total *Vibrio*. Green and yellow colonies were counted, as described by Froelich *et al*. (2015), and values were summed to determine total *Vibrio* abundance per unit shellfish tissue mass. CAV plates, prepared as instructed, were used to isolate presumptive colonies of *V. parahaemolyticus* (purple) and *V. vulnificus* (blue). All plates 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 oyster.

From each plate 10 colonies each (or as many as possible if less than 10 were present) of presumed *V. vulnificus* and *V. parahaemolyticus* were isolated using sterile approaches and placed into 100 μl of heart-infusion (HI) broth and incubated at room temperature overnight. Following this, cells were lysed to release DNA by incubation at 100°C for 10 min. Centrifugation at 10 000× *g* for 10 min separated the aqueous DNA from cellular material. Supernatants, to be used as PCR templates, were diluted in nuclease-free water and the undiluted, 10-1, and 10-2 isolate subaliquots were stored at −80°C until they were prepared for PCR amplification as described below.

### *Molecular confirmation of isolates and determination of potential pathogenicity*

Molecular species identification of both *V. vulnificus* (*vvhA*) and *V. parahaemolyticus* (*toxR*), and subsequent potential for 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.

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).

## Results

Total *Vibrio* concentrations between farmed and wild oysters did not vary statistically from site to site (ANOVA, $P > 0.05$, Table 2,A) nor from sampling date to sampling date (ANOVA, $P > 0.05$, Table 2,B). There was no difference in concentrations of total *Vibrio* between suspended and on-bottom oysters (*t*-test, $P = 1.00$), nor at the control site with both farmed and wild oysters being grown on bottom (t-test, $P = 0.52$, Fig. 3). There was also no difference in total *V. parahaemolyticus* concentrations between farmed or wild oysters at both the experimental (off vs on bottom) and control (both on bottom) sites (two-way ANOVA, $P > 0.05$, Fig. 4). Analysis of pathogenic *V. parahaemolyticus* was not performed due to too few samples containing these bacteria.

A significant difference was observed in total *V. vulnificus* concentrations, shown in Fig. 5, with off-bottom farmed oysters having fewer total *V. vulnificus* than wild oysters (t-test, $P = 0.0334$). This difference was not mirrored in the control site with both on-bottom farmed and on-bottom wild oysters (*t*-test, $P = 0.8379$). *Vibrio vulnificus* was found in 87.5% of samples in this study, with 91.7% of bottom grown and 81.3% of off-bottom farmed oyster samples containing the bacteria. Ten samples were devoid of confirmed *V. vulnificus*, four from on-bottom oysters and six from off-bottom oysters. Half of the off-bottom oyster samples that were devoid of *V. vulnificus* came from sampling at the Jarret Bay farmed site on 24 August 2018, meaning that three of the eight off-bottom oyster samples from that date did not have any confirmed *V. vulnificus*. The corresponding on-bottom site at Jarret Bay had confirmed *V. vulnificus* in 10 out of 10 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 analysed according to growing approach, i.e. by off-bottom and on-bottom oysters, however, 20.1% of on-bottom oysters were *vcgC*-positive and only 10.3% of off-bottom oysters were potentially pathogenic.

Like confirmed *V. vulnificus*, potentially pathogenic *V. vulnificus* and *percent* potentially pathogenic *V. vulnificus* were also lower in off-bottom farmed oysters than on-bottom wild oysters at the experimental sites

**Table 1.** Primer sequences.

| Gene target | Direction | Sequence (5′-3′) | Amplicon size (bp) | Source |
|---|---|---|---|---|
| *vvhA* | F | TTCCAACTTCAAACCGAACTATGAC | 205 | Panicker and Bej (Panicker and Bej, 2005) |
| | R | ATTCCAGTCGATGCGAATACGTTG | | |
| *vcgC* | F | AAAACTCATTGARCAGTAACGAAA | 146 | Warner and Oliver, (Warner and Oliver, 2008) |
| | R | AGCTGGATCTAAKCCCAATGC | | |
| *toxR* | F | GTCTTCTGACGCAATCGTTG | 368 | Kim *et al*. (Kim *et al*., 1999) |
| | R | ATACGAGTGGTTGCTGTCATG | | |
| *tdh* | F | GTAAAGGTCTCTGACTTTTGGAC | 269 | Bej *et al*. (Bej *et al*., 1999) |
| | R | TGGAATAGAACCTTCATCTTCACC | | |
| *trh* | F | TTGGCTTCGATATTTTCAGTATCT | 500 | Bej *et al*. (Bej *et al*., 1999) |
| | R | CATAACAAACATATGCCCATTTCCG | | |

**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.

| Site (A) | W/F | S/B | Average total *Vibrio* log(CFU/g) |
|---|---|---|---|
| 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 |

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

CIB, Cedar Island Bay; JB, Jarret Bay; NR, Newport River; FS, Farmed-Surface; FB, Farmed-Bottom; WB, Wild-Bottom; 'F' indicates farmed oysters and 'W' indicates wild oysters.

[*t*-test, $P = 0.0366$ (Fig. 6) and *t*-test, $P = 0.0342$ (not shown) respectively]. Again, this was not demonstrated at the control site [*t*-test, $P = 0.7832$ (Fig. 6) and *t*-test, $P = 0.8924$ (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 off-bottom oysters contained *vcgC*-positive *V. vulnificus*. Two samples contained 100% *vcgC*-positive *V. vulnificus*, both from the same sample site and day (Newport River-Farmed bottom and Newport River-Wild bottom on 8 July 2018). The salinity was 23 ppt and daily air temperature was 28°C.

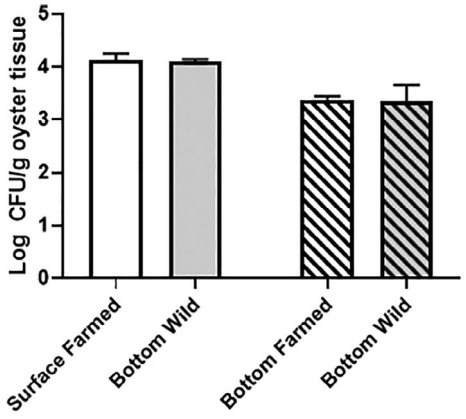


**Fig. 3.** Comparison of total *Vibrio* in farmed and wild oysters. Error bars are standard error of the mean. There was no significant different between farmed and wild oysters by group ($P > 0.05$).

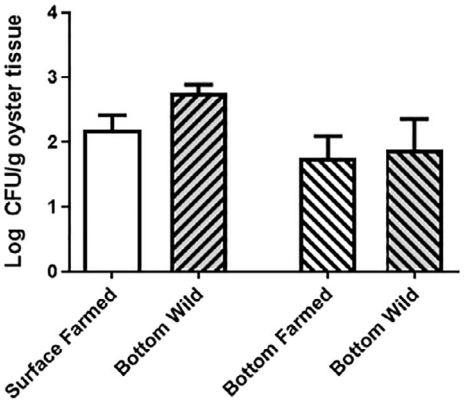


**Fig. 4.** Comparison of total *V. parahaemolyticus* in farmed and wild oysters. Error bars are standard error of the mean. There were no significant differences ($P > 0.05$).

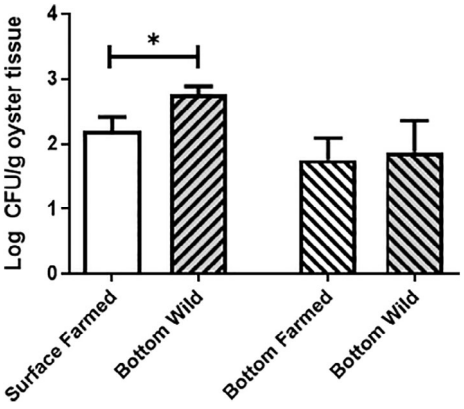


**Fig. 5.** Comparison of total *V. vulnificus* in farmed and wild oysters. Error bars are standard error of the mean. Asterisk indicates significant difference in mean ($P = 0.0334$).

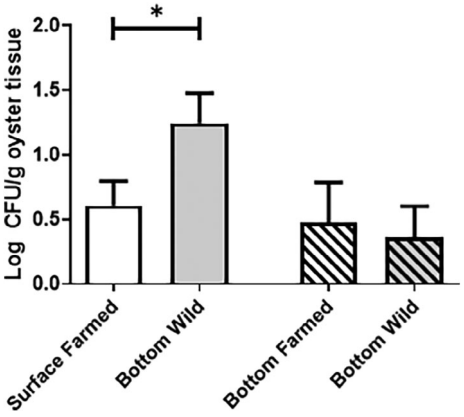


**Fig. 6.** Comparison of pathogenic *V. vulnificus* in farmed and wild oysters. Error bars are standard error of the mean. Asterisk indicates significant difference in mean ($P = 0.0366$).

Daily 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.

July 2018 brought heavy rainfall. In Carteret County, NC, rainfall total for the month of July was ca 12 in., making it the wettest July on record; 24 July 2018, alone had 3.51 in. of rain (Supporting Information Table S2). Rainfall had differing effects on off- and on-bottom oysters. Specifically, 24-h rainfall correlated positively with on-bottom oyster total *Vibrio* populations ($r^2 = 0.329$). About 3- and 7-day antecedent rainfall, however, has a negative impact on total *Vibrio* in oysters ($r^2 = -0.618$ and $-0.439$ respectively). Daily wind speeds negatively correlated with total *Vibrio*, *V. parahaemolyticus*, and *V. vulnificus* in surface oysters ($r^2 = -0.617$, $-0.649$ and $-0.625$ respectively). There was no significant correlation with wind and *Vibrio* populations in on-bottom oysters.

## Discussion

Oysters from farmed and wild sites were collected and analysed for human pathogenic *Vibrio* species, including *V. parahaemolyticus* and *V. vulnificus*. This experiment controlled for confounding factors by using three sites that were chosen because 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 on two separate dates. Each sampling day consisted of sampling both the off and on-bottom locations with eight individual samples of six oysters each. This resulted in 96 oysters being collected at each sampling date, 48 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 for control of aquaculture methodology.

There was no observable difference in the number of total *Vibrio* in farmed or wild oysters, regardless of aquaculture practice (Table 2 and Fig. 3). This appears to indicate that *Vibrio* will, either by uptake or by replication, reach some maximum carrying capacity in an oyster. Yet, wild oysters contained significantly more *V. vulnificus*, including pathogenic forms (Figs. 5 and 6). This suggests that oysters growing on the surface versus on-bottom can contain differing concentrations of specific *Vibrio* species, even when the total number of *Vibrio* is nearly identical. Because each species has its own separate conditions in which it will survive or thrive, this is not unexpected, and a similar phenomenon has been seen in eastern NC waters previously (Froelich *et al*., 2019). 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 (Figs. 3–6). 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.

*Vibrio parahaemolyticus* was confirmed in nearly all oyster samples (97.9% on-bottom and 100% of off-bottom oysters). Variances between off and on-bottom *V. parahaemolyticus* concentrations were not observed in this study, which is in contrast to a study by Cole *et al*. (2015) that, like this study, focused on the effects of off-bottom farming on *Vibrio* populations in a shallow, estuarine location. Cole *et al*. (2015) found higher total *V. parahaemolyticus* concentrations in off-bottom oysters. Unlike this study, the Cole *et al*. (2015) study was conducted in the Gulf Coast, over a longer time-scale (1 year) and deployed their own oysters for on-bottom oysters (in cages), indicating that they did not use wild oysters for their on-bottom studies. Our study was in agreement with Cole *et al*. (2015) on *V. vulnificus* dynamics, which indicated a higher trend in on-bottom populations of these bacteria. Cole *et al*. (2015) suggested that off- and on-bottom *Vibrio* discrepancies in oysters could be due to the higher concentrations of *Vibrio* in sediments (Johnson *et al*., 2012). Thus, oysters that are on bottom, and closer to sediments, are exposed to higher concentrations of bacteria. This theory is supported by research previously conducted by Fries *et al*. (2008) in the Neuse River Estuary, in which *Vibrio* spp. that were attached to sediment were a prominent proportion of the total *Vibrio* population. In that study, it was demonstrated that resuspension events could drive higher concentrations of *Vibrio* into the upper water column. In the current study, it was found that total *Vibrio* was negatively correlated with daily wind speed, an indicator of potential sediment resuspension events. However, the factors explaining these patterns may be more complex than just daily wind speed (i.e. sustained wind speed, gust speed, sustained wind direction, water column depth) and the timing of the data pairing may be inappropriate.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

**Table S1** Harvest site locations, salinities, and oyster types. CIB has two wild locations because oyster clusters were scarce in the original wild area we chose to sample. Salinities were within 3 ppt of each other except during a single extreme rainfall condition (JB, August 3, 2018). 'W' = wild, 'F' = farmed, 'S' = suspended, 'B' = on-bottom.

**Table S2** Daily wind speed, wind directions, termperature and precipitation