Resuscitation of Bacterial Biofilm by Sunlight: Effects on Different Enteropathogenic Bacteria

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A dissertation submitted to the Department of Mathematics and Natural Sciences in partial fulfillment of the requirements for the degree of
B.Sc. in Biotechnology.

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Declaration

It is hereby declared that

1. The thesis submitted is our original work while completing degree at BRAC University.
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.
4. We have acknowledged all main sources of help.

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Abstract

Bangladesh encounters seasonal cholera endemics biannually. *Vibrio cholerae* and Shiga toxin-producing *Escherichia coli* (STEC) is responsible for cholera and similar diarrheal disease outbreaks. Biofilms go through a cycle of formation and degradation influenced by various environmental conditions, and it is this degradation that contributes to increased numbers of planktonic bacteria in water bodies. In this study, the effect of sunlight on resuscitation of bacterial biofilm was investigated. Biofilm of *Vibrio cholerae*, three strains, and STEC were subjected to sunlight exposure during wet season, June to August. The results demonstrate that degradation of biofilm during these seasons, considered the off-season, occurs but is insignificant when compared to summer season, considered the peak season of bacterial infection. This can indicate that sunlight is a significant factor in biofilm resuscitation and seasonal outbreaks of bacterial diseases. Including these seasonal results, around the year data has been obtained for this study. However, replication would lead to a more insightful conclusion.

Keywords: Pathogenic bacteria, endemic, wet season

Dedication

This is dedicated to our family and faculty members for their ever-lasting support and encouragement.

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Contents

Acronyms

STEC- Shiga Toxin Producing E. coli

EPS- Exo-Polysaccharide

VPS- Vibro Polysaccharide

CVEC- Conditionally Viable Environmental Cell

VBNC- Viable But Not Culturable

OD- Optical Density

ELISA- Enzyme Linked Immunosorbant Assay

EIA- Enzyme Immunoassay

HUS- Hemolytic Urine Syndrome

CTX- Cholera Toxin

ER- Endoplasmic Reticulum

AFR6- ADP ribosylation factor 6

c-AMP- Cyclic Adinosine Monophosphate

PKA- Phospho Kinase

CFTR- Cystic Fibrosis Trans membrane Receptor

LPS- Lipopolysaccharide

STX- Shiga Toxin

ER- Endoplasmic Reticulum

LB- Luria Broth

LA- Luria Bertani Agar

QS- Quorum sensing

CAI-1- Cholerae autoinducer-1

AI-2- Autoinducer-2

VPS- Vibrio Polysaccharide

HCD- High Cell Density

LCD- Low Cell Density

LSR- Lipolysis Stimulated Lipoprotein Receptor

HapR- Hemagglutinin Protease Regulatory Protein

aW- Water Activity

TCBS- Thiosulfate-citrate-bile salts-sucrose

TSI- Triple Sugar Iron

CBB G-250- Coomassie Brilliant Blue G-250

CFU- Colony Factor Unit

Chapter 1: Introduction

1.1 Background

Cholera, a severe diarrheal disease caused by the bacterium Vibrio cholerae, has been responsible for several epidemic and endemic outbreaks in Bangladesh over the years. One of the factors contributing to the persistence and spread of cholera in this region is the ability of V. cholerae to form biofilms. These biofilms are complex communities of bacteria that adhere to surfaces and can resist environmental stresses, such as antibiotics and disinfectants.

In Bangladesh, V. cholerae is known to form biofilms on the surfaces of rivers, ponds, and other water sources, which are commonly used for drinking, washing, and bathing. This allows the bacteria to persist and spread within these water sources, leading to the contamination of food and water supplies and contributing to the continued occurrence of cholera outbreaks in the region. Biofilm formation by V. cholerae has been linked to increased antibiotic resistance and virulence, which can further complicate efforts to control and treat cholera in Bangladesh.

Even though the cells in the biofilms tend to go dormant, they naturally resuscitate into active planktonic forms, thus multiply, spread and cause cholera epidemics. Till now, different factors have been taken into account behind this natural revival of cells but sunlight has not yet been researched into as a key factor behind such seasonal epidemics of cholera.

It has been observed that cholera infections rise in warmer seasons and slowly decline in winter seasons. This can be due to the fact that the sunlight is not as strong as it is in warmer seasons and thus forces vibrio cholera to remain in a dormant state inside their biofilms.
- factors causing this
Since Vibrio cholerae is native to the aquatic ecosystem and some studies have demonstrated a correlation between outbreaks and fluctuations in climatic and aquatic conditions, it is commonly believed that cholera epidemics are caused by environmental factors that promote the growth of local bacterial reservoirs.Epidemiological evidence and genome sequence analysis of clinical isolates indicate that the vast majority of V. cholerae strains in aquatic ecosystems are not related to epidemics. It seems that only a subset of V. cholerae El Tor 'types' are responsible for the current epidemics.[1]

Recent outbreak is caused by pathogens including V. cholerae, Enterotoxigenic Escherichia coli (ETEC), Rotavirus, and Campylobacter. On the basis of an immediate evaluation of all available data, it was assumed that a number of factors, such as poor water and food quality, poor hygiene practices, and rising environmental temperature, which is conducive to the rapid reproduction of these pathogens, could have caused this early outbreak of diarrheal disease.[2]

1.2 Aim of Study

The aim for this study is to determine whether sunlight is a key factor in degrading biofilms to release planktonic form of bacteria in summer. If such is determined, it can be safe to conclude that sunlight in the wet season and not in the winter or summer season is one of the key factors that cause seasonal outbreaks of STEC induced diseases like cholera, typhoid

Chapter 2: Literature Review

2.1.1 Biofilms

Microorganisms form biofilms in hostile environments to survive in adverse conditions. A group of bacteria cluster and attach to a surface or each other in a self-produced extracellular polymeric substance (EPS) matrix composed of polysaccharides, proteins, extracellular DNA and other components. [3] Biofilm formation protects bacteria from harsh conditions, such as, presence of antibiotics, lack of nutrition, toxic chemicals, UV radiation, host immune system and predators, and so can be considered a survival mechanism for bacteria. [4] When pathogenic forms of these bacterial biofilms are found in drinking water, they pose a potential risk to human health since each biofilm aggregate may contain as many as 1.0×10^9 cells, which is sufficient to cause infection. [5] Biofilms can contribute to the epidemic and endemic cycles of infectious diseases by serving as reservoirs for infectious agents, providing protection from host defences and antibiotics, and facilitating the exchange of genetic material. Understanding the role of biofilms in infectious disease transmission and persistence can help to develop strategies to prevent and control infectious diseases. In an epidemic cycle, biofilms can serve as reservoirs of infectious agents, allowing the microorganisms to persist and potentially spread to new hosts. For example, biofilms in water distribution systems can harbour bacteria such as *Vibrio cholerae*, which can cause cholera outbreaks when contaminated water is consumed by humans. In an endemic cycle, biofilms can also contribute to the persistence of infectious agents within a population. These biofilms can allow the bacteria to persist and re-infect the host, contributing to the ongoing prevalence of the disease.
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2.1.2 Biofilm development

Biofilm development is a complex process triggered by a change in the environment. It can occur on any surface, both non-living and living, including water bodies, industrial pipes and even the human body. In an unfavourable environment, bacterial cells have regulatory systems that transmit signals which reprogramme and reorganise the cells gene expression to produce biofilm. In a biofilm, the bacteria are embedded in an EPS matrix which retains nutrients and water for the bacterial cells. The matrix keeps the biofilm hydrated, strong and sturdy, allows bacterial cells to interact with each other and exchange DNA and provides protection. [6] (citation 7 has been changed to six)

Biofilm development can be broken down into five stages. It starts when planktonic (free) bacteria reversibly attach to a surface. Then the bacteria irreversibly adhere to the surface forming a monolayer. Secretion of extracellular polymeric substances (EPS) begin which aid in the attachment. As cells continue to cluster, microcolonies begin to form with multiple layers. This is the start of the maturation stage. Further maturation occurs and the biofilm forms a three-dimensional, mushroom-like structure. Finally, the biofilm starts breaking down dispersing planktonic bacteria and the life cycle begins again. This could happen due to multiple factors, such as, excess of toxic products within the biofilm and lack of nutrients for the bacterial cells.

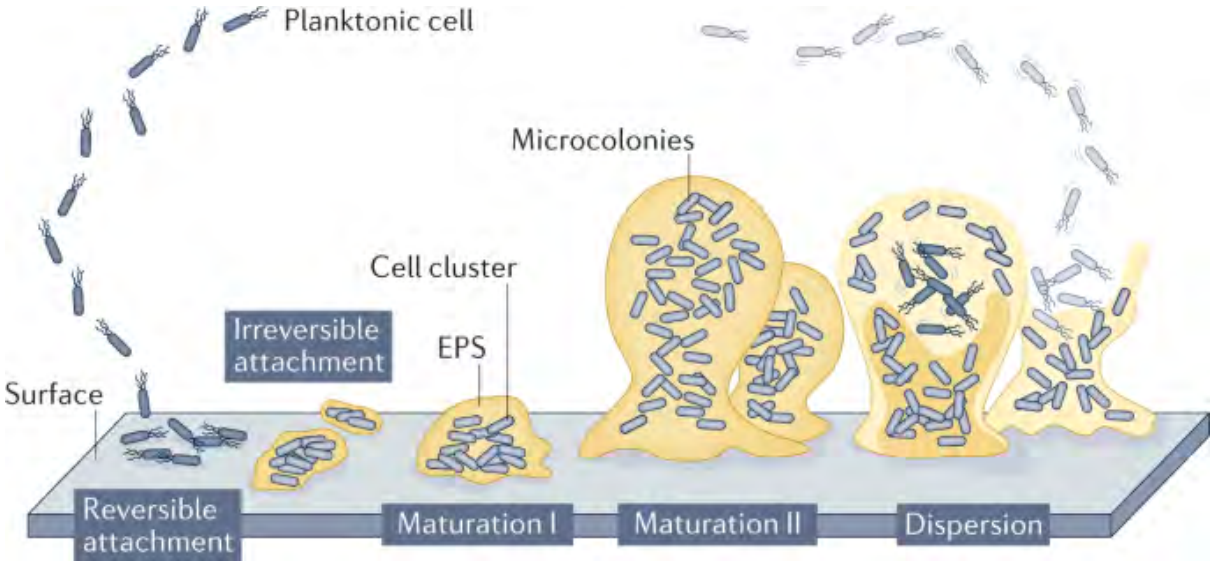

Figure: Life cycle of biofilm: formation on a solid surface [7]

2.2.1 Quorum Sensing and Biofilm Formation in *Vibrio cholerae* (A1)

Autoinducers used:
CAI-1 ((S)-3-hyroxytridecan-4-one) and AI-2 (4,5-dihydroxy-2,3-pentadione) are the two identified autoinducers of V. cholerae. They are produced by the CqsA and LuxS enzymes, respectively.
CqsA is conserved across all Vibrio species, indicating that CAI-1 is involved in intra-genus communication.[8]

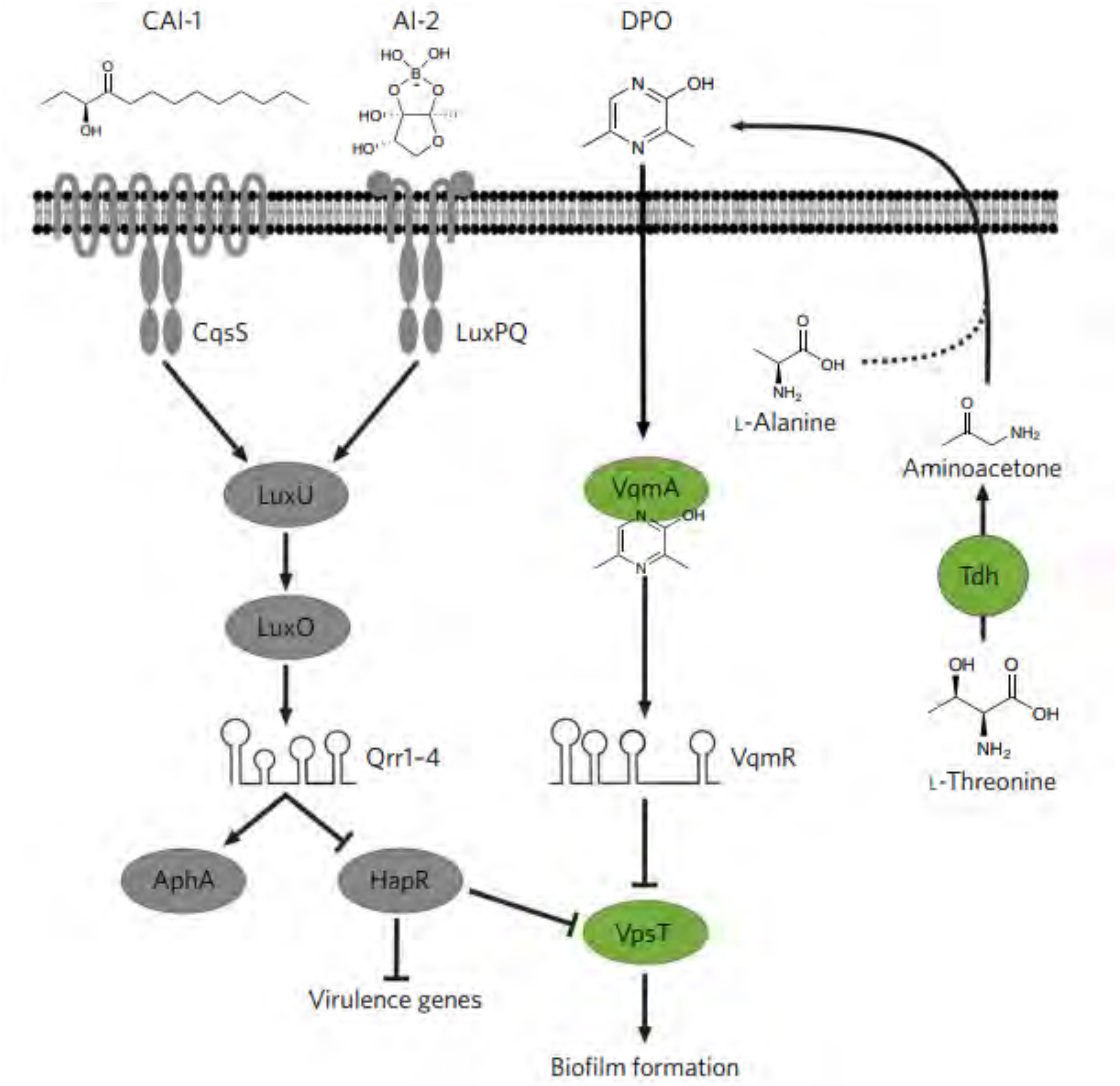

Figure: Dependent and independent QS pathway in cholerae. (**Papenfort, K., Silpe, J. E., Schramma, K. R., Cong, J. P., Seyedsayamdost, M. R., & Bassler, B. L. (2017, March 20))**

QS system summary

Gene expression is regulated by quorum sensing in response to changes in cell population density. Bacteria capable of quorum sensing generate and emit autoinducers, the concentration of which increases as cell density increases. Gene expression is altered upon detection of a minimal threshold stimulatory concentration of an autoinducer. Gram-positive and Gram-negative bacteria use quorum sensing communication circuits to control a variety of physiological functions. This consists of symbiosis, pathogenicity, competence, conjugation, antibiotic production, motility, sporulation, and biofilm formation. [9]

Bacteria exchange information with each other through chemical signaling molecules. Even in higher species, information from these molecules is essential for coordinating the activities of large cell groups. The generation, release, recognition and response of small hormone-like molecules known as autoinducers are essential for bacterial chemical transduction. This method, called quorum sensing, allows bacteria to monitor the environment of other bacteria and adjust their activities at the population level in response to changes in the amount and type of bacteria present in the community. Most of the ram-sensing-driven manipulations are ineffective when performed by a single bacterium working alone, but are beneficial when

performed by a large number of cells simultaneously. As a result, quorum sensing blurs the line between prokaryotes and eukaryotes by allowing bacteria to behave like multicellular organisms. [10]

LCD

LuxO represses HapR so that tcpP is expressed at the low cell densities observed during initial colonization of a host. This causes the expression of virulence factors that enable V. cholerae to colonize the small intestine, multiply, and produce cholera toxin (CT).

Toxin-coregulated pili (TCP) are necessary for the colonization of the intestine because they mediate bacterial-bacterial and bacterial-epithelial adhesion. The formation of TCPs is controlled by toxT, which induces the transcription of TCP genes (such as tcpA-F and tcpl) and CT genes (such as ctxA and ctxB).

The activity of TcpP, TcpH, ToxR, and ToxS receptors regulates transcription of toxT. HapR controls the expression of TcpP. It is believed that HapR activates the transcription of an upstream tcpP expression repressor. Solely at low cell densities are TCP and CT genes transcribed.[11]

HCD

At high cell density, the quorum-sensing regulator HapR represses not only expression of cholera toxin and the toxin coregulated pilus, essential virulence factors in human infection, but also synthesis of the Vibrio polysaccharide (VPS) exopolysaccharide-based matrix necessary for abiotic and biotic surface attachment. Here, we characterize a characteristic of V. cholerae quorum sensing that alters the interaction between the host and pathogen towards commensalism. V. cholerae HapR stimulates host intestinal serotonin production by inhibiting pathogen consumptive anabolic metabolism and, in particular, tryptophan uptake. In turn, this activates the intestinal innate immune signaling of the host to promote host survival.[12]

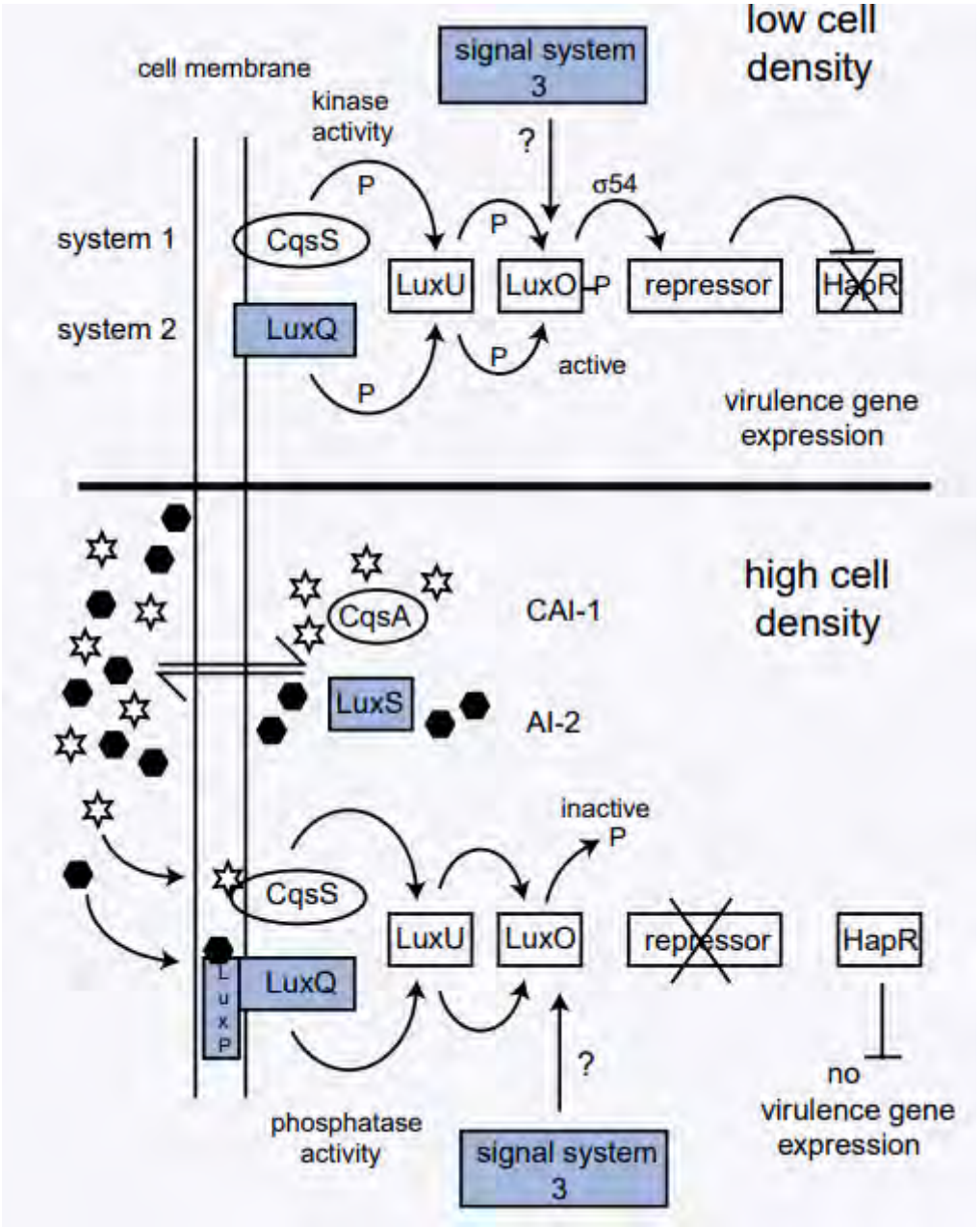

Figure: Differences in the state of low cell density and high cell density in Vibrio cholerae.

2.2.3 Quorum Sensing and Biofilm Formation in STEC

Autoinducers used

The autoinducers used by STEC are similar to those used by other bacteria, such as AI-2, but STEC also produces specific autoinducers that are unique to this pathogen.

One of the autoinducers produced by STEC is called AI-3[16], which is produced by the enzyme epinephrine/norepinephrine (Epi/NE) kinase. AI-3 has been shown to regulate the expression of virulence genes, including those involved in adhesion, motility, and toxin production.[13]

LCD

In low cell density of STEC, the concentration of autoinducers produced by the bacteria is low, and therefore the level of quorum sensing activity is also low. This means that the bacteria are not able to coordinate their behavior and gene expression in response to population density.

At low cell densities, STEC may be in a planktonic state, meaning that the bacteria are freely swimming in the environment and are not associated with surfaces or each other. This is a common state for many bacteria in aquatic environments.

In the absence of quorum sensing, STEC may not express certain virulence factors or may not form biofilms.

HCD

In high cell density of STEC, the concentration of autoinducers produced by the bacteria is high, and the quorum sensing system is activated. This allows the bacteria to coordinate their behavior and gene expression in response to changes in population density.

At high cell densities, STEC may form biofilms, which are structured communities of bacteria that adhere to surfaces and are encased in a matrix of extracellular polymeric substances (EPS). Biofilms provide several advantages to the bacterial population, such as protection from antibiotics, host immune defenses, and environmental stresses. In addition, the biofilm matrix may serve as a nutrient source for the bacteria.

High cell density of STEC can also lead to the expression of virulence factors, such as Shiga toxins, which are important for causing disease. The coordinated expression of virulence factors through quorum sensing allows STEC to act as a collective group rather than individual bacteria, which can increase its pathogenic potential.

Therefore, in high cell density of STEC, the bacteria may be more virulent and better able to cause disease due to the coordinated expression of virulence factors and biofilm formation.

2.3 Mutated V cholerae Strains

To have a better understanding on whether sunlight degrades the V. cholerae biofilm, two mutant strains have been used. 1. V.cholerae 1712. This is a Lux-O mutant. Here a natural strain is taken which lacks LuxO gene. The gene is then inserted into the strain in pLuxO form to mutate the natural strain. 2. V. cholerae 1877. This is a HapR mutant strain. Here a natural strain is taken and its HapR gene is permanently repressed. This causes a low cell density state in the strain and ensures the continuous formation of biofilm.

2.5 Pathogenic Significance of Biofilms

Biofilms are complex communities of microorganisms, such as bacteria, that attach to surfaces and are embedded within a self-produced extracellular matrix. Biofilms are commonly found in nature, on surfaces such as rocks, soil, and plant roots, as well as in human-made structures such as pipes, medical devices, and dental plaques.In the context of human health, biofilms can have significant pathogenic significance. The ability of microorganisms to form biofilms can facilitate the establishment and persistence of infections, as well as making them more difficult to treat. Here are a few examples:

Chronic infections: Biofilms can form on medical devices, such as catheters and artificial heart valves, and can contribute to the persistence of infections that are difficult to treat with antibiotics.[14]

Dental plaque: Biofilms in the form of dental plaque can lead to gum disease and tooth decay.[15]

Respiratory infections: Biofilms can form in the lungs of people with cystic fibrosis, contributing to chronic infections and lung damage.[16]
Chronic wounds: Biofilms can form in chronic wounds, such as diabetic ulcers, making them difficult to heal and increasing the risk of infection.[17]
Food safety: Biofilms on food contact surfaces can contribute to foodborne illness.[18]
In summary, biofilms can have significant pathogenic significance by facilitating the persistence of infections, making them more difficult to treat, and contributing to the spread of infectious diseases.

2.6.1 Diseases Caused by V. cholera

Vibrio cholerae is a bacterium that can cause cholera, a severe diarrheal disease. There are several serogroups of V. cholerae, but only serogroups O1 and O139 are known to cause epidemic cholera.
Cholera is primarily spread through contaminated water or food, particularly in areas with poor sanitation and hygiene. The symptoms of cholera include severe diarrhea, vomiting, and dehydration. Without proper treatment, cholera can be life-threatening.
V. cholerae can also cause other types of infections, including:Non-O1, Non-O139 Vibrio cholerae infections: These infections can cause a range of symptoms, including diarrhea, skin and soft tissue infections, and sepsis.[19]Septicemia: In rare cases, V. cholerae can cause septicemia, which is a life-threatening bloodstream infection.[20]Wound infections: V. cholerae can cause wound infections, particularly in people who have suffered injuries in saltwater or brackish water.[21]Gastroenteritis: V. cholerae can cause gastroenteritis, which is inflammation of the stomach and intestines that can cause diarrhea, vomiting, and stomach pain.[22]It's important to note that V. cholerae infections are relatively rare in developed countries, but they can be a serious public health concern in developing countries with poor sanitation and hygiene practices.

2.6.2 Diseases caused by STEC

The diseases caused by STEC can range from mild diarrhea to severe and potentially life-threatening conditions such as hemolytic uremic syndrome (HUS). Some of the diseases caused by STEC include:

Hemorrhagic colitis: This is a severe form of diarrhea, which is often bloody, that can be caused by STEC. It typically lasts for several days and can be accompanied by abdominal cramps and fever.[23]

Hemolytic uremic syndrome (HUS): This is a rare but serious condition that can occur as a complication of STEC infection. It can cause kidney failure, anemia, and a low platelet count, which can lead to bleeding and bruising.[24]

Thrombotic thrombocytopenic purpura (TTP): This is another rare but serious condition that can occur as a complication of STEC infection. It is characterized by blood clots in small blood vessels throughout the body, which can lead to organ damage and neurological symptoms.[25]

2.7 Biofilm and diseases

When in biofilm, microorganisms are in a protective EPS matrix. Hence, they can be resistant to the host’s immune system as well as antibiotics. Hence, they are a major contributor to the

development and persistence of various diseases. For instance, dental plaque biofilms can cause tooth decay and periodontitis[26]; biofilms in chronic wounds can impede healing and increase the risk of infection[27]; and biofilms on prosthetic joints[28] and heart valves[29] can cause chronic infections. Catheter-associated urinary tract infections[30] and biofilms in the lungs of people with cystic fibrosis are also associated with biofilms[31][32] . These infections can be difficult to treat and may cause serious health complications. Therefore, effective prevention and treatment strategies for biofilm-related infections are essential for reducing the burden of these diseases on human health.

2.8 Cholera Biofilm and epidemics

Biofilm formation by *V. cholerae* has been linked to cholera epidemics. In areas with endemic cholera, *V. cholerae* may persist in the environment in the form of biofilms. These biofilms can serve as a reservoir for the bacteria, which can then infect humans through contaminated water or food. In the context of cholera outbreaks, the formation of biofilm could potentially contribute to the prolonged existence and transmission of the disease. Cholera outbreaks are often associated with poor sanitation and hygiene, which can lead to the contamination of water sources with *V. cholerae.* Once introduced into the water, *V. cholerae* may form biofilms, allowing it to persist and spread through the population. Moreover, biofilms may serve as a source of *V. cholerae* for new outbreaks even after an outbreak has been controlled.
In Bangladesh, V. cholerae is known to form biofilms on the surfaces of rivers, ponds, and other water sources, which are commonly used for drinking, washing, and bathing. This allows the bacteria to persist and spread within these water sources, leading to the contamination of food and water supplies and contributing to the continued occurrence of cholera outbreaks in the region.

Each year, there is a surge in the cholera confirmed cases in March and early April. In March 2022, the number of cases reached a record high with patient counts of 1,300 or more per day in Dhaka hospital of icddr,b. In total, they treated more than 50,300 patients from 1 March 2022 to 10 April 2022 across its two hospitals in Dhaka and Chandpur.[33] The ECDC had confirmed 495,433 cholera cases with 29 deaths across Bangladesh by the end of April 2022, with the Rohingya Refugee camp in Cox's Bazar having reported 33,832 of the cases.[34]

2.9 Coomassie Stain and Dissolving Coomassie Stain with Glacial Acetic Acid

Coomassie stain

Coomassie Brilliant Blue is a cationic dye frequently used in protein staining techniques as it is capable of detecting proteins as small as 30-100 ng. The chemical formula of the dye is $C_{47}H_{50}N_3NaO_7S_2$, and it has a molar mass of 3 g/mol. [35]. The absorption maximum range of Coomassie Brilliant Blue dye lies approximately between 465 to 595 nm in the visible range of the electromagnetic spectrum [41]. It is used in colorimetric protein tests to detect proteins. The amount of the complex present in the solution is used as a proxy for the protein content by measuring the intensity of the blue colour upon stabilization. [36]

Biofilms are complex structures with a matrix and difficult to visualise without staining. The

dye can be used to quantitate the amount of biofilm as it stains the extracellular matrix, which is composed of proteins, polysaccharides, and other macromolecules.[37] [38][39] Since it is a cationic dye that has a net positive charge, it is attracted to the proteins in the extracellular matrix of the biofilm that have a net negative charge due to the presence of carboxyl and amino groups in their chemical structure. The subsequent binding gives rise to a dye-protein complex, the amount of which is correlated with intensity of staining.[40] The intensity of the staining can then be used to quantitate the amount of matrix material in the biofilm, and hence the amount of biofilm.

Glacial Acetic Acid

Glacial acetic acid (CH_3COOH) is a strong organic acid that has a low pH, which makes it an effective solvent for Coomassie Brilliant Blue dye. When glacial acetic acid is added to a Coomassie Brilliant Blue solution, the acid dissociates into hydrogen ions (H^+) and acetate ions ($CH3COO^-$).[42] The hydrogen ions increase the acidity of the solution, which protonated the dye molecules and increases their solubility in the acidic environment. The acetate ions, which are negatively charged, can also interact with positively charged sites on the dye molecules, increasing their solubility in the solution.

2.10 ELISA

ELISA, which stands for Enzyme-Linked Immunosorbent Assay, is an immunological assay used in the detection and quantification of proteins, antibodies, peptides, and other biomolecules in biological samples. ELISA is a widely used technique in research and clinical laboratories for a variety of applications, such as diagnosing infectious diseases, monitoring immune responses, and detecting biomarkers for cancer and other diseases.

There are 4 type of ELISA [43]-

1. Direct ELISA
2. Indirect ELISA
3. Sandwich ELISA
4. Competitive ELISA

For this experiment, only the OD measuring function of ELISA is used as it is a quick and relatively simple way to quantify the amount of biofilm present. The resulting signal is measured as an OD value at a specific wavelength which was 450 nm for this study. The OD value is proportional to the amount of target biomolecules present in the biofilm, and can be used to compare the relative amounts of biomolecules in different samples or to monitor changes in the amount of biomolecules over time.

2.11 Other Factors Affecting Biofilm Resuscitation

Biofilm resuscitation requires the availability of nutrients to support the growth and metabolism of dormant cells. Therefore, a lack of nutrients in the surrounding environment may inhibit resuscitation. Temperature can also affect biofilm resuscitation, as certain microorganisms may require specific temperature ranges to resume active growth. For example, psychrophilic microorganisms require cold temperatures for growth, while thermophilic microorganisms require high temperatures.

The pH of the surrounding environment may also influence biofilm resuscitation. Some microorganisms may require a specific pH range to resume active growth, while extreme pH levels can inhibit resuscitation. Oxygen is essential for resuscitation of aerobic microorganisms, as it is required for cellular respiration. Anaerobic microorganisms, on the other hand, may require an oxygen-free environment to resume active growth.

Quorum sensing, a process by which bacteria communicate with each other using signalling molecules, can also influence biofilm resuscitation. The presence of signalling molecules can promote the resuscitation of dormant cells by inducing the expression of genes involved in resuscitation. Physical disturbance, such as shear stress or fluid flow, can disrupt the biofilm structure and promote the resuscitation of dormant cells.

Chapter 3: Methods and Materials

3.1 Organisms

In this study, the following 4 different strains of enteropathogenic bacteria have been used:

1. Shiga toxin-producing Escherichia coli (STEC)
2. Vibrio Cholerae 1877 (HapR mutated)
3. Vibrio Cholerae 1712 (LuxO induced)
4. Vibrio Cholerae WT324

3.2.1 Bacterial Culture Media

In this experiment, Luria Broth (LB) and LB Agar were used as media. All of these organisms are gram-negative bacteria, and the LB medium is optimal for their proliferation. Other than that, preservation media consisted of 0.8% LB Agar. This was used to store bacterial supplies and was coated with paraffin oil.

The required organisms and media have been provided, revived, used and maintained with the standard procedure guide provided by the BRAC University Natural Science Laboratory.

3.2.2 Biochemical Tests

To confirm the bacterial isolates used in this investigation, a number of biochemical tests were conducted.

Thiosulfate-citrate-bile salts-sucrose (TCBS) agar media were used to confirm that the vibrio cholera isolates were indeed vibrio. After streak plating Vibrio cholerae strains on a TCBS agar media plate and incubating it at 37°C for 24 hours, if the green TCBS agar turned yellow, the strains were confirmed to be vibrio cholerae, whereas if they remained green or any other color, they were not. After plating with TCBS, each of the three V.cholerae strains produced yellow colonies, whereas STEC colonies remained green.

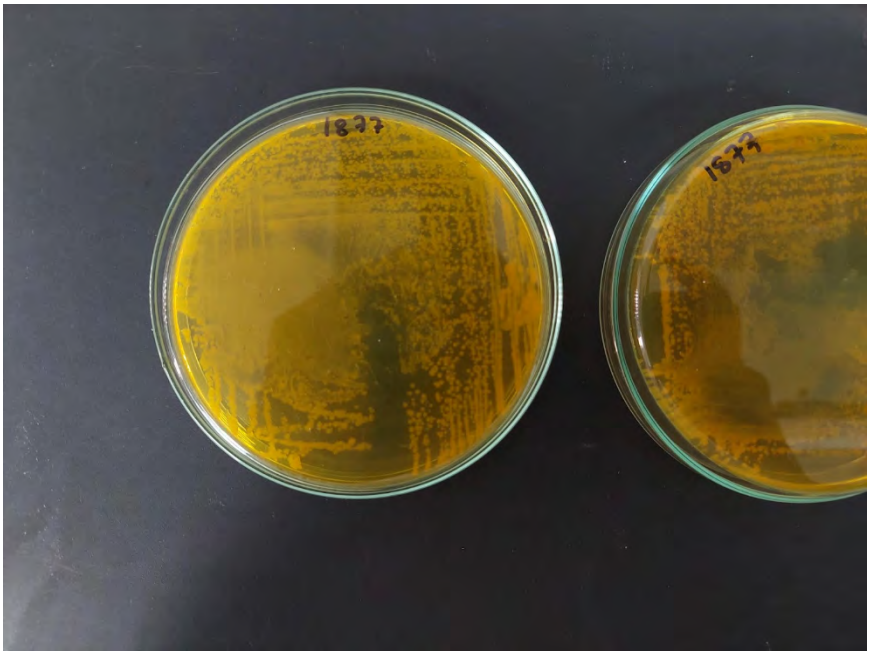

Figure: 1877 V cholerae growth on TCBS agar plate.

On triple sugar iron agar (TSI), the STEC was examined to confirm that it was E. coli. STEC colonies were transferred from a LA plate to TSI slant agar by stabbing then streaking the TSI slant agar with an inoculation needle. At 37 degrees Celsius, the inoculated TSI test-tube were incubated for 24 hours. STEC was determined to be present in the test-tube with a yellow slant and butt.

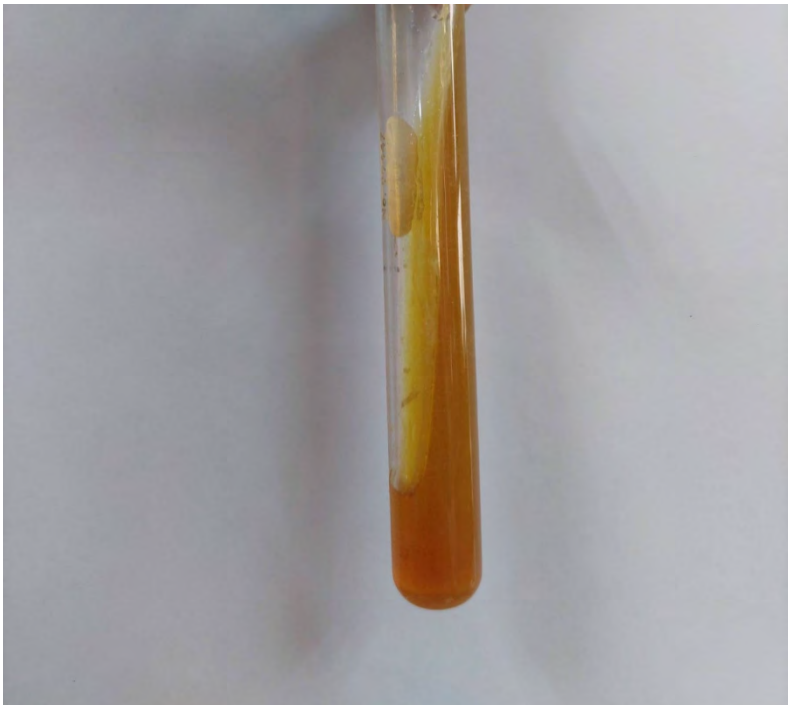

Figure: STEC inoculated in TSI media showing yellow slant and butt

When the STEC was streaked and incubated at 37°C for 24 hours, it produced yellow colonies on XLD agar media plates, confirming that it was an E.coli strain. The purpose of these periodic tests was to maintain the correct bacterial strains throughout the duration of the investigation.

3.3 Method Overview

Firstly, bacteria were revived from stock by plating in fresh LA media. From these, active cultures were prepared then transferred in vials/ELISA plates/ falcon tubes to form biofilm.
After formation of biofilm, they are divided into two groups, one of which is exposed to sunlight and the other of which is kept in the dark for the same amount of time. Four phases were then implemented to determine the effect of sunlight on resuscitation of biofilm. In each phase, different factors were measured or observed such as, the OD of the biofilms in 96-well ELISA plate and of stained biofilm, the cell count in the biofilms and the thickness of biofilm rings.

3.4 Reviving Bacterial Cultures

Using T1N1-preserved laboratory populations, the bacterial isolates were regenerated. By producing subcultures on LB agar plates using the streak plate method, the cultures from the stocks were revived. After 24 hours of incubation at 37°C, solitary colonies were isolated from the plates.

3.5 Creating Young Culture and Biofilm

Single colonies from agar plates were used to inoculate 1 ml of LB in Eppendorf tubes to generate a young culture. This was left overnight in a shaker incubator to produce the overnight culture.
Then, 0.5 mL of the overnight culture was added to 9.5ml of fresh LB in test tubes/falcon tubes placed in the shaker incubator until turbidity was observed. These cultures were then transferred to glass vials, falcon tubes, and ELISA plates and left for 72 to 96 hours to ensure biofilm formation. Thirdly, a half-submerged coverslip was inserted in the falcon containing the young culture.

3.6 Addition of New Media and Discarding Old Bacterial Culture

After 72 hours, the old cultures were discarded and new media was introduced.
The biofilm layer formed on the surface of the culture was meticulously removed using micropipette points. The old culture was then removed using a micropipette while preserving the biofilm ring on the vial surface. To remove the majority of surface bacteria and debris, the vials/falcon tubes were washed twice with sterile LB media.
Fresh LB was then added to the vials, typically 1000µL,, which was sufficient to submerge the biofilm rings in vials and 20 ml in falcon tubes to submerge the biofilm-coated coverslips.
After 96 hours of biofilm formation, the coverslips were removed from the old cultures in the falcon tubes, separated, and deposited in tubes containing fresh LB media. To remove the surplus biofilm layer from the cover slide, saline was used to rinse it. They were entirely immersed in the liquid media.

3.7 Exposure to Sunlight and Darkness

3.7.1 Phase 1: Glass Vials

During this phase, the 72-hour biofilms formed in the glass vials were divided into two groups and exposed to sunlight and darkness for six hours at intervals. In each group, there were four vials of for each of the four bacterial strains. One vial from each set was isolated on the first day in order to collect data during 0 hours of daylight and darkness. After six hours per day, the cultures were plated using the droplet method, and the vials were stained so that changes in the biofilm rings could be observed visually. This procedure was repeated three days in a row.

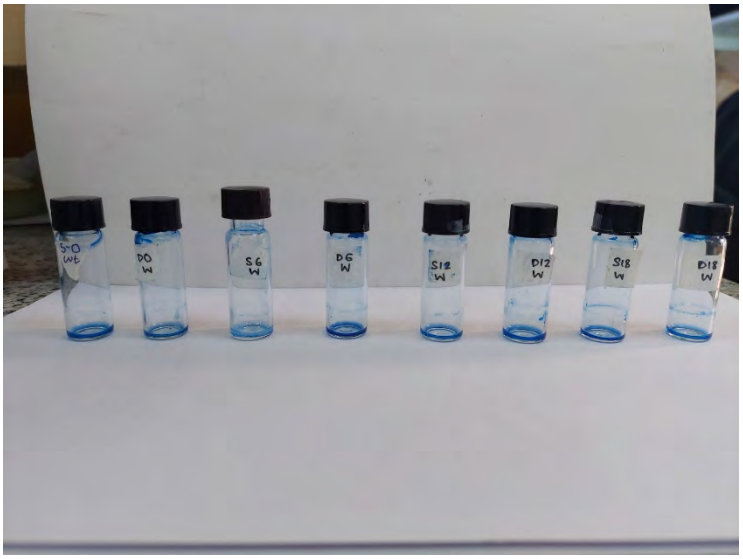

Figure: Changes of biofilm ring of Vibrio WT324 strain caused due to exposure to sunlight and darkness over 18 hours time frame. Observed blue rings have been stained with CBB G-250 overnight and washed with solution.

3.7.2 Phase 2: Optical Density of Dissolved Biofilm Rings

In this phase, the CBB G250-stained biofilm rings were dissolved in glacial acetic acid at a concentration of 33 percent. With the aid of a micropipette, enough glacial acetic acid was poured into the vials to dissolve the stain and submerge the biofilm rings. The vials were shaken so that the stain dissolves into the solution, resulting in a blue solution.

After dispensing 200µL of this solution into labeled wells of a non-autoclavable 96-well ELISA plate, the optical density (OD) was determined utilizing a MultiscanEX ELISA reader. The absorbance was set to 450nm.

Figure: CBB G-250 stained biofilm ring dissolved by 33% glacial acetic acid, forming a blue solution of different blue color spectrum according to the thickness of the biofilm rings used for phase 2 data collection.

3.7.3 Phase 3: Optical Density of Biofilms formed in ELISA Plates

Biofilm was formed on two sterile ELISA plates for 72 hours during this phase. One plate was exposed to sunlight for six hours, while the other was kept in darkness. This occurred daily for three days. Every 3 hours, the OD was measured using a MultiscanEX ELISA Machine. Each plate contained multiple replicates of the same biofilm in order to calculate the average OD value and minimize error.

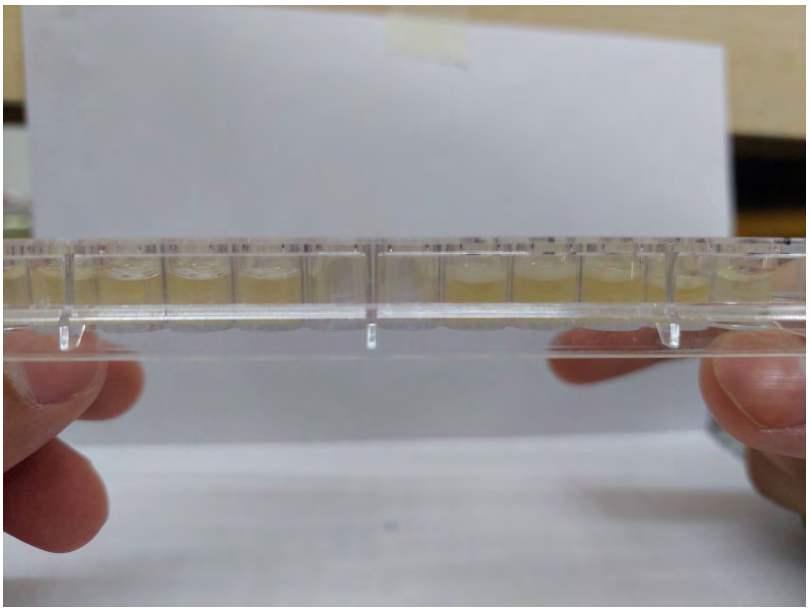

Figure: Active culture media in 96 well ELISA plate for biofilm formation.

3.7.4 Phase 4: Coverslips

In this phase, falcon tubes were implanted with coverslips containing biofilms that had been growing for 96 hours. Additionally, these falcon tubes were divided into two groups, one exposed to sunlight and one to darkness. Each bacterial biofilm had 6 falcons, 3 kept under sunlight and 3 kept in the dark. The biofilm was observed before exposure, 6 hours after exposure and 12 hours after exposure.

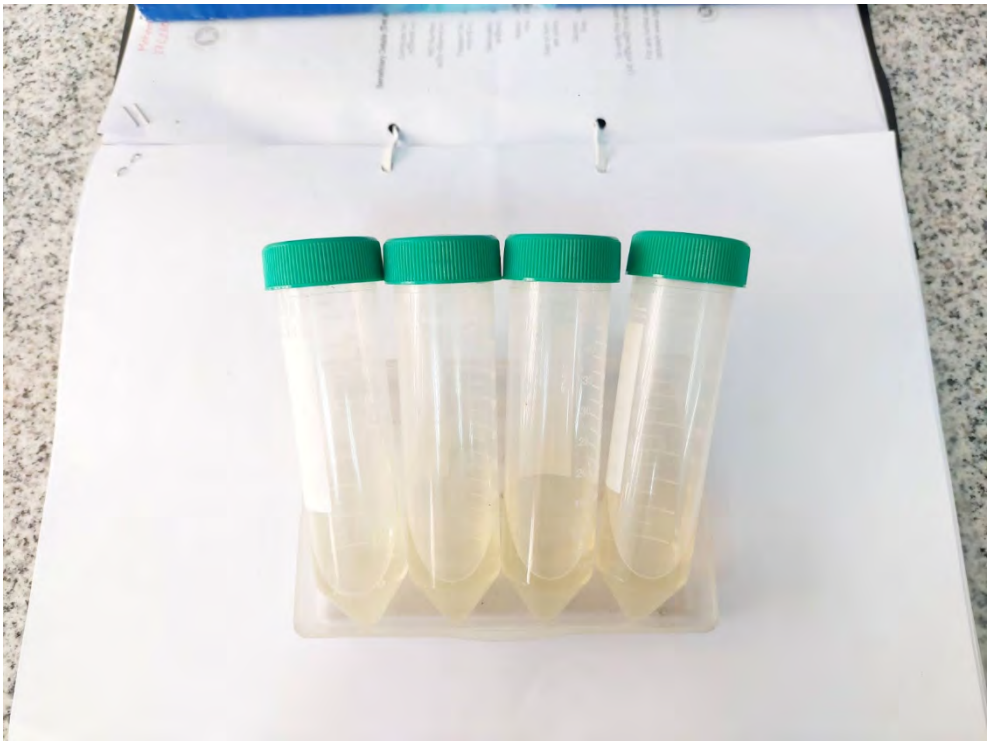

Figure: Exposure of coverslip containing biofilm inside falcon tubes containing LB media to sunlight as part of phase 3 data collection.

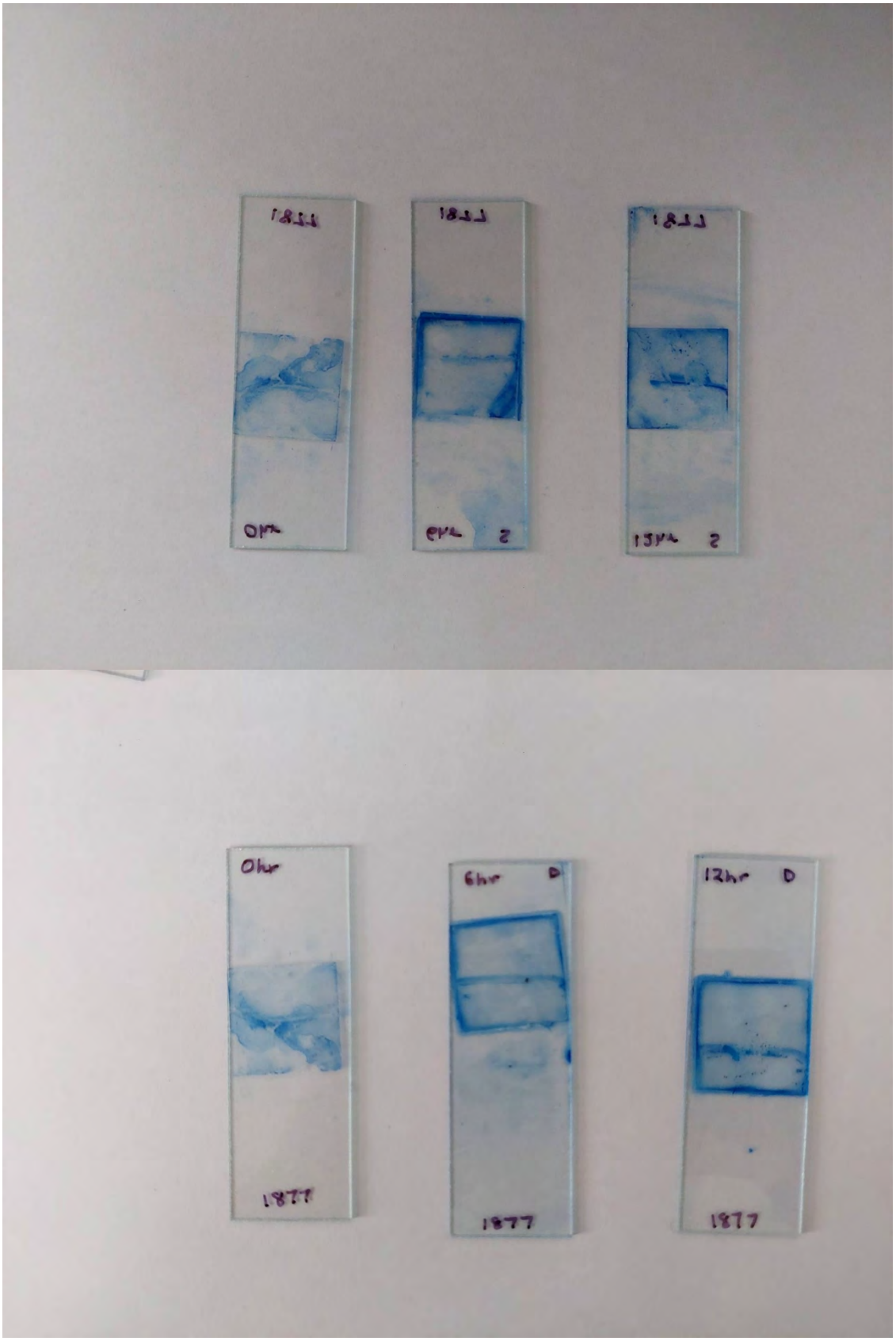

Figure: Biofilm layers on the coverslips of 1877 Vibrio cholerae strain before and after to exposure to sunlight and darkness over a period of 12 hours.

3.8 Plating of Exposed Biofilm Cultures

Initially, the surface biofilm was removed using a micropipette tip. Collecting approximately 100µL of culture from the glass vials and falcon tubes, serial dilution was performed. For serial dilution, 100µL of culture was added before plating four dilutions of each strain using the drop plate method. In each quadrant, three 10µL droplets of culture were added for each dilution. The cell count acquired through this method was instrumental in determining the number of microbes that emerged from the biofilm. The effects of sunlight exposure on the bacterial biofilms were determined by comparing the count during both hours of sunlight and darkness. Using the spread plate procedure, antibiotics were distributed on the petri dishes prior to plating. Antibiotic use ensured the proliferation of only the desirable bacterial strains. The antibiotics used and their respective dosages for each strain are listed below:

Bacterial strain	**Antibiotic used**	**Working Concentrations**	**Volume of antibiotic used**
V. cholerae 1877	Kanamycin	10mg/ml	100 µL
V. cholerae 1712	Metronidazole	0.02mg/ml	80 µL
V. cholerae WT324	Metronidazole	0.02mg/ml	80 µL
STEC	Vancomycin	1mg/ml	100 µL

Table : The table shows the name of the antibiotic and the amount used for the respective bacterial strains.

3.9 Staining Biofilm and Washing it

The vials and coverslips were rinsed carefully with sterile saline (0.9N NaCl), dried, and stained with Coomassie Brilliant Blue G-250 dye. The biofilm was stained overnight with a dye, and then rinsed with sterile saline to remove excess dye.

3.10 Dissolving the Stained Biofilm Rings

In phase 2, surplus dye was removed from the pigmented biofilm rings by rinsing them with sterile saline.

The rings were then treated with glacial acetic acid, which promptly dissolved the stains. The stained biofilm ring vials were dissolved and transferred to 96-well ELISA plates in order to measure the optical density (OD) of the biofilm rings.

In some cases, a second biofilm ring formed in the glass vials.

3.11 ELISA Reading of Biofilm Stains

Using glacial acetic acid, the stains on the biofilm rings formed in the vials were dissolved. Then, 200µL of the dissolved stain was added to the 96-well ELISA plate. The measurement

was taken with a MultiscanEX ELISA Machine. OD was measured for every 6 hours of exposure, each well was replicated.

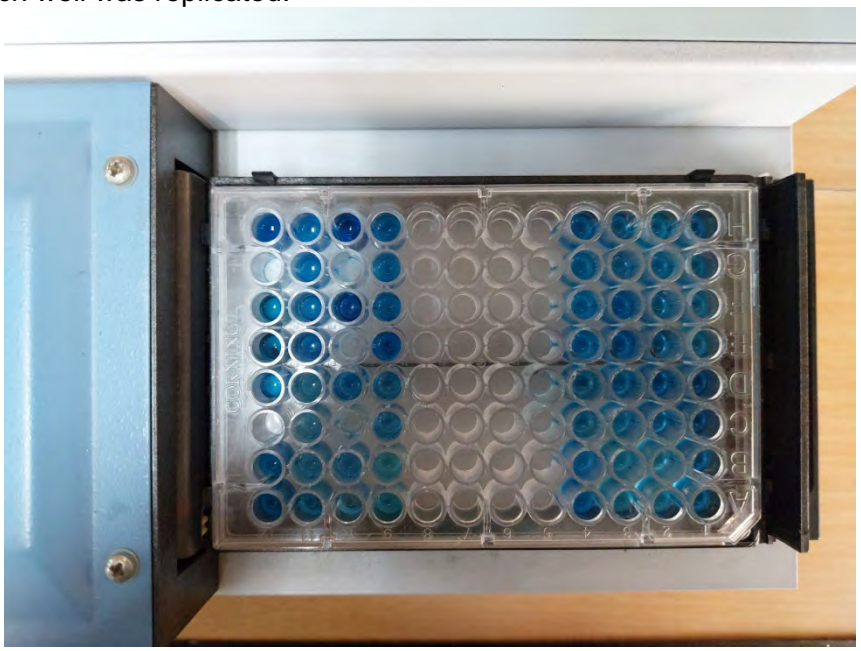

Figure:Dissolved biofilm stains in 96 well ELISA plate.

3.12 ELISA of Biofilms

The absorbance of the biofilms were measured, every 3 hours, at 450 nm wavelength using the MultiscanEX ELISA Machine by Thermo Scientific. As a control, some of the wells were filled with media.

Figure: Multiscan EX ELISA Machine by Thermo Scientific

3.13 Statistical Analysis

The statistical analysis was conducted using Microsoft Excel. The statistical differences between two groups were evaluated using the T-test for independent samples assuming that

the variances were equal. The significance level was deemed to be 0.05. In this investigation, two-tailed t Tests were conducted.

Chapter 4: Results

4.1.1 PHASE 1: Biofilm formed on Glass Vials

Average CFU per ml of bacteria after biofilm degradation in sunlight:

Time/ Bacterial strains	*V. cholerae WT324*	*V. cholerae 1712*	*V. cholerae 1877*	*STEC*
0	1.28E+06	4.70E+06	5.11E+07	9.87E+07
6	1.79E+06	2.14E+07	2.15E+07	1.88E+08
12	1.06E+09	4.87E+08	2.84E+08	7.90E+08

Table: CFU per ml of bacteria after biofilm degradation in sunlight

Average CFU per ml of bacteria after biofilm degradation darkness:

Time/ Bacterial strains	*V. cholerae WT324*	*V. cholerae 1712*	*V. cholerae 1877*	*STEC*
0	4.31E+05	4.15E+06	5.81E+07	5.30E+08
6	2.59E+06	1.03E+09	7.23E+08	5.33E+08
12	2.06E+06	7.84E+08	5.89E+08	8.37E+07

Table: CFU per ml of bacteria after biofilm degradation in darkness

4.1.2 Phase 1 Graphs and Regression Analysis

Organism: WT324

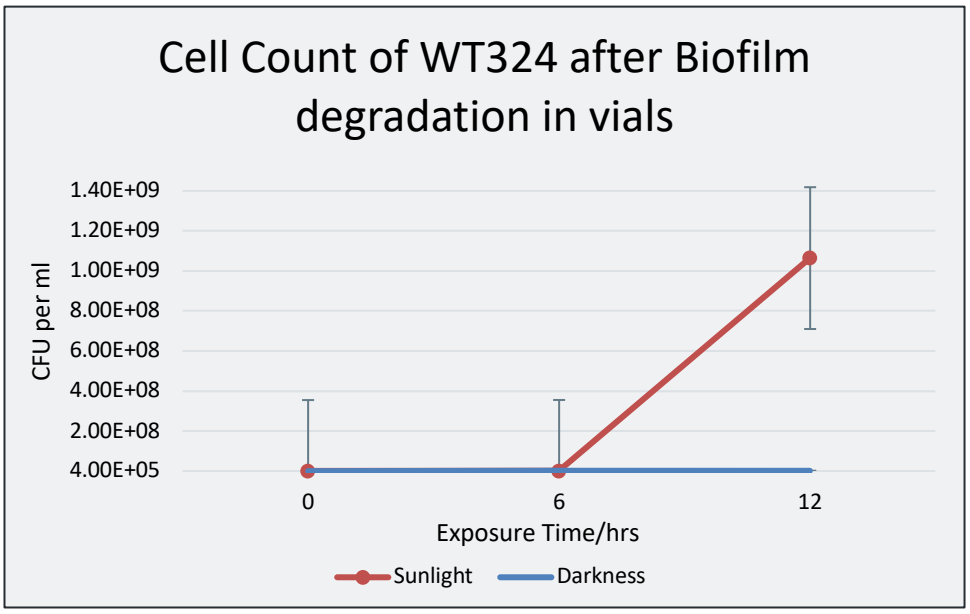

Figure: Graphical representation of cell count of WT324 after biofilm degradation in sunlight and in darkness taken from phase 1 data. X axis represents the exposure time in hours and Y axis represents CFU per ml

Organism	R value and interpretation	R^2 value and interpretation	Regression model and interpretation
WT324 SUNLIGHT	0.86623 Strong Positive Correlation	$R^2 = 0.7503625$ 75.04% of the variation of cell count of WT324 can be explained by the regression model.	y = 8.86E+07x - 1.8E+08 When the time of exposure is 0 hour, the cell count will be will be 1.8E+08 cfu/ml. With every 1 hour increase in exposure time, cell count will increase by 8.86E+07.
WT324 DARK	0.72438 Strong Positive Correlation	$R^2 = 0.524719$ 52.47% of the variation of cell count of WT324 can be explained by the regression model.	y = 1.36E+05x +8.78E+05 When the time of exposure is 0 hour, the cell count will be will be 8.78E+05 cfu/ml. With every 1 hour increase in exposure time, cell count will increase by 1.36E+05.

Table: R value, R square value, regression and their respective interpretations for WT324 exposed to wet season sunlight and darkness taken from phase 1 data

Organism: 1712

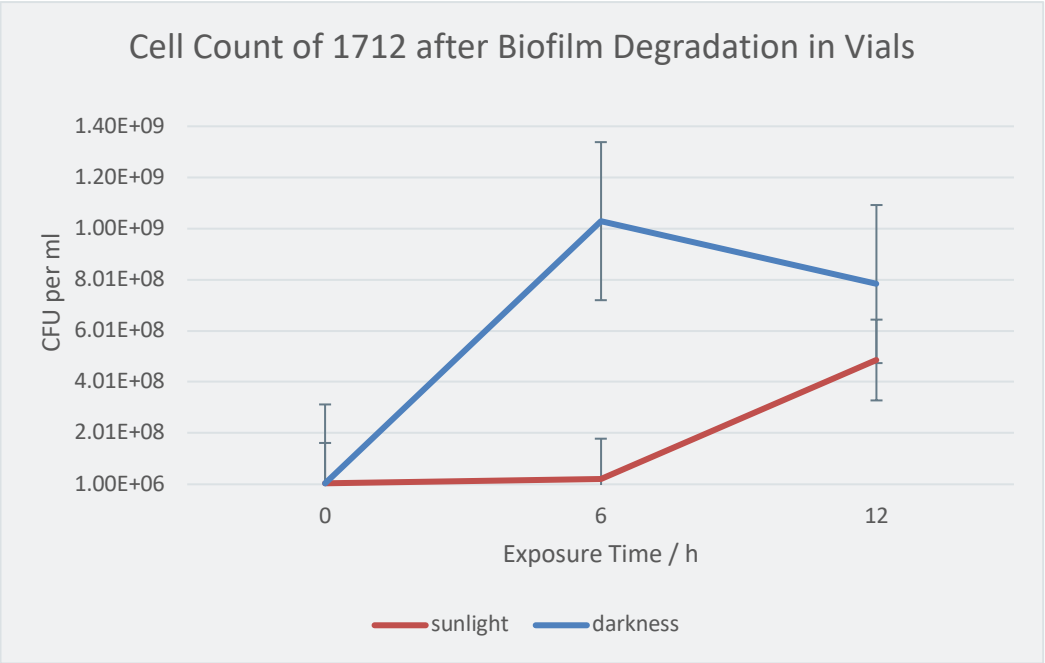

Figure: Graphical representation of cell count of 1712 after biofilm degradation in sunlight and in darkness taken from phase 1 data. X axis represents the exposure time in hours and Y axis represents CFU per ml

organism	R value and interpretation	R^2 value and interpretation	Regression model and interpretation
1712 SUNLIGHT	0.88087 Strong Positive Correlation	$R^2 = 0.775937$ 77.59% of the variation of cell count of 1712 can be explained by the regression model.	y = 4.02E+07x – 7.01E+07 When the time of exposure is 0 hour, the cell count will be will be 7.01E+07 cfu/ml. With every 1 hour increase in exposure time, cell count will increase by 4.02E+07.
1712 DARK	0.72805 Strong Positive Correlation	$R^2 = 0.53005$ 53.01% of the variation of cell count of 1712 can be explained by the regression model.	y = 6.50E+07x + 2.16E+08 When the time of exposure is 0 hour, the cell count will be will be 2.16E+08 cfu/ml. With every 1 hour increase in exposure time, cell count will increase by 6.50E+07.

Table: R value, R square value. regression and their respective interpretations for *1712* exposed to wet season sunlight and darkness taken from phase 1 data

Organism: 1877

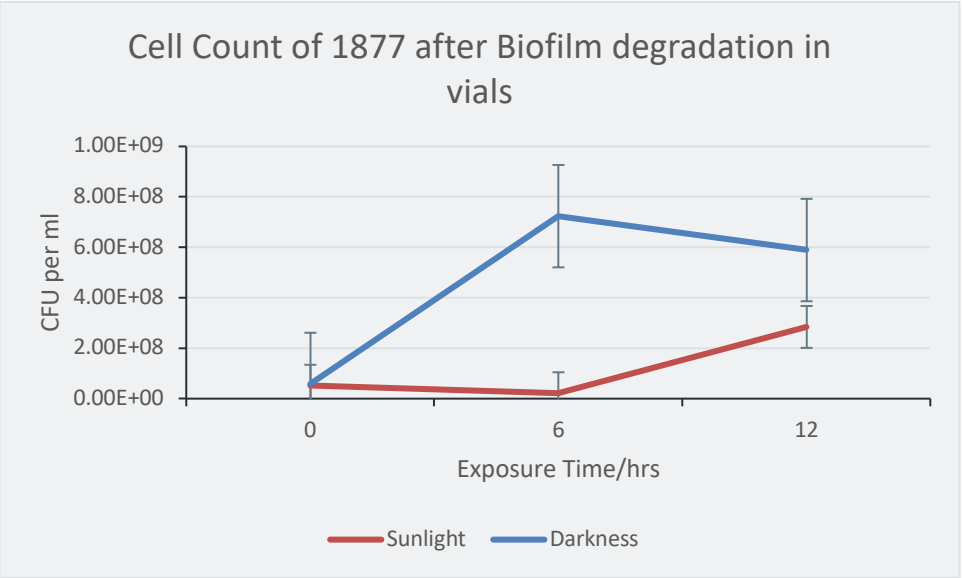

Figure: Graphical representation of cell count of 1877 after biofilm degradation in sunlight and in darkness taken from phase 1 data. X axis represents the exposure time in hours and Y axis represents CFU per ml

Organism	R value and interpretation	R^2 value and interpretation	Regression model and interpretation
1877 SUNLIGHT	0.81012 Strong Positive Correlation	$R^2 = 0.656297$ 65.63% of the variation of cell count of 1877 can be explained by the regression model.	y = 1.94E+07x + 2.35E+06 When the time of exposure is 0 hour, the cell count will be will be 2.35E+06 cfu/ml. With every 1 hour increase in exposure time, cell count will increase by 1.94E+07.
1877 DARK	0.754614 Strong Positive Correlation	$R^2 = 0.5694427$ 56.94% of the variation of cell count of 1877 can be explained by the regression model.	y = 4.42E+07x +1.91E+08 When the time of exposure is 0 hour, the cell count will be will be 1.91E+08 cfu/ml. With every 1 hour increase in exposure time, cell count will increase by 4.42E+07.

Table: R value, R square value, regression and their respective interpretations for 1877 exposed to wet season sunlight and darkness taken from phase 1 data

Organism: *STEC*

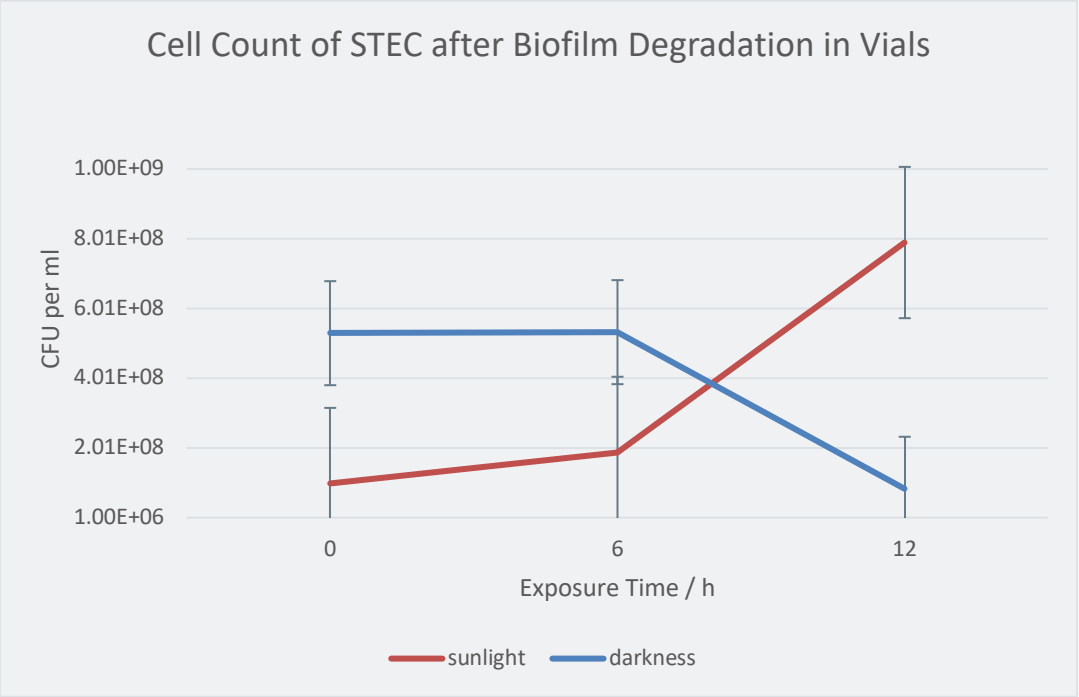

Figure: Graphical representation of cell count of STEC after biofilm degradation in sunlight and in darkness taken from phase 1 data. X axis represents the exposure time in hours and Y axis represents CFU per ml

organism	R value and interpretation	R^2 value and interpretation	Regression model and interpretation
STEC SUNLIGHT	0.91927 Strong Positive Correlation	$R^2 = 0.84506$ 84.51% of the variation of cell count of STEC can be explained by the regression model.	y = 5.76E+07x + 1.32E+07 When the time of exposure is 0 hour, the cell count will be will be 1.32E+07 cfu/ml. With every 1 hour increase in exposure time, cell count will increase by 5.76E+07.
STEC DARK	-0.86311 Strong Negative Correlation	$R^2 = 0.74496$ 74.50% of the variation of cell count of STEC can be explained by the regression model.	y = 6.05E+08 – 3.72E+07x When the time of exposure is 0 hour, the cell count will be will be 6.05E+08 cfu/ml. And with every 1 hour increase in exposure time, cell count will decrease by 3.72E+07.

Table: R value, R square value. regression and their respective interpretations for *STEC* exposed to wet season sunlight and darkness taken from phase 1 data

4.1.3 T-Tests for Optical Density of Biofilm Rings Stained by Coomassie Blue

Null Hypothesis: There is no significant difference between OD of Coomassie rings in wet season sun and wet season dark.

Alternative Hypothesis: There is significant difference between OD of Coomassie rings in wet season sun and wet season dark.

Organism	Time/ hour	p-value	Interpretation	NULL HYPOTHESIS	Overall remarks
WT324 SUN VS. WT324 DARK	0	0.446730151	0.44673 > 0.05	ACCEPT	ACCEPT
	6	0.52617393	0.52617 > 0.05	ACCEPT	
	12	0.152616067	0.152616 > 0.05	ACCEPT	
1712 SUN VS. 1712 DARK	0	0.8768	0.8768 > 0.05	ACCEPT	ACCEPT
	6	0.002	0.002 < 0.05	REJECT	
	12	0.5387	0.5387 > 0.05	ACCEPT	
1877 SUN VS. 1877 DARK	0	0.81908	0.81908 > 0.05	ACCEPT	ACCEPT
	6	0.010638	0.010638 <0.05	REJECT	
	12	0.43383	0.43383 > 0.05	ACCEPT	
STEC SUN VS. STEC DARK	0	0.1472	0.1472 > 0.05	ACCEPT	ACCEPT
	6	0.167	0.167 > 0.05	ACCEPT	
	12	0.1928	0.1928 > 0.05	ACCEPT	

Table: Statistical significance comparison between the cell count taken from Phase 1 data of biofilms exposed to Wet Season sunlight and Wet Season darkness By t-test

4.1.4 Interpretation of the Statistical Analysis of Phase 1 Data

Between 0 to 6 hours, the cell counts of WT324, 1712 and 1877 increased when exposed to sunlight whereas the cell count for STEC decreased. Between 6 to 12 hours, the cell count of all 4 organisms showed a significant increase. For all 4 organisms, upon exposure to darkness, the cell count increased from 0 to 6 hours with STEC having the smallest increase of the 4 organisms. Then from 6 to 9 hours, the OD decreased with 1877 demonstrating the smallest increase.

The regression model and its implications have been outlined in the table above. The results of the T-test shows that there is no significant difference between cell counts from bacterial biofilms of WT324, 1712, 1877 and STEC exposed to sunlight and darkness.

4.2.1 PHASE 2: OD of Biofilm Rings Stained with Coomassie Blue Dye

Average OD of biofilm exposed to sunlight:

	Time/ Bacterial strains	*V. cholerae WT324*	*V. cholerae 1712*	*V. cholerae 1877*	*STEC*
Set 1	0	0.1231667	0.11933	0.13767	0.16533
	6	0.1345	0.12317	0.109	0.14033
	12	0.125667	0.11317	0.09833	0.11233
	18	0.1185	0.0815	0.108	0.0955
Set 2	0	0.0835	0.08833	0.10667	0.04167
	6	0.108	0.1115	0.08883	0.04083
	12	0.0691667	0.0805	0.0875	0.03767
	18	0.0995	0.079	0.1248	0.03467
Set 3	0	0.082	0.09617	0.12233	n/a
	6	0.12	0.12867	0.08867	
	12	0.0695	0.0885	0.09	
	18	0.0965	0.08967	0.12733	

Table: Average OD of stained biofilm exposed to sunlight, obtained using ELISA at 450 nm

Average OD of biofilm exposed to darkness:

	Time/ Bacterial strains	*V. cholerae WT324*	*V. cholerae 1712*	*V. cholerae 1877*	*STEC*
Set 1	0	0.125333	0.14433	0.11983	0.052333
	6	0.12	0.12317	0.14533	0.041
	12	0.095833	0.1095	0.118	0.04267
	18	0.09	0.1015	0.13172	0.052
Set 2	0	0.121	0.10217	0.13167	0.05367
	6	0.143833	0.10217	0.108	0.04483
	12	0.1165	0.0875	0.16017	0.04033
	18	0.101667	0.09317	0.13745	0.047
Set 3	0	0.1281667	0.101	0.142	n/a
	6	0.1465	0.0985	0.11533	
	12	0.1345	0.085833	0.13533	
	18	0.1135	0.0975	0.12454	

Table: Average OD of stained biofilm exposed to darkness, obtained using ELISA at 450 nm

4.2.2 Phase 2 Graphs and Regression Analysis

Organism: *Wt324*

Set 1

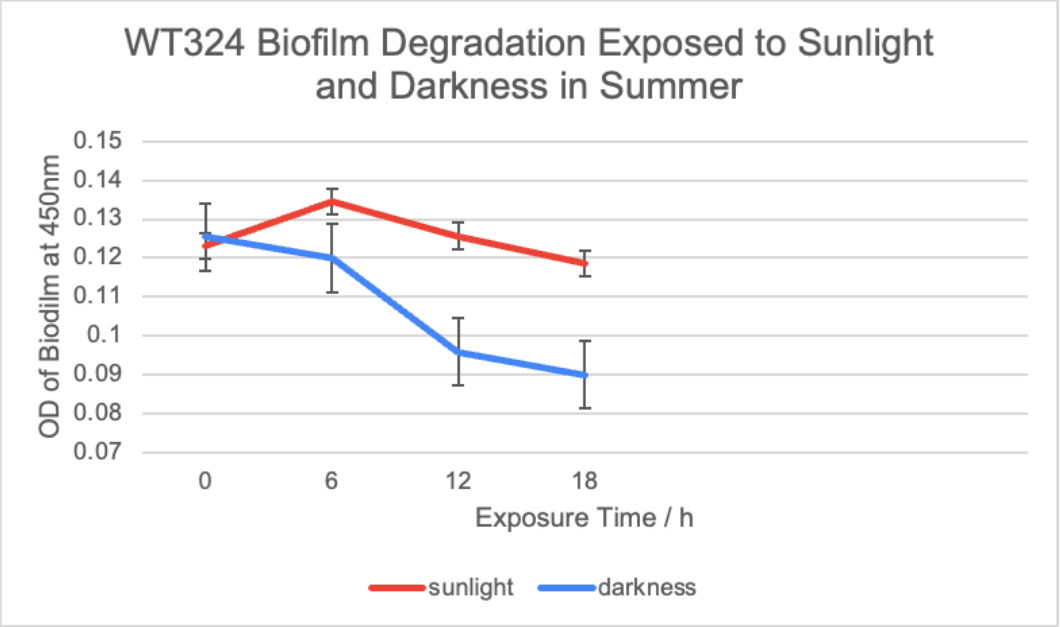

Figure: Graphical representation of OD of CBB G-250 stained biofilm rings of *Vibrio WT324* after biofilm degradation in sunlight and in darkness taken from phase 2 data. X axis represents the exposure time in hours and Y axis represents OD of coomassie blue stained biofilm rings

organism	R value and interpretation	R^2 value and interpretation	Regression model and interpretation
WT324 SUNLIGHT	-0.438672 Moderate Negative Correlation	$R^2 = 0.192433$ 19.24% of the variation in OD of WT324 biofilm stain can be explained by the time of exposure in the sun.	y = 0.1288833 - 0.000360556x When the time of exposure is 0 hour, the OD of the biofilm stain will be 0.1288833. And with every 1 hour increase in exposure time, the OD of biofilm stains will decrease by 0.000360556.
WT324 DARK	-0.961538 Strong Negative Correlation	= 0.924554556 92.46% of the variation in OD of WT324 biofilm stain can be explained by the time of exposure in the dark.	y = 0.12731667 - 0.00216944x When the time of exposure is 0 hour, the OD of the biofilm stain will be 0.12731667. And with every 1 hour increase in exposure time, the OD of biofilm stains will decrease by 0.00216944.

Table: R value, R square value. regression and their respective interpretations for *WT324* exposed to wet season sunlight and darkness taken from phase 2 data

Set 2

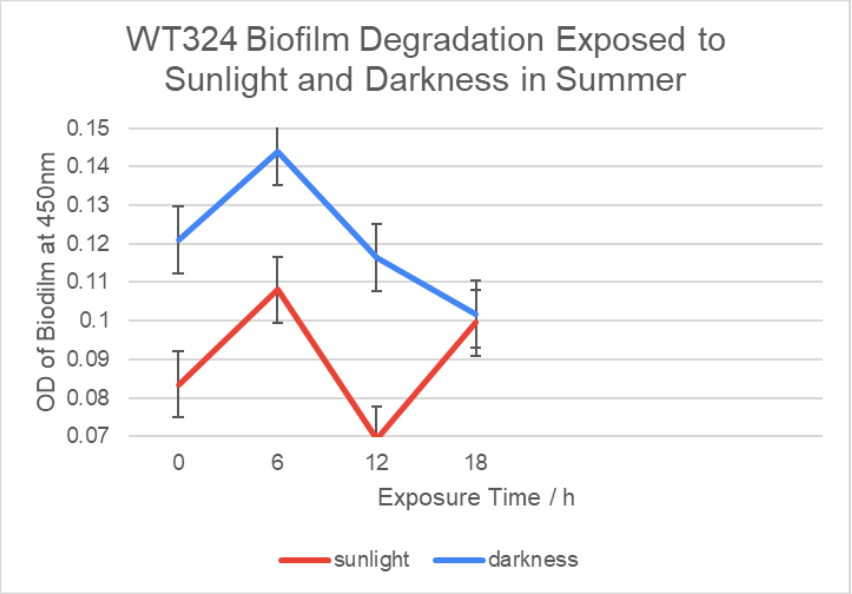

Figure: Graphical representation of OD of CBB G-250 stained biofilm rings of *Vibrio WT324* after biofilm degradation in sunlight and in darkness taken from phase 2 data. X axis represents the exposure time in hours and Y axis represents OD of coomassie blue stained biofilm rings

organism	R value and interpretation	R^2 value and interpretation	Regression model and interpretation
WT324 SUNLIGHT	0.068686976 Very Weak Positive Correlation	R^2 = 0.004717907 0.47% of the variation in OD of WT324 biofilm stain can be explained by the time of exposure in the sun.	y = 0.08866667 + 0.000152778x When the time of exposure is 0 hour, the OD of the biofilm stain will be 0.08866667. And with every 1 hour increase in exposure time, the OD of biofilm stains will increase by 0.000152778.
WT324 DARK	-0.63075428 Moderate Negative Correlation	= 0.397850964 39.79% of the variation in OD of WT324 biofilm stain can be explained by the time of exposure in the dark.	y = 0.13355 - 0.00142222x When the time of exposure is 0 hour, the OD of the biofilm stain will be 0.13355. And with every 1 hour increase in exposure time, the OD of biofilm stains will decrease by 0.00142222.

Table: R value, R square value. regression and their respective interpretations for *WT324* exposed to wet season sunlight and darkness taken from phase 2 data

Set 3

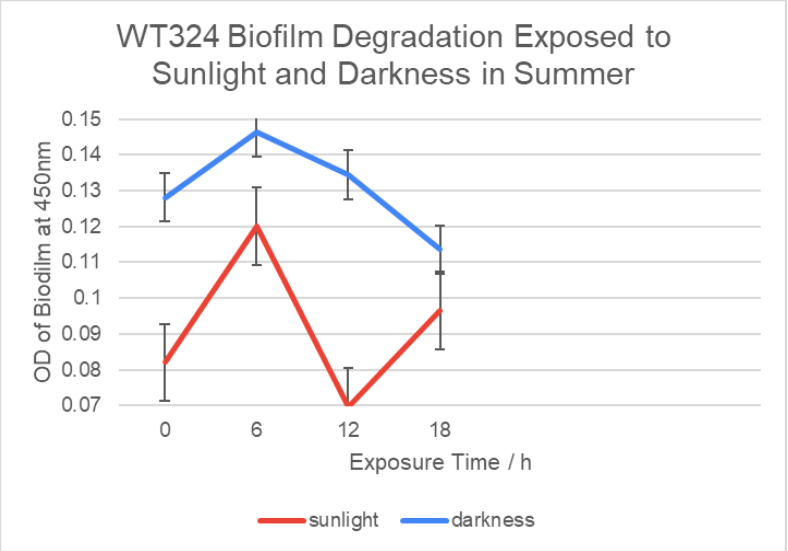

Figure: Graphical representation of OD of CBB G-250 stained biofilm rings of *Vibrio WT324* after biofilm degradation in sunlight and in darkness taken from phase 2 data. X axis represents the exposure time in hours and Y axis represents OD of coomassie blue stained biofilm rings

organism	R value and interpretation	R^2 value and interpretation	Regression model and interpretation
WT324 SUNLIGHT	-0.041677 Very Weak Negative Correlation	R^2 = 0.001736973 0.17% of the variation in OD of WT324 biofilm stain can be explained by the time of exposure in the sun.	y = 0.09305 - 0.00011667x When the time of exposure is 0 hour, the OD of the biofilm stain will be 0.09305. And with every 1 hour increase in exposure time, the OD of biofilm stains will decrease by 0.00011667.
WT324 DARK	-0.52618319 Moderate Negative Correlation	= 0.276868746 27.68% of the variation in OD of WT324 biofilm stain can be explained by the time of exposure in the dark.	y = 0.13906667 - 0.00093333x When the time of exposure is 0 hour, the OD of the biofilm stain will be 0.13906667. And with every 1 hour increase in exposure time, the OD of biofilm stains will decrease by 0.00093333.

Table: R value, R square value. regression and their respective interpretations for *WT324* exposed to wet season sunlight and darkness taken from phase 2 data

Organism: ***1712***

Set 1

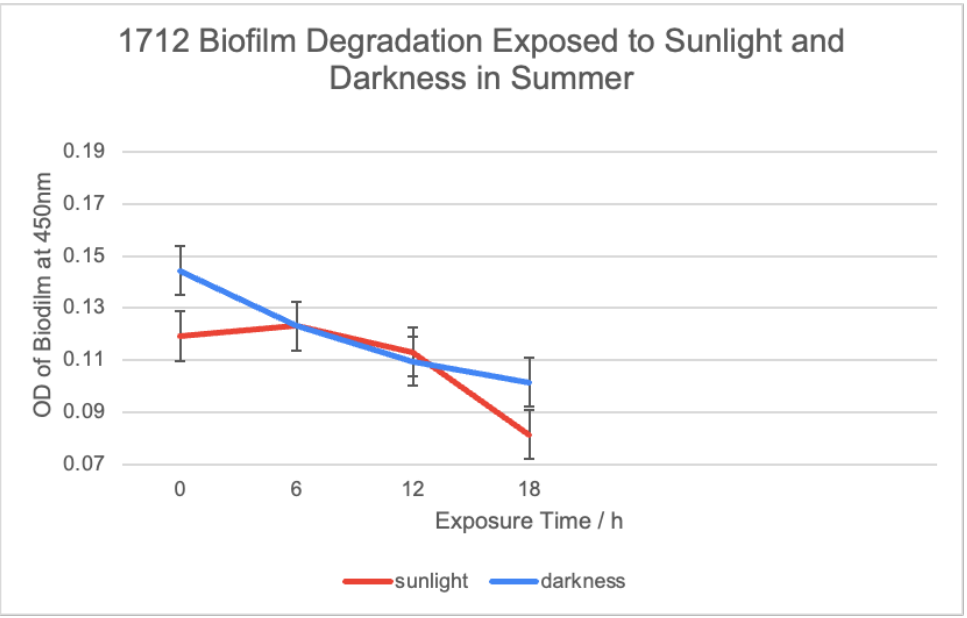

Figure: Graphical representation of OD of CBB G-250 stained biofilm rings of *Vibrio WT324* after biofilm degradation in sunlight and in darkness taken from phase 2 data. X axis represents the exposure time in hours and Y axis represents OD of coomassie blue stained biofilm rings

organism	R value and interpretation	R^2 value and interpretation	Regression model and interpretation
1712 SUNLIGHT	-0.840022141 Strong Negative Correlation	$R^2 = 0.705637$ 70.56% of the variation in OD of WT324 biofilm stain can be explained by the time of exposure in the sun.	y = 0.127816667 - 0.002058333x When the time of exposure is 0 hour, the OD of the biofilm stain will be 0.127816667. And with every 1 hour increase in exposure time, the OD of biofilm stains will decrease by 0.002058333.
1712 DARK	-0.979144391 Strong Negative Correlation	$R^2 = 0.958723738$ 95.87% of the variation in OD of WT324 biofilm stain can be explained by the time of exposure in the dark.	y = 0.14095 - 0.002369444x When the time of exposure is 0 hour, the OD of the biofilm stain will be 0.14095. And with every 1 hour increase in exposure time, the OD of biofilm stains will decrease by 0.002369444.

Table: R value, R square value. regression and their respective interpretations for *WT324* exposed to wet season sunlight and darkness taken from phase 2 data

Set 2

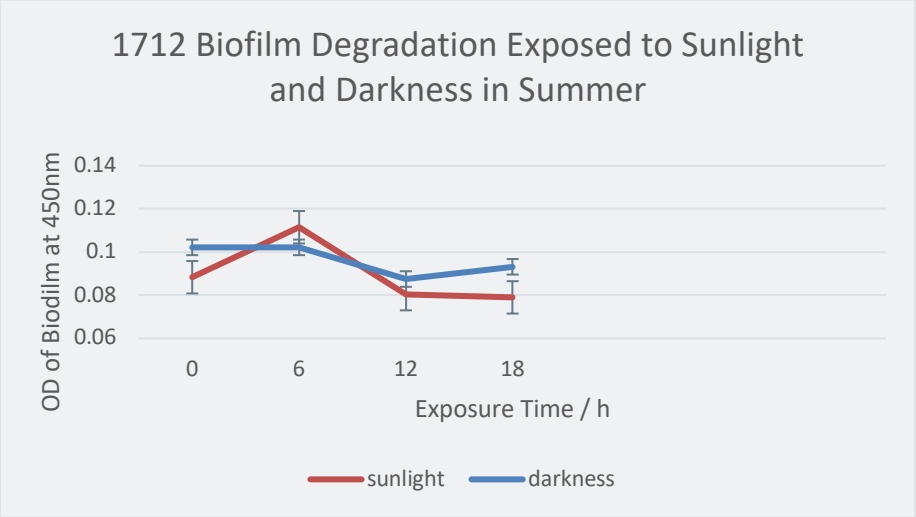

Figure: Graphical representation of OD of CBB G-250 stained biofilm rings of *Vibrio WT324* after biofilm degradation in sunlight and in darkness taken from phase 2 data. X axis represents the exposure time in hours and Y axis represents OD of coomassie blue stained biofilm rings

organism	R value and interpretation	R^2 value and interpretation	Regression model and interpretation
1712 SUNLIGHT	-0.507352886 Moderate Negative Correlation	$R^2 = 0.257406951$ 25.74% of the variation in OD of WT324 biofilm stain can be explained by the time of exposure in the sun.	y = 0.098683333 - 0.000983333x When the time of exposure is 0 hour, the OD of the biofilm stain will be 0.098683333. And with every 1 hour increase in exposure time, the OD of biofilm stains will decrease by 0.000983333.
1712 DARK	-0.74573834 Strong Negative Correlation	$R^2 = 0.556148781$ 55.61% of the variation in OD of WT324 biofilm stain can be explained by the time of exposure in the dark.	y = 0.1025 - 0.000694444x When the time of exposure is 0 hour, the OD of the biofilm stain will be 0.1025. And with every 1 hour increase in exposure time, the OD of biofilm stains will increase by 0.000694444.

Table: R value, R square value. regression and their respective interpretations for *WT324* exposed to wet season sunlight and darkness taken from phase 2 data

Set 3

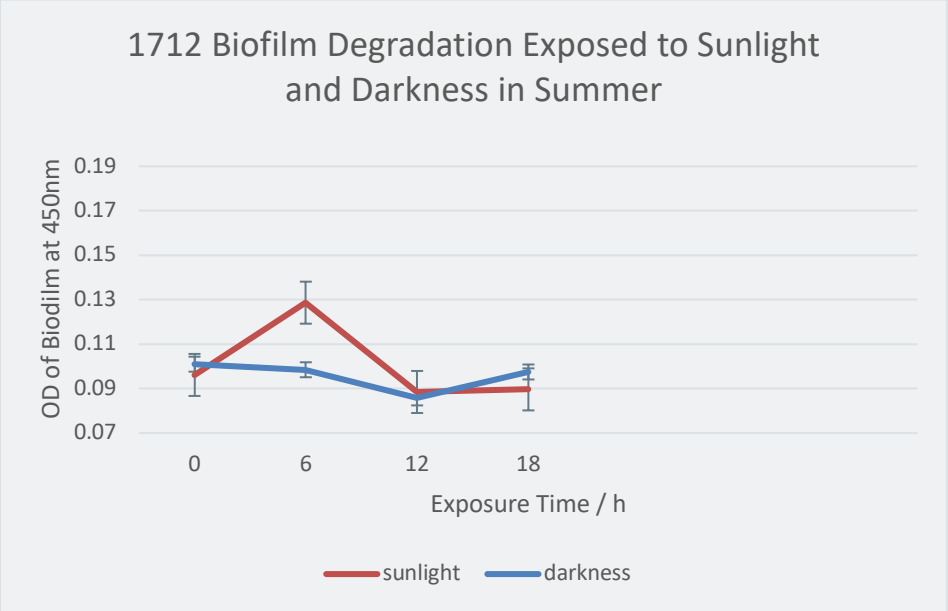

Figure: Graphical representation of OD of CBB G-250 stained biofilm rings of *Vibrio WT324* after biofilm degradation in sunlight and in darkness taken from phase 2 data. X axis represents the exposure time in hours and Y axis represents OD of coomassie blue stained biofilm rings

organism	R value and interpretation	R^2 value and interpretation	Regression model and interpretation
1712 SUNLIGHT	-0.407254911 Moderate Negative Correlation	$R^2 = 0.165856562$ 16.59% of the variation in OD of WT324 biofilm stain can be explained by the time of exposure in the sun.	y = 0.1097 - 0.000994444x When the time of exposure is 0 hour, the OD of the biofilm stain will be 0.1097. And with every 1 hour increase in exposure time, the OD of biofilm stains will decrease by 0.000994444.
1712 DARK	-0.443352423 Moderate Negative Correlation	$R^2 = 0.196561371$ 19.66 % of the variation in OD of WT324 biofilm stain can be explained by the time of exposure in the dark.	y = 0.099183333 - 0.000386111x When the time of exposure is 0 hour, the OD of the biofilm stain will be 0.099183333. And with every 1 hour increase in exposure time, the OD of biofilm stains will increase by 0.000386111.

Table: R value, R square value. regression and their respective interpretations for *WT324* exposed to wet season sunlight and darkness taken from phase 2 data

Organism 1877:

Set 1

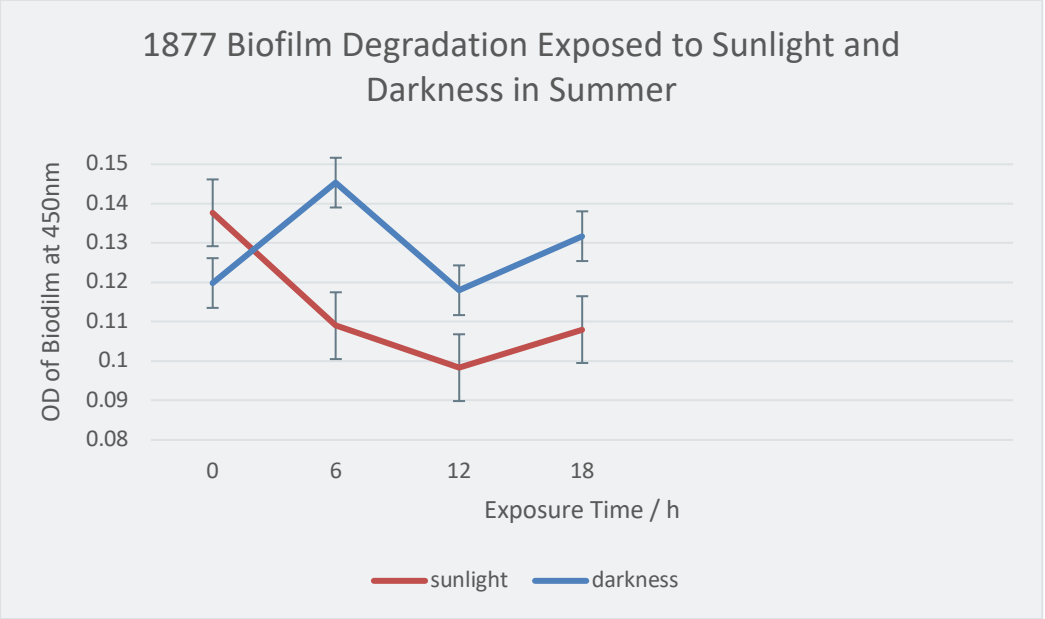

Figure: Graphical representation of OD of CBB G-250 stained biofilm rings of *Vibrio WT324* after biofilm degradation in sunlight and in darkness taken from phase 2 data. X axis represents the exposure time in hours and Y axis represents OD of coomassie blue stained biofilm rings

organism	R value and interpretation	R^2 value and interpretation	Regression model and interpretation
1877 SUNLIGHT	-0.758055989 Strong Negative Correlation	$R^2 = 0.574688$ 57.46% of the variation in OD of WT324 biofilm stain can be explained by the time of exposure in the sun.	y = 0.1282 - 0.001661111x When the time of exposure is 0 hour, the OD of the biofilm stain will be 0.1282. And with every 1 hour increase in exposure time, the OD of biofilm stains will decrease by 0.001661111.
1877 DARK	-0.085300807 Very Weak Positive Correlation	$R^2 = 0.007276228$ 0.73% of the variation in OD of WT324 biofilm stain can be explained by the time of exposure in the dark.	y = 0.127471212 - 0.000139141x When the time of exposure is 0 hour, the OD of the biofilm stain will be 0.127471212. And with every 1 hour increase in exposure time, the OD of biofilm stains will increase by 0.000139141.

Table: R value, R square value. regression and their respective interpretations for *WT324* exposed to wet season sunlight and darkness taken from phase 2 data

Set 2

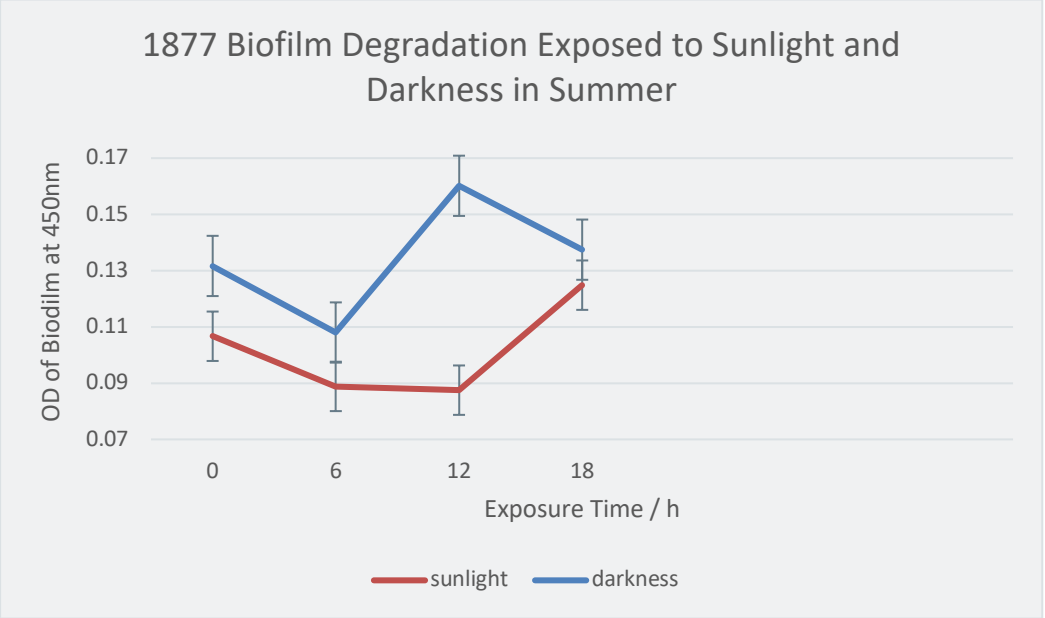

Figure: Graphical representation of OD of CBB G-250 stained biofilm rings of *Vibrio WT324* after biofilm degradation in sunlight and in darkness taken from phase 2 data. X axis represents the exposure time in hours and Y axis represents OD of coomassie blue stained biofilm rings

organism	R value and interpretation	R^2 value and interpretation	Regression model and interpretation
1877 SUNLIGHT	0.390521334 Weak Positive Correlation	$R^2 = 0.152506913$ 15.25% of the variation in OD of WT324 biofilm stain can be explained by the time of exposure in the sun.	y = 0.0939833 + 0.000886111x When the time of exposure is 0 hour, the OD of the biofilm stain will be 0.0939833. And with every 1 hour increase in exposure time, the OD of biofilm stains will increase by 0.000886111.
1877 DARK	0.418878816 Moderate Positive Correlation	$R^2 = 0.175459462$ 17.55% of the variation in OD of WT324 biofilm stain can be explained by the time of exposure in the dark.	y = 0.123892424 - 0.001158838x When the time of exposure is 0 hour, the OD of the biofilm stain will be 0.123892424. And with every 1 hour increase in exposure time, the OD of biofilm stains will increase by 0.001158838.

Table: R value, R square value. regression and their respective interpretations for *WT324* exposed to wet season sunlight and darkness taken from phase 2 data

Set 3

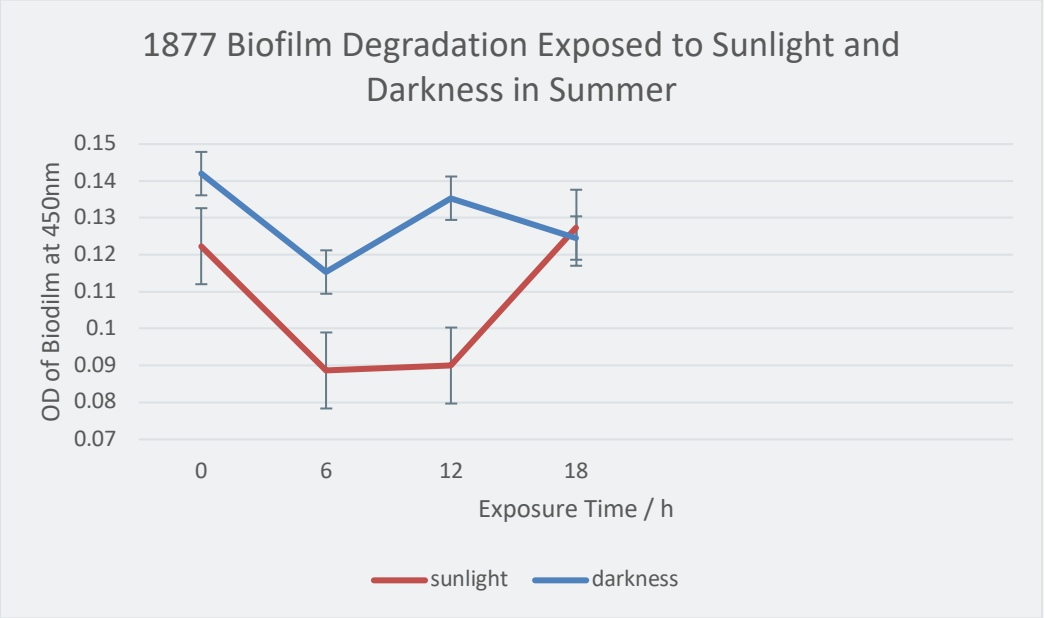

Figure: Graphical representation of OD of CBB G-250 stained biofilm rings of *Vibrio WT324* after biofilm degradation in sunlight and in darkness taken from phase 2 data. X axis represents the exposure time in hours and Y axis represents OD of coomassie blue stained biofilm rings

organism	R value and interpretation	R^2 value and interpretation	Regression model and interpretation
1877 SUNLIGHT	0.10233794 Weak Positive Correlation	$R^2 = 0.010473054$ 1.05% of the variation in OD of WT324 biofilm stain can be explained by the time of exposure in the sun.	y = 0.104633333 + 0.000272222x When the time of exposure is 0 hour, the OD of the biofilm stain will be 0.104633333. And with every 1 hour increase in exposure time, the OD of biofilm stains will increase by 0.000272222.
1877 DARK	-0.355081134 = Moderate Negative Correlation	$R^2 = 0.126082612$ 12.61% of the variation in OD of WT324 biofilm stain can be explained by the time of exposure in the dark.	y = 0.134157576 - 0.000539394x When the time of exposure is 0 hour, the OD of the biofilm stain will be 0.134157576. And with every 1 hour increase in exposure time, the OD of biofilm stains will decrease by 0.000539394.

Table: R value, R square value. regression and their respective interpretations for *WT324* exposed to wet season sunlight and darkness taken from phase 2 data

Organism: STEC

Set 1

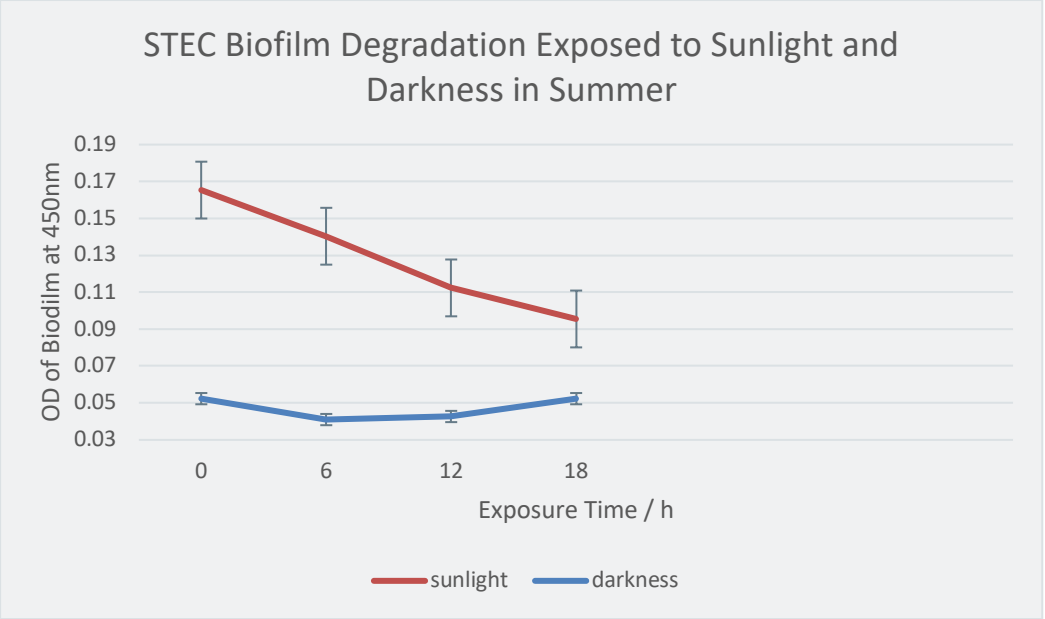

Figure: Graphical representation of OD of CBB G-250 stained biofilm rings of *Vibrio WT324* after biofilm degradation in sunlight and in darkness taken from phase 2 data. X axis represents the exposure time in hours and Y axis represents OD of coomassie blue stained biofilm rings

organism	R value and interpretation	R^2 value and interpretation	Regression model and interpretation
STEC SUNLIGHT	-0.995298372 Very Strong Negative Correlation	$R^2 = 0.990618849$ 99.06% of the variation in OD of WT324 biofilm stain can be explained by the time of exposure in the sun.	y = 0.164 - 0.003958333x When the time of exposure is 0 hour, the OD of the biofilm stain will be 0.164. And with every 1 hour increase in exposure time, the OD of biofilm stains will increase by 0.003958333.
STEC DARK	0.035271668 Very Weak Positive Correlation	$R^2 = 0.001244091$ 0.12% of the variation in OD of WT324 biofilm stain can be explained by the time of exposure in the dark.	y = 0.0468333 - 0.00002778x When the time of exposure is 0 hour, the OD of the biofilm stain will be 0.0468333. And with every 1 hour increase in exposure time, the OD of biofilm stains will decrease by 0. 00002778.

Table: R value, R square value. regression and their respective interpretations for *WT324* exposed to wet season sunlight and darkness taken from phase 2 data

Set 2

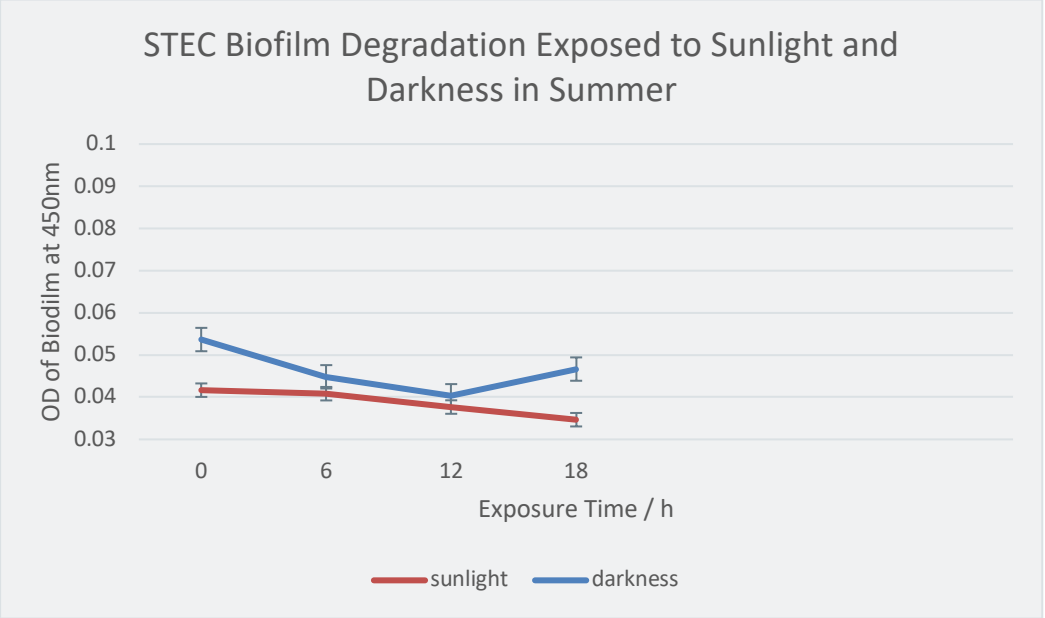

Figure: Graphical representation of OD of CBB G-250 stained biofilm rings of *Vibrio WT324* after biofilm degradation in sunlight and in darkness taken from phase 2 data. X axis represents the exposure time in hours and Y axis represents OD of coomassie blue stained biofilm rings

organism	R value and interpretation	R^2 value and interpretation	Regression model and interpretation
STEC SUNLIGHT	-0.975485907 Very Strong Negative Correlation	$R^2 = 0.951572754$ 95.16% of the variation in OD of WT324 biofilm stain can be explained by the time of exposure in the sun.	y = 0.042333 - 0.000402778x When the time of exposure is 0 hour, the OD of the biofilm stain will be 0.042333. And with every 1 hour increase in exposure time, the OD of biofilm stains will increase by 0.000402778.
STEC DARK	-0.594045984 Moderate Negative Correlation	$R^2 = 0.352890631$ 35.29% of the variation in OD of WT324 biofilm stain can be explained by the time of exposure in the dark.	y = 0.0502 - 0.000425x When the time of exposure is 0 hour, the OD of the biofilm stain will be 0.0502. And with every 1 hour increase in exposure time, the OD of biofilm stains will decrease by 0. 000425.

Table: R value, R square value. regression and their respective interpretations for *WT324* exposed to wet season sunlight and darkness taken from phase 2 data

4.2.3 T-Tests for Optical Density of Biofilm Rings Stained by Coomassie Blue

Null Hypothesis: There is no significant difference between OD of Coomassie rings in wet season sun and wet season dark.

Alternative Hypothesis: There is significant difference between OD of Coomassie rings in wet season sun and wet season dark.

Organism	Set	Time/ hour	p-value	Interpretation	NULL HYPOTHESIS	Overall remarks
WT324 SUN VS. WT324 DARK	1	0	0.111295	0.111295 > 0.05	ACCEPT	NULL HYPOTHESIS IS REJECTED
		6	0.030801	0.030801 < 0.05	REJECT	
		12	5.19242E-05	5.19242E-05 < 0.05	REJECT	
		18	3.703E-06	3.703E-06 < 0.05	REJECT	
	2	0	0.000207	0.000207 < 0.05	REJECT	
		6	0.005696	0.005696 < 0.05	REJECT	
		12	7.22767E-11	7.22767E-11 < 0.05	REJECT	
		18	0.337598	0.337598 > 0.05	ACCEPT	
	3	0	3.84996E-06	3.84996E-06 < 0.05	REJECT	
		6	0.015841	0.015841 < 0.05	REJECT	
		12	3.53602E-09	3.53602E-09 < 0.05	REJECT	
		18	0.000786	0.000786 < 0.05	REJECT	
1712 SUN VS. 1712 DARK	1	0	1.03797E-05	1.03797E-05 < 0.05	REJECT	NULL HYPOTHESIS IS REJECTED
		6	1.000	1.000		
		12	0.127860	0.127860 > 0.05	ACCEPT	
		18	1.02136E-06	1.02136E-06 < 0.05	REJECT	
	2	0	0.001125	0.0011 < 0.05	REJECT	
		6	0.047133	0.0471 < 0.05	REJECT	
		12	0.142776	0.1428 > 0.05	ACCEPT	
		18	0.0018298	0.0018 < 0.05	REJECT	
	3	0	0.006837	0.0068 < 0.05	REJECT	
		6	4.01721E-06	4.0172E-06 < 0.05	REJECT	
		12	0.053009	0.0530 > 0.05	ACCEPT	
		18	0.014761	0.0148 < 0.05	REJECT	
1877 SUN VS. 1877 DARK	1	0	2.67219E-05	2.67219E-05 < 0.05	REJECT	NULL HYPOTHESIS IS REJECTED
		6	3.67189E-11	3.6719E-11 < 0.05	REJECT	
		12	1.10574E-09	1.1057E-09 < 0.05	REJECT	
		18	2.05213E-08	2.0521E-08 < 0.05	REJECT	
	2	0	6.53474E-07	6.5347E-07 < 0.05	REJECT	
		6	1.6033E-05	1.6033E-05 < 0.05	REJECT	

		12	9.11384E-13	9.1138E-13 < 0.05	REJECT	
		18	0.73460788	0.7346 > 0.05	ACCEPT	
	3	0	5.61218E-06	5.6122E-06 < 0.05	REJECT	
		6	8.5919E-07	8.5919E-07 < 0.05	REJECT	
		12	0.011953795	0.0119 < 0.05	REJECT	
		18	0.773389	0.7734 > 0.05	ACCEPT	
STEC SUN VS. STEC DARK	1	0	2.1918E-07	2.1918E-07 < 0.05	REJECT	NULL HYPOTHESIS IS REJECTED
		6	1.716232E-14	1.7162E-14 < 0.05	REJECT	
		12	6.88883E-10	6.8888E-10 < 0.05	REJECT	
		18	1.08471E-07	1.0847E-07 < 0.05	REJECT	
	2	0	0.029656	0.0297 < 0.05	REJECT	
		6	0.304465633	0.3045 > 0.05	ACCEPT	
		12	0.1876698	0.1877 > 0.05	ACCEPT	
		18	2.28718E-05	2.2872E-05 < 0.05	REJECT	

4.2.4 Interpretation of the Statistical Analysis of Phase 2 Data

The graphs overall do not show a clear pattern over time. The OD increases and decreases randomly during each hour.

In Set 1, the OD of *Wt324* and *1712* biofilm exposed to sunlight increases from 0 hour to 6 hour then decreases till 18 hour. The OD exposed to darkness decreases from 0 hour till 18 hour. For *1877*, OD of the biofilm exposed to sunlight decreased from 0 to 12 hour then increased till 18 hour. When exposed to darkness, it increases from 0 hour till 6 hour, decreases till 12 hour then increases till 18 hour. When *STEC* biofilm is exposed to sunlight, its OD decreases from 0 hour till 18 hour whereas the OD of the biofilm exposed to darkness remains almost same throughout.

In Set 2, the OD of both sets of *Wt324* exposed to sunlight and darkness increases from 0 hour to 6 hour then decreases till 12 hour. From 12 hour to 18 hour, the OD of biofilm exposed to sunlight increases whereas the OD of the set exposed to darkness continues to decrease. For *1712*, when exposed to sunlight the OS increases from 0 hour to 6 hour, then decreases till 18 hour. Whereas, the OD of biofilm exposed to darkness decreases from 0 hour to 12 hour, then increases slightly from 18 hour. The OD of *1877* biofilm exposed to sunlight decreases from 0 hour till 12 hour then increases till 18 hour. The OD of *1877* biofilm exposed to sunlight decreases from 0 hour till 6 hour, increases till 12 hour then decreases again till 18 hour. For *STEC*, the OD of biofilm exposed to sunlight decreases from 0 hour till 18 hour. The OD of *STEC* biofilm exposed to darkness decreases from 0 hour till 12 hour, then increases till 18 hour.

In Set 3, the OD of *WT324* biofilm exposed to both sunlight and darkness increases from 0 hour till 6 hour, then decreases from 6 hour till 12 hour. The OD of biofilm exposed to darkness continues decreasing till 18 hour whereas the OD of biofilm exposed to sunlight increases. For *1712*, the OD of biofilm exposed to sunlight increases from 0 hours to 6 hours, decreases till 12 hour then remained almost unchanged till 18 hour. The OD of biofilm exposed to darkness decreases from 0 hour to 12

hour, then increases till 18 hour. For *1877*, the OD of biofilm exposed sunlight decreases from 0 hour to 6 hour, remains almost unchanged till 12 hour, then increases till 18 hour. The OD of biofilm exposed to sunlight decreases from 0 hour till 6 hour then increases from 6 hour to 12 hour, then decreases till 18 hour.

4.3.1 Phase 3: OD of biofilm formed in ELISA plates

Average OD of biofilm exposed to sunlight:

	Time/ Bacterial strains	*V. cholerae WT324*	*V. cholerae 1712*	*V. cholerae 1877*	*STEC*
Week 1	0	0.778887	0.928759	0.922778	0.820185
	6	0.722868	0.876037	0.870667	0.817741
	12	0.850093	0.873815	0.921056	0.762759
	18	0.935722	0.897759	0.977519	0.80363
Week 2	0	1.211528	1.150889	1.176542	1.319264
	6	1.195694	1.163847	1.117903	1.330458
	12	0.965833	1.006214	0.98958	1.236444
	18	1.14725	1.130028	1.093736	1.311472
Week 3	0	1.068722	1.056736	1.017208	1.179347
	6	1.061125	1.054486	1.032319	1.094764
	12	1.072139	1.059806	1.104389	1.175819
	18	1.069014	1.012708	1.033361	1.158278

Average OD of biofilm exposed to darkness:

	Time/ Bacterial strains	*V. cholerae WT324*	*V. cholerae 1712*	*V. cholerae 1877*	*STEC*
Week 1	0	0.70513	0.84387	0.910889	0.873852
	6	0.653604	0.768528	0.817222	0.802037

	12	0.736796	0.847463	0.868185	0.819463
	18	0.761815	0.820981	0.857852	0.791667
Week 2	0	1.328958	1.318167	1.253014	1.517944
	6	1.33875	1.323042	1.247458	1.510486
	12	1.378972	1.374	1.292458	1.51975
	18	1.187083	1.283319	1.039017	1.278597
Week 3	0	1.068722	0.955639	1.018958	1.120069
	6	1.057792	0.965194	1.011639	1.140639
	12	1.037292	0.994278	1.000736	1.143403
	18	1.039292	1.061417	1.032833	1.200708

4.3.2 Phase 3: Graphs and Regression Analysis

WT324

Set 1

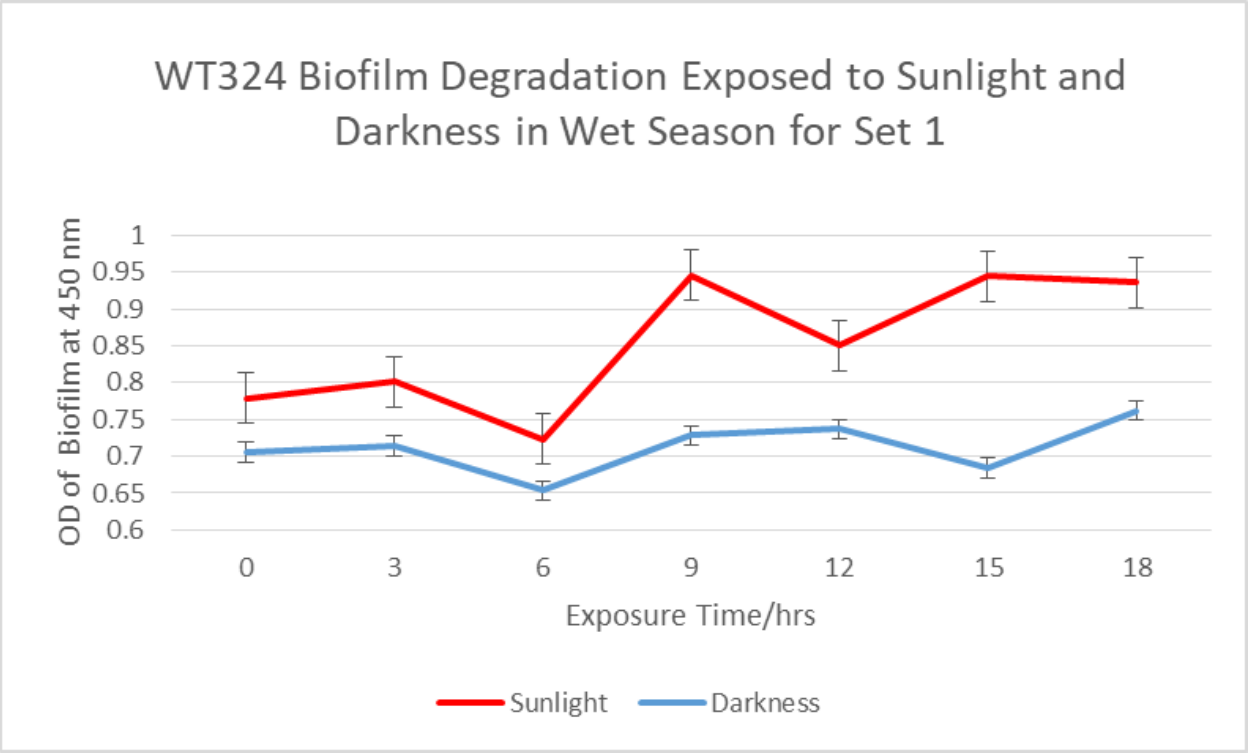

Figure: Graphical representation of OD of biofilms formed in 96 well ELISA plate of Vibrio WT324 after biofilm degradation in sunlight and in darkness taken from Set 1 of Phase 3 data. X axis represents the exposure time in hours and Y axis represents OD of the biofilm at 450 nm

Organism	R value and interpretation	R^2 value and interpretation	Regression model and interpretation
WT324 SUNLIGHT	0.755252474 Strong Positive Correlation	R^2 = 0.57040629949294 57.04% of the variation in OD of WT324 biofilm can be explained by the time of exposure in the sun.	y = 0.010516x + 0.759524 When the time of exposure is 0 hour, the OD of the biofilm will be 0.759524. With every 1 hour increase in exposure time, the OD of biofilm will increase by 0.010516.
WT324 DARK	0.422326971 Moderate Positive Correlation	R^2 = 0.17836007 17.84% of the variation in OD of WT324 biofilm can be explained by the time of exposure in the dark.	y= 0.002318x + 0.69102 When the time of exposure is 0 hour, the OD of the biofilm will be 0.69102. With every 1 hour increase in exposure time, the OD of biofilm will increase by

			0.002318.

Table: R value, R square value. regression and their respective interpretations for *WT324* exposed to wet season sunlight and darkness taken from Phase 3 data

Set 2

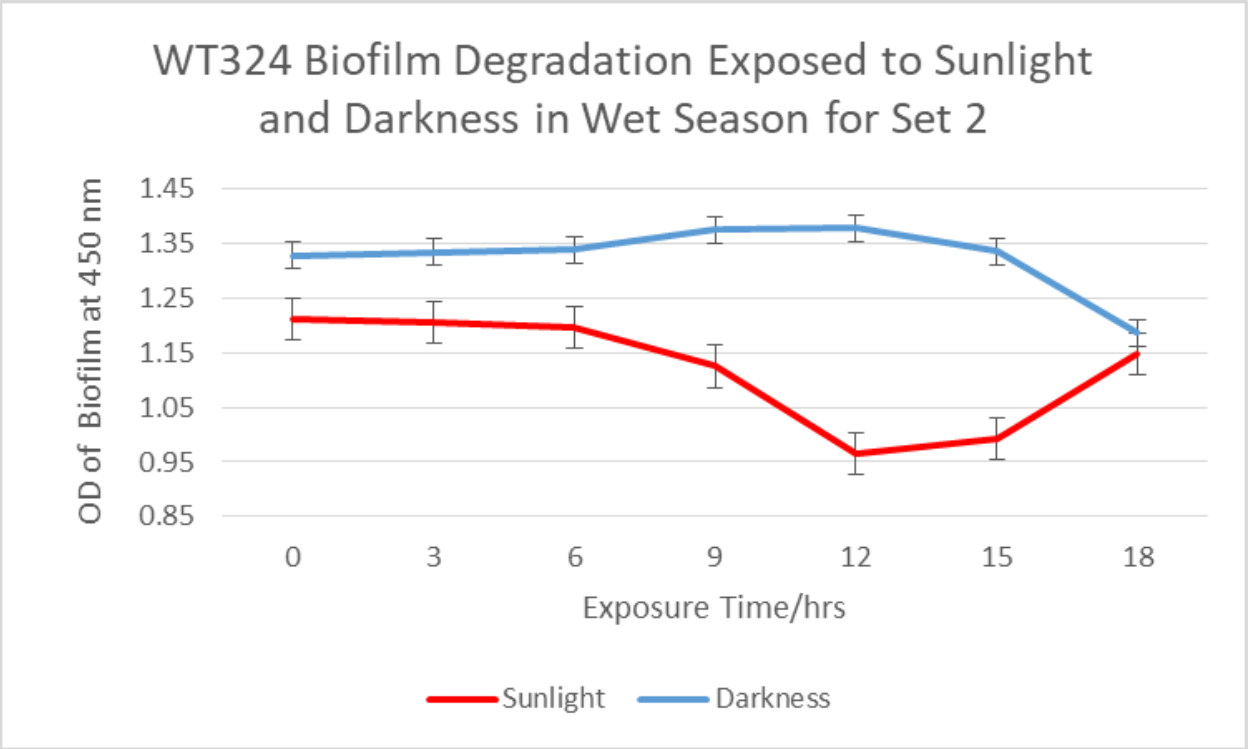

Figure: Graphical representation of OD of biofilms formed in 96 well ELISA plate of Vibrio WT324 after biofilm degradation in sunlight and in darkness taken from Set 2 of Phase 3 data. X axis represents the exposure time in hours and Y axis represents OD of the biofilm at 450 nm

Organism	R value and interpretation	R^2 value and interpretation	Regression model and interpretation
WT324 SUNLIGHT	-0.64346 Moderate Negative Correlation	R^2 = 0.414038178837768 41.40% of the variation in OD of WT324 biofilm can be explained by the time of exposure in the sun.	y = -0.010114x + 1.211668 When the time of exposure is 0 hour, the OD of the biofilm will be 1.211668. With every 1 hour increase in exposure time, the OD of biofilm will decrease by 0.010114.
WT324 DARK	-0.459455804549 Moderate Negative Correlation	R^2 = 0.2110996 21.11% of the variation in OD of WT324 biofilm can be explained by the time of	y = -0.004563x + 1.366621 When the time of exposure is 0 hour, the OD of the biofilm will

		exposure in the dark.	be 1.366621. With every 1 hour increase in exposure time, the OD of biofilm will decrease by 0.004563 .

Table: R value, R square value. regression and their respective interpretations for WT324 exposed to wet season sunlight and darkness taken from Phase 3 data

Set 3

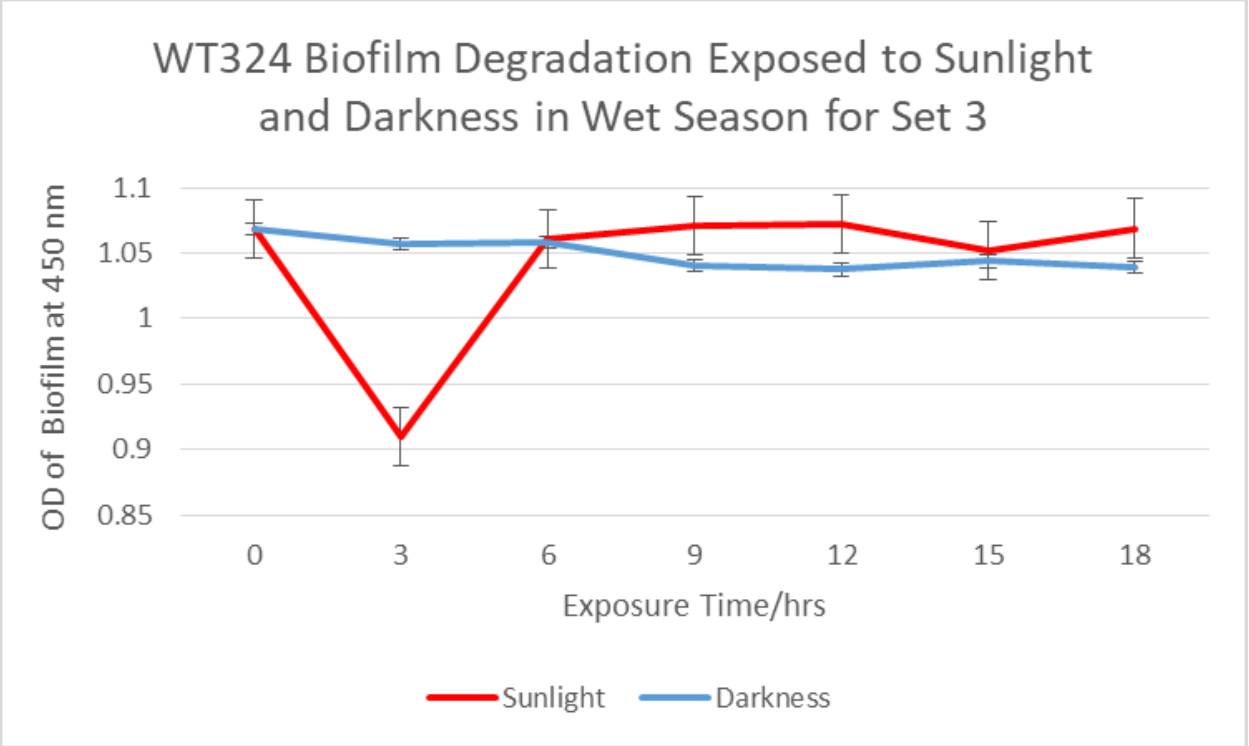

Figure: Graphical representation of OD of biofilms formed in 96 well ELISA plate of Vibrio WT324 after biofilm degradation in sunlight and in darkness taken from Set 3 of Phase 3 data. X axis represents the exposure time in hours and Y axis represents OD of the biofilm at 450 nm

Organism	R value and interpretation	R^2 value and interpretation	Regression model and interpretation
WT324 SUNLIGHT	0.386034 Moderate Positive Correlation	$R^2 = 0.149023$ 14.9% of the variation in OD of WT324 biofilm can be explained by the time of	y = 0.003527x + 1.011687 When the time of exposure is 0 hour, the OD of the biofilm will

		exposure in the sun.	be 1.011687. With every 1 hour increase in exposure time, the OD of biofilm will increase by 0.003527.
WT324 DARK	-0.8729589 Strong Negative Correlation	R^2 = 0.762057258 76.21% of the variation in OD of WT324 biofilm can be explained by the time of exposure in the dark.	y = -0.001601x + 1.063580 When the time of exposure is 0 hour, the OD of the biofilm will be 1.063580. With every 1 hour increase in exposure time, the OD of biofilm will decrease by 0.001601.

Table: R value, R square value. regression and their respective interpretations for *WT324* exposed to wet season sunlight and darkness taken from Phase 3 data

1712

Set 1

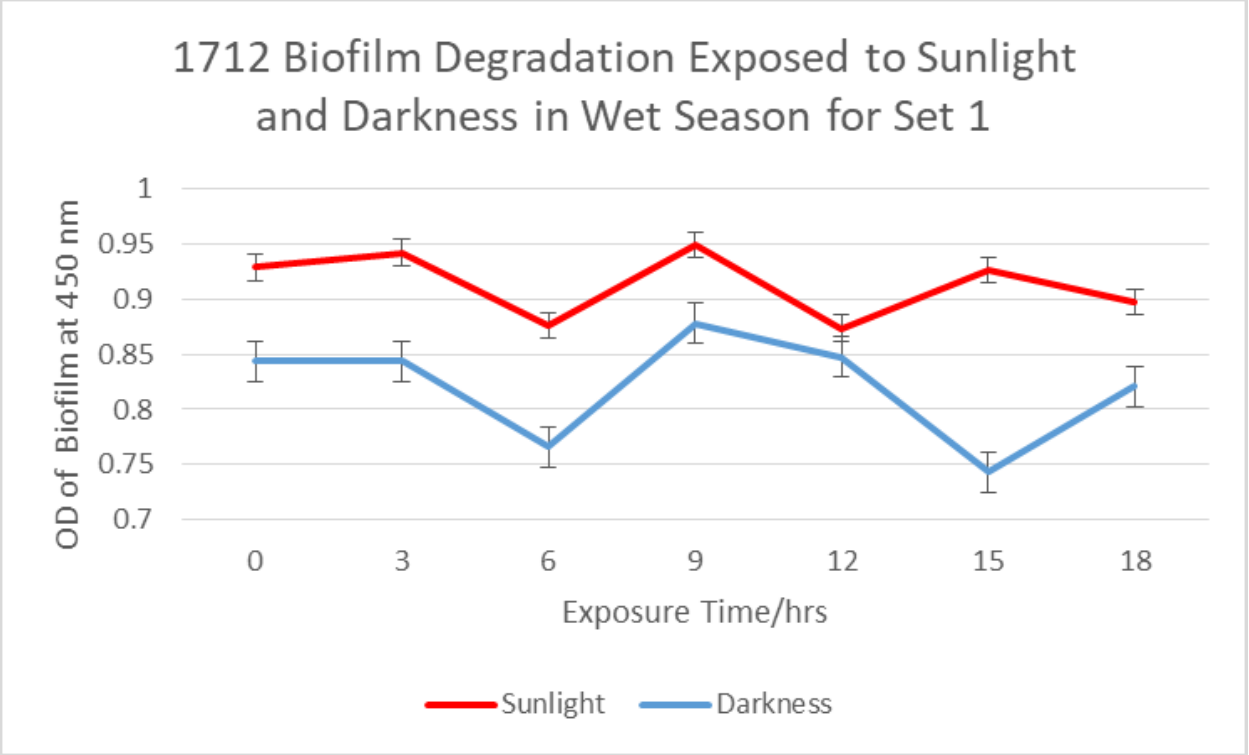

Figure: Graphical representation of OD of biofilms formed in 96 well ELISA plate of Vibrio 1712 after biofilm degradation in sunlight and in darkness taken from Set 1 of Phase 3 data. X axis represents the exposure time in hours and Y axis represents OD of the biofilm at 450 nm

Organism	R value and	R^2 value and	Regression model and

	interpretation	interpretation	interpretation
1712 SUNLIGHT	0.31645745 Weak Positive Correlation	R^2 = 0.100145319653809 10.01% of the variation in OD of 1712 biofilm can be explained by the time of exposure in the sun.	y = -0.001511x + 0.927111 When the time of exposure is 0 hour, the OD of the biofilm will be 0.927111 . With every 1 hour increase in exposure time, the OD of biofilm will decrease by 0.001511.
1712 DARK	-0.3015379 Weak Negative Correlation	R^2 = 0.090925079 9.093% of the variation in OD of 1712 biofilm can be explained by the time of exposure in the dark.	y = -0.002251x + 0.840813 When the time of exposure is 0 hour, the OD of the biofilm will be 0.840813. With every 1 hour increase in exposure time, the OD of biofilm will decrease by 0.002251.

Table: R value, R square value. regression and their respective interpretations for *1712* exposed to wet season sunlight and darkness taken from Phase 3 data

Set 2

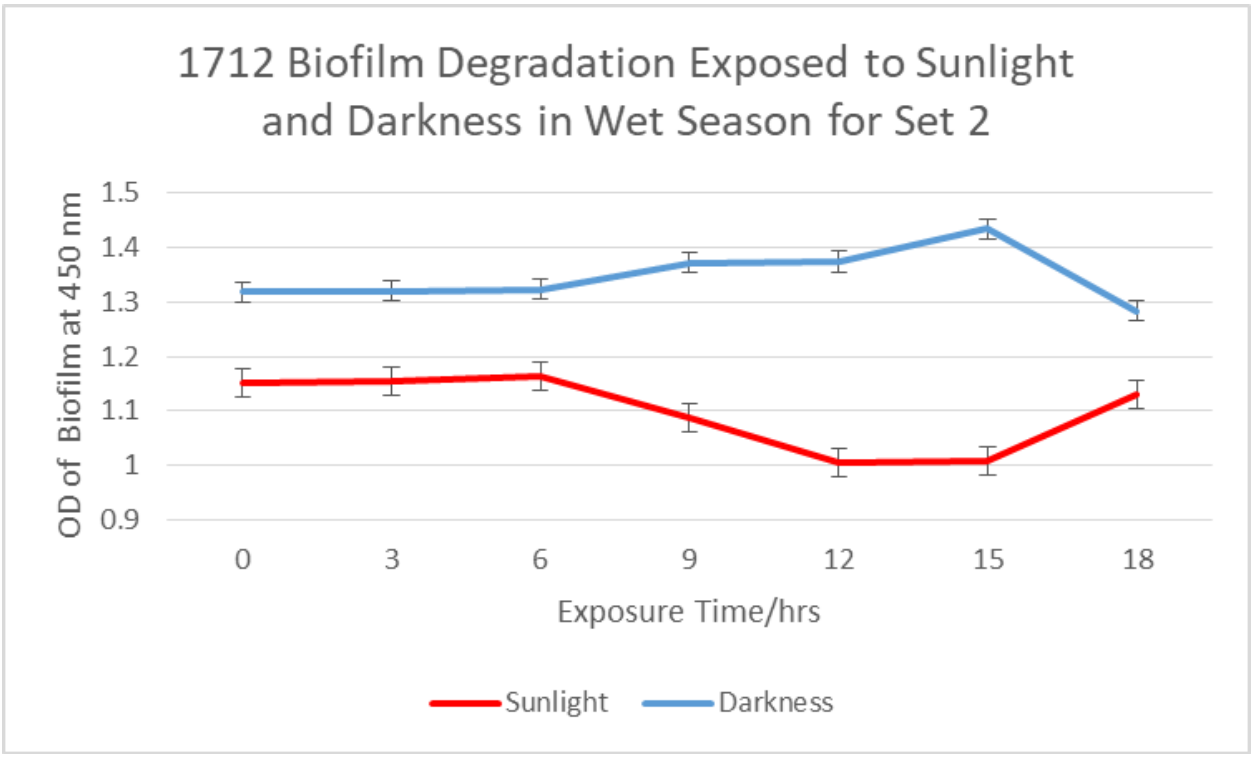

Figure: Graphical representation of OD of biofilms formed in 96 well ELISA plate of Vibrio 1712 after biofilm degradation in sunlight and in darkness taken from Set 2 of Phase 3 data. X axis represents the exposure time in hours and Y axis represents OD of the biofilm at 450 nm

Organism	R value and interpretation	R^2 value and interpretation	Regression model and interpretation
1712 SUNLIGHT	0.58025597 Moderate Positive Correlation	R^2 = 0.33669699 33.67% of the variation in OD of 1712 biofilm can be explained by the time of exposure in the sun.	y = -0.006135x + 1.155061 When the time of exposure is 0 hour, the OD of the biofilm will be 1.155061. With every 1 hour increase in exposure time, the OD of biofilm will decrease by 0.006135.
1712 DARK	0.266325 Weak Positive Correlation	R^2 = 0.070929 7.093% of the variation in OD of 1712 biofilm can be explained by the time of exposure in the dark.	y = 0.002053x + 1.327820 When the time of exposure is 0 hour, the OD of the biofilm will be 1.327820 . With every 1 hour increase in exposure time, the OD of biofilm will increase by 0.002053.

Table: R value, R square value. regression and their respective interpretations for *1712* exposed to wet season sunlight and darkness taken from Phase 3 data

Set 3

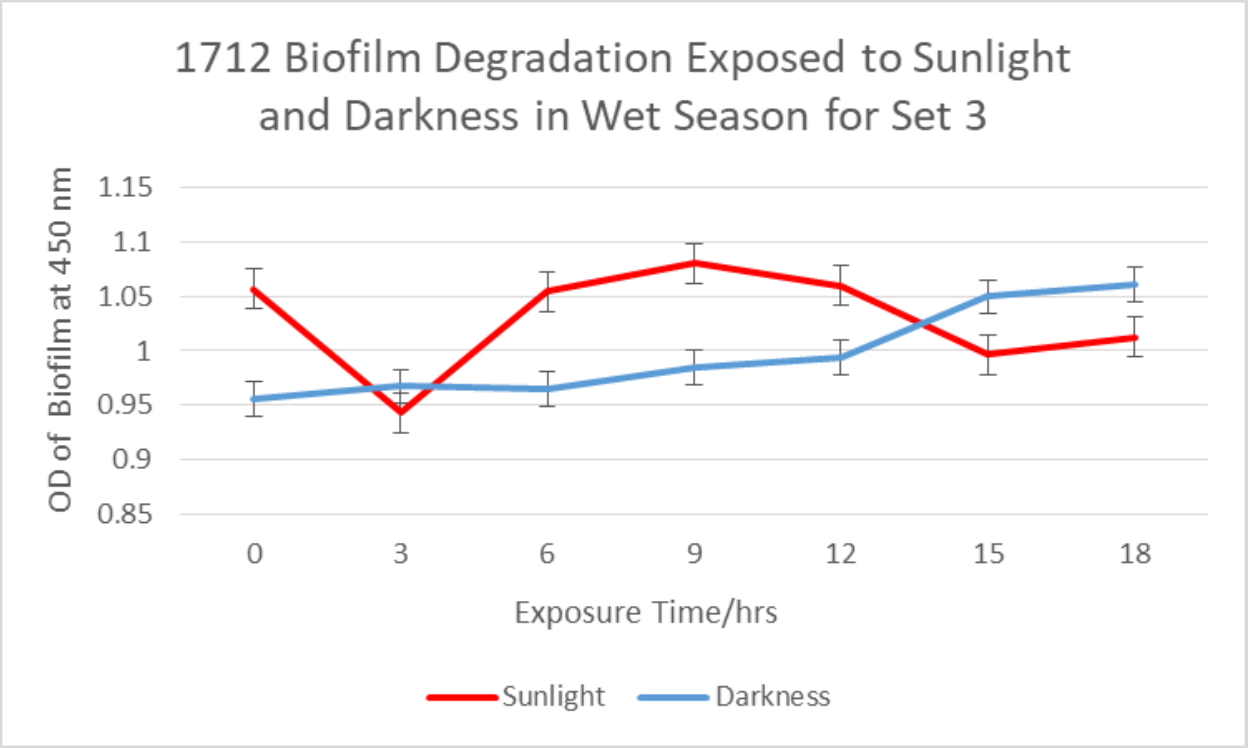

Figure: Graphical representation of OD of biofilms formed in 96 well ELISA plate of Vibrio 1712 after biofilm degradation in sunlight and in darkness taken from Set 3 of Phase 3 data. X axis represents the exposure time in hours and Y axis represents OD of the biofilm at 450 nm

Organism	R value and interpretation	R^2 value and interpretation	Regression model and interpretation
1712 SUNLIGHT	-0.032988655 Weak Negative Correlation	R^2 = 0.001088251359 0.109% of the variation in OD of 1712 biofilm can be explained by the time of exposure in the sun.	y = -0.000243x + 1.031265 When the time of exposure is 0 hour, the OD of the biofilm will be 1.031265. With every 1 hour increase in exposure time, the OD of biofilm will decrease by 0.000243.
1712 DARK	0.935176 Strong Positive Correlation	R^2 =0.874554 87.45% of the variation in OD of 1712 biofilm can be explained by the time of exposure in the dark.	y = 0.006082x + 0.942167 When the time of exposure is 0 hour, the OD of the biofilm will be 0.942167. With every 1 hour increase in exposure time, the OD of biofilm will increase by 0.006082.

Table: R value, R square value. regression and their respective interpretations for *1712* exposed to wet season sunlight and darkness taken from Phase 3 data

1877

Set 1

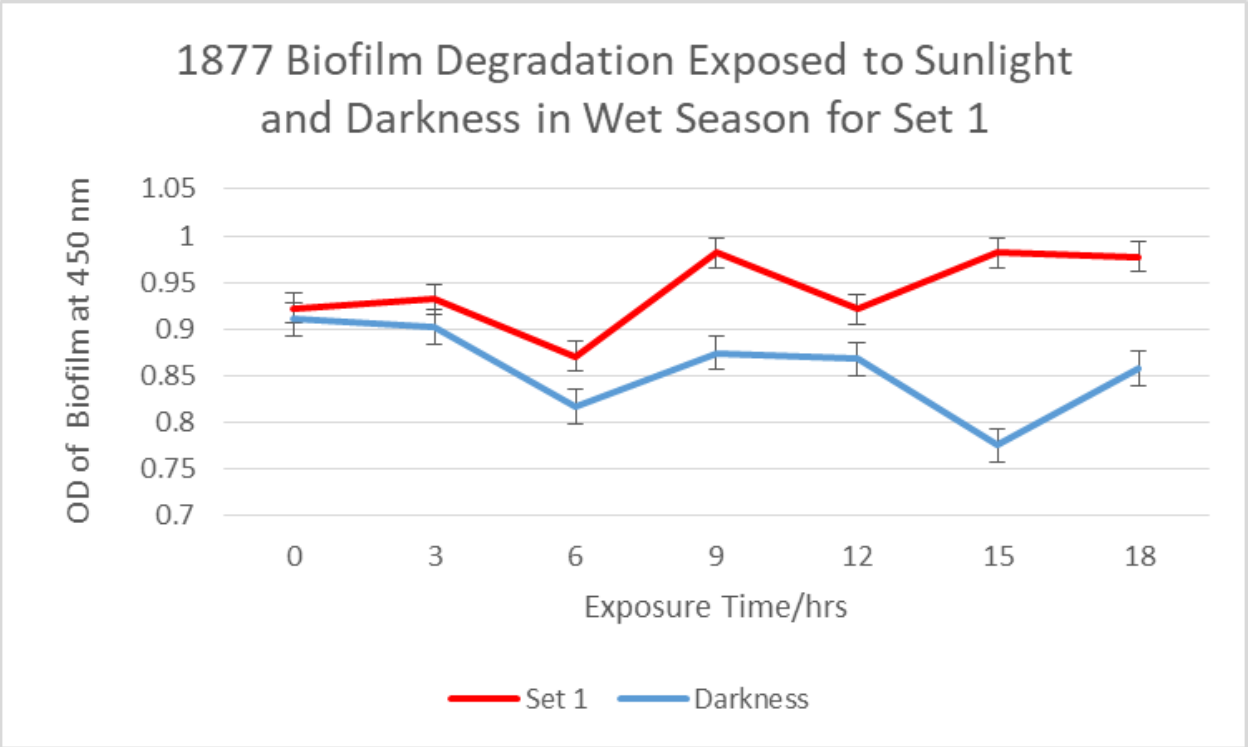

Figure: Graphical representation of OD of biofilms formed in 96 well ELISA plate of Vibrio 1877 after biofilm degradation in sunlight and in darkness taken from Set 1 of Phase 3 data. X axis represents the exposure time in hours and Y axis represents OD of the biofilm at 450 nm

Organism	R value and interpretation	R^2 value and interpretation	Regression model and interpretation
1877 SUNLIGHT	0.581678788 Moderate Positive Correlation	$R^2 = 0.3383502126$ 33.83% of the variation in OD of 1877 biofilm can be explained by the time of exposure in the sun.	y = 0.003741x + 0.907421 When the time of exposure is 0 hour, the OD of the biofilm will be 0.907421. With every 1 hour increase in exposure time, the OD of biofilm will increase by 0.003741.
1877 DARK	-0.586293968 Moderate Negative Correlation	$R^2 = 0.3437406167$ 34.37% of the variation in OD of 1877 biofilm can be explained by the time of exposure in the	y = -0.004312x + 0.896808 When the time of exposure is 0 hour, the OD of the biofilm will

		dark.	be 0.896808. With every 1 hour increase in exposure time, the OD of biofilm will decrease by 0.004312.

Table: R value, R square value. regression and their respective interpretations for *1877* exposed to winter sunlight and darkness taken from Phase 3 data

Set 2

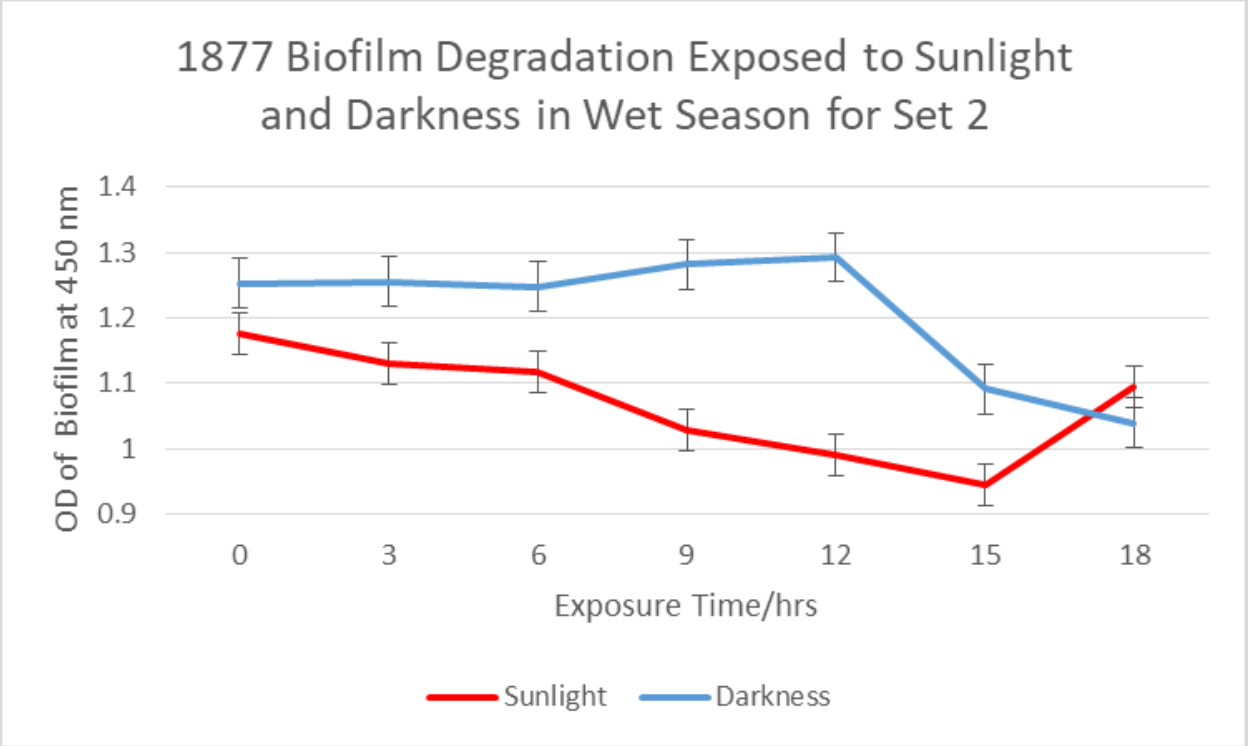

Figure: Graphical representation of OD of biofilms formed in 96 well ELISA plate of Vibrio 1877 after biofilm degradation in sunlight and in darkness taken from Set 2 of Phase 3 data. X axis represents the exposure time in hours and Y axis represents OD of the biofilm at 450 nm

Organism	R value and interpretation	R^2 value and interpretation	Regression model and interpretation
1877 SUNLIGHT	-0.6924838 Strong Negative Correlation	R^2 =0.479533866 47.953% of the variation in OD of 1877 biofilm can be explained by the time of exposure in the sun.	y = -0.008920x + 1.149103 When the time of exposure is 0 hour, the OD of the biofilm will be 1.149103. With every 1 hour increase in exposure time, the OD of biofilm will decrease by 0.008920.
1877	-0.7112317	R^2 = 0.505850557	y = -0.011033x +

DARK	Strong Negative Correlation	50.585% of the variation in OD of 1877 biofilm can be explained by the time of exposure in the dark.	1.307809 When the time of exposure is 0 hour, the OD of the biofilm will be 1.307809. With every 1 hour increase in exposure time, the OD of biofilm will decrease by 0.011033.

Table: R value, R square value. regression and their respective interpretations for *1877* exposed to winter sunlight and darkness taken from Phase 3 data

Set 3

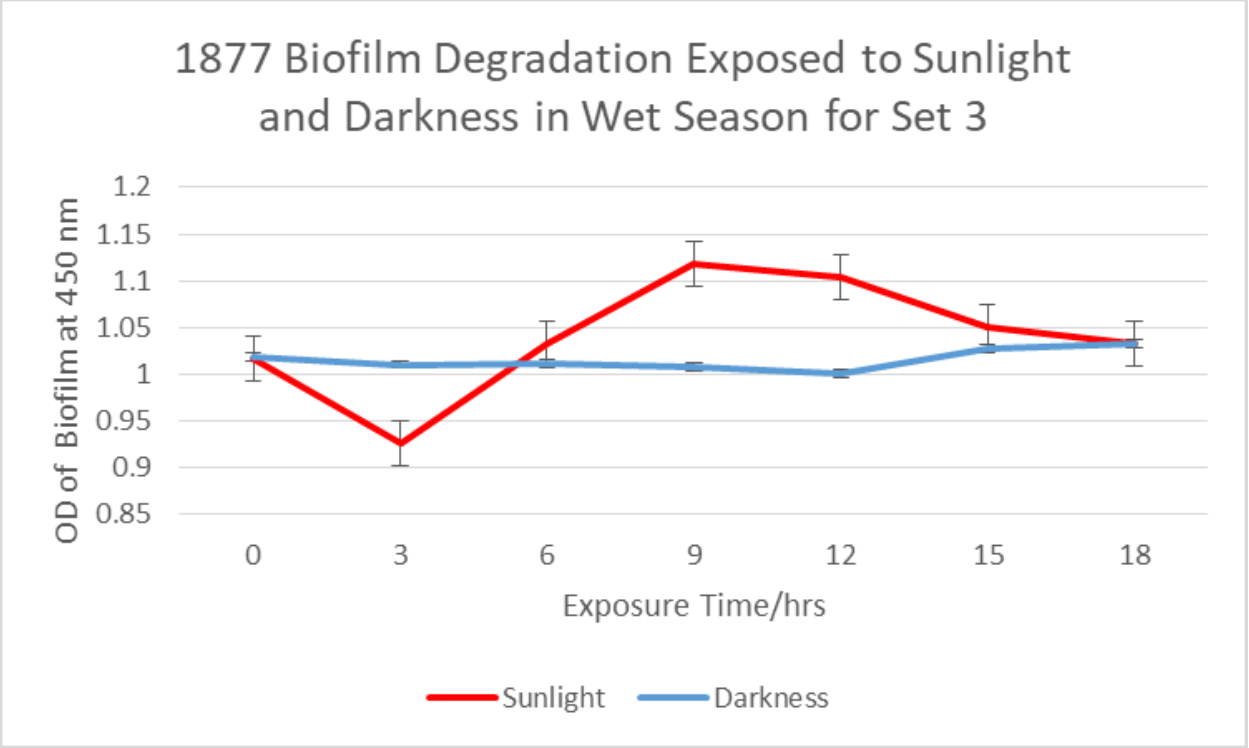

Figure: Graphical representation of OD of biofilms formed in 96 well ELISA plate of Vibrio 1877 after biofilm degradation in sunlight and in darkness taken from Set 3 of Phase 3 data. X axis represents the exposure time in hours and Y axis represents OD of the biofilm at 450 nm

Organism	R value and interpretation	R^2 value and interpretation	Regression model and interpretation
1877 SUNLIGHT	0.45363999584 Moderate Positive Correlation	R^2 =0.2057892458 20.58% of the variation in OD of 1877 biofilm can be explained by the time of exposure in the sun.	y = 0.004420x + 1.000510 When the time of exposure is 0 hour, the OD of the biofilm will be 1.000510. With every 1 hour increase in

			exposure time, the OD of biofilm will increase by 0.004420.
1877 DARK	0.44029495 Moderate Positive Correlation	R^2 = 0.19385964 19.39% of the variation in OD of 1877 biofilm can be explained by the time of exposure in the dark.	y = 0.000768x + 1.008827 When the time of exposure is 0 hour, the OD of the biofilm will be 1.008827. With every 1 hour increase in exposure time, the OD of biofilm will increase by 0.000768.

Table: R value, R square value. regression and their respective interpretations for *1877* exposed to winter sunlight and darkness taken from Phase 3 data

STEC

Set 1

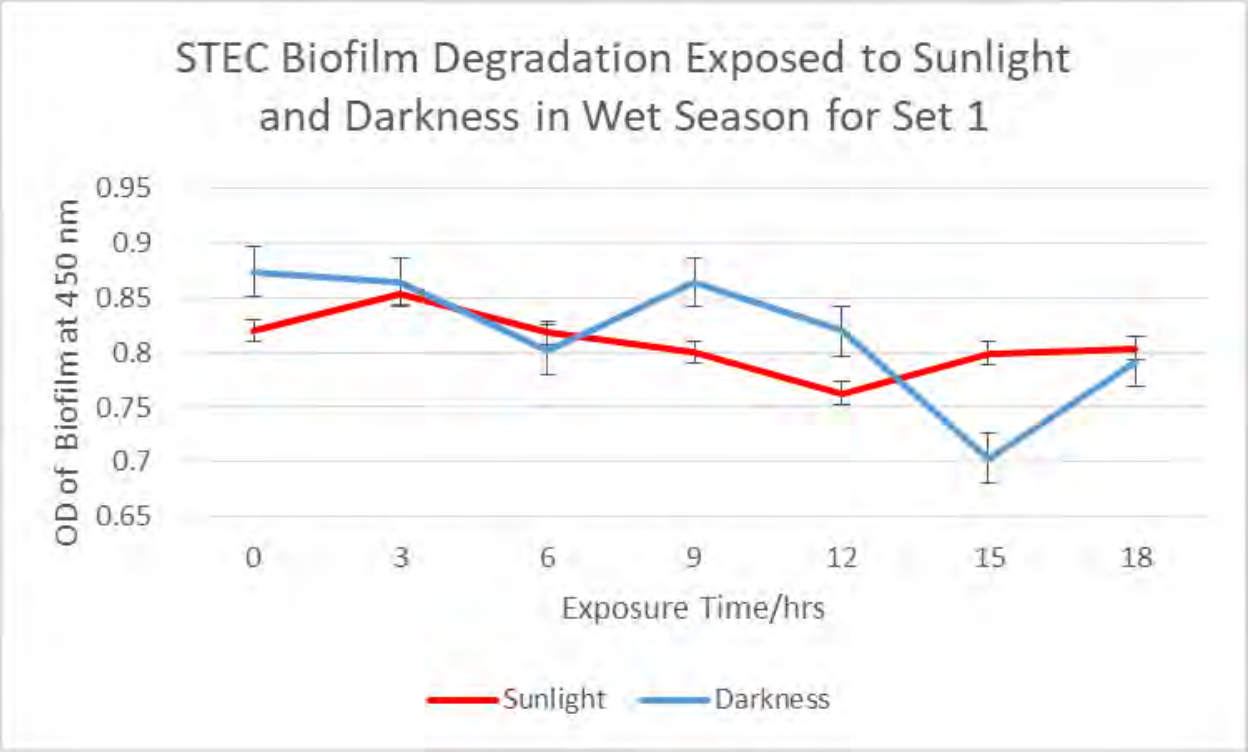

Figure: Graphical representation of OD of biofilms formed in 96 well ELISA plate of STEC after biofilm degradation in sunlight and in darkness taken from Set 1 of Phase 3 data. X axis represents the exposure time in hours and Y axis represents OD of the biofilm at 450 nm

Organism	R value and interpretation	R^2 value and interpretation	Regression model and interpretation

STEC SUNLIGHT	-0.603834788 Moderate Negative Correlation	R^2 =0.3646164516 36.46% of the variation in OD of STEC biofilm can be explained by the time of exposure in the sun.	y = -0.002529x + 0.832276 When the time of exposure is 0 hour, the OD of the biofilm will be 0.832276. With every 1 hour increase in exposure time, the OD of biofilm will decrease by 0.002529.
STEC DARK	-0.7104108 Strong Negative Correlation	R^2 =0.50468354 50.468% of the variation in OD of STEC biofilm can be explained by the time of exposure in the dark.	y = -0.006554x + 0.875976 When the time of exposure is 0 hour, the OD of the biofilm will be 0.875976. And with every 1 hour increase in exposure time, the OD of biofilm will decrease by 0.006554.

Table: R value, R square value. regression and their respective interpretations for *STEC* exposed to winter sunlight and darkness taken from Phase 3 data

Set 2

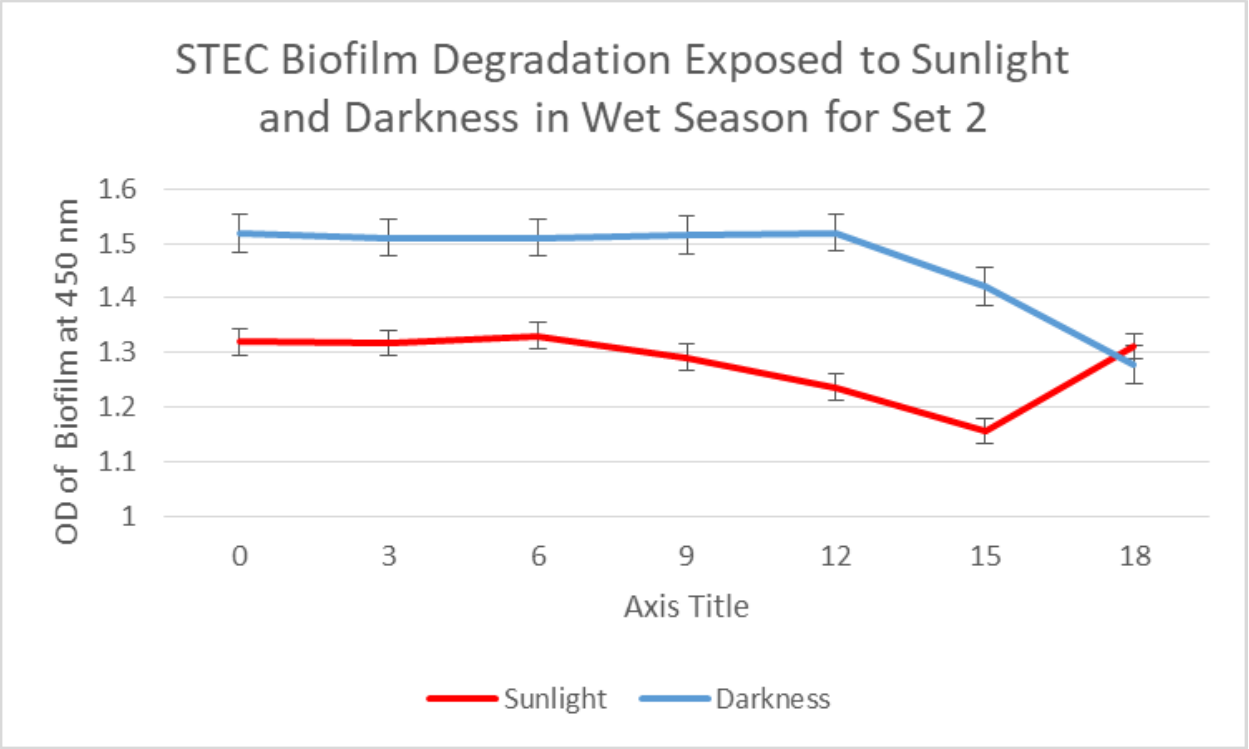

Figure: Graphical representation of OD of biofilms formed in 96 well ELISA plate of STEC after biofilm degradation in sunlight and in darkness taken from Set 2 of Phase 3 data. X axis represents the exposure time in hours and Y axis represents OD of the biofilm at 450 nm

Organism	R value and interpretation	R^2 value and interpretation	Regression model and interpretation
STEC SUNLIGHT	-0.539606967 Moderate Negative Correlation	R^2 =0.29117568 29.12% of the variation in OD of STEC biofilm can be explained by the time of exposure in the sun.	y = -0.005254x + 1.327645 When the time of exposure is 0 hour, the OD of the biofilm will be 1.327645. With every 1 hour increase in exposure time, the OD of biofilm will decrease by 0.005254.
STEC DARK	-0.7559833135 Strong Negative Correlation	R^2 =0.57151077 57.15% of the variation in OD of STEC biofilm can be explained by the time of exposure in the dark.	y = -0.010557x + 1.562852 When the time of exposure is 0 hour, the OD of the biofilm will be 1.562852. And with every 1 hour increase in exposure time, the OD of biofilm will decrease by 0.010557.

Table: R value, R square value. regression and their respective interpretations for *STEC* exposed to winter sunlight and darkness taken from Phase 3 data

Set 3

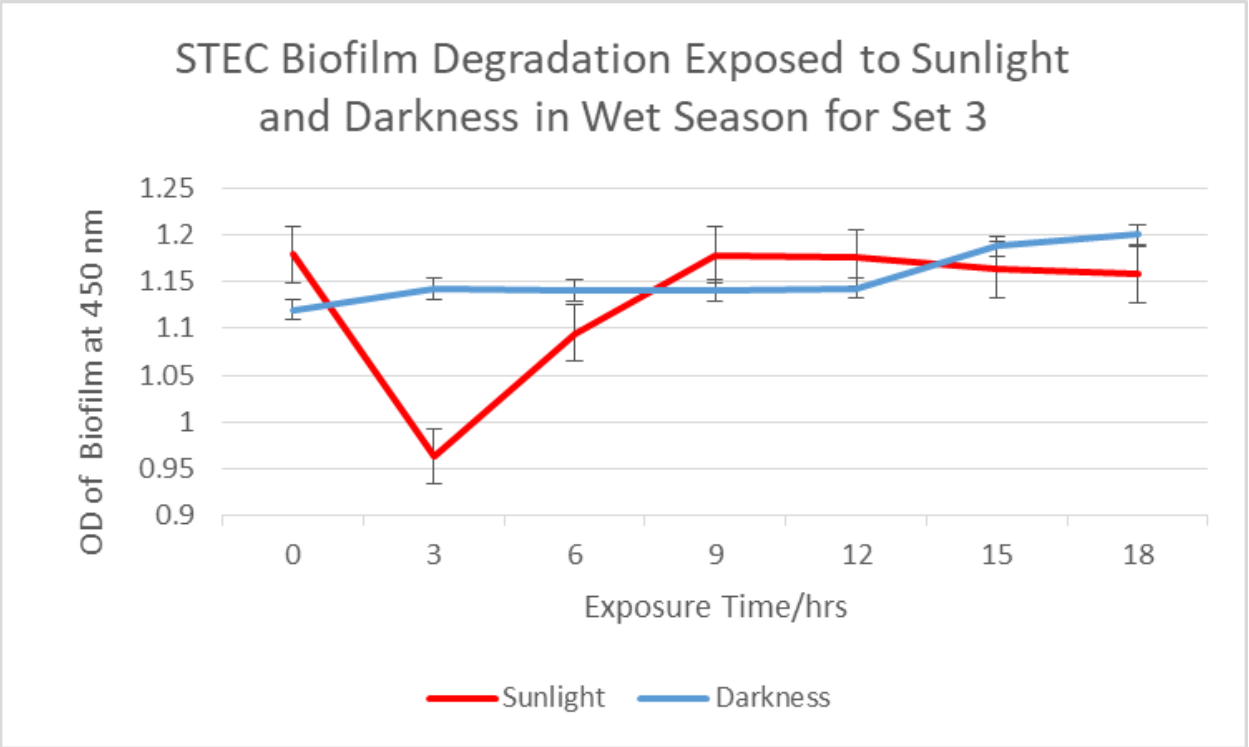

Figure: Graphical representation of OD of biofilms formed in 96 well ELISA plate of STEC after biofilm degradation in sunlight and in darkness taken from Set 3 of Phase 3 data. X axis represents the exposure time in hours and Y axis represents OD of the biofilm at 450 nm

Organism	R value and interpretation	R^2 value and interpretation	Regression model and interpretation
STEC SUNLIGHT	0.405623 Correlation	R^2 =0.16453 16.45% of the variation in OD of STEC biofilm can be explained by the time of exposure in the sun.	y = 0.004971x + 1.085691 When the time of exposure is 0 hour, the OD of the biofilm will be 1.085691. With every 1 hour increase in exposure time, the OD of biofilm will increase by 0.004971.
STEC DARK	0.8881283227 Correlation	R^2 =0.788771917585 78.88% of the variation in OD of STEC biofilm can be explained by the time of exposure in the dark.	y = 0.003988x + 1.117829 When the time of exposure is 0 hour, the OD of the biofilm will be 1.117829. And with every 1 hour increase in exposure time, the OD of biofilm will increase by 0.003988.

Table: R value, R square value. regression and their respective interpretations for *STEC* exposed to winter sunlight and darkness taken from Phase 3 data

4.3.3 Phase 3: T-TESTS FOR BIOFILM OD TAKEN BY ELISA EXPOSED TO SUNLIGHT AND DARKNESS DURING WET SEASON:

Null Hypothesis: There is no significant difference between OD of biofilm kept in sunlight and darkness during wet season

Alternative Hypothesis: There is a significant difference in OD of biofilm kept in sunlight and darkness during wet season

Organism	Set	Time/hour	p-value	Interpretation	NULL HYPOTHESIS	Set wise remarks	Overall Remarks
WT324 SUN VS WT324 DARK	1	0	0.097427222	0.097427 > 0.05	ACCEPTED	50/50	ACCEPTED
		6	0.08268176	0.0826818> 0.05	ACCEPTED		
		12	0.018866029	0.018866 < 0.05	REJECTED		
		18	0.000209776	0.00020978 < 0.05	REJECTED		
	2	0	3.61461E-10	3.6146E-10 < 0.05	REJECTED	REJECTED	
		6	4.6679E-12	4.6679E -12 < 0.05	REJECTED		
		12	9.58889E-31	9.58889E-31 < 0.05	REJECTED		
		18	0.250410075	0.25041 > 0.05	ACCEPTED		
	3	0	1	1 > 0.05	ACCEPTED	ACCEPTED	
		6	0.889433617	0.8894> 0.05	ACCEPTED		
		12	0.197979283	0.19798 > 0.05	ACCEPTED		
		18	0.355349671	0.35535 > 0.05	ACCEPTED		
1712 SUN VS 1712 DARK	1	0	0.000361808	0.0003618 < 0.05	REJECTED	REJECTED	
		6	1.01611E-06	1.01611E-06 < 0.05	REJECTED		
		12	0.326232359	0.32623> 0.05	ACCEPTED		
		18	0.009480811	0.0094808 < 0.05	REJECTED		

	2	0	5.04678E-31	5.04678E-31 < 0.05	REJECTED	REJECTED	REJECTED
		6	2.9303E-24	2.9303E-24 < 0.05	REJECTED		
		12	9.78461E-49	9.78461E-49 < 0.05	REJECTED		
		18	6.44613E-19	6.44613E-19 < 0.05	REJECTED		
	3	0	7.44101E-05	7.44101E < 0.05	REJECTED	REJECTED	
		6	0.001026952	0.001026952< 0.05	REJECTED		
		12	0.031696834	0.031697 < 0.05	REJECTED		
		18	0.133354481	0.13335> 0.05	ACCEPTED		
1877 SUN VS 1877 DARK	1	0	0.061260927	0.061261 > 0.05	ACCEPTED	REJECTED	REJECTED
		6	1.19234E-10	1.19234E-10< 0.05	REJECTED		
		12	0.000376537	0.00037654 < 0.05	REJECTED		
		18	2.93422E-09	2.934218E-09 < 0.05	REJECTED		
	2	0	1.15997E-10	1.15997E-10 < 0.05	REJECTED	REJECTED	
		6	5.81773E-19	5.81773E-19 < 0.05	REJECTED		
		12	5.38519E-39	5.38519E-39 < 0.05	REJECTED		
		18	0.049833182	0.049833 < 0.05	REJECTED		
	3	0	0.946604259	0.946604 > 0.05	ACCEPTED	ACCEPTED	
		6	0.39419574	0.394196> 0.05	ACCEPTED		
		12	1.07094E-05	1.07094E-05 < 0.05	REJECTED		

		18	0.983844267	0.9838442 0.05	ACCEPTED		
STEC SUN VS STEC DARK	1	0	0.008612401	0.0086124 < 0.05	REJECTED	50/50	REJECTED
		6	0.478856604	0.47886 > 0.05	ACCEPTED		
		12	0.025084398	0.025084 < 0.05	REJECTED		
		18	0.675008619	0.6750086> 0.05	ACCEPTED		
	2	0	1.09184E-19	1.09184E-19 < 0.05	REJECTED	REJECTED	
		6	1.9884E-21	1.9884E-21 < 0.05	REJECTED		
		12	1.89019E-48	1.89019E-48 < 0.05	REJECTED		
		18	0.409798765	0.409799> 0.05	ACCEPTED		
	3	0	8.12286E-05	8.12286E05 < 0.05	REJECTED	50/50	
		6	0.048545693	0.048546 < 0.05	REJECTED		
		12	0.11645488	0.1165 > 0.05	ACCEPTED		
		18	0.072090699	0.07209 > 0.05	ACCEPTED		

4.3.4 Interpretation of the Statistical Analysis of Phase 3 Data

In Set 1, the OD of *WT324 ,1712, 1877* and STEC biofilms exposed to sunlight showed subsequent increases and decreases throughout the 18 hour period, with *WT324* showing a sharp increase between 6 to 9 hours. The OD of biofilms exposed to darkness showed subsequent increases and decreases throughout the 18 hour period, which were smaller than those observed for sunlight.

In Set 2, the OD of biofilms of all 4 organisms exposed to sunlight decreased consistently and then increased. For WT324 and 1712, the decrease occurred from 0 to 12 hours with an increase from 12 to 18 hours; for 1877 and STEC, the decrease occurred from 0 to 15 hours with an increase from 15 to 18 hours. For biofilm exposed to darkness, the OD of all 4 organisms exhibited minimal variance with slight increases during the initial hours and then showed a decrease where STEC showed the greatest decline.

In Set 3, for biofilm exposed to sunlight, the OD of biofilms showed a decrease between 0 to 3 hours

and an increase from 6 to 9 for all organisms. For WT324, the OD varied slightly until 18 hours and for 1712, there was an increase until 9 hours, followed by a decline until 15 hours and an increase until 18 hours. For 1877 and STEC, the OD increased until 9 hours followed by a decline until 18 hours. The OD of biofilms exposed to darkness exhibited overall negligible variation with 1712 and STEC showing an overall increase during the 18 hour period.

4.4.1 Phase 4: Biofilm Formed on Coverslips

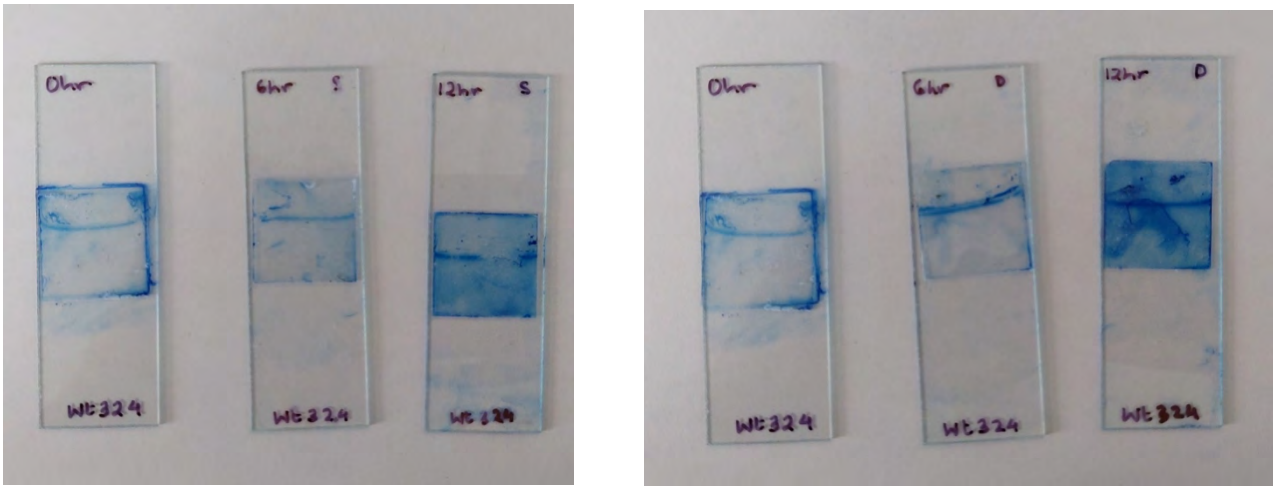

Figure: Stained WT324 biofilms formed on cover slip

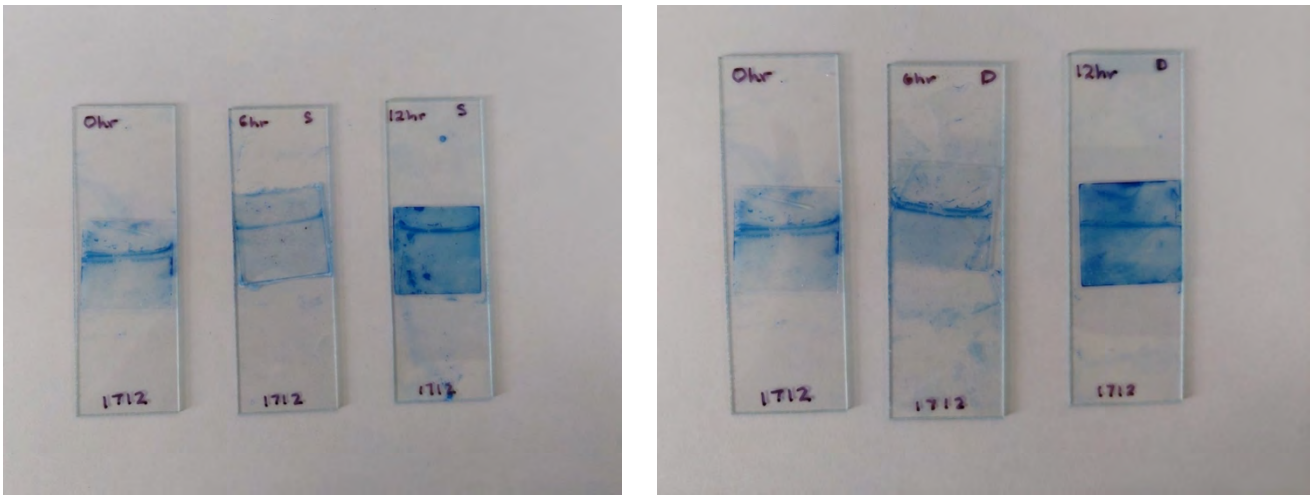

Figure: Stained 1712 biofilms formed on cover slip

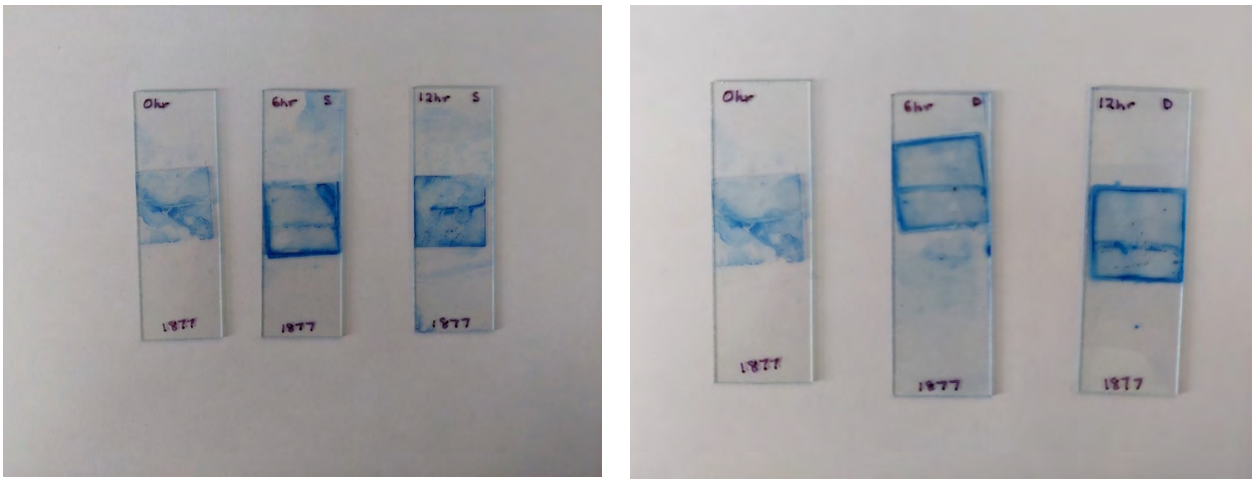

Figure: Stained 1877 biofilms formed on cover slip

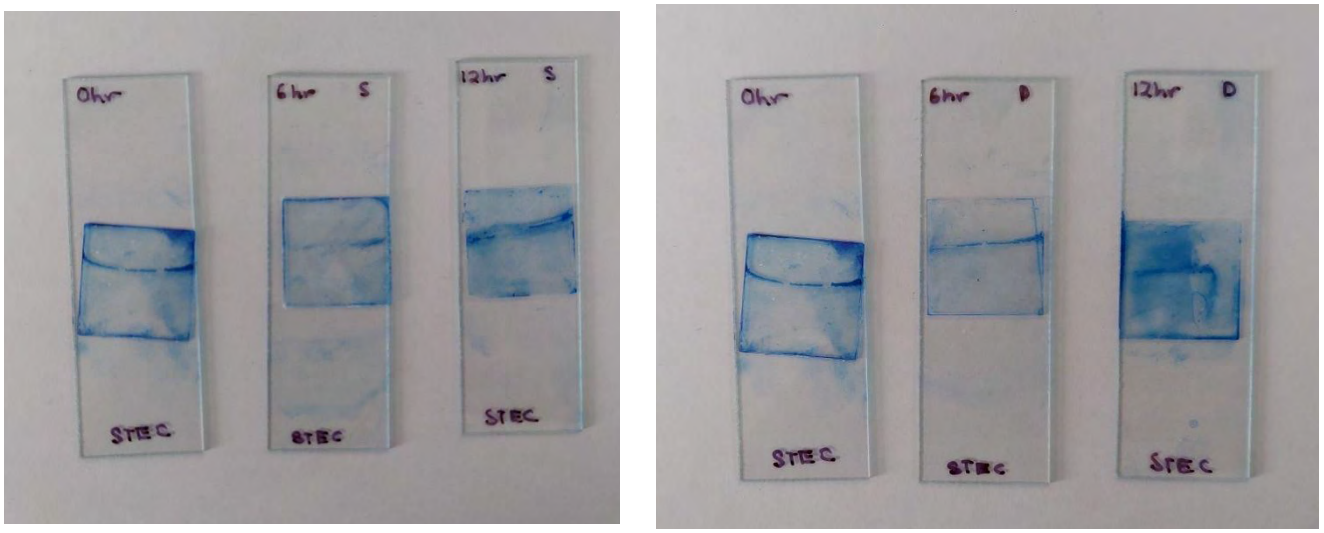

Figure: Stained 1877 biofilms formed on cover slip

4.5.1 Comparison between the Data of Biofilms Exposed to Sunlight and Darkness in Summer and Winter

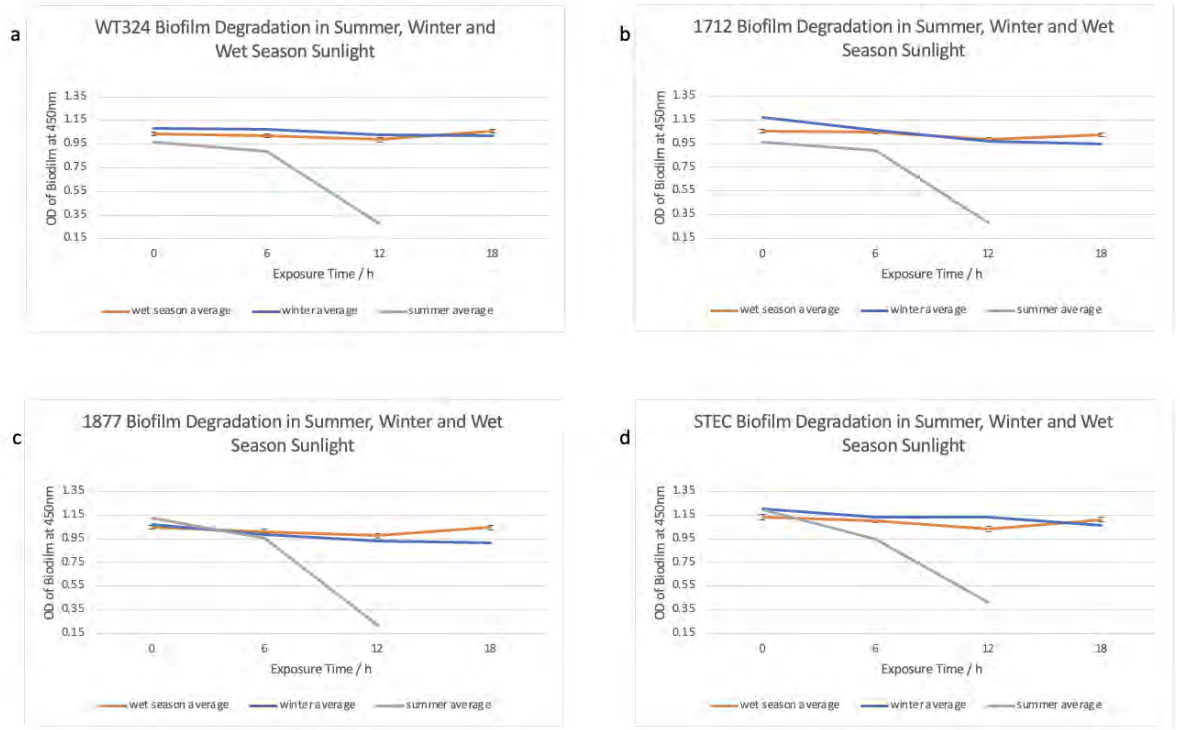

Figure: The image depicts the changes in OD of 4 different bacterial strains exposed to sunlight over a period of 12 hours in summer, winter and wet season, where the biofilms were formed on 96-well ELISA plates. Image (a) shows the changes in OD obtained after exposing WT324 Vibrio biofilm to 12 hours of sunlight in summer, winter and wet season. Similarly, image (b) shows the changes obtained after exposing 1712 Vibrio, image (c) from 1877 Vibrio, and finally, image (d) from STEC. Here the X axis represents the exposure time in hours in sunlight and the Y axis represents the optical density (OD) of biofilms at 450 nm wavelength.

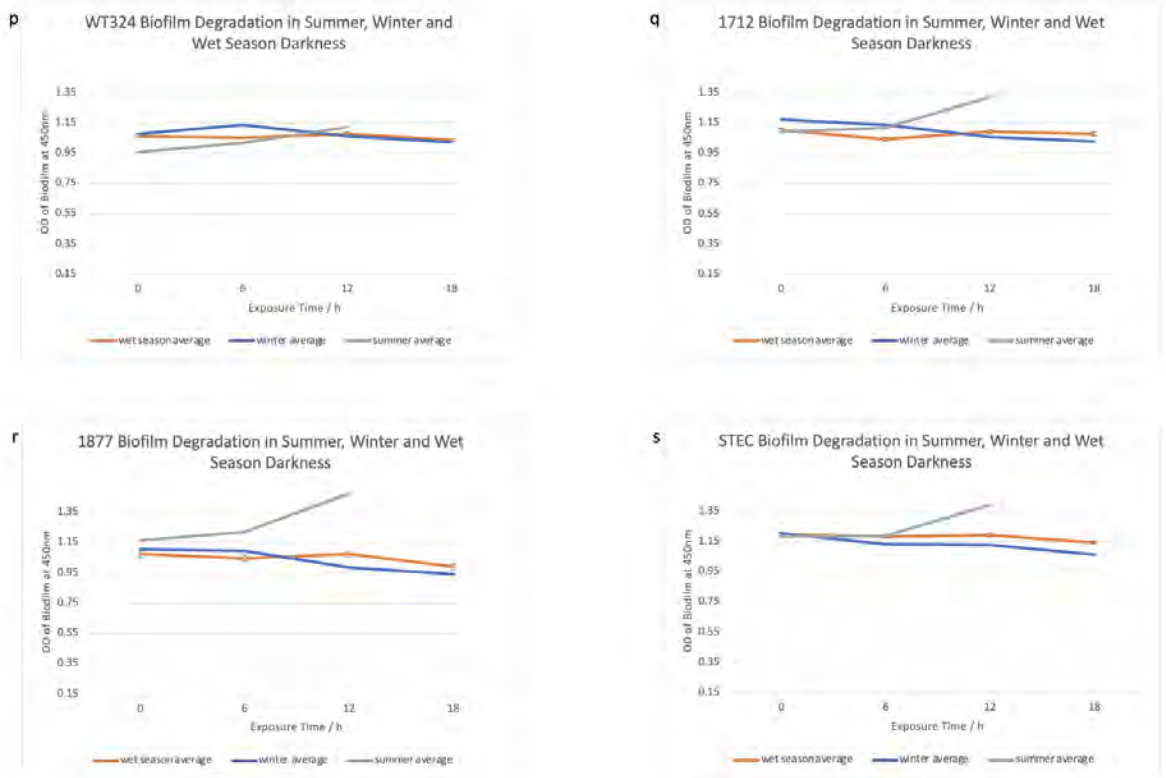

Figure: The image depicts the changes in OD of 4 different bacterial strains exposed to darkness over a period of 12 hours in summer, winter and wet season, where the biofilms were formed on 96-well ELISA plates. Image (p) shows the changes in OD obtained after exposing WT324 Vibrio biofilm to 12 hours of darkness in summer, winter and wet season. Similarly, image (q) shows the changes obtained after exposing 1712 Vibrio, image (r) from 1877 Vibrio, and finally, image (s) from STEC. Here the X axis represents the exposure time in hours in sunlight and the Y axis represents the optical density (OD) of biofilms at 450 nm wavelength.

4.5.2 T-tests for Comparison between OD of Biofilms that were formed in 96-Well ELISA Plates Exposed to Sunlight in Summer Season and Sunlight in Winter Season

Null Hypothesis: There is no significant difference between OD of biofilm exposed to summer sun and wet season sun
Alternative Hypothesis: There is a significant difference between OD of biofilm exposed to summer sun and wet season sun

Organism	Time/ hour	p-value	Interpretation	NULL HYPOTHESIS
Summer WT324 Vs. Wet Season WT324	0	0.5863	0.5863 > 0.05	ACCEPT
	6	4.0900E-10	4.0900E-10 < 0.05	REJECT
	12	4.0213E-09	4.0213E-09 < 0.05	REJECT
Summer 1712 Vs. Wet Season 1712	0	0.0043	0.0043 < 0.05	REJECT
	6	1.7067E-13	1.7067E-13 < 0.05	REJECT
	12	5.3713E-20	5.3713E-20 < 0.05	REJECT
Summer 1877 Vs. Wet Season 1877	0	0.2663	0.2663 > 0.05	ACCEPT
	6	0.3912	0.3912 > 0.05	ACCEPT
	12	4.9045E-26	4.9045E-26 < 0.05	REJECT
Summer STEC Vs. Wet Season STEC	0	0.9880	0.9880 > 0.05	ACCEPT
	6	0.1195	0.1195 > 0.05	ACCEPT
	12	5.3290E-07	5.3290E-07 < 0.05	REJECT

Table: Statistical Significance Comparison Between OD Of Biofilms That Were Formed On 96 Well Elisa Plates Exposed To Sunlight In Summer Season And Sunlight In Wet Season By T-test

4.5.3 Interpretation of the Graphical Representation and the T-test Comparing the Degradation of Biofilms by Summer Sunlight Exposure and Winter Sunlight Exposure

Wet season data shows degradation of biofilm due to sunlight, however, when compared to summer data it is observed that the degradation in summer is vastly more significant.

For WT324, the null hypothesis at 0 hour is accepted as OD of biofilm at 0 hour is similar. From 0 to 12, there is a significant decrease which is why T-test results at 6 hour and 12 hour show that the alternative hypothesis is accepted, i.e. there is a significant difference between OD of biofilm exposed to summer sun and wet season sun

For 1712, there is a significant difference between OD of biofilm exposed to summer sun and wet season sun as shown by both the graph and t-tests.

For 1877 and STEC, there is a subtle decrease from 0 hour to 6 hour, however there is a giant decrease from 6 hour to 12 hour. This is corroborated by the t-test as the hypothesis is accepted at 0 hour and 6 hour but rejected at 12 hour.

Chapter 5: Discussion

5.1 Key findings

Two qualitative data collection methods were used; staining biofilm rings in vials (part of phase 2) and staining biofilm grown on coverslips (phase 4). In both cases, insufficient empirical evidence was discovered due to limitations discussed in 5.2.

In phase 1 and phase 2, the quantitative results obtained were not sufficient enough to conclusively validate or refute our hypothesis. Consequently, it can be inferred that the utilization of these protocols does not contribute to the substantiation of our hypothesis and for further continuation of this project focus should be on phase 4 protocol. This is because growing biofilm in 96-ELISA plate and measuring its OD after exposure to both sunlight and darkness has consistently provided satisfactory data.

Comparing the wet season sunlight and dark data sets using statistical analysis revealed that there is a significant difference between the data obtained from sunlight exposure and the data obtained from darkness exposure. However, when compared with the summer data, it is found that there is a much more significant difference between the summer sunlight exposed data sets and the wet season sunlight data sets. This indicates that even though the sunlight in the wet season might be able to cause degradation of bacterial biofilm, it is not nearly as much as the degradation that occurs during summer, which is the peak outbreak season. Wet season data, as observed by comparison graphs, are similar to winter data.

5.2 Limitations

Culture plates solely comprising the intended bacterial strains were not successfully obtained due to persistent contamination, leading to a negative impact on bacterial cell count.
Moreover, each LA plate was inoculated with 4 dilutions of each bacteria which provided suboptimal growth conditions for each dilution.
Some bacteria cultured in LB media within vials produced a second biofilm ring. This sporadic phenomenon affected the measurements taken of the OD of dissolved and stained biofilm rings.
This study only examines the effect of sunlight on the degradation of bacterial biofilm. However, additional factors other than sunlight may also impact degradation of biofilm. While the protocol ensured identical treatment of bacterial biofilms (excluding exposure to sunlight) to obtain accurate results, there were conditions such as the weather, humidity and temperature that could not be controlled. A change in these parameters could have an effect on the degradation of biofilms, thereby influencing the results obtained from this study.

5.3 Future Prospect of this Study:

For this study, data has previously been collected in summer and winter seasons. This study collected data in the time between those two seasons - in the wet season.
Data pertaining to this hypothesis has been collected over the course of a year. It is imperative to continue the experiment and gather data for a minimum of another year to ascertain a definitive conclusion.

5.4 Future Research on the Topic:

This study looks into the effect of sunlight on biofilm. Further in-depth research can be done to find out exactly which component of sunlight causes the change. Other factors can be looked into for biofilm resuscitation and why they act in winter and not summer.
The wet season has sunlight similar to the summer season but it still does not bring about the same level of resuscitation of biofilm - this could be due to the fact that direct sunlight is not continuous in wet season as it is interrupted by rain and clouds. This could be further investigated.
Additionally, broadening the bacterial strains and including other seasonal pathogens can be an additional research project.

Chapter 6: Conclusion

In this study, it was found that during the wet season, exposure to sunlight caused a measurable level degradation of biofilm which was considerably less in comparison to the degradation experienced during the summer. This aligns with the absence of reported cholera outbreaks during the wet season of 2022. The inferred conclusion supports the hypothesis that exposure of biofilms to sunlight specifically during the summer season results in considerable degradation. The revival of biofilm by sunlight is considered a significant contributing factor to the seasonal occurrence of enteropathogenic bacterial diseases and hence, there are annual outbreaks of such infections during summer season.

Chapter 7: References

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