Temperature and Oxygen Abuse of *Crassostrea virginica* and its Effect on *Vibrio* Bacteria

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Abstract
Vibrio infections are one of the leading causes of seafood-borne gastroenteritis. As observed by the CDC, from 2008 to 2012, there was a noted 43% increase in *Vibrio* population occupying U.S. coastal waters[3]. Factoring in an estimated 4500 cases of *Vibrio* infection each year in the United States with varying degrees of severity, the absence of attention placed on the removal of *Vibrio* from harvested bivalves is concerning. In this project, *Crassostrea virginica* were incubated for durations up to a week at different temperatures and levels of oxygen availability. By doing so, preliminary patterns may be established correlating the viability of *Vibrio parahaemolyticus* and *Vibrio vulnificus* under different environmental constraints. Through this, it may be possible to arrive at the most optimal conditions at which *Crassostrea virginica* may be stored post-harvest to minimize the contamination by *Vibrio* bacteria. Through research, it was shown that at lower incubation temperatures, *Vp* proved nonviable while *Vv*, although still significantly lowered, presented noticeable counts. At highly temperatures, *Vp* vastly outnumbered *Vv*, with cases of extend incubation showing no representation by *Vv*. In an anaerobic environment, *Vp* counts were noticeably lowered while *Vv* counts expressed no identifiable pattern.

Introduction

Vibrio parahaemolyticus (*Vp*) and *Vibrio vulnificus* (*Vv*) are bacterium under the same family as *Vibrio cholera*[6,7]. Under the genus "*Vibrio*", indicating crescent-like cell structure, *parahaemolyticus* and *vulnificus* are gram-negative, facultative aerobic, halophiles. Naturally inhabiting the coastal waters of the United States and Canada, both species of *Vibrio* were historically present at low levels in cold water and higher levels in areas along the Gulf coast and California. Although not harmful upon proper treatment, *Vibrio* bacteria prove harmful otherwise. This is most commonly associated with the consumption of raw bivalves.

As filter feeders, *Crassostrea virginica* (Eastern Oysters) retain *Vibrio* bacteria naturally inhabiting the waters. Upon consumption of contaminated seafood, the presence of *Vp and Vv* results in acute seafood-borne gastroenteritis. With an incubation period of approximately 24 hours, symptoms include diarrhea, vomiting, nausea, abdominal cramps and fever[7]. Although treatment may be unnecessary for a *V. parahaemolyticus* infection, this is not the case for *V. vulnificus*. Traditionally, less common, a *V. vulnificus* infection may result in far more drastic symptoms. Upon consumption of contaminated bivalves, the bacterium may enter and infect the bloodstream, providing symptoms including fever, chills, septic shock and blistering skin lesions. If left untreated, this infection may prove life threatening[7]. In rare occasions, *Vp* and *Vv* infections may occur through open wounds. According to the Center for Disease Control and Prevention, there is an estimated 4500 cases of *Vp* infections each year in the United States[6]. Although cases of *Vv* infections are not as commonplace, these two species of *Vibrio* are highly similar, causing similar symptoms and being transferred through the same medium. *Vv* has a mortality rate of 53% in healthy humans and 67% in persons with liver impairment[7]. *Vp* has a mortality rate of between 30-50%[6].

According to research done by the CDC, from 2008 to 2012, there was a noted 43% increase in *Vibrio* populations occupying U.S. coastal waters[3]. Largely attributed to rising water temperatures, there is a significant increase of *Vibrio* populations in northern waters. Furthermore, with low doubling times and a transportation period of up to 10 hours prior to treatment, the process of harvesting *Crassostrea virginica* (eastern oysters) is concerning.

Currently, the procedure enforced by the FDA in the treatment of oysters postharvest is depuration. In the process of depuration, harvested oysters are washed with clean seawater over the course of 6-8 days. The bivalves filter the clean seawater through their system and bacterial contaminations are excreted into the water. The contaminated water is then filtered, exposed to UV light for sterilization and cycled back to the bivalves[1]. With the persistence of *Vibrio* infections, this procedure has shown unfavorable results. In addition to this, it should be noted that the primary focus of this procedure is the removal of *Escherichia coli*, not *Vibrio* bacteria.

The primary purpose of this research project was to examine the behavioral patterns of *Vp* and *Vv* under different experimental environments. By examining the correlation between the levels of bacterial growth and levels of oxygen availability and temperature, standards may be arrive at in which it is most optimal to store harvested oysters to minimize levels of *Vibrio* bacteria.

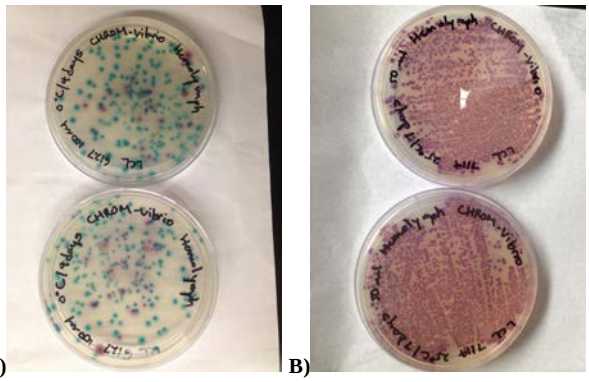
Materials and Methods

Crassostrea virginica samples were collected throughout the research period. Each week, ten oysters were collected from the Sound School Regional Vocational Aquaculture Center oyster beds along the New Haven Harbor. Upon collection, the samples were washed extensively to ensure the removal of bacteria and other contaminants on the surface of the shellfish. The samples

Table 1: Colony-forming unit counts standardized to per 100 grams of homogenized oyster tissue. It should be noted that although these values may seem abundant, experimentally, counts were performed only on 10-20 grams of homogenized oyster tissue. Upon standardization to 100 grams of homogenized tissue, all experimental values were amplified.

Temperature (Celsius)	Incubation Period	CFU/100g V.v. (50 uL aliquot)	CFU/100g V.p. (50 uL aliquot)	CFU/100g V.v. (100 uL aliquots)	CFU/100g V.p. (100 uL aliquots)
-20	1 days	0 ±0	12682.22 ±6002.92	0 ±0	10568.51 ±3001.46
-20	2 days	0 ±0	13112.28 ±6206.48	0 ±0	4370.76 ±6162.77
-20	4 days	0 ±0	5173.19 ±7345.93	0 ±0	2586.59 ±3672.96
-20	7 days	0 ±0	3556.19 ±5049.79	0 ±0	0 ±0
0	1 day	259259.26 ±78518.52	0 ±0	939814.81 ±6574.07	26455.03 ±26203.70
0	2 days	237179.49 ±172307.69	25641.03 ±18076.92	275641.03 ±27179.49	22435.89 ±13589.74
0	4 days	495927.17 ±296123.62	49592.72 ±54551.99	859607.09 ±77915.69	135002.39 ±27275.99
0	7 days	43346.01 ±12310.27	8669.20 ±12310.27	0 ±0	4334.60 ±6155.13
3	1 days	788663.4 ±68243.53	56333.10 ±11427.57	2852869.14 ±73957.31	337998.60 ±68324.00
3	2 days	409284.33 ±25179.88	26692.46 ±25179.88	471953.58 ±94357.83	40038.68 ±0
3	4 days	646693.05 ±211045.79	0 ±0	1529677.78 ±131888.07	21763.71 ±13182.59
3	7 days	1739262.47 ±198160.52	123644.25 ±123644.25	2749023.86 ±64130.15	98915.40 ±0
25	1 day	336223.98 ±56417.24	100867.95 ±100867.95	472993.06 ±88671.95	1817888.99 ±749494.55
25	2 days	4723.35 ±6707.15	221997.30 ±221997.30	4723.35 ±0	238529.01 ±10013.50
25	4 days	50145.35 ±7911.82	50145.35 ±50145.35	72432.17 ±15767.93	7906250 ±165479.65
25	7 days	82277.47 ±64724.94	36165517.24 ±1189366.48	150842.02 ±32362.47	25231756.21 ±594683.24

were then stored together under the desired experimental conditions of either -20°C, 0°C, 3°C, 25°C, or 37°C. The oysters were partially wrapped in a moist paper towel to maintain humidity. The durations at which the samples were stored under the desired experimental conditions were one, two, four, and seven days. At each of these desired incubation periods, two oysters were removed from the sample pool. These oysters were then washed and shucked. Following the shucking of the oysters, the oyster tissue was placed into a sterile blender and the amount of oyster tissue used was noted. 100 mL of artificial seawater was then added to the blender and the contents were blended for a minute for homogenization. The homogenized oyster tissue was then transferred over to CHROMagar-*Vibrio* plates in two aliquots of 50 µL and 100 µL. For the trials at 37°C, only 150 µL aliquots were used as these trials were performed during the process of determining the best procedure to follow.



A) **B)**

Figure 1:
A) CHROMagar-*Vibrio* plates demonstrating an abundance of *Vv*.
B) CHROMagar-*Vibrio* plates demonstrating an abundance of *Vp*.

CHROMagar-*Vibrio* is a specialized media that is both selective and differential[2]. The media itself is selective for *Vibrio* bacteria, not allowing for growth of other bacteria. Incorporated in the media are different chromogenic indicators that target specified enzymatic pathways of the different species of *Vibrio*. Resulting from this, as different species of *Vibrio* grow on a CHROMagar-*Vibrio* plate, the different species will trigger the different chromogenic indicators, each expressing a different color to allow for differentiation.

Upon inoculation of the homogenized oyster tissue onto a CHROMagar plate, the plates were incubated at 37°C for 24 hours. Following this incubation period, the bacterial growth on each plate was quantified through the count of colony-forming units (CFU). Obtained CFU counts were translated to CFU/100 grams of homogenized oyster tissue to standardize experimental values.

Polymerase Chain Reaction (PCR) and gel electrophoresis were performed to ensure that the bacterial colonies quantified were, in fact, *Vp* and *Vv*. The primers used were VPM1, VPM2, Tox 130, and Tox 200.

The effects of oxygen availability were examined through a trial at 25°C. Following the same procedure, the sample group of ten oysters were harvested and cleaned. For incubation, these samples were stored at 25°C in a GasPak, resulting in an anaerobic environment. Post-incubation treatment and quantification of *Vibrio* followed the same procedure described for temperature variability.

Results and Discussion

Table 1 shows all experimentally collected data standardized to CFU/100 grams for trials run at -20, 0, 3, and 25°C. The lowest incubation temperature examined in this experiment was -20, intending to simulate a standard freezer. Under these conditions, as expected, there was minimal to no growth for both species of *Vibrio*. Similarly, at 0°C and 3°C, with the samples stored on ice and in a

refrigerator respectively, minimal growth was noted. However, an interesting pattern was observed at lower temperatures when comparing the viability of the two species. As seen in Figure 2, at 3°C, there was a difference in the viability of the two species of *Vibrio*. *Vv* thrived significantly better than *Vp*. This pattern was consistent at -20°C and 0°C as well.

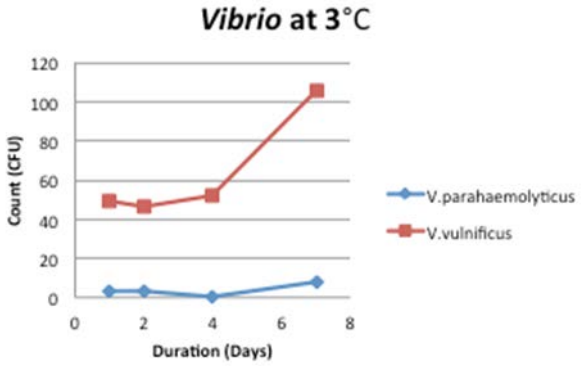


Figure 2: A graph comparing the growth of *Vp* to *Vv* at 3°C. noticeable

Table 2: Experimental trials performed at 37°C. The execution of these trials was done using 150 µL aliquots during the process of determining the best procedure. All values were standardized to CFU/100 grams.

Temperature (Celsius)	Incubation Period	CFU/100g V.v.	CFU/100g V.p.
37	1 day	4366.81 ±6157.21	543668.12 ±71004.37
37	2 days	936739.66 ±17274.94	591240.88 ±275182.48
37	4 days	283018.87 ±44654.09	14182389.94 ±400000
37	7 days	0 ±0	96296296.3 ±1246913.58

At the higher experimental temperatures of 25°C and 37°C, another interesting observation was noted. Representing room and body temperature respectively, both species of *Vibrio* should've replicated exponentially, theoretically. *Vp* encompassed this idea and replicated exponentially. However, *Vv* failed to do so. Interestingly, at extended durations of incubation, there was little to no presence of *Vv*.

Table 3: Experimental trials performed at 25°C under anaerobic conditions.

Temperature (Celsius)	Incubation Period	CFU/100g V.v. (50 uL aliquots)	CFU/100g V.p. (50 uL aliquots)	CFU/100g V.v. (100 uL aliquots)	CFU/100g V.p. (100 uL aliquots)
ANA (25)	1 day	13788.82 ±0	592919.25 ±19442.24	31024.84 ±4895.03	513633.54 ±141404.35
ANA (25)	2 days	176352.71 ±124769.54	1344689.38 ±259826.32	202070.81 ±88323.31	1774549.09 ±129913.16
ANA (25)	4 days	529201.43 ±56095.35	4921573.3 ±449027.41	886412.39 ±56161.50	6363647.19 ±93536.35
ANA (25)	7 days	26008.97 ±36759.34	216741.41 ±85829.60	47683.11 ±6155.46	247085.20 ±18379.67

In the absence of oxygen availability, differing growth patterns were observed in *Vp* and *Vv*. Shown in Figure 3, *Vp* saw a noticeable decline in bacterial population after incubation under an anaerobic environment for extended durations. This decline can be noted started at four days of incubation. *Vv*, however, showed no consistent pattern in response to the absence of oxygen. The data point representing samples under anaerobic conditions for four days is inconsistent with the other data points. However, this was concluded to be an outlier and no general response pattern could be observed to mark the point as significant.

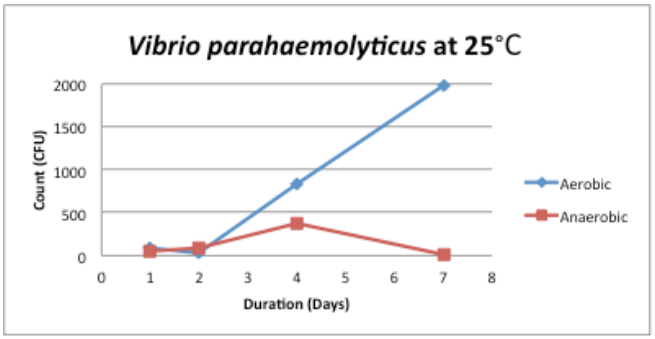


Figure 3: A graph comparing the growth of *Vp* under aerobic and anaerobic conditions.

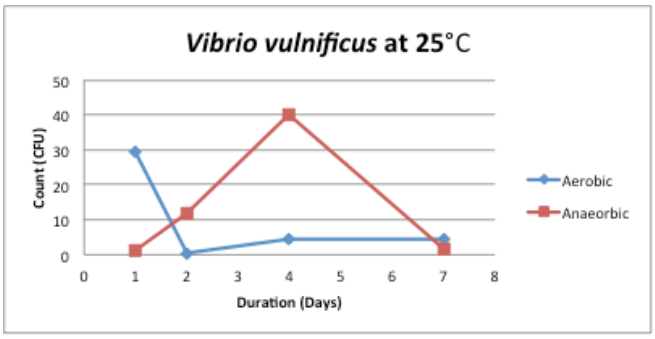


Figure 4: A graph comparing the growth of *Vv* under aerobic and anaerobic conditions.

Conclusion

The purpose of this research project was to attempt to determine the temperatures and levels of oxygen availability at which would prove most stressful for *Vibrio parahaemolyticus* and *vulnificus*. By doing so, it would be possible to arrive at an optimal procedure to limit the replication of *Vibrio* bacteria in eastern oysters during post-harvest transportation and treatment. Although this goal was not arrived at, through the observation and analysis of the collected data, a preliminary pattern for the behavior of *Vibrio* bacteria under environmental stress was discovered. Being facultative aerobes, *Vv* showed little to no alterations in patterns of growth under anaerobic conditions. *Vp,* on the other hand, showed significantly lowered rates of growth after four days of incubation at 25°C under anaerobic conditions. At -20°C, as expected, neither species produced representable counts. *Vv* was completely undetected, while *Vp* provided insignificant counts, varying between one and two CFU. Under standard refrigeration temperatures of 0°C and 3°C, neither species of *Vibrio* grew optimally. After an

experimental period of 24 hours under these conditions, *Vp* was noted to be nonviable. However, interestingly, *Vv* was still able to grow under these conditions, although providing drastically lower counts. On the other hand, at the highest experimental temperature of 37°C, it was observed that *Vv* was not present after extended periods of incubation. In comparison, *Vp* replicated at an exponential rate. Noting that the incubation temperature of 37°C was intended to simulate the conditions inside the human body, this result was unexpected, but not inconsistent as the same observation was noted for both trials at 25°C and 37°C. The reasoning behind this is unknown. However, one possibility could be that *Vv* is incapable of competing with *Vp* for resources, as at higher experimental temperatures, the CHROMagar plates were highly confluent, resulting in increased competition for space and nutrients. In addition to this, the results of PCR and electrophoresis did confirm that the counts recorded were, in fact, for *V.p.* and *V.v.* and not *V. cholerae.*

Reflecting on the data produced, many minor inconsistencies may be noted. However, the scope in which this project was performed must be taken into account. Due to constraints in time and resources, multiple trials under each experimental condition were not performed, resulting in less than desirable statistical variation.

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Biography

Lawrence Lam (2014) of Brooklyn, New York is a Forensic Science and Biology double major with a Pre-dental concentration. As a senior at the University of New Haven, he has been working as a microbiology lab assistant since Fall of 2013. Following his graduation from the University of New Haven, Lawrence intends to pursue a graduate degree in dentistry and work towards a career as a forensic odontologist.