EXPERIMENTATION AND DATA COLLECTION INVOLVING ALLEE EFFECTS
AND VIRULENCE DETERMINATIONS OF
VIBRIO CHOLERAE

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ABSTRACT

The proposed research focuses on the growth and potential risk associated with virulent spread of Vibrio cholerae. The thesis project is based upon a mathematical and computational simulation of pathogen dynamics that is validated via experimentation and data collection focused on the transmission and pathogenic evolution of Vibrio cholerae. While the larger scope of the project is focused around the importance of understanding Vibrio cholerae dynamics to create control and management strategies that may prevent future outbreaks, this thesis focuses on the experimentation and subsequent data collection surrounding the growth mechanisms of the bacterium under various environmental conditions. In addition, experimentation focuses on assessing virulence changes at different stages under varying virulence-inducing conditions that mimic those of a human host. This thesis discusses the methods used during experimentation, the rationale behind their selection, and the significance of the data collected in the larger scope of the project.
DEDICATION

Para mis papás Norma y Jose Rojas. Sin su ayuda y sacrificio no hubiera tenido la oportunidad de estudiar. Este logro es de los tres. Los amo.
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LIST OF ABBREVIATIONS

AMP, Adenosine Monophosphate

cAMP, Cyclic Adenosine Monophosphate

CFTR, Cystic fibrosis Transmembrane Conductance Regulator

CT, Cholera Toxin

CTXφ, Cholera Toxin Phage

ELISA, Enzyme-Linked Immunosorbent Assay

HRP, Horseradish Peroxidase

LB, Luria-Bertani

MIC, Minimum Inhibitory Concentration

pH, Potential Hydrogen

PBS, Phosphate Buffer Solution

PMB, Polymyxin B

SEM, Standard Error of the Mean
CHAPTER I
INTRODUCTION

A Gram-negative bacterium, the waterborne pathogen *Vibrio cholerae* remains a pandemic threat in several developing countries around the world for its ability to colonize and attack the human intestines [1, 2]. Despite the numerous advances made in pathogenic studies, *Vibrio cholerae*’s dynamic growth characteristics remain largely misunderstood systematically and quantitatively. While several models exist on the dynamics of waterborne diseases, they have limitations in the way in which they describe pathogenic transmission and spread because they often assume that pathogen growth requires a human presence. However, new evidence suggests that waterborne pathogens, such as *Vibrio cholerae*, are capable of surviving and replicating in various aquatic environments without human involvement [3].

Given this new information and advances in mathematical modeling and computation, this study seeks to generate in-vitro data to be used in mathematical models to better understand the interactions between environment, host, and pathogen. The two main areas of focus for this study involve the observation of growth patterns in environment simulating media as well as virulence assessment through cholera toxin (CT) detection. The information collected will be used to create control and management strategies in case of a cholera outbreak via mathematical computation.
It is important to note that not all the data acquired during experimentation will be used in mathematical modeling. However, the data presented includes important observations and conclusions regarding growth dynamics that are important in understanding cholera dynamics as whole. Since much is still unknown about the growth dynamics of *V. cholerae*, this study includes observations through relevant experimentation for biofilm formation, motility observations, and antimicrobial resistance testing that fall outside of the two main areas of focus for this project. Regardless, their inclusion into this study is important in understanding cholera dynamics and offers insight for future development of cholera models.
CHAPTER II

LITERATURE REVIEW

Allee Effects & Growth Dynamics

One possible explanation for the growth and persistence of *Vibrio cholerae* in varying water conditions previously not considered may be the biological phenomenon known as the Allee Effect. While quadratic growth modeling defined by lag, log, stationary, and death phases exists as the most common model to describe population changes, this study considers a cubic growth model that introduces Allee effect correlations that relate individual cell fitness to overall population density [3]. The Allee effect refers to the biological phenomenon that correlates the population density of an ecosystem directly with the average individual fitness of a population or species living in that ecosystem [1]. Allee effects were first discovered and demonstrated in a goldfish experiment where Warder Clyde Allee found that low populations cause species to lose fitness at low population densities and proceed into extinction [3]. Upon discovering certain species characteristics are dependent on the abundance of the species, Allee proposed a threshold density in which a species falling below the threshold value shows reduced fitness while species’ density above the threshold density value shows increased population fitness. Species fitness is critical for survival as higher fitness often ensures stable reproductive cycles that result in more offspring which result in the prolonged survival of the species. On the contrary, low population fitness exhibits the opposite correlation and results in
extinction. In bacterium such as *Vibrio cholerae*, a minimum population density is sometimes
required to initiate expression of virulence characteristics which eventually lead to large scale
infections [3,4].

The experimentation surrounding this study demands a concrete understanding of the
expected results and outcomes of density dependent phenomena. Previous experiments show
that weak Allee effects occur when a critical density is reached as per capita growth becomes
negative for a small population size while a strong Allee effect induces a sigmoidal relationship
between a population’s initial size and an inflection point that occurs at the critical density. A
sigmoidal relationship is one in which exponential growth is observed followed by stagnation
and eventual decline [5, 6, 7]. Furthermore, modeling of the sigmoidal relationship observed
during the study will require the establishment of a function that represents the observed
phenomena. Past studies have relied on the use of the Weibull function shown in Figure 2.1
presented as $p = 1 - e^{-\left(\frac{x}{\lambda}\right)^k}$. The usage of the Weibull function establishes an exponential
decay relationship that allows the modeling of the sigmoidal relationship. However,
interpretation of the model as an invasion probability relation results in a binomial distribution
that is not appropriate for the present research as it does not consider the environmental
conditions of growth for the bacterium that are essential in establishing future control and
management strategies in the greater scope of this project [5]. Instead, the function:

$$f(B, E)$$

presented by Dr. Jin Wang establishes both the growth of bacterial density, $B$, and the
environmental conditions (temperature, salinity, pH, etc.) in which they grow, $E$. The results
obtained from repeated experiments will be used to determine a specific form for the proposed
function as well as varying parameter values associated with growth under the proposed environmental conditions previously mentioned. A sensitivity analysis for the parameters found will be assessed using the near-random Latin Hypercube Sampling method. From the determinations made, pathogen dynamics based on the baseline environmental conditions will be connected to *Vibrio cholerae* transmission amongst human hosts to investigate the impact of the environment in the transmission, spread and persistence of waterborne disease.

![Figure 2.1 Probability of Establishment Fitted Weibull Curve [5]](image)

When conducting the experimental portion of this project, it is important to remember certain aspects that may require special consideration when investigating Allee effects. One such aspect that may require further attention is known as dispersal. Dispersal is known as the moving of a species from its site of origin to a potential replication or breeding site. Theoretical studies have predicted that dispersal can have a dual effect on population survival and spread by either preventing the colonization of new territories due to insufficient population members or by draining a source population when dispersal is too fast [7]. Using the basis of the study
conducted by Smith which programmed strong Allee Effects into *E. coli*, the research team will seek to investigate Allee effects in *Vibrio cholerae* while taking the growth patterns of the dispersion study into account as shown in Figure 2.2. By using source (central area of growth) and target (surrounding areas of growth) patches, Smith et al were able to model and predict spread rates that contribute to understanding the dynamics that control population spread. The growth rate correlations and sigmoidal growth patterns of the dispersion study suggests cooperative species with a strong Allee effect may follow unique spread dynamics, which may be dictated by the environment [6, 8]. In addition, Smith et al concluded that the populations with initial densities above a critical threshold maximize their total growth at low dispersion rates where spread does not occur, as opposed to fast spread that may result in unsuccessful replication. The conclusions drawn may offer a possible explanation as to why during biological invasions, spread is initially slow but tends to increase over time [6, 8].

**Figure 2.2** Density of bacterial populations that exhibit a strong Allee effect with varying initial densities [9]
Defining the observation of an Allee effect requires the comparison of density per capita growth rates. The positive relationship between per-capita growth and population density when population density is low defines the strength of the Allee effect observed. Figure 2.3 outlines the relationship between strong Allee effect observations and weak ones as presented by Kramer et al, 2009. Figure 2.3 shows what Allee effects look like when graphed in terms of per capita growth rate and probability of establishment. While the studies discussed thus far have all relied on the probability of establishment plot, for this study the per-capita growth rate plot is considered. In terms of per-capita growth rate, strong Allee effects are observed when a negative per-capita growth rate increases initially but ultimately returns to a critical threshold. Weak Allee effects are described in a similar fashion except the initial growth rate is not negative.

Increasing evidence suggests Allee effects play vital roles in low populations densities but populations may never experience densities low enough to observe Allee correlations in laboratory settings [14]. To provide small enough population sizes to observe Allee effects may require large differences in inoculum sizes during experimentation.
Figure 2.3 Strong and weak Allee effects in low populations [14]

Given that the larger scope of the project consists of creating management and control strategies in case of cholera outbreak, data collected from the Allee experiments is to be used mainly for Dr. Wang’s model. The key difference between Dr. Wang’s model and other models lies in the lack of knowledge surrounding disease dynamics at the host level. Additionally, much is still unknown about the effects environmental differences have on pathogen dynamics at the host level. As such, the current study examines the impact of inoculum size on key characteristics of *Vibrio cholerae* using two key conditions that are intended to mimic disease dynamics at both host and environment levels. The two conditions of interest in this study are human host and ocean since these two conditions most broadly represent the spread of *V.*
cholerae from growth to host. The challenge lies in replicating the conditions of interest as they relate to *V. cholerae* growth dynamics while ensuring growth and survivability.

It is expected that environmental differences will influence bacterial growth even if only to a minimal degree. As such, it is important the extent to which environmental variables influence growth, and how the bacteria respond to the severity of each change. As previously mentioned, to alter the conditions of each environment, three key variables are considered: salinity, pH, and temperature. Past studies have shown significant changes in *V. cholerae* growth as a function of both temperature and salinity with salinity influence increasing after environmental temperatures exceeded 15°C [9]. This suggests that warm climates and summer seasons may provide ideal temperatures for *V. cholerae* survival in brackish and ocean conditions [9]. Furthermore, salinity appears to be a key determinant in *V. cholerae* growth, with some studies suggesting optimal growth occurs in moderate salinities when temperatures are between 15°C and 25°C, but also possible in temperatures below 10°C with surviving cells remaining viable in highly saline environments until more favorable conditions return [10]. To mimic the environmental conditions *in vitro*, M9 served as a base medium that was adjusted for desired salt and pH while being supplemented with 0.4% glucose, 0.4% casamino acids, 2mM MgSO₄, and .01mM CaCl₂.

The influence of temperature and salinity on *V. cholerae* growth, while obvious, may not be the only two factors worth considering for bacterial growth. Agar and growth media nutrients have also been deemed as important factors to consider in *V. cholerae* growth with significant correlations between plate counts and nutrient concentrations detected at high nutrient concentrations [11]. Interestingly, it has been reported that Gram-negative bacteria
like *Vibrio cholerae* undergo cell division and an increase in cell numbers through reductive division as a survival mechanism during times of nutrient deprivation [15]. Given the discontinuity in nutrient concentrations observed in aquatic environments, combined with spatial and seasonal variations in nutrients, HEPES buffer was selected for its ability to maintain physiological pH levels steady in cell cultures while yielding more viable cells compared to TES buffered media, providing a more nutritionally rich environment at a pH range of 7.2 – 7.4 [12, 13]. The importance in maintaining nutrient rich environments during this study is focused on limiting and minimizing unexpected variations between experiments. Without enough nutrients, it becomes more difficult to differentiate the causes of change in population densities, considering that previous experiments performed between nutrient starved cells and healthy cells showed initial cell divisions began at approximately the same time [15]. Other options like bicarbonate buffers tend to be CO₂ dependent and often result in skewed pH levels if cultures are handled for long periods of time outside of the incubator. The importance in maintaining pH levels steady during this project does not stem from growth concerns regarding *V. cholerae* but rather due to the colonization and toxin secretion that occurs in the human intestine due to changes in pH [16].

**Assessment of Virulence**

The first leg of this project focuses on Allee effects and growth dynamics of *V. cholerae* as a product of different environments. The second half, however, involves understanding the virulence changes that occur in both the human body and ocean conditions to bridge the knowledge gap between bacterial growth and virulent transmission. In fact, little is known
regarding differentiation between the more than 200 *V. cholerae* serogroups that occur in nature. However, it’s commonly accepted that the O1 and 0139 serogroups might be largely responsible for most of the cholera pandemics that have occurred in recent time [21, 22]. While cholera may have been present in ancient populations, modern history dates the first cholera outbreak at the beginning of the 19th century with the 1st pandemic occurring in 1817 from its endemic area in South-East Asia [23]. Most recently, *V. cholerae* 0139 was found in Bangladesh and India removing previous assumptions that the O1 serogroup was the only serogroup able to expand into a pandemic [24]. However, since 2005, cholera cases in Bangladesh have been reported as a product of the 01 serotype entirely [25]. It is not well understood why the O1 serogroup is associated with large scale infections, but due to its consistent association with virulence, this specific serogroup will be the only group discussed throughout.

For this portion of research, the primary focus is centered on assessing the changes of virulence to distinguish different infectious stages of Vibrios and quantifying their evolution in human and ocean conditions. *V. cholerae* transmission from ocean conditions to human conditions relies on lifestyle changes that function by regulating phenotypes such as biofilm formation and motility as a means of survival in conditions that vary by salinity, pH, and temperature [16, 17]. *V. cholerae* cells are considered highly motile, which greatly influences virulence at early stages of infections. Studies have concluded that motility of *V. cholerae* correlates directly to the expression of Cholera toxin (CT) which penetrates the mucous layer in the human body and allows access to the underlying epithelial cells in duodenal conditions [17,18]. Moreover, environmental changes in the human body, from high viscosity mucous to less viscous mucous (water-like) results upon damage to the intestinal lumen as a result of the
expression of CT at high densities [29]. *V. cholerae* seems to detect increases in viscosity as it swims which trigger noticeable increases in virulent gene expression [29]. However, to be an effective pathogen, *V. cholerae* must repress virulence gene expression and initiate motility to escape from human defense mechanisms or risk dying to host adaptive immunity [29, 30].

Expression of signaling molecules that increase motility have been shown to directly correlate with higher cell densities that also facilitate cellular processes and downregulation of CT [33].

Since Cholera possesses many features that differ from other types of infections, little is known about the function of CT in phenotypic expression. Attempts have been made in understanding the role cholera-toxin phage (CTXφ) plays in the pathogenesis of *V. cholerae* as it transfers its DNA into bacterial cells to form cholera toxin, whose severity is far greater than that of ingested environmental Vibrios and causes the diarrhea symptom commonly present in cholera infections [18, 19]. Cholera toxin operates by stimulating membrane bound adenylate cyclase and binding specifically to cell surface glycolipid receptors known as G_M1 gangliosides [26]. Consisting of two subunits, A and B, CT increases the levels of cyclic AMP within host cells resulting in an electrolyte imbalance caused by the trans-membrane conductance regulator (CFTR) that decreases the influx of sodium ions and leads to mass water efflux through the intestinal cells, producing the severe diarrhea and vomiting often seen in Cholera infection cases [27]. The CFTR protein is a cAMP activated Cl⁻ channel found in the lining of epithelial cells located in mammalian airways, intestines, pancreas, and testis [28].

The noted influence of cell densities on virulence expression relates the second portion of this study to the Allee effect experimentation discussed during the first portion. Quorum sensing presents a possible explanation to *V. cholerae*’s selective expression of virulence factors
to maintain a balance between biofilm formation and motility to increase the spread of new colonies [4, 17, 30, 31]. Quorum sensing is the regulation of gene expression in response to fluctuations in cell-population density [32]. The expression of virulence in V. cholerae is controlled by several systems and regulatory cascades that are initiated by changes in the environment and detected by the cells through population density [29]. Most notably, quorum sensing in the intestines is important to establish colonization of bacterial agents which are responsive to stimuli like pH and bile production [17, 29]. Expression of certain regulators, like hapR (a negative virulence regulator), is repressed at low cell densities but becomes active during late stages of colonization when cell numbers are high, negatively affecting virulence gene expression [33]. Studies suggest the shift in temperature to 37°C and the highly acidic pH V. cholerae encounters upon entry into the stomach may result in the expression of specific proteins that affect gene expression to enhance survival and promote production of virulence factors [29]. For example, the secretion of the protease enzyme, prtV, is a product of the expression of the hapR regulator in human conditions functioning to produce an inflammatory response in the blood and epithelial tissue. The same enzyme, in aquatic environments, prevents protozoal grazing at high cell densities and regulates the transcription of other enzymes that cleave biofilm proteins [33]. Furthermore, observations encountered in recent studies of virulence operons indicated that five or more operons were responsive to pH under aerobic growth conditions and anaerobiosis at neutral pH. This implies that low pH encountered in the stomach after ingestion, followed by pH neutralization and reduction of oxygen, serve as environmental indicators used to correctly time virulence expression and colonization [29].
Another key aspect to consider in the virulence assessment of *V. cholerae* is the impact of salinity. The transition between the aquatic environment and the human intestine exposes the *V. cholerae* cells to varying salt concentrations with evidence suggesting that motility and biofilm formation are inversely regulated in an osmolarity dependent relationship [29]. It’s argued that one of the functions of CT is to generate a high Na⁺ environment in the lumen of the intestines, with studies showing the clear relationship between CT and electrolyte levels in the intestinal lumen [36]. Studies also show that *V. cholerae* is able to sense changes in sodium levels and respond with transcriptional mutations that trigger virulence gene expression, as is the case with the *nqr* sodium ion gradient and the virulence regulator toxT [29].

To emulate the virulence inducing conditions of the small intestine, AKI media will be used. AKI media is considered a highly favorable medium for stimulating the production of CT when prepared with 1.5% Bacto™ peptone, 0.4% yeast extract, 0.5% NaCl, and 0.3% NaHCO₃ at a pH of approximately 7.4 – 7.6 with cultures grown at 37°C [34]. Furthermore, AKI media resembles the sodium bicarbonate levels found in the upper small intestine which is believed to be another virulence inducer in *V. cholerae* [35]. To test the differences in toxicity between human and ocean conditions, the same HEPES buffer media from Allee experimentation will also be used for ELISA testing.

ELISA stands for Enzyme-Linked Immunosorbent Assay and is used to measure either antigen or antibody through experimental design [37]. For experimentation, the G₄M₁ ganglioside previously discussed is to be used as a key metric in the detection of CT with toxin production expressed in µg of toxin per culture OD₆₀₀ [38]. Production of CT will be analyzed *in vitro* by using a polyclonal anti-CT HRP-conjugated antibody grown in the specific media of
interest (Human or Host) with polystyrene microwell plates coated in $G_{M1}$ diluted with PBS/Tween. Purified CT will serve as a control measure [39, 40, 41]. HRP catalyzes a color change of the TMB substrate. The reaction is quenched with the addition of $H_2SO_4$, and CT production is measured via absorbance. Ideally a microtiter plate reader should be read at an $OD_{450}$ absorbance [41]. The purified CT readings will serve to create a calibration curve to determine the amount of CT present in the different media conditions tested. Plate layouts considered during experimentation in can be found in Appendix E.

Developing a full understanding of *V. cholerae* virulence also requires consideration of antibiotic resistance. Experimentation using the antibacterial polymyxin B is a common measure of stress resistance. Polymyxin B (PMB) resistance is important to consider as the pandemic of 1960 caused by the El Tor strain was reported as resistant to PMB, allowing for an easier distinction between the O1 and El Tor strain [42]. Most notably, studies show the expression of biofilm regulating genes are expressed in correlation with the PMB sensitivity of bacterial cells grown in LB media in the O1 serotype [43]. For this experiment, a simple PMB minimal inhibitory concentrations (MIC) assay will be used to assess the sensitivity to PMB as it pertains to the human and environmental conditions tested in all previous experiments performed in this project. Antibacterial resistance, of course, is an important variable that may need further consideration when developing risk and management models. While not critical to the success of this project, understanding the environmental effects *V. cholerae* exhibits when exposed to antibacterial agents may offer a better understanding of pandemic events.
Statistical Significance

Considering that the data collected in this project is to be used for mathematical modeling, the statistical significance of the data must be evaluated. By using standard error of the mean and regression statistical tools, the relationship strength between some variables in the data can be determined prior to usage in modeling and simulation. Mainly, it is important to ensure the repeatability of the data collected during this study and show that the relationships presented herein are statistically significant to warrant consideration in predictive models. This does not mean that every data set collected will be used in modeling as incorporation of data is discretionary in nature to be determined by Dr. Jin Wang in future research and computer models.

Standard Error of the Mean

The complexity of this investigation requires the use of several data analysis methods to better understand the expected results and determine a confidence level for said results. Two of the methods to consider for this investigation are the standard error of the mean and the p-value used in regression statistics. The standard error of the mean (SEM) tells us how many mean variations occur during experimentation. If the effects of random changes between sample readings are significant, then the SEM will be higher. If there is no change in the data points as experiments are repeated, then the SEM will approach zero [44]. For this study, the SEM of the ELISA experiments will be determined to assess differences amongst triplicate wells to create a confidence interval for the data tested. Major changes in SEM during ELISA are expected only in cases of contamination or procedural error.
Regression Statistics

Regression analysis provides an equation for the statistical relationship observed between variables. The p-value tests the null hypothesis that one of the equation coefficients is equal to zero, indicating a statistically insignificant relationship between the variable and the outcome presented. P-values below 0.05 indicate a strong relationship between that specific variable and the result of the data tested. Similarly, large p-values mean changes in the variable analyzed have little to no relationship with the associate output observed [45]. For the purposes of this study, the main relationships analyzed will be optical density and CT production as a function of pH, temperature, and salinity.
CHAPTER III

METHODOLOGY

In order to simulate \textit{in vivo} conditions, the media will be carefully prepared in terms of pH, temperature, and salinity. As previously discussed, both human and ocean conditions require certain buffer changes that differ to those of traditional culture media preparation. While uncommon for most studies, the methodology for making the media buffer will be presented in this study given its relevance to the expected outcomes being investigated.

\textbf{Human Host Buffer (pH 7.1, 150 mM NaCl, 37°C)}

1. 5.957g of HEPES free acid was added to approximately 90 ml of pure water.

2. 0.359g of NaCl was added to the solution.

3. After the NaCl addition the solution was titrated to a pH of 7.26 at an approximate temperature of 25°C.

4. During titration a monovalent strong base or acid was used as needed.

5. Make up water was added to the solution until volume reached 100 ml.

6. At a temperature of 37°C the buffer pH should read as 7.1.

\textbf{Ocean Buffer (pH 8.1, 600 mM NaCl, 30°C)}

1. 5.957g of HEPES free acid was added to approximately 90 ml of pure water.
2. 2.304g of NaCl was added to the solution.

3. After the NaCl addition the solution was titrated to a pH of 8.17 at an approximate temperature of 25°C.

4. During titration a monovalent strong base or acid was used as needed.

5. Make up water was added to the solution until volume reached 100 ml.

6. At a temperature of 30°C, the buffer pH should read as 8.1.

Allee Effects and Growth Rates

1. Overnight cultures of *V. cholerae* strain El Tor c6706 were incubated in Luria Bertani (LB) broth at 37 °C for 16-24 hours.

2. Cultures were then transferred to centrifugation tubes for centrifugation to pellet cells and to remove rich media supernatant.

3. The pellets were resuspended in 1 mL phosphate buffer solution (PBS) to be used for dilutions.

4. Host conditions media (pH 7.1, 150 mM NaCl, 37 °C), based on human duodenal conditions, and aquatic condition media (pH 8.1, 600 mM NaCl, 30 °C) were prepared with HEPES buffer as previously mentioned with their respective NaCl concentrations.

5. The media was then titrated to the pH values that would adjust to their intended values at their respective temperatures.

6. 1mL 30% glucose, 0.1 mL 1M MgSO₄, 5 μL 1M CaCl₂, and 1 mL casamino acids were added to the HEPES media per 50 mL prior to preparing dilutions.
7. For growth rate: Resuspended cells were checked for optical density (OD) at 600 nm, which was used to calculate the amount of the overnight culture to be used to make a 0.05 OD sample for the respective conditions in HEPES media.

8. This 0.05 OD sample was used for various ten-fold serial dilutions from $10^{-3}$ to $10^{-10}$ dilution factors. Samples were incubated while shaking at their respective conditions’ temperature.

9. OD values were tracked at regular intervals to record growth over time. LB agar plates were inoculated with 100μL of specific dilution samples and incubated for 12 hours to determine the number of colony forming units (CFU) for calculating estimated number of cells for each dilution.

**Effects of Inoculum Size on Swimming Motility**

1. Resuspended cells were prepared at 0.1, 0.075, and 0.05 OD, and the 0.05 OD sample was used for 1:2 dilutions ($5 \times 10^{-1}$, $2.5 \times 10^{-1}$, $1.25 \times 10^{-1}$, $6.25 \times 10^{-2}$, $3.125 \times 10^{-2}$, $1.5625 \times 10^{-2}$, $7.8125 \times 10^{-3}$) using PBS.

2. Soft agar (0.3% agar) plates were used to assess swimming motility for all ten dilutions in both ocean and host conditions.

3. Plates were subsequently inoculated with 2μL within soft agar.

4. Swimming agar plates contained 0.5% Bacto™ peptone, 0.3% Bacto™ agar, 0.3% yeast extract, and their respective molar concentrations of NaCl.

5. Using a ruler, the size of a colony’s diameter was measured to assess changes in motility.
Effects of Inoculum Size on Swarming Motility

1. Resuspended cells were prepared at 0.1, 0.075, and 0.05 OD, and the 0.05 OD sample was used for 1:2 dilutions ($5 \times 10^{-1}$, $2.5 \times 10^{-1}$, $1.25 \times 10^{-1}$, $6.25 \times 10^{-2}$, $3.125 \times 10^{-2}$, $1.5625 \times 10^{-2}$, $7.8125 \times 10^{-3}$) using PBS.

2. Swarming plates (0.5% agar) plates were prepared to assess motility for all ten dilutions in both ocean and host conditions.

3. Plates were subsequently inoculated with 2μL on the agar surface.

4. Swimming agar plates contained 0.5% tryptone, 0.5% Bacto™ agar, 0.25% Bacto™ yeast extract, and their respective molar concentrations of NaCl.

5. Using a ruler, the size of a colony’s diameter was measured to assess changes in motility.

Effect of Inoculum Size on Biofilm Formation in *V. cholerae* grown in host and ocean conditions

1. Resuspended cells were prepared at 0.1, 0.075, and 0.05 OD, and then 0.05 OD sample was used for 1:2 dilutions ($5 \times 10^{-1}$, $2.5 \times 10^{-1}$, $1.25 \times 10^{-1}$, $6.25 \times 10^{-2}$, $3.125 \times 10^{-2}$, $1.5625 \times 10^{-2}$, $7.8125 \times 10^{-3}$) in their respective media.

2. 96-well microtiter plates were used for incubating sextuplet sets of culture samples for all ten dilutions in both host and ocean conditions.

3. The outer surrounding 36 wells were filled with media only or sterile $dH_2O$ to limit edge effects and determine background staining.
4. Plates were then incubated at 37°C for 24 hours or at 30 °C for 48 hours, after which they were emptied, then subjected to crystal violet (CV) staining used 0.1% CV for 15 min.

5. After washing with \( dH_2O \), wells were left to dry before being filled with 225\( \mu \)L 30% acetic acid to dissolve stained biofilms.

6. The acetic acid mixture was transferred to fresh 96-well plates to be read at 590 nm using a BioTek Microplate Reader.

**Reading of Optical Density**

Optical Density measurements occur frequently when performing experiments with density dependent phenomena. A 1/10 dilution was prepared in the following form and measured at either 30-minute, 60-minute, or 120-minute time intervals.

1. Spectrometer wavelength was set at 600nm and zeroed with 900\( \mu \)L of deionized water.
2. 100\( \mu \)L of bacterial culture was added aseptically to a cuvette.
3. 900\( \mu \)L of deionized water were added to the same cuvette. It is important to that water be added after culture to allow for better mixing and results reading.
4. Cuvettes were added into the spectrometer and reading was recorded.
5. Cultures were placed back in incubation until the established time of experiment was completed.
Polymyxin B MIC Assay

1. Overnight cultures of *V. cholerae* strain El Tor c6706 were incubated in Luria Bertani (LB) broth at 37 °C for 16-24 hours.

2. Either pH 7.1 or pH 8.1 HEPES buffer was used to simulate ocean and human conditions.

3. Cultures were then transferred to centrifugation tubes for centrifugation to pellet cells and to remove rich media supernatant.

4. Instead of pellet resuspension, the culture was washed with the HEPES media and centrifuged for approximately five minutes.

5. Resuspension followed with 1000μL of HEPES buffered media.

6. The culture was then incubated on a shake table at 37°C and 200 rpm.

7. Polymyxin B dilutions were made via a microscale to make 6:1 μg/mL dilutions.

8. After approximately four hours of growth, the inoculum OD was measured and resuspended before a final suspension.

9. Inoculum 1:2 dilutions (1 × 10^8, 5 × 10^7, 2.5 × 10^7, 1.25 × 10^7, 6.25 × 10^6, 3.125 × 10^6, 1.5625 × 10^6, 7.8125 × 10^5) were prepared in preparation for the assay.

10. Assay additions occurred in aseptic hood environment wiped down with EtOH.

11. In the hood, 170μl of inoculum and 30μl of diluted PMB was added to the appropriate wells.

12. The plate was incubated at 30°C - 37°C overnight.

13. After overnight incubation, a plate reader measured absorbance at 600nm.

14. Important notes:
a. Do not label plate until all items are cleaned and under the hood to maintain sterility.

b. When adding inoculum and PMB, keep plate as covered as possible to prevent contamination.

c. When using a multi pipette check the tips before placing either inoculum or PMB in each well to insure equal amount of uptake in each tip.

**Toxin Secretion and Virulence Determination Using ELISA Test**

1. Prepping Reagents

   a. Overnight cultures of *V. cholerae* El Tor C6706 were incubated in Luria Bertani (LB) broth at 37 °C for 16-24 hours.

   b. GM1 was diluted in PBS at a concentration of 1µg/mL and stored for plate coating. Plate designs can be found in Appendix E.

   c. Purified cholera toxin (CT) was diluted in water (1mg/1mL) and subsequent dilutions were prepared in PBS (100 ng/mL, 10ng/mL, 1 ng/mL, 0.1 ng/mL).

   d. Antibodies were diluted in 15 ml conicals with PBS/Tween Blocking Buffer.

   e. To stop the reaction, 0.2 M H₂SO₄ was made up from 2M H₂SO₄.

2. Plate coating

   a. 100µg of GM₁ was added to the appropriate number of wells (according to plate design).

   b. The plate was then covered with a plate sealer and allowed to incubate at room temperature overnight.
c. After overnight incubation the plate was washed three times with PBS/Tween as follows:

i. The plate was inverted over a sink and then gently tapped onto an absorbent towel to remove overnight contents.

ii. A multi pipette was used to add 200µL of PBS/Tween to the inner 60 wells.

iii. After 3 minutes of standing, a wash step was repeated two more times.

iv. For storage, 150µL of PBS/Tween was added and left in the plate. The plate was then stored in the refrigerator.

3. Bacterial growth

a. Cultures were transferred to centrifugation tubes for centrifugation to pellet cells and to remove rich media supernatant.

b. Resuspended cells were prepared with the 0.05 OD sample used for 1:5 dilutions 
\((1 \times 10^8, 2.5 \times 10^7, 6.25 \times 10^6, 1.5625 \times 10^6, 3.9 \times 10^5, 9.8 \times 10^4, 2.4 \times 10^4, 6.1 \times 10^3)\) or 1:12 dilutions 
\((1 \times 10^8, 8.3 \times 10^6, 6.9 \times 10^5, 5.7 \times 10^4, 4.75 \times 10^3)\) in HEPES buffered media representative of either human or host conditions.

i. For the initial run, AKI media was used (1.5% Bacto™ peptone, 0.4% yeast extract, 0.5% NaCl, and 0.3% NaHCO₃ at a pH of approximately 7.4 – 7.6).

c. After dilutions were made, cells were incubated for approximately 3 to 4 hours.

d. Upon completion of incubation, cells were centrifuged. The supernatant was kept and the pellet discarded.
4. ELISA Test
   
a. After washing, the remaining blocking sites were filled with 150µL of PBS/Tween.

b. The plate was then left to incubate at room temperature for 30 min.

c. After incubation, the plate underwent three more washing steps.

d. 100µL of supernatant from each bacterial dilution was added to the appropriate number of wells.

e. 100µL of CT was added at each concentration in the appropriate wells.
   
i. Note: CT should be run in duplicate minimum.

f. 100µL of negative PBS controls were added to the appropriate wells.

g. The plate was covered and incubated for approximately one hour at 37°C.

h. The contents were removed, and another 3 wash steps were performed.

i. Next, 150µL of PBS blocking buffer was added and the plate was incubated at 35°C for another hour.

j. The contents were removed, and three more washing steps took place.

k. 100µL of polyclonal antibody at the 1:500 concentration was added to each well and the plate was left to incubate at 37°C for another hour.

l. After incubation, three more wash steps occurred.

m. 100µL of TMB was added to each well and the plate was left to incubate until blue color changes are spotted in the controls (approximately 5-10 minute wait).

n. Once color changes appear, the reaction was stopped using 50 µL of the 0.2M H₂SO₄.

o. The plate was read in a plate reader at an OD₄₅₀.
CHAPTER IV

RESULTS

Allee Effects and Growth Rates – Non-Contaminated Runs

Trial 1 – 8/1/2018

The basis of this experiment was centered around understanding the growth changes V. cholerae exhibits in response to different environmental conditions. Figure 4.1 is one of twelve experiments performed attempting to explain growth density as a product of Allee effect observations. As seen on Figure 4.1, sigmoidal growth is obviously observable with ODs correlating directly with inoculum size as was expected. Growth curves show similar growth in both human and ocean conditions albeit human condition curves showed more accelerated initial growth. No definite Allee effects were observed with only two samples showing increased growth after a critical point was reached occurred at both smallest inoculum sizes in human and ocean conditions.

Salinity concentration was raised from the procedure listed in the methodology chapter to establish baseline growth conditions to insure V. cholerae would grow in the HEPES buffer before more variable manipulations could take place. Additionally, the first run was performed using CM9 media considering contents of media required for growth were comparable to those of HEPES for an initial run. Bacterial CFU counts were obtained through spread plating and showed no signs of contamination in the media.
Figure 4.1 *Vibrio cholerae* C6706 Trial 1 – 300 mM NaCl vs. 600 mM NaCl, 37°C

**Trial 2 – 8/3/2018**

A follow up run maintained the high salt concentration for human environment media producing similar results to those seen in figure 4.1. Figure 4.2, visually, follows a similar growth pattern to trial 1 with no distinct Allee effects present in the growth curves. As noted previously in chapter 2, strong Allee effects are dictated by per-capita growth relative to a critical threshold density. In human conditions, at low concentrations, the second experiment performed seems have slower initial growth despite inoculum size. The most interesting observation stems from the middle inoculum in ocean conditions since this appears to be the only sample that maintained growth after a critical density was reached but the observation is inconclusive since
Reinforcing the original observations of the first run, inoculum size presents a strong correlation to growth as dictated by the red and green lines presented in Figure 4.2, representing host and ocean conditions respectively. Although, not as pronounced as in the first run, inoculum size has a noticeable effect on initial growth and eventual density decrease of cells. The differences in temperature, pH, and salinity on growth were not as pronounced in this run as in the first run.

Figure 4.2 *Vibrio cholerae* C6706 Trial 2 – 300 mM NaCl vs. 600 mM NaCl, 37°C

**Trial 3 – 8/7/2018**

The third trial varied the growth of bacteria in ocean conditions by altering incubation temperature to 30°C. Figure 4.3 shows growth patterns that decrease by inoculum size and
media type confirming the observations seen in the previous two trials. As in the previous two experiments, large inoculums exhibit stronger initial growth as is expected due to the large number of starting cells. Unlike the previous two experiments, lag time varies significantly with stagnated occurring at variable times. However, a noticeable trend between human host conditions and growth continues from the previous two experiments with human conditions exhibiting rapid initial growth but eventual decrease in growth. Spread plates performed during the experiment showed no contamination.

Figure 4.3 *Vibrio cholerae* C6706 Trial 3 – 300 mM NaCl vs. 600 mM NaCl, 37°C
The fourth growth curve of Allee effects observations presented on Figure 4.4 continues previous trends observed thus far. Inoculum size is once again a key factor in density dependent growth. The main difference from previous growth curves is the temperature in which the bacteria cultures were incubated. Following the exact temperature specifications for the environmental conditions of primary interest, observations during this experiment were focused around keeping pH stable. PH values during this experiment were found to have dropped from 7.1 and 8.1 to 6.3 and 6.25 respectively. The drastic change in pH resulted in a proposed change of media from CM9 to HEPES for trials 5 and beyond.

Besides the temperature changes, experimental growth followed previous trend with inoculum size correlating directly with growth. Ocean conditions, again, were seen to exhibit the longest lag time in growth produced the only two curves that increased in density after the critical density point was reached.
Figure 4.4 *Vibrio cholerae* C6706 Trial 4 – 300 mM NaCl, 37°C vs. 600 mM NaCl, 30°C

**Trial 6 – 8/24/2018**

The run presented in Figure 4.5 was the continuation of temperature variation testing exhibited in trial 4. The importance behind the changes to temperature incubation was a direct result of the ideal conditions of interest as they relate to *V. cholerae* growth. Moreover, trial 5 experimentation was altered so salinity reflected that of human blood, as 300mM NaCl is relevant to human skin while 150mM NaCl resembles human blood which is a much better representation of the intestinal conditions of the human body. Salinity changes were not the
only alterations to this experiment as HEPES media was introduced to maintain pH testing consistent with the appropriate condition temperature.

The observations from this experiment when compared to previous runs produced vastly different growth curves indicating salinity and temperature effects on growth might be greater than originally thought. Inoculum size still correlated with increased bacterial density. Ocean conditions exhibited poor growth overall and deviated greatly from the initial curves observed in trials 1 – 4. Our collaborative research group theorized that changes in media may have limited nutrition for growth. However, HEPES supplemented with glucose and case amino acids was considered appropriate for V. cholerae density-dependent experimentation as was discussed in the literature review. Another possible explanation for the vast difference in results might be a synergistic effect involving temperature, pH, and salinity that is amplified compared to a singular change in any one variable. One thing is certain, inoculum size is a key contributor to growth dynamics regardless of environmental conditions.
Trial 7 – 8/27/2018

Trial 7 of the *V. cholerae* growth experiments, presented by Figure 4.6, was an identical run to that of trial 6 with the only variation being increased time intervals for OD readings. Upon examination of the growth curve, larger optical density measurements are instantly visible for human conditions. No major alterations to the testing indicate possible poor preparation of media contributed to much higher densities than previously observed. Regardless, no contamination was found on streak plates and as such the data from this run, while confusing, still demonstrates the influence differing conditions have on growth dynamics.
Conversely, Allee effect correlations are somewhat observed in ocean condition at 0.05 inoculum as density growth rate decreases at what is already a low density.

Figure 4.6 *Vibrio cholerae* C6706 Trial 7 - 150 mM NaCl, pH 7.1, 37°C vs. 600 mM NaCl, pH 8.1, 30°C

**Trial 9 – 9/06/2018**

A third identical trial to that of trial 6 was performed. Instead, readings after 13.5 hours were unusually low compared to previous runs as shown in Figure 4.7. No contamination was observed in streak plates dictating low cell counts may have been a product of improper growth of cultures in LB broth. Furthermore, besides the high optical density observations noted again for human conditions, no Allee correlations were observed as growth rates never decline at low
density thresholds. Temperature, pH, and salinity effects, while still present, are also difficult to assess given the strange outcome of the growth curve. Inoculum size continues to be influential in growth at all density levels.

Figure 4.7 *Vibrio cholerae* C.6706 Trial 9 – 150 mM NaCl, pH 7.1, 37°C vs. 600 mM NaCl, pH 8.1, 30°C

**Trial 10 – 9/14/2018**

The penultimate of the non-contaminated runs, trial 10 was focused on smaller inoculum sizes at longer periods of time as is shown by figure 4.8. Readings were taken at longer intervals and ODs recorded every two hours. Changes in growth due to longer growth periods and inoculum changes were not as pronounced as expected. It is believed Allee effects
are more visible at lower densities, but little evidence points to an Allee effects present in this trial. Differences in environmental conditions seemed to do little to nothing related to growth. Perhaps the flaw in this trial was extending OD reading intervals instead of increasing their frequency over the 48hr observation period. Inoculum size, while hard to notice, still appears to be the most influential variable in bacterial growth.

Figure 4.8 *Vibrio cholerae* C6706 Trial 10 – 150 mM NaCl, p.H 7.1, 37°C vs. 600 mM NaCl, pH 8.1, 30°C – Extended Dilutions

**Trial 12 – 10/05/2018**

Figure 4.9 presents the growth curve results of trial 12. Diluted CFU counts discussed in trial 10 were further diluted to observe density changes over long periods of time. The results
of the trial concluded as expected with no bacterial growth occurring at the smallest inoculum sizes. Human conditions presented the most growth although an Allee effect correlation appears to manifest at ocean conditions. However, such an observation is difficult to establish as an Allee correlation given that no previous growth had been recorded. It is more plausible that the OD was erroneous as is often the case at very low absorbances. Growth was limited almost exclusively to human conditions suggesting highly saline conditions may prove too difficult to adapt to for bacterial growth at small inoculum size. The findings may also suggest moderate temperatures provide more suitable growth environments for bacterial cultures regardless of salinity or pH.

Figure 4.9 Vibrio cholerae C6706 Trial 12 – 150 mM NaCl, pH 7.1, 37°C vs. 600 mM NaCl, pH 8.1, 30°C – Extended Dilutions
Contaminated Runs

Trials 5, 8, and 11 were not included in the findings due to contaminants found on spread plates. Ideally, certain conclusions could still be reached from the data collected from contaminated runs. However, it is too difficult to distinguish between changes caused by variable manipulation and changes caused by contamination. The growth curves for the contaminated runs can be found in Appendix B.

Effects of Inoculum Size on Biofilm Formation, Swarming Motility, & Swimming Motility

Figures 4.10 and 4.11 present the findings of swimming motility experimentation while Figures 4.12 and 4.13 present the findings of swarming motility using the 1:2 dilutions from previous growth experiments. Swimming and swarming plates were used to test the effects environmental differences had on V. cholerae behavior. For measurements, diameters were measured in millimeters with values less than 3mm being omitted from records. Ocean conditions required longer growth times than host conditions with the rate of swimming motility under host conditions being considerably higher than in ocean conditions (Host: 1/67 mm/hr vs. Ocean: 0.222 mm/h). Inoculum size proved an important factor in motility with a significant difference between $1.25 \times 10^7$ and $6.25 \times 10^6$ dilutions. This finding indicates inoculum size has a much stronger effect for swarming phenotypes specifically in host conditions. In fact, the smallest inoculum size under host conditions established detectable motility growth within the allotted study time, while motility in the ocean condition required an additional ten-fold increase in inoculum size before exhibiting detectable growth. The rate of swarming motility in V. cholerae under host conditions is significant when compared to ocean
conditions (0.45 mm/h vs. 0.09 mm/h). Inoculum size did not appear to have an effect on swarming motility under ocean conditions. Perhaps the most interesting finding stems from differences in swarming phenotypes which were observed based on environmental conditions and inoculum size. There was a distinct swarm size breakpoint based on inoculum size for host and ocean conditions. Furthermore, Figures 4.14 and 4.15 show biofilm formation correlates directly with inoculum size in host and ocean conditions, with less overall biofilm being produced in ocean conditions.

Figure 4.10 Effects of Inoculum Size on Swimming Motility – Host Condition
Figure 4.11 Effects of Inoculum Size on Swimming Motility – Ocean Condition

Figure 4.12 Effects of Inoculum Size on Swarming Motility – Host Condition
Figure 4.13 Effects of inoculum size on Swarming Motility – Ocean Condition
Figure 4.14 Effects of Inoculum Size on Biofilm Formation in *V. cholerae* – Host Condition
Figure 4.15 Effects of Inoculum Size on Biofilm Formation in *V. cholerae* – Ocean condition

Morphologies of swarm colonies varied between host and ocean conditions. Environmental conditions appeared influential in affecting swarming motility phenotypes and introducing Allee effects. Selective differences in swarm size are clearly noticeable based on inoculum size and incubation period. Vast differences were also visible in appearance between swarm formed under host versus ocean conditions from the same inoculum size. A color difference was observed between the white color observed in, formation of rings and upward growth in host conditions as compared to the cream color, smooth and uniform appearance in ocean conditions. The difference in incubation time is necessary to illustrate swarm morphology, since the swarming in ocean conditions is significantly slower. Phenotypic
expression photographs of swarming plates in both human and host conditions can be found in Appendix C.

**Virulence Results**

**ELISA Testing**

The ELISA experiments conducted were designed to test the level of CT secreted by *V. cholerae* in different environments. Tables 4.1 and 4.2 show both the plate design and absorbance readings from each experiment. Initial analysis of the first plate shown on Figure 4.1 indicates higher CT levels detected in AKI media and human host media (7.1 pH, 150mM, 37°C). CT presence correlates directly with inoculum size with AKI media having the largest amount of CT present at the lowest inoculum size. The results from the second experiment shown on Figure 4.2 also show noticeable differences in CT detection based on media. Ocean media, once again, has the lowest CT levels although not by much. Inoculum size also correlates directly to the amount of CT detected in each well. Neither plate appears to have any contaminants as PBS wells show mostly as static in all six wells. The purified CT control from both experiments shall serve as a calibration curve to present the absorbance findings in terms of density.
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**Key**

- **CT/PBS**
- **AKI Media**
- **Moat**

Table 4.1 ELISA Assay Experiment 1 Plate Layout and Absorbances
### Polymyxin B MIC Assay

The polymyxin B MIC assay was designed to test antibacterial resistance in human and ocean conditions. Figures 4.16 and 4.17 show the results of the assay in human and ocean simulating media respectively. Figure 4.16 presents the preliminary data for antibiotic resistance in human conditions. Similarly, Figure 4.17 present the minimum inhibitory concentrations for polymyxin B for *V. cholerae* at ocean conditions. Table 4.3 shows the MICs for human conditions and Table 4.4 shows the MICs for ocean conditions.

#### Table 4.2 ELISA Assay Experiment 2 Plate Layout and Absorbances

<table>
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<th></th>
<th>Moat</th>
<th>10&lt;sup&gt;8&lt;/sup&gt;</th>
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<th>10&lt;sup&gt;5&lt;/sup&gt;</th>
<th>10&lt;sup&gt;4&lt;/sup&gt;</th>
<th>10&lt;sup&gt;3&lt;/sup&gt;</th>
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<th>10ng</th>
<th>1ng</th>
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<td>0.05</td>
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<td>OVRFL W</td>
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<td>2.06</td>
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<td>0.05</td>
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<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
<td>Moat</td>
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</table>
Figure 4.16 Antibiotic Resistance in Human Conditions

Table 4.3 MIC of Polymyxin B at Human Conditions

<table>
<thead>
<tr>
<th>Culture</th>
<th>MIC (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05</td>
<td>400</td>
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<tr>
<td>2×10^7</td>
<td>400</td>
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<tr>
<td>4×10^6</td>
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<tr>
<td>8×10^5</td>
<td>200</td>
</tr>
<tr>
<td>1.6×10^5</td>
<td>200</td>
</tr>
<tr>
<td>3.2×10^4</td>
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<tr>
<td>6.4×10^3</td>
<td>100</td>
</tr>
<tr>
<td>1.28×10^3</td>
<td>100</td>
</tr>
</tbody>
</table>
Figure 4.17 Antibiotic Resistance in Ocean Conditions

Table 4.4 MIC of Polymyxin B at Ocean Conditions

<table>
<thead>
<tr>
<th>Culture</th>
<th>MIC (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05</td>
<td>400</td>
</tr>
<tr>
<td>2x10^7</td>
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</tr>
<tr>
<td>4x10^6</td>
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<td>8x10^5</td>
<td>400</td>
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<tr>
<td>1.6x10^5</td>
<td>100</td>
</tr>
<tr>
<td>3.2x10^4</td>
<td>100</td>
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<tr>
<td>6.4x10^3</td>
<td>10</td>
</tr>
<tr>
<td>1.28x10^3</td>
<td>0</td>
</tr>
</tbody>
</table>
In both conditions, the highest inoculum sizes have the highest inhibitory effect although ocean conditions maintain a higher MIC through four dilutions while the same four inoculum sizes show a decrease in MICs after the second dilution in human conditions. Lower antibiotic resistance is observed in ocean conditions indicating differences in MIC may not only be affected by inoculum size, but also by the environments themselves.
Allee Effects, Growth Dynamics, & Virulence

The research conducted studied the important dynamic relationships that exist between different variables. By creating environments that simulated growth of the bacteria in human and ocean conditions, certain *V. cholerae* responses were observed to better understand the growth patterns that occur as a result. The growth patterns observed during this study will contribute to defining the *V. cholerae* fitness in the context of phenotypes associated with virulence. For the determination of future control and management strategies using mathematical models, the data gathered in this study can be utilized for calculating predictive epidemiological effects in pandemic events.

In the context of growth dynamics, bacterial cultures were grown in different media to demonstrate the role pH, salinity, and temperature have on transmission. Allee effects were discussed and defined as the correlation between population density and the average individual fitness of a cell. While twelve growth experiments were performed, only nine were produced without contamination and used for results analysis. The growth curves observed were compiled into Figure 5.1 and calibrated according to the starting CFU count for each experiment. Colony forming units per milliliter (CFU/mL) were determined by triplicate counting from designated dilutions. In the context of Allee effects, Figure 5.1 provides little
evidence to make conclusions regarding Allee effects. However, the significance of this graph involves the future mathematical modeling that will be used to create control and management strategies for cholera outbreaks. With the use of Figure 5.1, the number of V. cholerae colonies per milliliter can be predicted based on time and environment. Few studies consider the growth dynamics of V. cholerae beyond a single environment making the data found in Figure 5.1 especially valuable for mathematical modeling. Additionally, the time scale in which experiments were conducted is useful in understanding the growth of V. cholerae as it transitions from an aquatic environment to a human host.

![Figure 5.1 Composite Representation of Non-Contaminated Allee Experiments](image)

**Figure 5.1 Composite Representation of Non-Contaminated Allee Experiments**
Figure 5.1, while useful for modeling and computation, does not provide enough evidence on its own to suggest Allee effects were observed. As noted previously in chapter 2, strong Allee effects are dictated by per-capita growth relative to a threshold density. The values found in Figure 5.1 were re-calculated to express the per-capita growth density of the original nine growth curve experiments as shown in Figure 5.2. However, since there were many instances in which smaller inoculum sizes produced no visible growth, only five of the nine runs are included in Figure 5.2.

An analysis of Figure 5.2 suggests weak Allee effects were present at nearly every inoculum size in both human and ocean conditions. The presence of Allee effects suggest that the growth and persistence of V. cholerae cells depends not only on the environment, but also on the fitness of individual cells. Furthermore, the presence of Allee effects in both environmental conditions suggests that a reduction in cell density does not necessarily indicate the certain extinction for an entire population. The data presented in Figure 5.2 is important to consider when creating control strategies for cholera outbreaks as fewer cases of cholera infection may not mean the bacterium is eradicated.
The specific role of each variable tested should also be considered when analyzing the growth patterns created by \textit{V. cholerae}. NaCl concentration changes performed after trial 4 were made to mimic the concentration of salt found in human blood (150mM NaCl) instead of human skin (300mM NaCl) which resulted in vastly different growth patterns than those observed during the initial calibration runs. Considering that temperature changes made during trial 4 produced few changes in density growth from trial 3 to trial 4 suggests salinity may have a greater impact on growth patterns than temperature. The p-values obtained from the regression statistics performed on the data from figure 5.1 support this idea. Table 5.1 shows the p-values for each variable considered during experimentation. While all variables were
found to be statistically significant, the lower p-value found for salinity compared to 
temperature implies salinity is a more significant for *V. cholerae* growth than temperature is. In 
addition, after saline concentrations were altered in trial 4, all trials afterward saw a decrease in 
*V. cholerae* growth at ocean conditions further reinforcing the impact salinity has on growth.

Table 5.1 P-values of Environmental Variables Tested

<table>
<thead>
<tr>
<th>Variable</th>
<th>P-value</th>
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<tr>
<td>Salinity</td>
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<tr>
<td>pH</td>
<td>8.57E-12</td>
</tr>
<tr>
<td>Inoculum Size</td>
<td>5.97E-16</td>
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</table>

Inoculum size was found to be perhaps the most important variable in determining 
growth regardless of condition simulated. The p-value for inoculum size presented in Table 5.1 
confirms that inoculum size is the most important variable in *V. cholerae* growth. Even at 
extremely small inoculum sizes, correlations persisted between inoculum and growth observed 
in either of the two conditions. While it was expected that Allee effects would be more easily 
observable at smaller densities, the only predictive measure of growth observed beyond 24-
hour incubation was inoculum size. As growth allotted time was extended beyond 24-hours into 
48 hours, the impact of salinity, pH, and temperature was more difficult to observe revealing 
obvious correlations between inoculum size and extended growth time.
PH changes observed during trial 4 resulted in the replacement of CM-9 media with HEPES buffered media. The media changes resulted in growth pattern differences from those observed in earlier runs. Potential procedural errors may be responsible for the changes in growth as was discussed in the literature. At the same time, incubation of bacterial cultures at the corresponding environmental temperatures shifted growth downward. The observations from trial 6 provided insight into the responses of *V. cholerae* when undergoing multiple changes. The significant changes in absorbance readings because of multiple environmental changes imply alterations to pH, salinity, or temperature are more impactful when more than one variable is altered. This finding is supported by the results of phenotypic testing on biofilm formation, swarming motility, and swimming motility.

During experimentation, dose-dependent responses were observed for all three phenotypes. However, bacterial growth, motility, and biofilm formation were all significantly influenced by the simulated environment in which testing occurred. For example, the same inoculum size exposed to host conditions led to exponentially faster growth with higher bacterial yield than the respective ocean conditions. In fact, less than 10 CFU/mL supported growth in human conditions but not in ocean conditions during 40-hour incubation. The difference in incubation times also played a role in phenotypic expression, specifically in swarm morphology, where swarming motility was found to be considerably lower in ocean conditions. Overall, the growth data found in the first leg of experimentation contributes to defining and understanding the fitness of *V. cholerae* in the context of phenotypic virulent expression.

Virulence was assessed through ELISA assays designed to test the impact of environmental conditions in CT secretion. Figure 5.3 is a graph of the absorbance readings
found during the ELISA assay. However, using a calibration curve of the CT controls, Figure 5.4 shows a conversion of the absorbance readings from each assay into the density of CT present per inoculum size. Larger inoculum sizes correlate with higher CT detection. Additionally, differences in media also show a direct correlation to CT levels with AKI having the highest quantities of CT present followed by human and ocean respectively. Interestingly, human conditions had higher levels of CT than AKI media for the second largest inoculum size. The results suggest that *V. cholerae* transitioning from an aquatic environment to a host leads to a significant increase in the levels of CT secreted. Moreover, the results observed from motility and biofilm experimentation suggest phenotypic responses and CT production changes act similarly when transitioning from ocean to host, depending largely on population density. The importance of inoculum size in CT production indicates that virulence is not only a function of the environment but also of the cell interactions that take place within the population. Since the secretion of CT was observed to be a density dependent outcome, the Allee effects observed during growth experimentation might explain the sudden phenotypic changes that occur as *V. cholerae* transitions from ocean to host. Similarly, from the observed experiments, both Allee effects and quorum sensing provide a plausible explanation for the differences seen in CT secretion when *V. cholerae* grows in different environments.
Figure 5.3 ELISA Assay Results with 95% Confidence Bands
Figure 5.4 ELISA Assay Results in ng/mL with 95% Confidence Bands

**Conclusions**

Weak Allee effects were present in most of the growth experiments performed based on the per-capita growth rates plotted in Figure 5.2. Population density was found to be critical in both growth and virulence experiments with inoculum size correlating directly with both growth and CT production. Environmental differences also showed a direct impact on growth and virulence. In the growth experiments, human conditions resulted in greater overall growth based on inoculum size. During virulence experimentation, ELISA assays detected higher levels of CT production in human versus ocean conditions. Biofilm and motility experiments showed
not only more rapid growth in human conditions, but also increased levels of biofilm production in human conditions and slower motility rates in ocean conditions.

Besides the presence of Allee effects the most significant findings from the study come from the virulence experiments. CT production and antibacterial resistance was found to be dependent not only on environmental variables, but also inoculum size. When looking at the absorbances from ELISA experiments, the presence of Allee effect observations in growth experimentation offer a possible explanation for the differences in CT detected in different environments. Furthermore, p-values indicate strong correlations amongst V. cholerae growth, pH, salinity, temperature, and inoculum size. However, the inoculum size p-value indicated cell density is more important for growth than the environmental variables tested. Still, the importance of salinity, pH, and temperature on growth and CT secretion is well established.

The data found in this study offers a more robust understanding of V. cholerae dynamics as the bacterium passes from aquatic environments to human hosts. The Allee effect growth experiment composite figure offers time scale data that is limited especially when trying to research between-host growth. Likewise, few studies exist such as Figures 5.3 and 5.4 that present CT production in terms of inoculum size and environmental conditions. The information presented in this thesis may serve to create more robust control and management strategies for cholera outbreaks than those currently in place.

**Future Work**

Future work should be based around further virulence testing. The determinations gathered from the virulence experiments conducted in this study, while significant, require
further experimentation to fully understand *V. cholerae* growth dynamics at the host level. The growth experiments performed during this study might also benefit from longer incubation periods to determine the presence of Allee effects over greater periods of time and at lower densities. Finally, the statistical analysis performed with *p*-values should be further expanded to include multiple data sets across several media types, environmental variables, and inoculum sizes to better understand the relationships between each component tested.
REFERENCES


40. Saha, P K, and Nair, G B. “Production of Monoclonal Antibodies to the Non-Membrane-Damaging Cytotoxin (NMDCY) Purified from Vibrio cholerae O26 and Distribution of NMDCY Among Strains of Vibrio cholerae and Other Enteric Bacteria Determined by Monoclonal-


APPENDIX A

V. CHOLERA ALLEE GROWTH CURVES

(NON – CONTAMINATED)
8-7-2018 V. C. C6706 - 37 DEGREES C FOR 300MM AND 30 DEGREES C FOR 600MM - GROWTH CURVE

8-10-2018 V. C. C6706 - 37 DEGREES C FOR 300MM AND 30 DEGREES C FOR 600MM - GROWTH CURVE
8-24-2018 V. C. C6706 - 37 DEGREES C FOR 150MM AND 30 DEGREES C FOR 600MM - GROWTH CURVE

8-27-2018 V. C. C6706 - 37 DEGREES C FOR 300MM AND 30 DEGREES C FOR 600MM - GROWTH CURVE
9-14-2018 V. C. C6706 - 37 DEGREES C FOR 150MM AND 30 DEGREES C FOR 600MM - GROWTH CURVE

9-6-2018 V. C. C6706 - 37 DEGREES C FOR 150MM AND 30 DEGREES C FOR 600MM - GROWTH CURVE
APPENDIX B

V. CHOLERA ALLEE GROWTH CURVES

(CONTAMINATED)
8-15-2018 V. C. C6706 - 37 DEGREES C FOR 300MM AND 30 DEGREES C FOR 600MM - GROWTH CURVE

8-28-2018 V. C. C6706 - 37 DEGREES C FOR 150MM AND 30 DEGREES C FOR 600MM - GROWTH CURVE
8-28-2018 V. C. C6706 - 37 DEGREES C FOR 150MM AND 30 DEGREES C FOR 600MM - GROWTH CURVE
APPENDIX C

EFFECTS OF INOCULUM SIZE ON BIOFILM FORMATION,

SWARMING MOTILITY, & SWIMMING MOTILITY
Biofilm Formation

Absorbance (OD_{560})

CFU/ml

Absorbance (OD_{560})

CFU/ml
Effects of Inoculum Size on Swimming Motility – Host Conditions

Host Conditions

Effects of Inoculum Size on Swimming Motility – Ocean Conditions

Ocean Conditions
Effects of Inoculum Size on Swarming Motility – Host Conditions

![Graph showing the effects of inoculum size on swarming motility under host conditions.](image)

Effects of Inoculum Size on Swarming Motility – Ocean Conditions

![Graph showing the effects of inoculum size on swarming motility under ocean conditions.](image)
Phenotypic Differences in Swarming Motility by Environmental Condition

A

Host Conditions

24h

2.5x10⁷

5x10⁷

2x10⁷

6.25x10⁶

7.8x10⁵

1.6x10⁷, 120h

12h

24h

Ocean

2h

1.6x10⁷

120h

2x10⁷

5x10⁷

2.5x10⁶

6.25x10⁵

7.8x10⁴

1.6x10⁷

1.6x10⁶, 120h

B

Ocean Conditions

C

Host Conditions
APPENDIX D

POLYMIXIN B MIC ASSAY RESULTS
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<td>03</td>
<td>17</td>
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</tbody>
</table>
Run 1 – Results

Absorbance vs. Polymyxin B Concentration (µg/mL)

- 0.05 µg/mL
- 2x10^7 µg/mL
- 4x10^6 µg/mL
- 8x10^5 µg/mL
- 1.6x10^5 µg/mL
- 3.2x10^5 µg/mL
- 6.4x10^4 µg/mL
- 1.28x10^3 µg/mL
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<th></th>
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Run 2 – Results

Polymyxin B Concentration (µg/mL) vs. Absorbance

- 0.05
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- 8x10^5
- 1.6x10^5
- 3.2x10^4
- 6.4x10^-3
- 1.28x10^-3
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Run 3 – Results

5/14/19 - V.C. C6706 PMB MIC - Ocean Conditions

Absorbance (OD)

Polymixin B Concentrations (µg/mL)
APPENDIX E

ELISA TOXIN ASSAY RESULTS

& PLATE DESIGNS
ELISA Assay Experiment 1 Plate Layout and Absorbances

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Key

- 7.1
- 8.1
- CT/PBS
- AKI
- Media
- Moat

88
ELISA Assay Experiment 2 Plate Layout and Absorbances

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Key

|        | 7.1 | 8.1 | CT/PBS | AKI | Media | Moat |

Note: The table shows absorbance values for different concentrations and conditions.
VITA

Erick Rojas was born in Hidalgo, Mexico, to parents Jose Rojas and Norma Rojas. He is an only child who attended Joseph Wheeler High School. After graduation, Erick attended Berry College where he earned Bachelor of Science Degree in Biochemistry in 2017. After working for in the pharmaceutical industry at Dendreon Corp., Erick decided to further his education after being mentored by several chemical engineers. Erick accepted a graduate assistantship with the Academic Affairs department in 2017 and began his master’s studies in chemical engineering at the University of Tennessee at Chattanooga. Soon after, Erick accepted a second graduate assistantship working with his advisor Dr. Bradley Harris. Erick plans on graduating in August 2019. Erick currently works for ADM Southern Cellulose in Chattanooga, TN, where he will remain in his co-op position until December 2019.