2019

Elucidating the Role of Oxygen and Biotype in the Environmental Persistence of Vibrio Cholerae

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ELUCIDATING THE ROLE OF OXYGEN AND BIOTYPE ON THE ENVIRONMENTAL PERSISTENCE OF *VIBRIO CHOLERAE*

by

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A thesis submitted in partial fulfillment of the requirements
for the Honors in the Major Program in Biological Sciences
in the College of Medicine
and the Burnett Honors College
at the University of Central Florida
Orlando, FL

Spring Term, 2019

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ABSTRACT

Vibrio cholerae is a facultative pathogen that represents the etiological agent of the severe diarrheal disease cholera. The first six cholera pandemics were caused by the classical biotype, which was then replaced by the El Tor biotype responsible for the seventh cholera pandemic. The pathogen encounters abiotic fluctuations within the aquatic environment throughout the year. To cope with environmental conditions that are not conducive to growth, such as fluctuations in nutrient availability or temperature, *V. cholerae* enter a protective state known as viable but nonculturable (VBNC). Cells found in VBNC are characterized by their inability to be cultured through standard detection methods and by drastic changes in their metabolism and morphology. Even though there are numerous abiotic stressors in the environment, we are specifically interested in how oxygen concentrations induce entry into VBNC. We recently determined that aeration promotes faster entry into the VBNC state for the O395 biotype of *V. cholerae.* In this study, we investigate how aeration differentially induces entry into VBNC for different biotypes. By creating a microcosm for *V. cholerae*, we examined culturability between the classical and El Tor biotypes in both aeration and static (non-aeration environments) at 5°C and 30°C. From the conditions we tested, we found that aeration accelerates entry into VBNC for the El Tor biotype. In this study, it appears that the classical biotype approaches the VBNC state more rapidly. Overall, this study deepens our understanding of the complex ecological variables that enable *V. cholerae* to persist during unfavorable environmental conditions.

DEDICATION

This thesis is dedicated to Mariah, Taylor, Josh, Sabrina, Jennifer, and Jacob. Thank you for being a constant source of laughter and happiness and for encouraging me to reach for the stars.

ACKNOWLEDGEMENTS

I would like to thank Dr. Moreno for the guidance and mentorship he has offered me throughout my undergraduate research career over the last two years. Thank you for the opportunity to work in your research lab, which has fueled my passion to pursue a doctoral degree.

I also would like to sincerely thank my honors thesis committee for your continued support and patience. Thank you for believing in me throughout the thesis process and for always being available for suggestions and advice.

Additionally, I would like to thank my lab mates for the constant support every day. Thank you for your flexibility and your willingness to answer my questions. A special thank you to Geethika Reddi for serving as my graduate student mentor.

Lastly, I am especially thankful for the unwavering support and love from my parents, Mitchell and Barbara Freiberg, my sister, Emily Freiberg, and my grandmother, Harriet Simon. Thank you for encouraging me to pursue my dreams and for helping me overcome any obstacles.

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ACRONYMS

VBNC - Viable but Nonculturable

ASW – Artificial Sea Water

RPM – Rotations per Minute

CFU – Colony Forming Unit

LB - Luria-Burtani

ddH_2O: Double-Deionized Water

CHAPTER 1 – INTRODUCTION

Vibrio cholerae is a facultative pathogen that inhabits brackish environments. In its natural environment, *V. cholerae* interacts with a wide range of other aquatic inhabitants, including copepods [1,2,3,4], shellfish [5,6], and cyanobacteria [7,8]. Associations with other marine dwellers, such as the attachment to copepod surfaces and fluctuations within abiotic variables such as pH, temperature, and nutrient availability have been shown to affect the survival of *V. cholerae* in the environment [9-13]. In the natural environment, *V. cholerae* can be found as free-living single cells or they can even clump together to form a biofilm [14]. This complex combination of biotic and abiotic factors found in the marine environment collectively influence the growth and survival of *V. cholerae* [15].

V. cholerae is the etiological agent of cholera, a severe diarrheal disease that causes death by dehydration [16]. Individuals in developing areas with limited resources and poor sanitation, particularly South Asia, Africa, and Latin America, are most susceptible to cholera outbreaks [17]. Widespread cholera outbreaks, known as pandemics, have been exclusively caused by the classical and the El Tor biotypes. Cholera remains a serious threat to the human population as infections can lead to death if not treated. Cholera affects millions of individuals and is responsible for over 100,000 deaths annually [18]. The disease is primarily spread through human consumption of contaminated food or water with human feces. Once ingested, *V. cholerae* must successfully travel through the stomach and colonize within the small intestine to cause infection [19]. Intravenous and oral hydration serve as the main cholera treatment strategies, and the tetracycline antibiotic has also been shown to be an effective cholera treatment [20,21].

When significant changes in the environment provide the ideal conditions for *V. cholerae* to proliferate, only the classical and El Tor biotypes are known to cause cholera pandemics. The El Tor biotype was first isolated in 1937 and is responsible for the most recent cholera pandemic that occurred in Indonesia in 1961 [22]. This highly adapted biotype reveals genotypic and phenotypic differences from the classical biotype, which it replaced in 1992. The classical biotype served as the causative agent of the first six cholera pandemics between 1817 and 1961, which was then replaced by the El Tor biotype responsible for the seventh pandemic in 1961. While the El Tor represented the main source of cholera outbreaks between 1961 and 1992, the classical biotype was still responsible for isolated incidents until 1992. Interestingly, clinical isolates have been identified that are of the El Tor background but reveal traits characteristic of the classical biotype [23]. Both the classical and El Tor biotype belong to the O1 serogroup responsible for cholera epidemics.

In endemic regions such as Bangladesh, cholera outbreaks are strongly correlated with environmental changes. Cholera outbreaks in these regions exhibit biannual peaks and are associated with increased temperatures and heavy rainfalls [24]. These seasonal variations in the climate are associated with fluctuations in the aquatic environment, due to changes in the pH, salinity and introduction of chemical runoffs [24]. Additional consequences of seasonal cholera outbreaks include significant changes in oxygen concentrations and turbidity levels [25]. Another naturally-occurring event that can disrupt dynamics of the bacterium's environment is global warming. Global warming, which is caused by a global increase of carbon dioxide emissions, leads to the gradual increase in atmospheric temperatures [26]. Regarding the aquatic ecosystem, global warming represents the main source of declining oxygen levels. Human activities, such as

waste production and consumption of nitrogen-rich chemical fertilizers, also influence the balance of the aquatic ecosystem. The process of depositing these harmful chemicals into bodies of water has been associated with deoxygenation [27]. These drastic changes within the aquatic environment occur throughout the year and disrupt the balance of the aquatic ecosystem in such a way that *V. cholerae* face adverse growth conditions.

Since environmental conditions fluctuate and do not always promote active growth, pathogens have adapted different survival strategies to persist during unfavorable conditions, including changes in temperature, pH, and oxygen concentrations. During environmental conditions that are adverse for survival, *V. cholerae* cells enter a protective dormant state called Viable but Nonculturable (VBNC) state. Cells in this state are characterized by drastic morphological change to coccoid morphology, which deviates from the traditional rod shape characteristic of *V. cholerae* [28]. Cells in VBNC maintain decreased metabolic activity, drastic morphological changes and antibiotic resistance [29]. Notably, VBNC cells require less energy and exploit slower metabolism to survive during nutrient-limiting conditions. *V. cholerae* found in this persistent state remain a threat to the human population due their ability to resuscitate and infect the human host under the appropriate environmental conditions. Investigating entry into VBNC is significant because a large proportion of *V. cholerae* are found in this protective state [30-32]. Cells in the protective VBNC state are more resistant to unfavorable environmental conditions. For example, antibiotics that are targeted towards cell components of active cells will not be as effective against VBNC cells that aggregate together and secrete an extracellular matrix [29].

Inherent to the VBNC state, *V. cholerae* cannot be detected using standard microbiological techniques, such as recovery of cells after plating on LB media [33]. The loss of culturability on LB agar can be measured through decreasing CFU measurements. Unculturability of human pathogens is significant because such water can inaccurately be deemed safe to drink, even though it is actually contaminated with bacteria. Cells found in VBNC require additional detection methods, such as through a Live/DEAD kit or LPS-specific antibodies, to confirm the viability of these cells [34]. Through the Live/DEAD screening, cells that are alive are green and cells that are dead are red through fluorescent microscopy. Cells can further be classified based on whether or not they are culturable. Dead cells take up a red staining known as propidium iodide, which is only able to penetrate within the membrane of impaired cells. Cells that are viable and culturable display a green rod-shape. Meanwhile, cells that are viable but non-culturable are a green coccoid-shape, which is representative of cells in the VBNC state [35]. Various cellular markers that can be used to confirm that VBNC cells are indeed alive include integrity of the cell membrane, uptake of labeled amino acids, and DNA protection [36]. Initially, VBNC was regarded as a dormant state as it remained unclear how the two states differed from one another. Current literature indicates that the main difference between VBNC and dormancy is that cells in VBNC are able to maintain decreased metabolic activity, while dormant cells reveal undetectable metabolic levels [37]. Thus, VBNC can also be referred to as a metabolically active but non-culturable state (ABNC) [26].

V. cholerae regularly encounter numerous abiotic stressors in their natural environment. Fluctuations in these environmental variables, including oxygen concentrations, affect *V. cholerae* growth and survival. Interestingly, bacterial cells in VBNC are able to aggregate into

biofilms in response to adverse environmental conditions, such as oxygen limitation [14]. To explain, when fluctuations in the external environment result in decreasing oxygen concentrations that are not suitable for *V. cholerae* growth, cells can respond through formation of biofilm. The biofilm represents a stable local environment and contains microbial cells that are enclosed within an extracellular polysaccharide matrix, which serves as both a mechanical and selective chemical barrier for the bacterial cells against external environmental pressures [38].

Biofilms can form on different abiotic or biotic surfaces and even form water channels for nutrients and wastes to travel in and out [39-41]. Once environmental conditions become favorable again and represent suitable growth conditions, *V. cholerae* cells are able to detach from the protective biofilm complex and actively grow. *V. cholerae* have developed other mechanisms in response to oxygen limitations. If given the appropriate environmental conditions, *V.* cholerae can also respond to such growth conditions by simply migrating to areas that are oxygen-rich through aerotaxis [42]. In addition to other human pathogens, *V. cholerae* reveals the unique ability to adapt and to persist in environments with different oxygen concentrations [43]. *V. cholerae* can be described as facultative anaerobes that utilize different mechanisms to persist during fluctuations within the aquatic environment and to successfully colonize the human host [44]. Within the aquatic environment, *V. cholerae* survive in varying oxygen concentrations.

The ability to respond to different oxygen concentrations could be biotype-specific due to different gene expression patterns. El Tor biotype contains higher levels of gene expression for

biofilm formation, chemotaxis, and transport of amino acids, peptides, and iron than the classical biotype [45]. Hence, the ability for cells to aggregate into biofilms to survive during oxygen limitation may be dependent on the biotype of *V. cholerae.* It is of scientific interest to understand how the classical and the El Tor biotype differentially induce entry into VBNC. To date, there hasn't been another observed instance of such rapid replacement of one epidemic-causing strain by a newly emerged strain for any other bacterial pathogen [46]. While the two biotypes reveal distinct differences between the regulation of virulence factor production between the two biotypes, it remains unclear how these biotypes differentially enter VBNC. The classical O395 strain has been used for research efforts regarding virulence in previous cholera outbreaks [47,48]. Interestingly, previous research indicates that this classical strain approaches the VBNC state faster than other strains [49]. The El Tor biotype strain N16961, which is responsible for the recent cholera pandemic, reveals different entry patterns into VBNC [50]. When the two biotypes are cocultured, it has been demonstrated that the El Tor strains exhibit a clear competitive growth advantage starting from the late stationary phase with the ability to eventually take over the entire culture [51]. Such studies highlight that the classical and the El Tor biotype may respond to unfavorable environmental conditions in different ways. We will be examining how the O395 and N16961 strains enter VBNC specifically in estuarine environments, where cholera outbreaks are known to originate [52].

Recently, we examined that oxygen concentration influences culturability of the O395 strain of *V. cholerae.* Our preliminary studies reveal that aeration increases the rate at which cells lose culturability, when compared to non-aerated (static) environmental conditions. Through this discovery, we are especially interested in elucidating the role of oxygen concentration between

biotypes on *V. cholerae* persistence. To investigate these associations, we will measure loss of culturability. We are specifically interested in measuring the rates at which cells reach the unculturable state that is characteristic of VBNC. To our knowledge, this is the most environmentally-representative study that aims dissect each individual environmental variable through continuous monitoring of the culturability of long-term *V. cholerae* samples into VBNC. One obstacle that has contributed to the limited research regarding VBNC is the lack of reproducible results [53]**.** When inducing the VBNC state through laboratory methods, it is necessary to first determine the individual abiotic factors involved with entry into the VBNC state before elucidating the synergistic role. It is crucial to understand the exact role of each individual environmental variable towards the entry of VBNC to ensure stable, reproducible results without any confounding variables. An additional concern towards the lack of reproducible results is due to the use of different compositions of media available [53]. To demonstrate, other studies that have investigated the VBNC state used water samples from the natural aquatic environment. In one study, cells were introduced into fresh water, brackish water and salt water samples that were collected [35]. While such water samples most closely simulate the aquatic environment, these media sources do not contain known nutrient compositions and it is therefore not possible to produce valid, reproducible results [53]. Consequently, we prepared our long-term cultures of *V. cholerae* in nutrient-free artificial seawater (ASW).

Investigating the exact conditions that influence development of VBNC is critical to understand the underlying molecular and genetic mechanisms involved in triggering this persistent state. Associations with protozoans and zooplankton in the aquatic environment are also known to support entry into VBNC [54]. Due to the complex combination of biotic and

abiotic factors found in the marine environment that collectively influence the growth and survival of *V. cholerae*, it is necessary to elucidate the role of each individual factor towards the onset of VBNC [55]. After all, our overarching research interests lie in understanding the hierarchal influence of ecological factors that enable pathogens to persist in the environment during adverse environmental conditions.

Since traditional water sampling and standard quality tests cannot detect the presence of VBNC cells, it is likely that the presence of *V. cholerae* is inaccurately measured. Considering that diarrheal diseases are globally ranked as the third highest cause of morbidity [56], unculturability of common aquatic pathogens like *V. cholerae* is a serious threat to humans because the bacteria remain undetectable. When humans consume water that is contaminated with *V. cholerae*, the cells have the potential to resuscitate under the appropriate conditions to give rise to another cholera outbreak [57,58]. In the small intestine, *V. cholerae* are exposed to an anoxic environment with different pH and temperature than found in their natural environment to allow for resuscitation. The food industry also poses a concern regarding VBNC, in which improper sanitation and the pressure of CO_2 in packing are recognized to induce the state [59]. Additionally, VBNC pathogens have been reported to be implicated in the onset of latent infections, such as tuberculosis [60].

In this project, we will develop a deeper understanding of how *V. cholerae* respond to different oxygen concentrations related to climate change and global warming through continuously monitoring the culturability of long-term *V. cholerae* samples. Additionally, we will determine whether biotype fosters entry into VBNC at different rates. Overall, our efforts will expand knowledge about how oxygen and biotype contribute to pathogenic persistence between epidemics.

CHAPTER 2 - METHODOLOGY

Bacterial Strains and Preparation

The two *V. cholerae* biotypes used in this study include the classical O395 and the El Tor N16961. The stocks for these strains were prepared in 50% (v/v) glycerol stock and were stored in the -80°C freezer.

Preparation for Long-Term Culture

A three-zone streak was performed for the O395 and N16961 strains on nutrient-rich Luria-Burtani (LB) agar plates and stored in the 37°C incubator for 18 hours. After incubation, an isolated colony was collected utilizing a P200 micropipette and then suspended in 200 mL of LB broth for 16 hours in the 37°C shaking incubator at 200 RPM until the cells reached the exponential growth phase. At this point, the cells were centrifuged using an F10-6 x 500y fixed-angle rotor at 4000 RPM for 10 minutes in 4°C. During this process, the samples were washed twice with 200 mL of nutrient-free artificial sea water (ASW) and then finally transferred into 100 mL of ASW in a 500 mL Erlenmeyer flask for the 30-day time period. Flasks were sealed with a cotton and tinfoil.

Experiment Setup

Cells under aerated-conditions were stored in a temperature-controlled, Thermo Scientific MaxQ4000 shaking incubator at 250 RPM**.** Cells under static conditions were stored stationary at the appropriate temperature, either in a 4°C cooler or 30°C incubator.

Enumeration of Culturable Cells

To understand how *V. cholerae* approached VBNC, serial dilutions were conducted every 5 days and the CFU/ml were measured for each condition over the 30-day timeline. For each serial

dilution, 200 μL of the desired sample were obtained from the flask and were diluted in ASW in a 1:10 ratio. Specifically, 100 μL of the original sample were transferred to a separate microcentrifuge tube containing 900 μL ASW. After each 10-fold dilution, the sample was vortexed for 5-7 seconds to ensure that the sample was equally spread throughout. The serial dilutions were platted on LB agar plates using sterile wooden sticks and partitioned off into quadrants, with each quadrant containing 10 μL of each individual dilution factor. After incubation for 18 hours at 37°C, colony growth was recorded for any dilution factor that revealed growth between 7 to 50 colonies. Dilution factors that were more concentrated displayed too many colonies to count, while more diluted sections did not reveal any colony growth at all.

Determination of Loss of Culturability

As the cells in the flask continued to lose culturability, very few colonies grew on the LB agar. When no more visible colonies grew, a 1 mL sample from the original flask was obtained and pelleted down in a bench-top microcentrifuge at 13.0 RPM for one minute. The supernatant was removed and the pellet was re-suspended in 100 μL of ASW. No colony growth in this most concentrated form indicated that the sample had completely lost culturability.

Data Visualization

Data obtained from the serial dilutions was organized using the GraphPad Prism 8 software. The time (days) along the x-axis was compared against the CFU/mL on the y-axis, increasing by a logarithmic scale of 10-fold. To calculate the CFU/ml, the 10 μL (0.01 mL) sample that was platted was multiplied by the dilution factor. The observed number of colonies would then be divided by this value. Error bars were included to indicate any standard deviation between the

three trials. Several graphs were created to establish a relationship between each unique condition and entry into VBNC.

Media Preparation

The Luria-Bertani (LB) broth was prepared to obtain a final concentration of 1% tryptone, 0.5% yeast extract, 0.5% NaCl. The LB agar was made to achieve the same final concentrations, with the addition of 1.5% agar for the media to solidify. The artificial sea water (ASW) was prepared using NaCl to achieve the desired salinity, HEPES to maintain the appropriate pH, and various trace metals. To prepare 1 L of ASW with a 1.5% salt concentration and 7.5 pH, 15 g NaCl, 11.92 g HEPES, 1.5 mL $NaHCO_3$, 5 mL $CaCl_2$, 100 µL K_2HPO_4, 10 mL NH_4Cl, 50 µL NaBr, 5 mL KCl, 100 mL $MgSO_4$, 1 mL Na_2B_4O7, 10 µL NaI, 10 µL LiCl, and 100 µL $SrCl_.$.

CHAPTER 3 – RESULTS

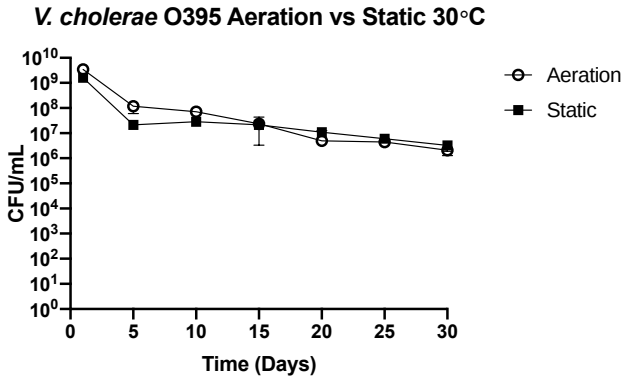

Figure 1: Effect of oxygen on the culturability of the O395 strain at 30°C.

Approximately 1 x 10^9 cells mL^{-1} of *V. cholerae* O395 cultured in artificial sea water were stored in a Thermo Scientific MaxQ4000 shaking incubator at 250 RPM (aeration) or in a Thermo Scientific Heratherm Refrigerated Incubator (static) at 30°C. Shown is the decline of culturable counts over time after incubation compared between aerated (empty circles) and static (full squares) conditions. The number of culturable cells was determined through serial dilutions on LB agar every 5 days for the 30-day timeline. Data points represent the mean of three trials (n = 3). Error bars represent the standard deviation.

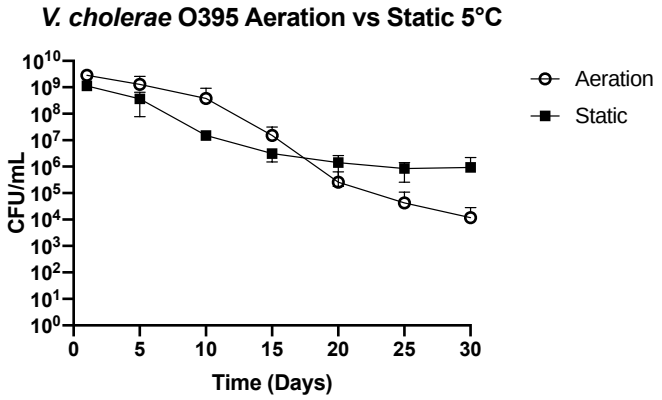

Figure 2: Effect of oxygen on the culturability of the O395 strain at 5°C.

Approximately 1 x 10^9 cells mL^{-1} of *V. cholerae* O395 cultured in artificial sea water were stored in a shaking Thermo Scientific MaxQ4000 incubator at 250 RPM (aeration) or in a stationary Thermo Scientific Heratherm Refrigerated Incubator (static) at 5°C. Shown is the decline of culturable counts over time after incubation compared between aerated (empty circles) and static (full squares) conditions. The number of culturable cells was determined through serial dilutions on LB agar every 5 days for the 30-day timeline. Data points represent the mean of three trials (n = 3). Error bars represent the standard deviation.

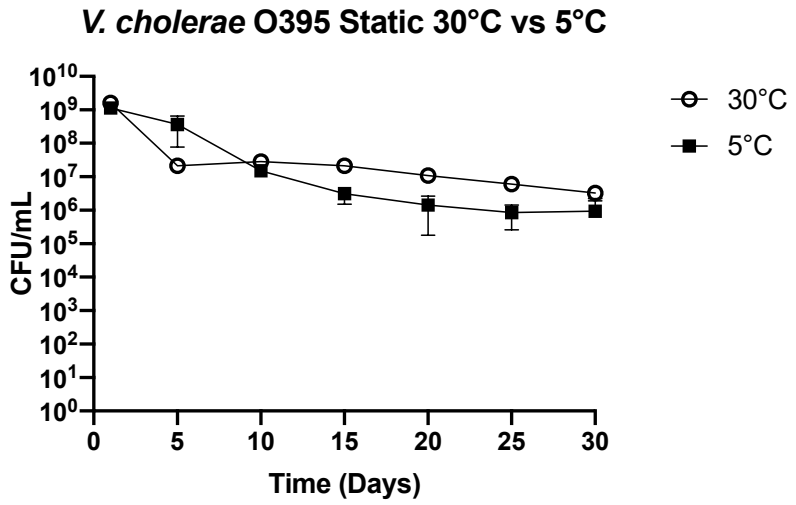

Figure 3: Effect of oxygen limitation on the culturability of the O395 strain.

Approximately 1 x 10^9 cells mL^{-1} of *V. cholerae* O395 in artificial sea water were stored in a stationary Thermo Scientific Heratherm Refrigerated Incubator at both 30°C (empty circles) and 5°C (full squares) to represent oxygen limitation (static). Shown is the decline of culturable counts over time after incubation compared between temperatures. The number of culturable cells was determined through serial dilutions on LB agar every 5 days for the 30-day timeline. Data points represent the mean of three trials (n = 3). Error bars represent the standard deviation.

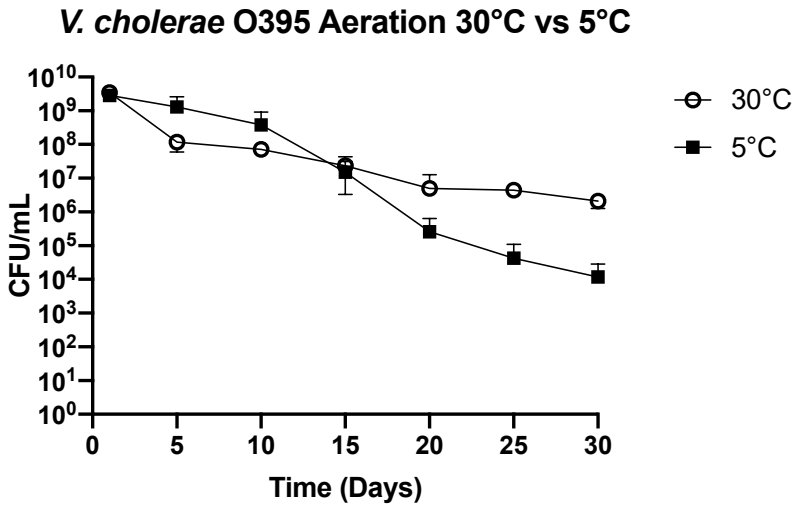

Figure 4: Effect of aeration on the culturability of the O395 strain.

Approximately 1 x 10^9 cells mL^{-1} of *V. cholerae* O395 in artificial sea water were stored in a shaking Thermo Scientific MaxQ4000 incubator at 250 RPM at both 30°C (empty circles) and 5°C (full squares) to represent aeration. Shown is the decline of culturable counts over time after incubation compared between temperatures. The number of culturable cells was determined through serial dilutions on LB agar every 5 days for the 30-day timeline. Data points represent the mean of three trials (n = 3). Error bars represent the standard deviation.

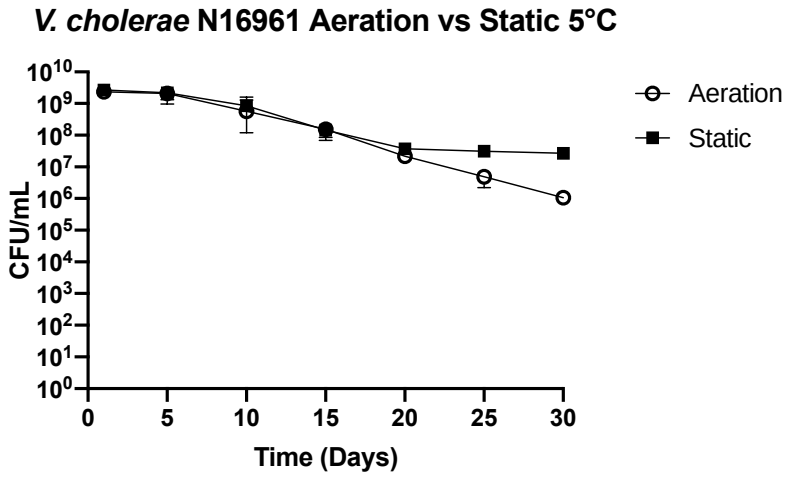

Figure 5: Effect of oxygen on the culturability of the N16961 strain at 5°C.

Approximately 1 x 10^9 cells mL^{-1} of *V. cholerae* N16961 cultured in artificial sea water were stored in a shaking Thermo Scientific MaxQ4000 incubator at 250 RPM (aeration) or in a stationary Thermo Scientific Heratherm Refrigerated Incubator (static) at 5°C. Shown is the decline of culturable counts over time after incubation compared between aerated (empty circles) and static (full squares) conditions. The number of culturable cells was determined through serial dilutions on LB agar every 5 days for the 30-day timeline. Data points represent the mean of three trials (n = 3). Error bars represent the standard deviation.

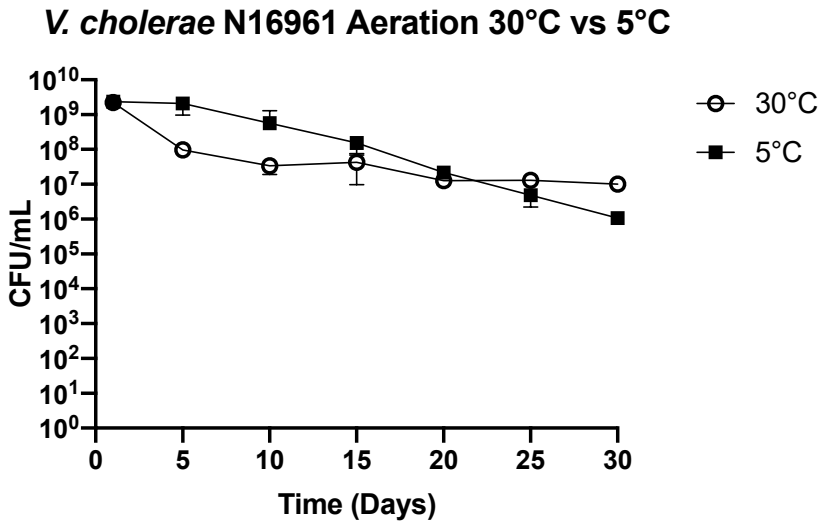

Figure 6: Effect of aeration on the culturability of the N16961 strain.

Approximately 1 x 10^9 cells mL^{-1} of *V. cholerae* N16961 in artificial sea water were stored in a shaking Thermo Scientific MaxQ4000 incubator at 250 RPM at both 30°C (empty circles) and 5°C (full squares) to represent aeration. Shown is the decline of culturable counts over time after incubation compared between temperature. The number of culturable cells was determined through serial dilutions on LB agar every 5 days for the 30-day timeline. Data points represent the mean of three trials (n = 3). Error bars represent the standard deviation.

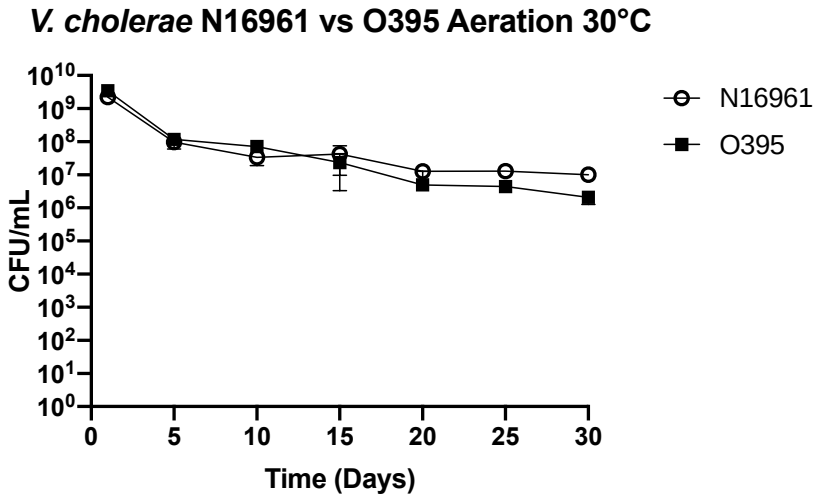

Figure 7: Effect of biotype on the culturability at 30°C.

Approximately 1 x 10^9 cells mL^{-1} of *V. cholerae* N16961 (El Tor biotype) and O395 (classical biotype) in artificial sea water were stored in a shaking Thermo Scientific MaxQ4000 incubator at 250 RPM at 30°C to represent aeration. Shown is the decline of culturable counts over time after incubation compared between N16961 (empty circle) and O395 (full square) under aerated conditions at 30°C. The number of culturable cells was determined through serial dilutions on LB agar every 5 days for the 30-day timeline. Data points represent the mean of three trials (n = 3). Error bars represent the standard deviation.

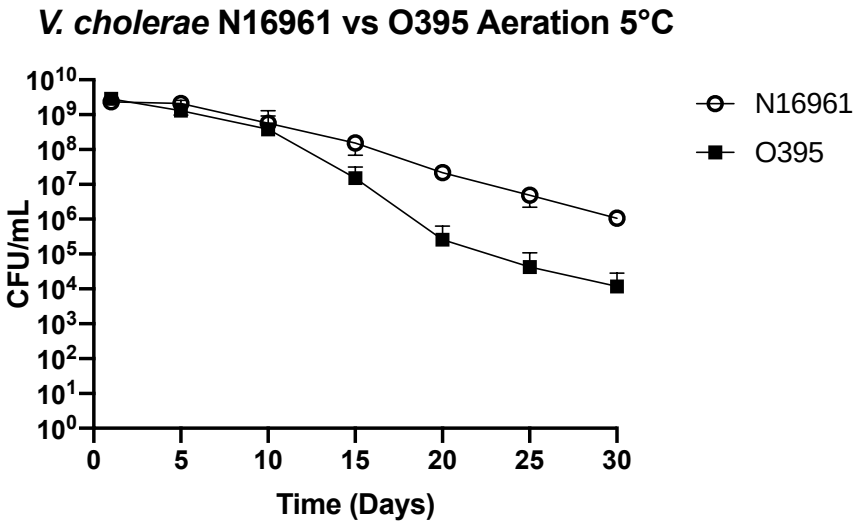

Figure 8: Effect of biotype on the culturability at 5°C.

Approximately 1 x 10^9 cells mL^{-1} of *V. cholerae* N16961 (El Tor biotype) and O395 (classical biotype) in artificial sea water were stored in a shaking Thermo Scientific MaxQ4000 incubator at 250 RPM at 5°C to represent aeration. Shown is the decline of culturable counts over time after incubation compared between N16961 (empty circle) and O395 (full square) under aerated conditions at 5°C. The number of culturable cells was determined through serial dilutions on LB agar every 5 days for the 30-day timeline. Data points represent the mean of three trials (n = 3). Error bars represent the standard deviation.

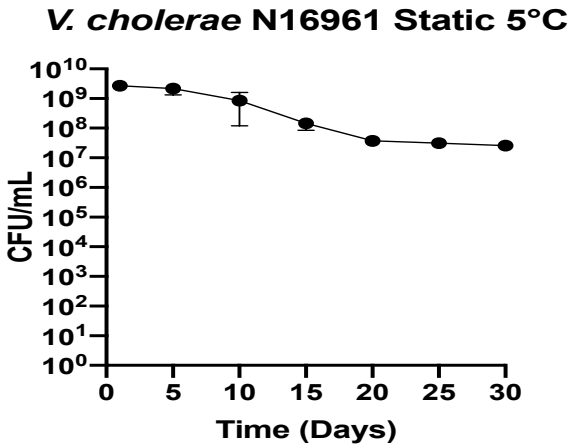

Figure 9: Effect of oxygen limitation on the culturability of the N16961 strain at 5°C.

Approximately 1 x 10^9 cells mL^{-1} of *V. cholerae* N16961 in artificial sea water were stored in a stationary Thermo Scientific Heratherm Refrigerated Incubator at 5°C to represent oxygen limitation. Shown is the decline of culturable counts over time after incubation compared under oxygen-limiting conditions. The number of culturable cells was determined through serial dilutions on LB agar every 5 days for the 30-day timeline. Data points represent the mean of three trials (n = 3). Error bars represent the standard deviation.

CHAPTER 4 – DISCUSSION

During unfavorable environmental conditions, *V. cholerae* are able to persist in their natural environment through entry into VBNC. Numerous variables, including pH, temperature, and salinity, within the aquatic ecosystem influence entry into this persistent state. Of these variables, we have investigated the role of oxygen concentration and biotype towards the onset of VBNC in *V. cholerae.* The results of this study reveal that aeration increases the rate at which cells enter VBNC and that the classical biotype loses culturability faster than the El Tor biotype.

First, we explored the influence of oxygen concentrations on *V. cholerae* culturability. We started by comparing loss of culturability for the O395 strain between aerated and static environments at 30°C. As revealed in Figure 1, these two environments revealed similar patterns as cells lost culturability. The largest difference in culturability was observed at Day 5, where there is a one log difference between the aerated and static environments. We then analyzed how the aerated versus static environments differentially promote entry for the O395 strain into VBNC at 5°C, which is illustrated in Figure 2. At the end of the 30-day timeline, cells in the aerated environment lost an additional 2 logs of culturability when compared to the static (non-aerated) environment.

We further examined how oxygen limitation (static) influences entry into VBNC for the O395 strain by comparing the loss of culturability between 30°C and 5°C. As displayed in Figure 3, both temperatures reveal comparable patterns as the cells lost culturability. We then studied how entry into VBNC is differentially induced between 30°C and 5°C in aerated environments. Figure 4 shows that the cells in the 5°C environment lost an additional 2 logs of culturability

over the 30-day timeline when compared to the 30°C environment. This finding suggests that aeration serves to increase the rate at which cells lose culturability.

We were also interested in understanding how oxygen concentrations influence loss of culturability for the N16961 strain of *V. cholerae.* Figure 5 reveals data from the culturability assay that we conducted for this strain under aerated and static conditions. At the end of the 30-day timeline, we recognized that aeration promotes faster loss of culturability for the N16961 strain as well. At this point, we compared how entry patterns into VBNC differ between the 30°C and 5°C in aerated environments for this strain. As revealed in Figure 6, the 5°C aerated environment promoted faster loss of culturability by a difference of one log at the end of the 30-day timeline.

After elucidating how oxygen concentrations differentially promote loss of culturability, we then explored the role that biotype plays on the entry into VBNC. The two biotypes of interest were the *V. cholerae* O395 (classical biotype) and the *V. cholerae* N16961 (El Tor biotype). We first compared the loss of culturability between the two biotypes in an aerated environment at 30°C, as revealed in Figure 7. Over the course of the 30-day timeline, both biotypes revealed similar patterns for loss of culturability. We then analyzed the loss of culturability between the two biotypes in an aerated environment at 5°C, as displayed in Figure 8. Through this culturability plot, we were able to identify that the classical biotype approached VBNC at a faster rate than the El Tor biotype. Specifically, the classical biotype lost an additional 2 logs compared to the El Tor biotype by the end of the 30-day timeline.

Additionally, we are interested in understanding how oxygen limitation influences how the *V. cholerae* N16961 strain enters VBNC. Currently, we have only been able to measure the loss of culturability under static conditions at 5°C, as indicated in Figure 9. We originally planned to also investigate the *V. cholerae* N16961 strain under static conditions in 30°C. Due to contamination issues, we were not able to conclude that portion of the project in this timeline. Contamination for this condition was first observed at Day 10 in trial 1 and trial 2. Once recognized, we attempted to identify the source of contamination by platting the unknown colony on Thiosulfate-citrate-bile salts -sucrose (TCBS) agar, which is used to selectively isolate *V. cholerae* species. We did not observe any colony growth on the TCBS media. We also platted our blank artificial sea water (ASW) control on LB agar, which resulted in colony growth and we were therefore not able to proceed. The growth of unidentifiable cells could be attributed to pipetting error and aseptic technique. If the pipette was not properly cleaned with ethanol before entering the culture and/or the flask was not properly flamed, the cell culture could have become contaminated. Another source of error could result from improper dishwashing and autoclaving procedures. Moving forward, we have increased the time for sterilization in the autoclave. Additionally, it is possible that the contamination was introduced during the preparation of the artificial sea water, which would result in contamination for the long-term cultures involved in downstream experimentation.

For each condition, we prepared our long-term cultures in 1.5% salinity, pH 7.5 artificial sea water (ASW). This specific salinity and pH may not be representative of all estuarial and coastal waters, as there are even environmental fluctuations that disrupt the salinity and pH within a single estuarial system. To illustrate, the Gautami-Godavari estuary exhibits low

surface salinities (0 to 8‰) during monsoon conditions, compared to the pre-monsoon salinities of 25 to 30‰ [61]. However, the preparation of ASW serves as an appropriate representative model due to its reproducibility and consistent composition [53].

The two temperatures that we incubated our long-term ASW cultures in were 5°C and 30°C, which represent the extreme ranges in the aquatic environment where *V. cholerea* are found. There is a direct association between increasing temperature and the number of cholera cases. For instance, even a 5°C increase can double or quadruple the risk of infection [62]. Previous research indicates that incubation at 4°C is capable of inducing VBNC in the laboratory [63]. Such an extreme water temperature as 4°C is unlikely for estuaries, which have average water temperatures between 20.2 and 24.3°C [64].

A potential limitation of this study is the difficulty of maintaining non-aerated conditions throughout the entire study when analyzing oxygen limitation. When we took the static cultures out of storage every five days for serial dilutions, we temporarily introduced oxygen into the flask. In order to account for this concern, we could detect the presence of oxygen by periodically measuring oxygen levels over the course of the study.

While this study only investigated how oxygen and biotype influence entry into VBNC, this data can be used towards understanding the hierarchal influence of ecological factors that enable pathogens to persist in the natural environment. Ultimately, we hope to develop models to better understand and to predict future cholera outbreaks based on environmental variables.

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