Potential Causative Agents of Biofilm-Induced Cholera: An In Vitro Approach

By

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Master of Science in Biotechnology

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Declaration

It is hereby declared that

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2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material that has been accepted or submitted, for any other degree or diploma at a university or other institution.
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Abstract:

Cholera, while easily treatable, is extremely common across many parts of the world, especially in the regions close to water bodies. The bacteria responsible, *Vibrio cholerae,* has an interesting affinity to forming biofilms, as well as a seasonal pattern of epidemics. Despite only having two serogroups considered virulent among 200, this disease still has a global estimate of 1.3-4 million cases each year. Various studies point toward the presence of extracellular DNA, as well as the cyclic variation of bacteriophage prevalence against planktonic bacteria as causative agents towards biofilm formation. This research aims to link both of these factors together as a causative agent, and determine how it may play out within the environment as well. Compiling a set of four experiments, this study aims to point out the effect of bacterial growth under Free whole genome DNA, free fragmented genome DNA, phage soup, co-culture, as well as fresh water samples retrieved at different times of the year. For the experiments that involve the author, among an initial 15 strains chosen, 3 best strains, and one optional strain was chosen based on their species, growth factor, as well as susceptibility to bacteriophages. Upon conducting the necessary tests on these bacterial strains, it was observed that there is a directly proportional correlation of biofilm growth relative to the DNA present within the solution, regardless of the source, strain or type of the DNA. This proves that DNA extracted through the phage-mediated lysis of planktonic bacteria can also have a significant effect on the formation of biofilms.

Key words: Cholera, *Vibrio Cholerae,* Biofilm, Bacteriophage, eDNA, seasonality, Bacterial co-culture, environmental biofilm

Dedicated to

This study in its entirety is dedicated to our beloved parents who provided us with inspiration, motivation and support when we thought of giving up.

To our Friends, Peers and Well Wishers who provided us with advice and encouragement to finish this study.

To the scientists and artists across the globe, with their endless dedication and creativity, stopping at nothing to further the limits of possibilities, and inspiring us with their inventions and creations.

And lastly to Almighty God for your guidance,strength, protection,power of mind and most importantly giving us a happy and healthy life. All these we offer to you.

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List of Acronyms:

CFU: Colony Forming Unit
PFU: Plaque Forming Unit
OD: Optical Density
LB: Luria Bertani
RPM: Revolutions Per Minute
NC: Negative Culture
BC: Base Culture
BCC: Base Co-Culture
CC: Co-Culture
BP: Bacteria - Phage

Chapter 1: Introduction

1.1. Vibrio Cholerae:

Despite being easily treatable, Cholera has a global estimate of 1.3-4 million cases, and 21,000 - 143,000 deaths annually. This situation is worsened by the fact that an estimated 90-95% cases go unreported, and patients may not show visible symptoms to begin with (Ali et al., 2015). The causative agent of Cholera, *Vibrio Cholerae* is a Gram - negative bacteria that belongs to the Vibrionaceae family. While it is mostly found in closed-off water bodies, evidence suggests that it can even grow in coastal and salty areas as well. Overall, there are about 200 serogroups of *Vibrio Cholerae,* among which only two serogroups, namely O1 and O139 are considered "Virulent/epidemic strains". Structurally, all of them follow the same "comma" shape across all of their variations, and are only classified based on the O antigen of their liposaccharides (Morris & Acheson, 2003, Sack et al., 2004).

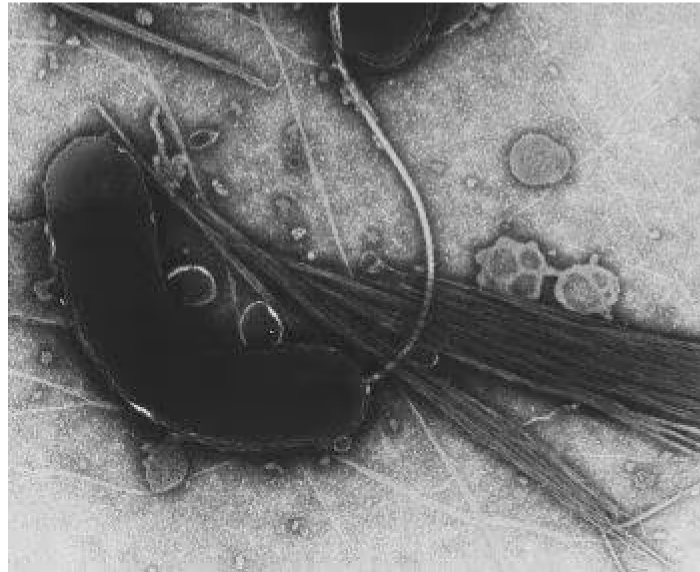

Fig 1.1: Electron Microscope view of *Vibrio Cholerae*; (Sajeev Handa, 2023)

Common symptoms of Cholera infections include fever, runny stool, fever, dehydration, vomiting, abdominal pain, muscle cramps etc. with fever more frequently occurring in patients younger than 2 years of age, and muscle cramps occurring more frequently in those above. Additionally, the risk factors mostly tie towards the consumption of raw food and unboiled water. It is suggested that while the death rate is significantly low, it can occur due to the excessive dehydration, if unreported (Fukuda et al., 1995).

1.2. Biofilm:

Biofilms serve as visible evidence of the nature of microbial cell clustering, upon contact with a solid surface. Initially observed by Antoine Van Leeuwenhoek in 1970, which was established further through Heukelekian and Heller's observations, biofilms are known to occur when the planktonic microbes clump together in an organized manner, live within what is referred to as "Extracellular Polymeric Matrix" and create a rigid structure (Jamal et al., 2018). Ideally, the life cycle of a biofilm follows 4 major steps.

1. **Attachment to surface:** Using multiple factors to their advantage, microbial cells attach to a solid surface. Factors include the microbial adhesion, physical force, hydrophobicity of the surface etc. (Garrett et al., 2008, Tribedi & Sil, 2013)
2. **Formation of Micro-colonies:** Upon successful attachment and stabilization, the Extracellular Polymeric Substances (EPS) sends out a chemical signal, initiating the formation of many micro-colonies. Through a series of carefully coordinated actions, stable and crucial substrate exchange is ensured within the colonies. More precisely, fermentative bacteria has to produce acid and alcohol, which is consumed by acetogenic bacteria, finally producing acetate and being consumed alongside $CO2$ and Hydrogen by the methanogens. Upon a successful series of communication, further development can be established. (Davey & O'toole, 2000)
3. **Maturation phase:** During this stage, formation of EPS is considered extremely important. Therefore, anti-inducer signals are utilized for colony communication. Additionally, EPS facilitating genes are expressed, water filled voids are formed within the matrix, and a proper distribution system is established within the biofilm environment itself (Parsek & Singh, 2003).
4. **Detachment:** Finally, after many rounds of multiplication, the biofilm reaches a considerably large size. This leads to the "dispersion" of sessile cells, in order to turn them into planktonic or "Motile" cells again. Mainly, this is done to ensure that the planktonic bacteria will move to a different surface area, and repeat the cycle (Otto, 2013).

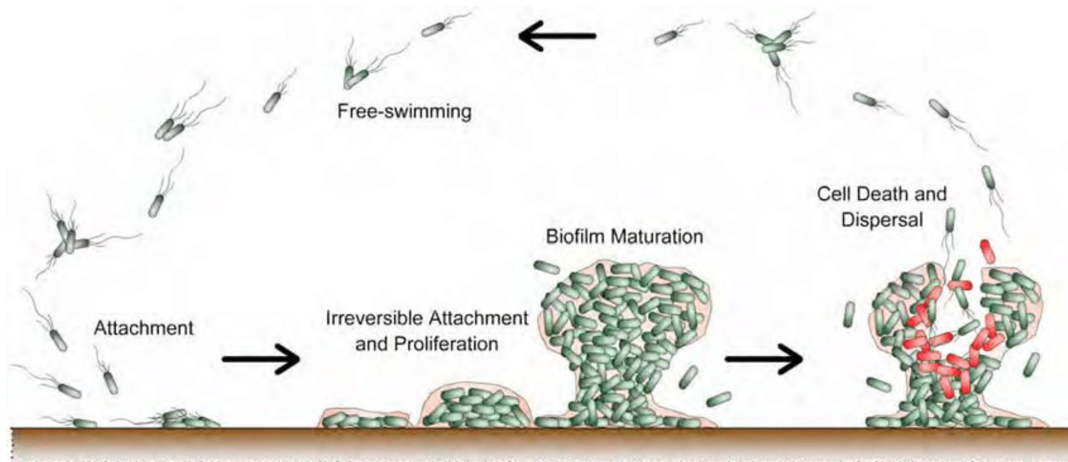

Fig 1.2: Depiction of the biofilm life cycle (Barraud, 2007)

1.3. Bacteriophages:

Bacteriophages, also referred to as "Phage" are highly specific virus strains that only target and kill bacterial cells. Structurally, they all follow a core principle of an outer capsid layer covering an inner phage DNA. Most of the times, they are species specific, but they can also be found in strain specific variants (Won't affect different strains of the same species), Ideally, a bacteriophage will attach onto the outer layer of the host, and follow either a lytic, or a lysogenic cycle, both of which directly or indirectly will result in the inevitable death of the bacterial host (Kasman, 2022).

The life cycle of bacteriophages follow a few common stages; Infection, attachment and entry. During the lysogenic cycle, this follows repression, integration and replication, which repeats the third stage. On the other hand, during the Lytic cycle, these stages follow the transcription, translation and replication stage, then packaging, and finally lysis, repeating the first step. The core difference between the two stages is that during the lytic cycle, the phage DNA directly affects the bacterial DNA, disintegrating it and repurposing the fragments to replicate its own parts. On the other hand, the phage DNA integrates itself to the bacterial DNA, achieving a dormant state during the lysogenic cycle (Campbell, 2003).

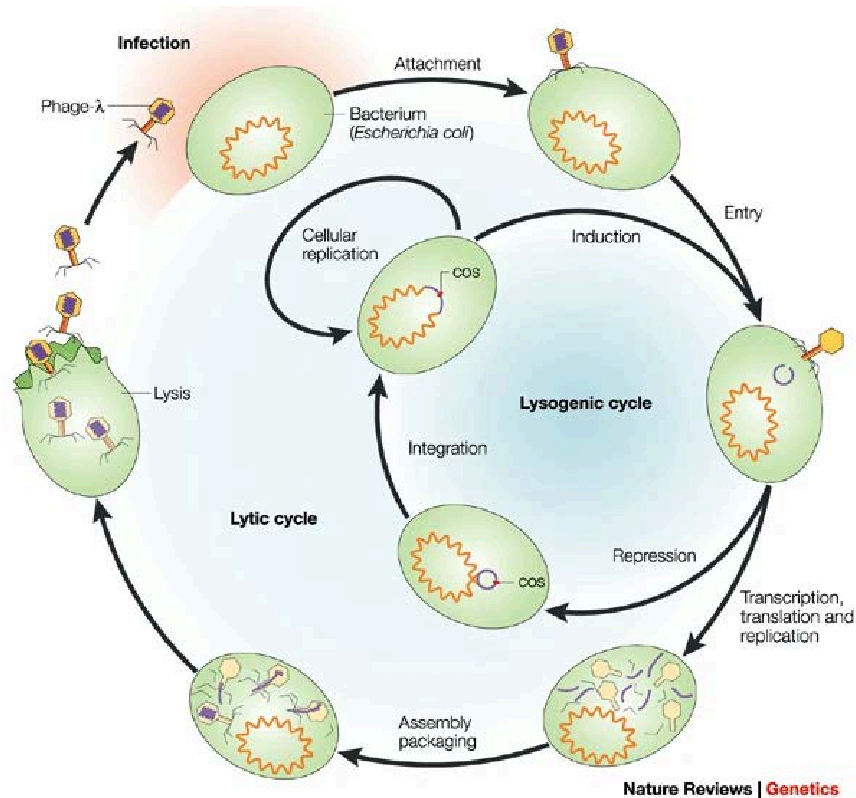

Fig 1.3: Bacteriophage Life Cycle; (Campbell, 2003)

1.4. Significance of *Vibrio cholerae* within a biofilm:

Several studies have shown that *Vibrio cholerae* can exist within both a planktonic and biofilm lifestyle. This is important in terms of understanding its pathogenicity, due to the fact that their biofilms have been observed both in the intestinal tract of patients, as well as the environment (Kierek & Watnick, 2003). While the adhesion factor to surface is the core driving factor of this phenomenon, there are also a few additional factors that facilitate this.

For example, the biofilm inducing activities of the microbe are known to be certain gene regulators such as cyclic diguanylic acid (c-di-GMP) as well as five encoded, PiLZ domain containing proteins. Both of these factors have been shown to affect the pathways leading to biofilm formation, and if deleted, appear to show signs of reduced biofilm activity overall (Silva & Benitez, 2016).

1.5. The cyclic nature of Cholera, lytic phage and potential factors of influence:

For the most part, the statistical data on *Vibrio cholerae* revolve around the infectivity of Cholerae across the year, as well as the isolation pattern of bacterial samples. Studies ranging across multiple years show that the most isolates in both clinical and environmental samples lie in months revolving around monsoon or heavy rain (March - May, September - January). It also appears to follow a cyclic pattern throughout the year, where there are almost no isolates, or symptoms in between the peaks (Alam et al., 2006, Baracchini et al., 2017, Mookerjee et al., 2015).

On the other hand, another isolated study conducted over a 3 year period suggested that if there is prevalence of a phage capable of lysing one serogroup, there will be little to no bacteria present of that particular serogroup. This study also depicts a similar pattern to the bacterial prevalence, just in reverse, leading to the suggestion that phage present in the environment may play a role in the seasonality of *Vibrio cholerae* (Faruque et al., 2005).

1.6. Objective of this study:

While the effect of extracellular DNA on biofilm formations has been a researched topic for decades, the correlation between the seasonality of *Vibrio cholerae* and environmental factors that lead to the production of eDNA remain relatively uncharted. Statistical studies regarding this matter, if any, remains far and few between. Therefore, it is imperative to understand the potential links between the factors leading to the seasonality of *Vibrio cholerae*.

Therefore, this study aims to:

- Compile the data collected from multiple thesis groups researching major branches of this project
- Present the data collected from this group's own research conducted on a major branch
- Correlate all of the present data to the established data on biofilm formation, as well as the seasonality of *Vibrio cholerae.*

Chapter 2: Materials and Methods

2.1: Materials

2.1.1 - Containers:

Petri plates, Glass vials 4ml, Falcon tubes (15ml/50ml), conical flask, test tubes

2.1.2 - Machines:

Micropipette (100-1000ul, 20-200ul), 37C incubator, 37C shaker incubator, Spectrophotometer, Biosafety Cabinet

2.1.3 - Growth medium:

Luria Bertani Broth (Ready-Made), Luria Bertani Agar (LB Broth + 1.5% Agar), Luria Bertani Soft Agar Media (LB Broth + 0.6% Agar), Thiosulfate Citrate Bile Salt (TCBS) media (Ready-Made), MacConkey Agar media (Ready-made).

2.1.4 - Reagents:

0.9% NaCl, 0.1% Crystal violet, 100% Glacial Acetic Acid.

2.2: Methodology:

2.2.1: Revival of bacterial strains:

From an assortment of random known and unknown isolated samples, the bacteria was revived and cultured into petri plates filled with Luria Bertani Agar media..

2.2.2: Verification of Bacterial strains:

For both known and unknown strains, species verification was done by sub-culturing them through a multitude of selective media (TCBS, MacConkey, XLD agar etc.), and the species of the strains were verified. Verified strains were stored at room temperature, inside vials containing Luria Bertani Soft Agar media.

2.2.3: Young Culture formation:

Verified strain single colonies from the solid culture were then inoculated into autoclaved Luria broth medium, and cultured for 3 hours to form young culture.

2.2.4: Initial screening through biofilm formation:

Inside a properly cleaned biosafety cabinet, young culture solutions were then placed in a ratio of Young culture 1:1 Fresh Luria Broth inside a 4ml vial, and let sit at room temperature for 48-72 hours.

2.2.5: Phage Selection:

A: Selection and enrichment: Phage strains that correlate to the best biofilm forming strains were selected, and enriched. Single colonies from the bacterial culture were inoculated in Luria broth for 2 hours. After that, it was treated with appropriate volume of bacteriophage solution, and kept for an appropriate amount of time. Finally, the resulting solution was centrifuged at 10,000 rpm for 10 minutes, and the supernatant was filtered through a 0.22u filter to isolate the phage from bacterial debris.

B: Verification of enriched phage: After enrichment, phage efficiency was tested by performing Double layer assay and spot tests on the targeting bacteria. Efficient performing phages were selected for further experimentation.

2.2.6: Phage-affected biofilm formation:

Upon selecting the best performing bacteria, and the countering phage strains, 4ml vials were filled in a series of combinations containing one target bacterial strain immune to the phage, one bacterial strain susceptible to phage, the targeting phage in question, as well as fresh Luria Broth. Outcomes were tested for multiple strains.

2.2.7: Clean up, Observation and Documentation:

At first, the liquid solution alongside the planktonic bacterial growth was poured out. After that, the vials were carefully washed with 0.9% NaCl solution three times. 0.1% Crystal Violet

solution was poured till it covered the visible biofilm rings, and let sit for 20 minutes. The solution was then poured out again, and the vials were washed another 3 times with 0.9% NaCl. Finally, the rings are dissolved in 2ml 100% Glacial Acetic Acid solution. Resulting liquids were placed under the Spectrophotometer, and the Optical Density was measured at 600 nm. Results of this research were recorded, and placed in parallel with the other aforementioned research results.

Chapter 3: Results

3.1: Previous Studies:

3.1.1: Biofilms Grown in Different Conditions:

Initially, a group of students researched the effect of growing the bacterial biofilm under these following parameters:

- Bacteria cultured in regular Luria Bertani (LB) Broth
- Bacteria cultured in Phage Soup
- DNAse I + Bacteria Culture in Phage soup
- Protease + Bacteria cultured in Phage soup.

The results (Optical Density) are as follows:

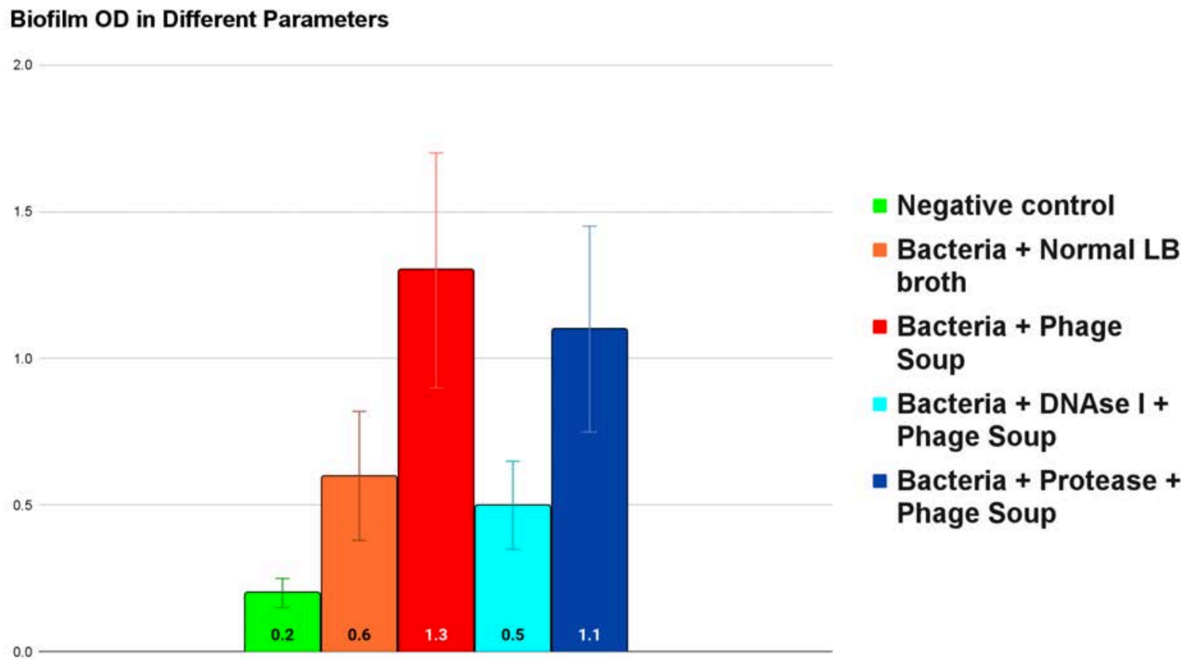

Fig 3.1: Average optical density of biofilms grown under different parameters

3.1.2: Biofilms Grown under the influence of isolated DNA:

After acquiring the results from the first batch of students, another batch of students observed the effects of growing biofilms under whole and fragmented DNA isolated from the selected bacterial strains.

Results indicate that on average, the biofilm's growth is noticed between the strains in an almost-consistent pattern form, ranging from 20-100%, regardless of whether they are treated with whole genome, or fragmented. Of course, a few peaks can be noticed as well.

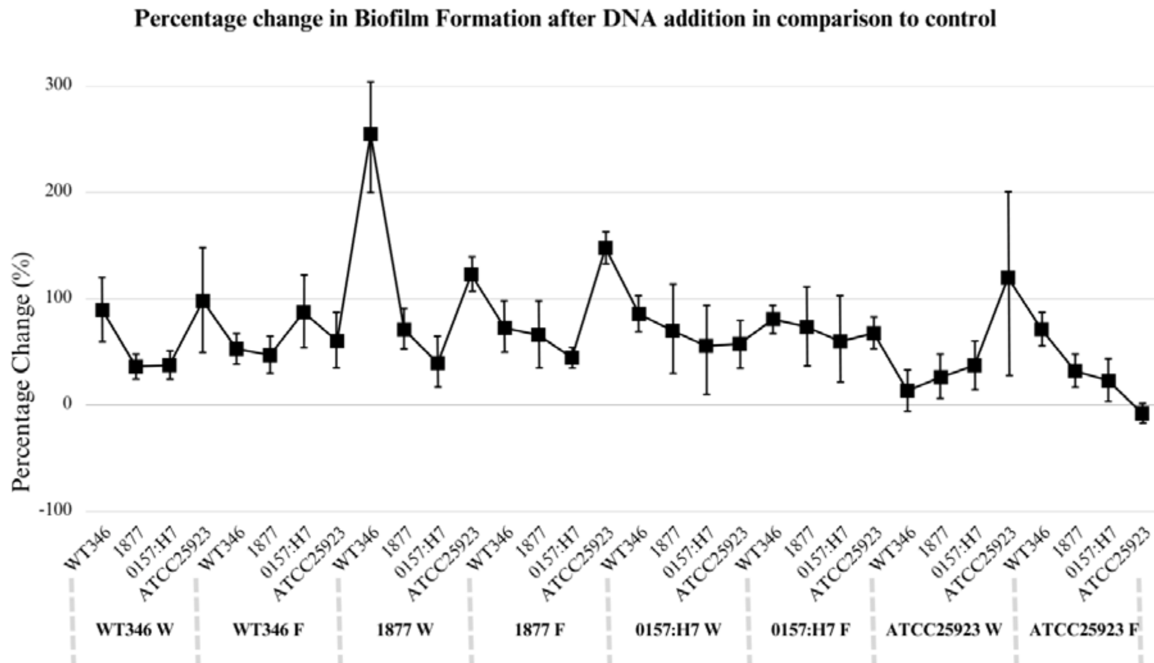

Fig 3.2: Percentage change in Biofilm formation after DNA addition, compared to control

3.1.3: Bacterial Biofilm Growth in Environmental Water Sample:

Additionally, another team of students conducted an experiment by growing a few select bacterial strains within water samples collected throughout a period of time. Results are as follows:

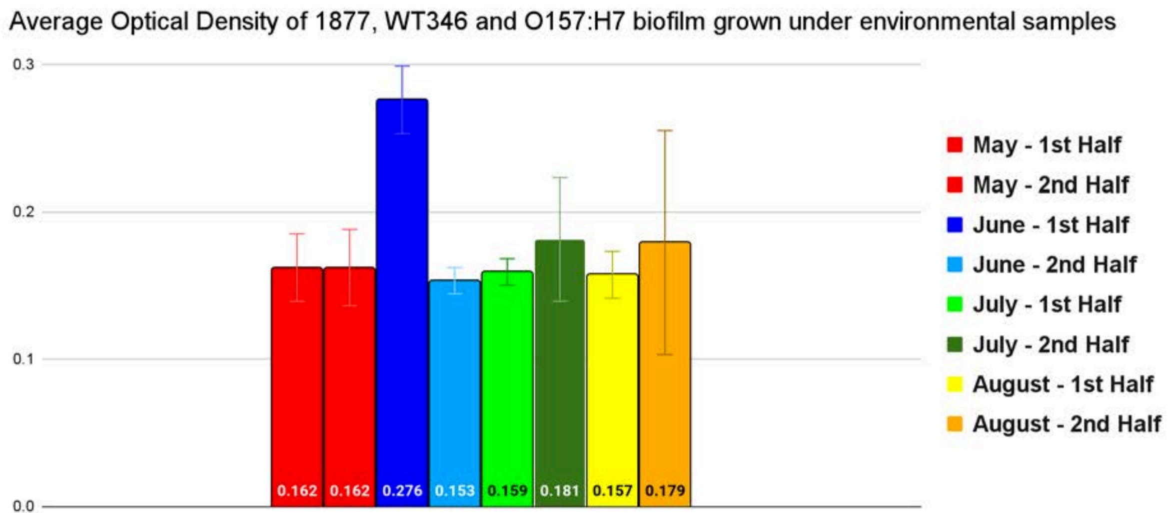

Fig 3.3: Average optical density of 1877, WT346 and O157:H7 across May 2023 - August 2023

3.2: Author's work; Biofilm Co-Culture growth:

3.2.1: Initial Biofilm Selection:

Among a retrieved stock of 15 different strains ranging across multiple species, 4 bacterial strains were grown. Namely, WT346 and 1877 *Vibrio Cholerae* strain, and O157:H7 and 5.1 *E.Coli* strain.

Fig 3.4: Biofilm growth observed among many strains in order to select the best ones.

3.2.2: Bacteriophage Selection, and potency verification:

Alongside the stock of bacteria, two strains of phage (JSF25; countering both WT346 and 1877, and 5.1K; Countering 5.1 strain) were selected, and Double-Layer Assay was conducted to test out their efficacy.

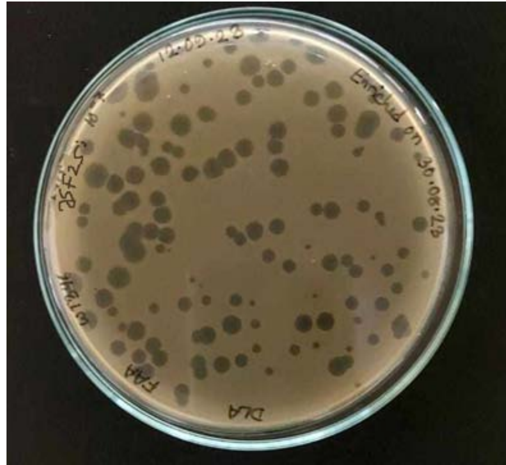

Fig 3.5: Double Layer Assay conducted on WT346 to initially test the viability of the bacteriophage, as well as cfu (Colony forming Unit)

3.2.3: Co-Culture Growth under Bacteriophage Influence:

Under these following parameters, biofilm growth results were retrieved:

- **NC:** Negative culture, sole Luria Broth - 2ml.

- **BC:** Base culture, Bacterial young culture - 0.7ml + Luria Broth -1.3ml

- **BCC:** Co-Culture. Target bacteria young culture - 0.7ml + Phage-susceptible bacteria young culture - 0.6ml + Luria Broth - 0.7ml

- **CC1:** Co-Culture stage 1: Target bacteria young culture - 0.7ml + Phage-susceptible bacteria young culture - 0.3ml + Targeting bacteriophage - 0.3ml + Luria Broth - 0.7ml

- **CC2:** Co-Culture stage 2: Target bacteria young culture - 0.7ml + Phage-susceptible bacteria young culture - 0.6ml + Targeting bacteriophage - 0.3ml + Luria Broth - 0.4ml

- **BP:** Bacteria-Phage: Phage-susceptible bacteria - 0.3ml + Targeting bacteriophage - 0.3ml + Luria Broth - 1.4ml

The average OD is presented in the bar graph to denote a trend in growth, error bars denoting the standard deviation between observations. Additionally, the OD results seen within the vials themselves are presented in the chart, and color-coded based on the visible growth on the vial after washing. For example: Green cells denote vials with no visible growth, Light blue cells denote vials with thin biofilm growth, Violet cells denote vials with heavy visible biofilm growth.

a) Target Bacteria: O157; Susceptible Bacteria: WT346; Bacteriophage: JSF25:

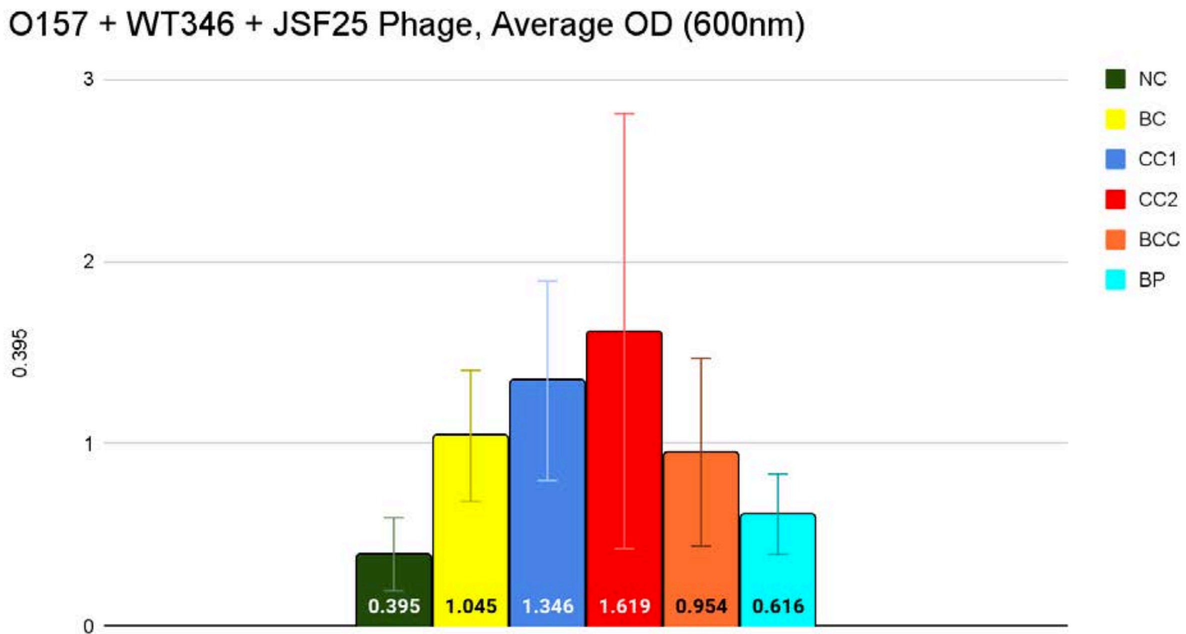

Fig 3.6: Average optical density of O157:H7 Biofilms grown under WT346 strain's influence

O157 + WT346 + JSF25 Phage, Triplicated OD (600mm)			
NC	0.164	0.497	0.523
BC	0.799	0.879	1.458
CC1	0.783	1.382	1.873
CC2	0.732	1.152	2.974
BCC	0.474	0.89	1.497
BP	0.373	0.671	0.805

Chart 3.1: Detailed overview of the O157:H7 Biofilm observations under WT346 strain's influence

b) Target Bacteria: O157; Susceptible Bacteria: 1877; Bacteriophage: JSF25:

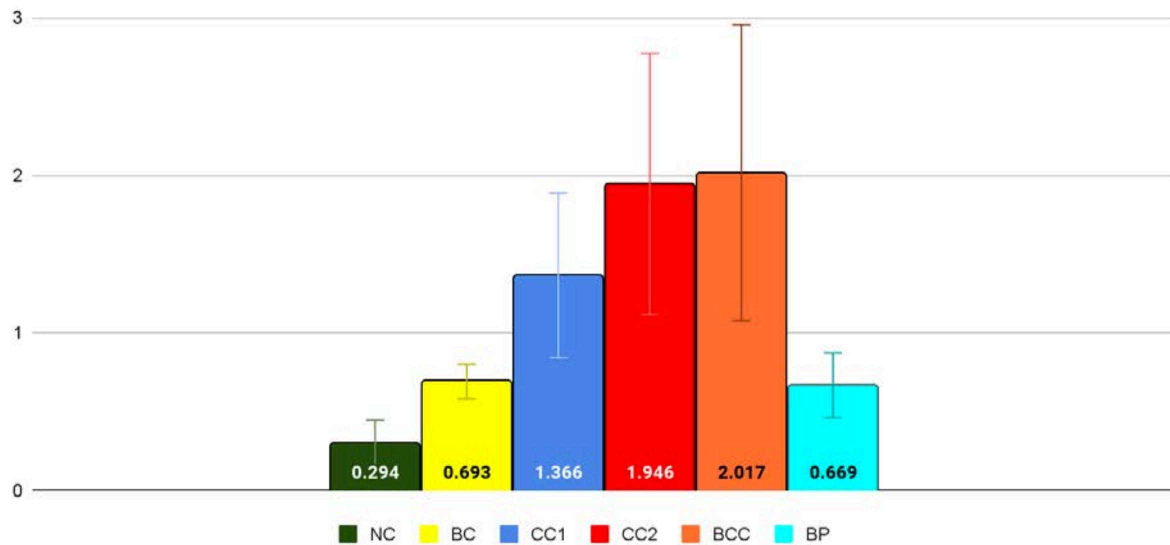

Fig 3.7: Average optical density of O157:H7 Biofilms grown under 1877 strain's influence

Parameters	O157 + 1877 + JSF25 Phage, Triplicated OD (600mm)		
NC	0.184	0.229	0.469
BC	0.584	0.693	0.801
CC1	0.844	1.366	1.888

CC2	1.116	1.946	2.776
BCC	1.914	2.017	2.119
BP	0.45	0.699	0.857

Chart 3.2: Detailed overview of the O157:H7 Biofilm observations under 1877 strain'

c) Target Bacteria: 1877; Susceptible Bacteria; 5.1; Bacteriophage: 5.1K:

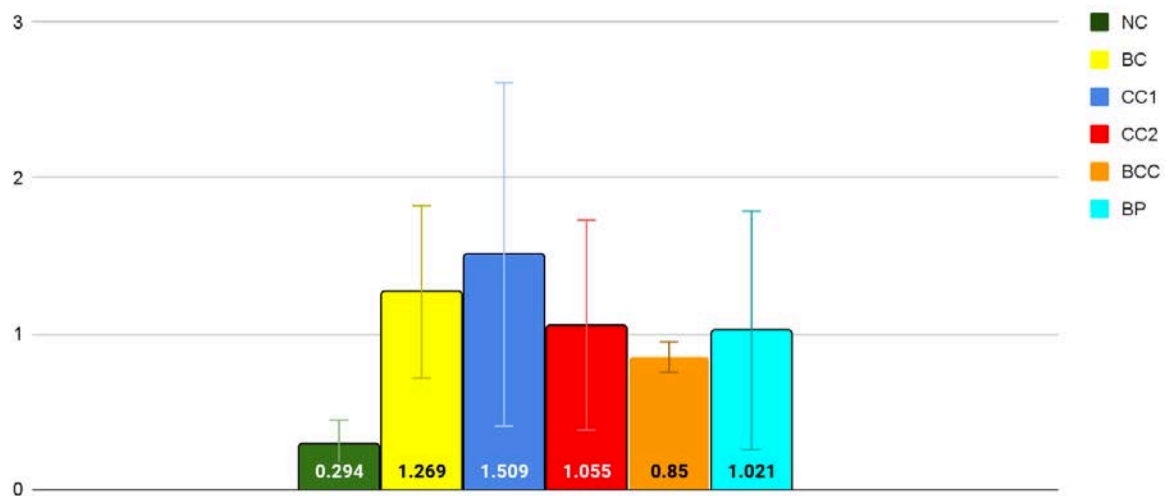

Fig 3.8: Average optical density of 1877 Biofilms grown under aforementioned parameters

Parameters	1877 + 5.1 + 5.1K Phage, Triplicated OD (600mm)		
NC	0.184	0.229	0.469
BC	0.716	1.269	1.822
CC1	0.837	0.909	2.78
CC2	0.381	1.055	1.728
BCC	0.753	0.85	0.946
BP	0.301	0.937	1.826

Chart 3.3: Detailed overview of the 1877 Biofilm observations under aforementioned parameters

d) Target Bacteria: WT346; Susceptible Bacteria; 5.1; Bacteriophage: 5.1K:

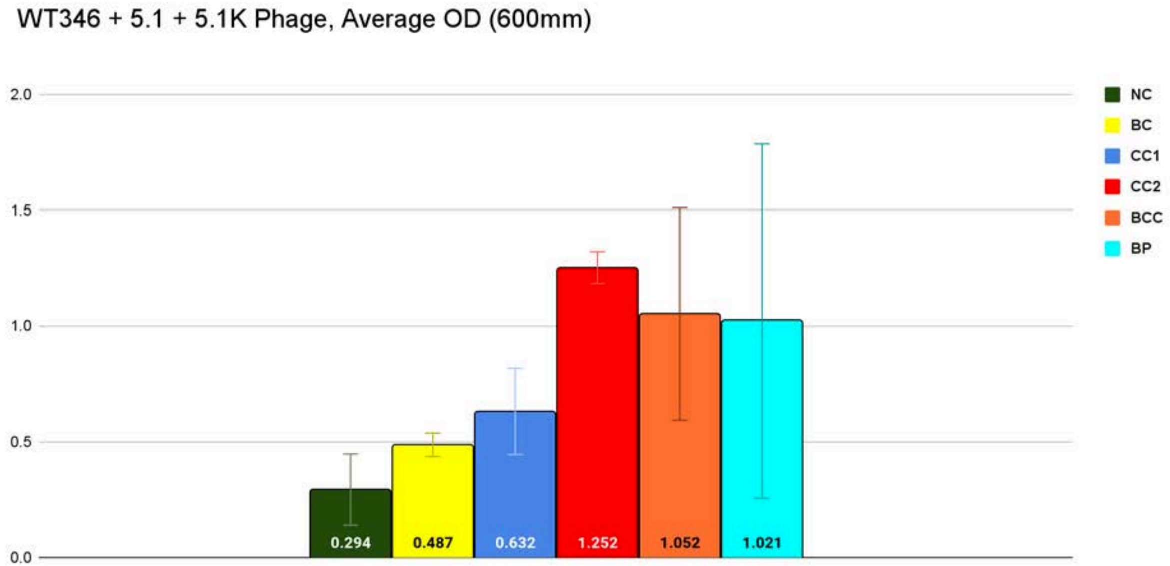

Fig 3.9: Average optical density of WT346 biofilms grown under aforementioned parameters

WT346 + 5.1 + 5.1K Phage, Triplicated OD (600mm)			
NC	**0.184**	**0.229**	**0.469**
BC	**0.436**	**0.487**	**0.537**
CC1	**0.445**	**0.632**	**0.818**
CC2	**1.183**	**1.252**	**1.32**
BCC	**0.593**	**1.052**	**1.51**
BP	**0.301**	**0.937**	**1.826**

Chart 3.4: Detailed overview of the WT346 biofilm observations under aforementioned parameters

Chapter 4: Discussion

4.1: Setting the Timeline:

In order to fully understand the project's goal, the timeline must be understood first. The first mentioned project was conducted by using a combination of multiple bacterial species, and their countering phage, as well as DNAse and Protease in order to properly establish the connection between eDNA and biofilm growth. Here, DNAse, or Deoxyribonuclease I is an enzyme capable of degrading both single stranded and double stranded DNA (Samejima & Earnshaw, 2005). Therefore, adding DNAse to an enriched phage solution would effectively kill off all the extracellular DNA present within the solution.

On the other hand, protease is an enzyme known to cause hydrolysis between peptide bonds, thereby degrading the loose protein content within the affected region. Naturally, adding protease to an enriched phage solution would kill off loose proteins in the solution. (López-Otín & Bond, 2008)

As observed from the results, on average the bacteria grown in phage solutions had a much higher density than that grown in regular LB broth. Additionally, while treating the solution with protease resulted in marginally lower Optical Density (Lower biofilm density), treating the solution with DNAse resulted in a drastic drop instead, going below the regular biofilm growth rate even. This proves that the DNA present in the solution is one of the determining factors in biofilm production. This result made the way for 3 subsequent branches of the same project, specializing in different aspects of the biofilm's growth.

4.2: The Continuation:

Upon receiving the confirmation of DNA's effect on Biofilm's growth, three simultaneous projects were carried out. One focused on the direct effect of gDNA in the formation of biofilms, another focused on the effect of bacterial co-culture towards biofilm growth, and finally, one that focused on the effect of water samples collected across multiple months on biofilm growth. This was carried out in order to understand, respectively, the extent of effect (Dose-dependency, species-dependency, fragmentation etc.) of bacterial genomic DNA as extracellular DNA, the extended effect of bacteriophage as an indirect catalyst in biofilm growth (Dose-dependency,

co-culture effect etc.), and finally, the understanding of natural factors in the formation of biofilms.

The group focusing on gDNA influence tested the effects of both whole and fragmented DNA on the growth of biofilms. More specifically, their parameters include a pure negative culture, bacterial culture grown without influence, bacterial culture treated with 20ul whole/fragmented genome and dilutions of said genome till 10-3. It was observed that for the most part, there is a trend of decrease in the optical density as the dilutions grow thinner. Additionally, from the image presented, it is seen that the difference between fragmented and whole genome's effects, aside from a few spikes, remain mostly the same. This reinforces the original project's idea of DNA being a core influencing factor, and also points out that the state of the DNA matters less than the concentration of DNA within the solution.

The group focusing on biofilm growth under environmental samples mainly pointed out the current situation of eDNA in the selected areas. According to their findings, and as per the data presented, it appears that there may have been a heightened concentration of extracellular DNA during the first half of June 2023, and the second half of July 2023, otherwise the water DNA levels stayed mostly the same.

4.3: Author's Work:

Keeping the current information in hand, the effect of extracellular DNA as well as some minor factors influencing them has been established. As observed from the results, the co-culture trend lines can indicate multiple things upon initial investigation.

First of all, negative culture (NC) was maintained to never contain any form of bacteria, and thus has the lowest Optical Density value amongst all of them. BC is the observation of target bacteria's biofilm formation capacity without influence. BCC aims to implement a ratio of both young cultures correlating to the phage influenced counterpart, in order to understand the capability of both bacterial strains adjoined growth. Susceptible bacterial strain was implemented in increasing volumes in CC1 and CC2, while the target bacterial culture as well as the targeting phage solution's volume was kept the same regardless of the parameters. Finally, BP aims to establish the growth capacity of susceptible bacteria, with equal volume bacteriophage alongside it.

The initial concentration of 1877, WT346, O157:H7 and 5.1 was estimated to be 2.43 x 10^6 CFU/ml, 1.86 x 10^6 CFU/ml, 4.58 x 10^6 CFU/ml and 4.26 x 10^6 CFU/ml respectively. Since the bacteriophage were enriched multiple times after conducting the Double-Layer Assay, the concentration during the experiment would be much greater than the initial findings. This was maintained to ensure as little susceptible bacteria can survive during the experiment as possible, mimicking the patterns seen in the environment. Regardless, the initial PFU for JSF25 was estimated at 1.21 x 10^9 PFU/ml, and 5.1K at 2.57 x 10^9 PFU/ml.

For the sake of consistency across the board, the end volume within each vial was maintained at 2ml, utilizing fresh LB to fill up any leftover volume. This allows for a bit more headspace for fresh nutrients to enter the solution. In order to correlate with the results found from the other research branches, the base solution's Optical Density has to be lower than the Phage-influenced solutions. Finally, growth mediums such as Luria Bertani (All forms), MacConkey Agar etc. were autoclaved as per the instructions before usage.

Looking at the results, we can determine the potential meaning of each variable tested out:-

O157 + WT346 + JSF25 Phage:

Initially, to test out the viability of this method, an isolated experiment was carried out using O157 as the target bacteria, and WT346 as the susceptible bacteria. As it can be seen, the BC (Base O157 Culture) has shown growth in all three vials, and an average Optical Density of 1.045. Adding 0.6ml WT346 to the base culture showed growth in only two of the three vials, with an average OD of 0.954 and a peak of 1.497, marginally different from the base culture's peak value of 1.458. This makes it likely that WT346 is not influencing the conjoined biofilm formation to a significant degree.

Upon adding 0.363 x 10^9 JSF25 bacteriophage and 0.558 x 10^6 WT346 Young culture to the base (CC1), the optical density increases to 1.346 on average, with the peak being 1.873, much higher than the base culture's peak. This leads to the idea that while adding WT346 as a base may not have influenced much in the growth, the addition of phage influences it to a noticeable amount.

Adding 0.363 x 10^9 bacteriophage and 1.116 x 10^6 WT346 young culture (CC2), a considerable shift can be seen, with an average OD of 1.619, and a peak of 2.974. This reinforces the

previously derived idea, that the involvement of bacteriophage significantly affects the growth of the biofilm.

Finally, upon growing the same volume of WT346 (0.558×10^6) and JSF25 (0.363×10^9), it was seen that only one vial showed biofilm growth, and the other two showed no growth at all. It can be assumed that for both of the vials, the phage was successfully capable of wiping out almost all bacterial content, and the third one could potentially have mutated bacterial strains, making them resistant to the phage.

O157 + 1877 + JSF25 Phage:

Upon receiving the results of the first combination, three other combinations were carried out, one of which involves O157 as the target bacteria, and 1877 as the susceptible Bacteria..

Similar to the experiment with WT346, the trendline between BC, CC1, and CC2 represents an upward shift, much similar to the previously tested variable, with the averages going from 0.577 in BC to 0.995 in CC1 and 1.386 in CC2. The observed peaks also follow a similar trend, with CC1 having a noticeable increase, and CC2 having a significant one.

However, the BCC results paint an interesting picture for this combination. Upon adding 2.43×10^6 1877 to the base culture, there was a significant shift in the biofilm's growth, with an average of 2.017 and a peak of 2.119. This is considerably higher than the base culture (0.693, peak of 0.801), leading to a few possibilities.

First of all, the quorum sensing efficiency between the biofilms might be greatly enhanced from the addition of 1877. Second of all, there may be an anomaly in the growth due to unknown or invisible factors (Contamination, mutation etc.). Additionally, the environmental condition around the vial's growth (Current room temperature, general weather etc.) may have an effect on the bacteria's growth as well. Further replication of the process may lead to a more solidified conclusion.

Finally, BP showed growth in only one vial, leading to the same conclusions as the last parameter.

1877 + 5.1 + 5.1K Phage:

Since there was no retrieved bacteriophage for O157, a new *E.Coli* strain with an established bacteriophage had to be utilized for further experimentation. Since this bacteria is only used as susceptible bacteria for experiments, the principle stays the same.
Upon observing the results, a bit of a different picture is painted. Here, the biofilm growth in CC2 (1.055 Average, 1.728 Peak) seems to be lower than CC1, and BC (1.269 Average, 1.822 Peak) itself. Regardless, the shift between BC and CC1 remains consistent to that of the previous parameters, leading to the potential assumption that CC2 may have been an anomaly. The peak on BC and CC1 is also relatively high (1.822 and 2.78 respectively), which is somewhat consistent with the previous parameter, which indicated 1877 to have a really high growth rate.
Since the three parameters were tested simultaneously, BP for 5.1 and 5.1K phage has the same results for both this and the next parameter. It is observed that two out of the three vials show biofilm growth, and an average OD of 1.021. This could mean that 5.1 inherently mutated faster than 5.1K could lyse them, 5.1K might follow a lysogenic cycle rather than a lytic cycle at the start of its infection phase, or potentially there was contamination from other strains in the vials.
Finally, BCC showed less optical density than that of BC (0.85 vs 1.269, respectively) and it can be assumed that most likely, 5.1 isn't a factor on its own contributing to the growth of 1877.

WT346 + 5.1 + 5.1K Phage:

Similar to the first two parameters, the BC (0.487 average), CC1 (0.632 average) and CC2 (1.252 average) seem to show an upward curve, further reinforcing the hypothesis. However, BCC (1.052 average) appears to, while lower than CC2, is higher than BC. This leads to the assumption that while the growth of WT346 is naturally increasing as DNA concentration in the solution gets higher, 5.1 on its own appears to be cooperating with WT346 during regular growth, accelerating the biofilms structure by a considerable amount.
Further experimentation and testing may prove insightful in finding out more reasons behind this.

4.4: Challenges and Setbacks:

Many challenges were faced across the duration of this project. The biggest of all was the shifting of the weather. The author's part of the project started in February 2023 and ended in

March 2024. During this time, the weather and subsequently the temperature has shifted many times over, making it harder for some bacteria to grow, while making it easier for other bacteria to grow. This resulted in the need for constant adaptation, improvisation, and tweaking of the plans set in place, due to the fact that the biofilms were being grown at room temperature.
Another major hurdle was the relocation of the laboratory. Around August-September 2023, the lab relocation had been announced, meaning all crucial procedures within the lab would be shut down till late January 2024. This had hampered the progress quite a bit, since the entire procedure had to be redone, under a different weather altogether.
Additionally, the malfunctions of certain crucial machines i.e. ELISA led to a considerable delay in results obtained, and some of the work needed to be interpreted in the spectrophotometer.
Finally, albeit maintained at a minimal rate at all costs, contamination caused by many working parties within the same lab was also an issue.

4.5: Future Prospects:

Despite the success found within the results, there is much more that can be done in order to improve the consistency, and decrease the error margin during experiments. First of all, adding more samples to culture and conducting tests would result in a greatly diverse set of results, leading to more accuracy and better observation. Secondly, a smaller time frame between experiments could also lead to better accuracy, due to there no longer being a need to test growth changes relative to the weather. This could also be solved by culturing the biofilms within a neutral area (i.e. Inside a cabinet) to mitigate the effects of room temperature.
Additionally, more workforce may be employed to minimize time spent in experiments. While the other projects were conducted by two people each, the author’s work was carried out by himself. Having a bigger, coordinated team would help greatly in the efficiency of the procedures carried out.

Chapter 5: Conclusion:

Vibrio cholerae, and by extension Cholera is an important disease to keep track of due to its seasonal nature, and affinity towards forming biofilms. Previously, research showed that extracellular DNA plays a role in the growth of biofilms, as well as that bacteriophages play a

role in the prevalence of planktonic bacteria within a specific area. However, there seems to be little study linking the two factors together, which would hold the environmentally present bacteriophages responsible as one of the factors responsible for the seasonality of the bacteria. This study attempts to tie together the results of four separate experiments, including one of author's own in order to better understand the extent of effect environmental extracellular DNA, as well as bacteriophages may have in the seasonality of biofilms. Utilizing a detailed set of methodology for each separate experiment, it was determined within a select set of samples, that not only does extracellular DNA plays a crucial role in the formation of biofilms, but also bacteriophage, if placed in an environment where it can access it's host bacteria can also contribute to the formation of biofilms, albeit indirectly. Outside of a few outliers, the experiments indicated that the presence of DNA, be it through phage lysis or regular extraction, will always result in biofilm growth proportional to that of the amount of DNA present.

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