EFFECT OF FREE DNA OF ENVIRONMENT SURFACE WATER ON BACTERIAL BIOFILM FORMATION

Submitted by

Ajora Saha

19236001

Riti Sen

19236012

Suhanin Binte Habib

19236017

A thesis submitted to the Department of Mathematics and Natural Sciences in partial fulfillment of the requirements for the degree of Bachelor of Science in Biotechnology

Department of Mathematics and Natural Sciences

BRAC University, Bangladesh

November, 2023

Declaration

It is hereby declared that

1. The thesis submitted is our own original work while completing Bachelor of Science 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 which 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.

Student's Full Name & Signature:

Student Full Name: Ajora Saha

Student ID: 19236001

Student Full Name: Riti sen

Student ID: 19236012

Student Full Name : Suhanin Binte Habib

Student ID: 19236017

Approval

The thesis titled **"Effect of free DNA of environment surface water on bacterial biofilms formation"** submitted by Ajora Saha , Riti Sen, Suhanin Binte Habib
has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Bachelor in Biotechnology on [30th November,2023].

Examining Committee:

Supervisor:
(Member)

_____

Iftekhar Bin Naser, PhD
Associate Professor,
Biotechnology Program
Department of Mathematics and Natural Sciences,
BRAC University

Program Director:
(Member)

_____

Munima Haque, PhD
Associate Professor,
Biotechnology Program
Department of Mathematics and Natural Sciences
BRAC University

	_____
Departmental Head:	A F M Yusuf Haider, PhD
(Chair)	Chairman and Professor,
	Department of Mathematics and Natural Sciences,
	BRAC University.

Ethics Statement

This material is an original work, which has not been previously published elsewhere. It is our own research and analysis in a truthful and complete manner. The paper properly credits all the sources used .

Abstract

Bacteriophages, commonly referred to as bacterial viruses, have long been recognized as natural adversaries of bacteria. They find application in medical settings for eliminating bacterial infections. The prevalence of bacteriophages in the environment experiences seasonal fluctuations, with an increase contributing to elevated bacterial infections and subsequent reduction in bacterial numbers, and vice versa. Bacteriophages inject their genetic material into bacterial cells, leading to replication within. Upon reaching maturity, these viral predators rupture the bacterial cell, releasing all its contents, including free DNA, into the surrounding environment. Faced with such adverse conditions, bacteria often respond by forming biofilms to protect themselves from these viral invaders. This study seeks to explore whether the free DNA introduced by Bacteriophages influences the formation of bacterial biofilms. Biofilms of a number of vibrio cholerae strains that cause diseases cholera and diarrhea during the months of May to June were subjected to free DNA. Methods of data collection and its effects were observed and analyzed using appropriate statistical analysis. The resulting data and statistical analysis suggests that there is an increase of Bacteriophage in the water which releases free DNA in the environment that causes induction of the biofilms. However, in order to provide any conclusive evidence, round the year study including more samples is required. So the presence of free DNA appears to have a discernible impact on biofilm production, highlighting its potential role in influencing bacterial behavior and community dynamics.

Acknowledgement

First and foremost, we would like to express our sincere gratitude to Almighty God for providing us with the perseverance, patience, and knowledge needed to finish this project within the desired time span.

We convey our special thanks to Professor A. F. M. Yusuf Haider, PhD, Chairman, Department of Mathematics and Natural Sciences, BRAC University, who always has tremendous support for every student. We express our gratitude to our supervisor, Dr.Iftekhar Bin Naser, PhD, Assistant Professor, Department of Mathematics and Natural Sciences, BRAC University, for his encouragement and continuous guidelines for this research. We sincerely express our appreciation and admiration for his patience and for providing a sense of direction whenever we encountered complications throughout this phase. We feel to acknowledge our indebtedness to him for his insightful corrections and directions which shaped the present work as it is today. We are indebted to all the extraordinary people of the Life Science Laboratories, BRAC University. Without their all-time support this would not be completed. We would like to specially recognize Asma Afzal, Lab Officer, and Tanzila, Nadira and Ashiq, Lab Assistants, Life Science Laboratories, BRAC University for being so patient and helpful towards us for the entirety of the project. Also , we would like to convey our gratitude to Fariya Anny , research assistant of Department of Mathematics and Natural Sciences for guiding us to perform the stages in our experiment .Without their all-time support this would not be complete. We sincerely thank our beautiful family who trusted in us and are always there for us. Grateful and indebted to all of these people.

Sincerely,

Ajora Saha,

Riti sen,

Suhanin Binte Habib,

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List of Acronyms

OD- Optical Density

T1N1- Tryptone Salt Agar

ELISA- Enzyme-Linked Immunosorbent Assay

LB- Luria Broth

LA- Luria Bertani Agar

TCBS- Thiosulfate-citrate-bile salts-sucrose

MAC - MacConkey agar

S- Sample

ADW-Autoclave Distilled Water

EPS- Extracellular Polymeric Substance

UV- Ultraviolet Ray

V.cholerae- Vibrio cholerae

ER- Endoplasmic Reticulum

ARF6- ADP ribosylation factor 6

E.coli- Escherichia coli

UTIs- Urinary tract infections

CV- Crystal Violet

Chapter 1. Introduction:

1.1 Background

Many bacteria create a structured layer of protective encasement, called a biofilm that adheres to microorganisms within and is made up of a complex polymeric substances (EPS) matrix. This is the underlying reason for the cholera and diarrhea outbreak in Bangladesh . Bacteria form biofilm in adverse conditions and remain dormant until a favorable environment is created for multiplication and infection in a host body. Biofilm formation is also one of the survival mechanisms of bacteria. Some of the reasons for bacterial biofilm formation are adverse environments (excessive heat or cold), lack of nutrients, invasion of bacteriophages, etc. (Naser et al., 2017) . However, by different methods, these cells can naturally revive into the active planktonic form, reproduce, and create cholera epidemics (Naser et al., 2017).

Toxigenic *Vibrio cholerae* persists in cholera-endemic areas mostly in a biofilm-associated condition, in which the bacteria are fixed in an exopolysaccharide matrix. In Bangladesh from May to May cholera outbreaks are seen to increase whereas it decreases from the month June. Many factors cause these seasonal outbreaks and many factors have been investigated in order to validate this periodic increase and decreased infections caused by the causative agents of these diseases. Bacterial biofilms have been a topic of focus in order to explain this. One of the main factors is the increase of bacteriophage can trigger the collapse of epidemics. The large amount of phages reduce the bacterial number in the environment and increase the amount of free DNA. This release of free DNA in the environment may induce the planktonic bacteria to form biofilms and let them hide from the pages and thus survive. Although, different other factors like irradiation, bacteriophage infection, chemical treatment, sunlight , temperature have been inspected as possible reasons that can cause cholera bacterial biofilms and releases the

planktonic bacteria which then causes infections and in a larger scale, epidemics. However, one of the viable causes that induce bacterial biofilms and causes breakdown of seasonal epidemics of cholera in Bangladesh can be an increase of bacteriophage that releases free DNA in an environment that is yet to be explored.

According to the previous hypothesis, where cholera infections decrease starting from the last of June , it can be presumed that in that time of the season there is decrease of bacteriophage in the environment which releases less or very few free DNA in the environment that does not form the biofilm rings that will release the planktonic bacteria which causes the infection as free DNA is not available in the environment from the period of July to August. As a result, infectious bacteria like Vibrio cholera remain dormant inside the biofilm structures unable to cause cholera and diarrhea respectively during the particular time of the season.

1.2 Aim of the study

There was a previous report that confirmed that the amount of bacteriophages vary in a seasonal manner. The aim of the study is to investigate whether the surface water of different months may have any effect on the formation of bacterial biofilms. The objective is whether or not bacterial DNA provided by the bacteriophages is responsible for biofilm formation as bacteriophage numbers rise, free DNA levels rise as well resulting in an increase in biofilm formation.

Chapter 2. Literature Review:

2.1.1 Biofilm

A biofilm is an assemblage of microbial cells that is irreversibly associated with a surface and enclosed in a matrix of primarily polysaccharide material. Noncellular materials such as mineral crystals, corrosion particles, clay or silt particles, or blood components, depending on the environment in which the biofilm has developed, may also be found in the biofilm matrix. Biofilm-associated organisms also differ from their planktonic counterparts with respect to the genes that are transcribed. Biofilms may form on a wide variety of surfaces, including living tissues, indwelling medical devices, industrial or potable water system piping, or natural aquatic systems. The variable nature of biofilms can be illustrated from scanning electron micrographs of biofilms from an industrial water system and a medical device, respectively (Figures 1 and 2) .The water system biofilm is highly complex, containing corrosion products, clay material, freshwater diatoms, and filamentous bacteria. The biofilm on the medical device, on the other hand, appears to be composed of a single, coccoid organism and the associated extracellular polymeric substance (EPS) matrix. (Rodney M. Donlan, 2002)

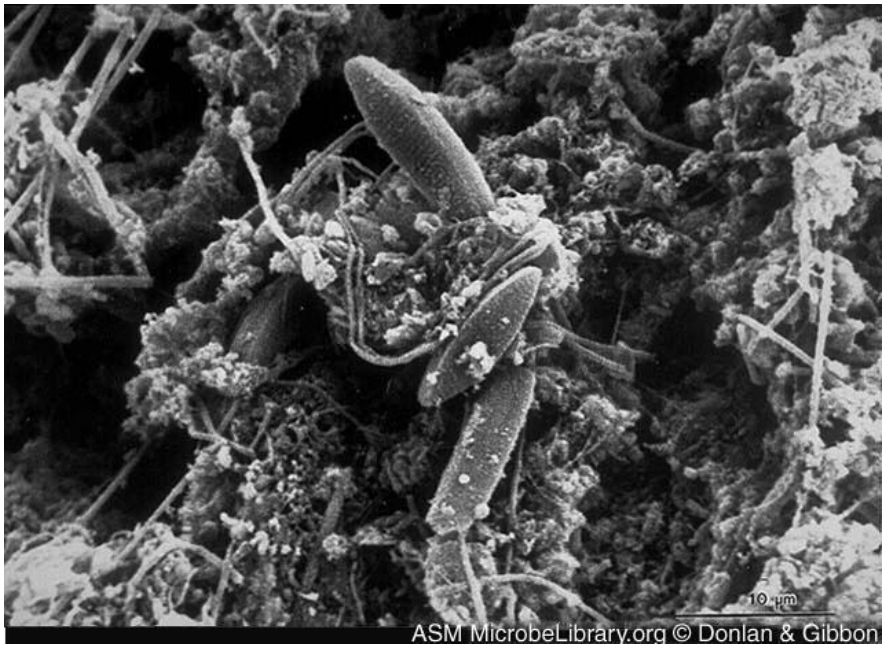

Figure 1: Scanning electron micrograph of a native biofilm that developed on a mild steel surface in an 8-week period in an industrial water system.

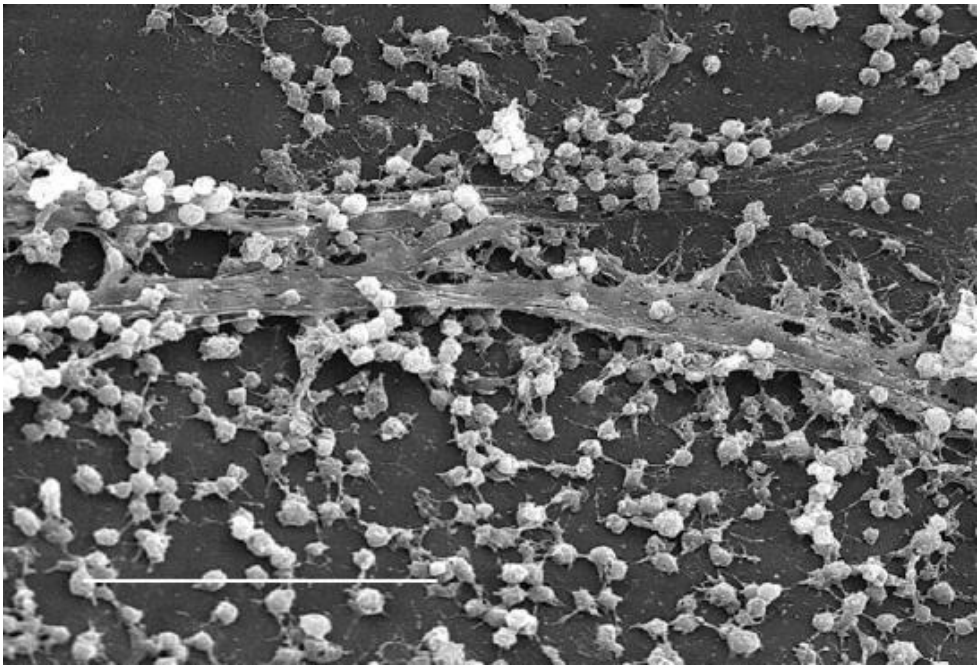

Figure 2: Scanning electron micrograph of a staphylococcal biofilm on the inner surface of an indwelling medical device.

Evidence shows that pathogenic *Vibrio cholerae* biofilm production aids the pathogen's persistence in the environment, where adhesion to surfaces in aquatic settings plays a critical part in the pathogen's epidemic cycles. Within biofilms, local microenvironments may be very varied, and organisms struggle for space under a variety of circumstances, including nutrition constraint, fluid movement, desiccation, toxic chemical gradients, and UV irradiation, and pH and temperature fluxes. As a result, biofilm development is a simple microbial survival strategy in which microorganisms, including pathogens, dwell in a dynamic equilibrium in which cell clusters grow, mature, and detach to spread to other surfaces (Hall-Stoodley & Stoodley, 2005). A biofilm three dimensional structure can be made up of one or more than one type of bacteria. They can be formed on both living and non-living surfaces and they can be found anywhere from lake water, raw food, sewage lines to kitchen sinks, animal teeth and laboratory tools. Commonly, biofilms are referred to as slime. However, inside this slime a unique and complex system develops that is stable and has a significant role in microbes' survival and pathogenesis.

2.1.2 Biofilm Development

Biofilms are made up of proteins, polysaccharides, lipids, and DNA, and they form a protective matrix around bacteria, ensuring their integrity and survival. Microorganisms take up around 10% to 30% of the biofilm volume. Water makes up around 97 percent of the biofilm, and it is responsible for the flow of nutrients essential for bacterial life. In the environment, the functional consequences of bacterial life in biofilms have been associated with enhanced protection from shear stress, desiccation, toxic compounds and protozoan grazing. Moreover, retention of enzymes in the biofilm matrix was proposed to improve efficiency and diversity of organic matter decomposition, and biofilm formation on plant roots and fungal cells may promote bacterial nutrient acquisition and transport, respectively .Pathogenic biofilms that form on plants may also have serious disease consequences. While (motile) planktonic cells are primarily found in water columns and soil pores, the predominant forms of microbial life in natural environments are linked to highly diverse biofilm communities in aquatic environments (including sediments, submerged surfaces, as free-floating flocs and on higher organisms), sediments and soil (e.g. on litter, plant roots and soil particles). Likewise, biofilms dominate in industrial microbial

applications, such as cleaning of wastewater and bioremediation of soil and water. A common denominator of bacterial biofilms is the distinction between surface-attached and non-surface-attached bacterial aggregates, despite new evidence showing that these share similar phenotypes. For both of these phenotypes the bacteria create microenvironments which in turn influence bacterial community and behavior in an interdependent and dynamic manner. (Paul Stoodley, Darla M. Goeres, Mette Burmølle, Philip S. Stewart,and Thomas Bjarnsholt , 2022). Five main steps can be characterized in the complex but well-regulated process of biofilm development: (i) Surface swarming, which is made possible by surface sensing carried out by planktonic bacteria's flagella; (ii) the stage of attachment, whereby the bacterial adhesions carry out the initial reversible attachment, which indicates loosely adhering to the surface and detaching, and the subsequent irreversible attachment, which is more precise and stable adhesion; (iii) the excretion of EPS matrix, which is a sign that a biofilm has been formed and was produced by recently attached bacteria; (iv) the maturation of the biofilm, which involves bacterial cell interactions that result in the development of micro-colonies; (v) the structure of the biofilm spreading after planktonic bacteria are released, causing the biofilm to form at new locations (Alhede et al., 2011) , (Armbruster & Parsek, 2018) .

Biofilm formation is a process whereby microorganisms irreversibly attach to and grow on a surface and produce extracellular polymers that facilitate attachment and matrix formation, resulting in an alteration in the phenotype of the organisms with respect to growth rate and gene transcription. (Rodney M. Donlan, 2001)

In our experiment, we formed bacterial biofilm in solid surfaces like glass and the method how bacteria forms and adheres their biofilm to a solid surface is shown below:

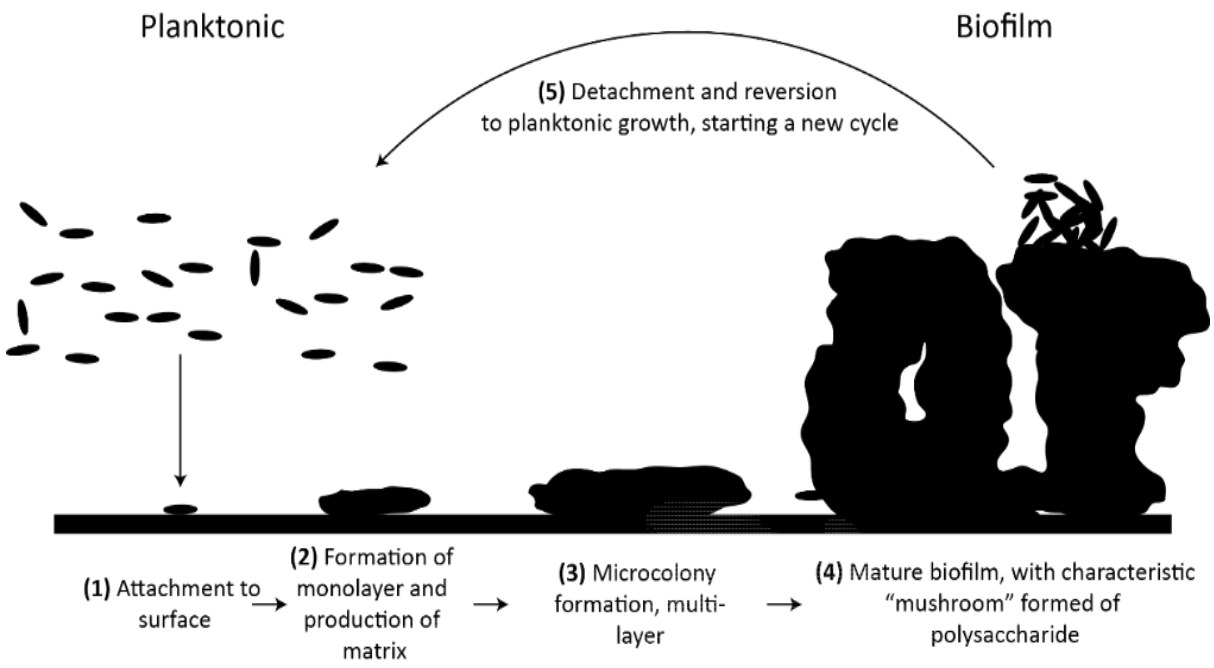

Figure 3: The process of biofilm formation

This is the schematic representation of biofilm formation. The formation begins with a reversible attachment of the planktonic cells (brown ovals) followed by the adhesion to the surface (gray) (1). The bacteria then form a monolayer and irreversibly attach by producing an extracellular matrix (2). Next, a microcolony is formed where multilayers appear (3). During later stages, the biofilm is mature, forming characteristic "mushroom" structures due the polysaccharides (4). Finally, some cells start to detach and the biofilm (shown in yellow) will disperse (5). (Vasudevan, 2014).

The mechanism of biofilm formation is triggered and regulated by quorum sensing, hostile environmental conditions, nutrient availability, hydrodynamic conditions, cell-to-cell communication, signaling cascades, and secondary messengers. Antibiotic resistance, escape of microbes from the body's immune system, recalcitrant infections, biofilm-associated deaths, and food spoilage are some of the problems associated with microbial biofilms which pose a threat to humans, veterinary, and food processing sectors. (Braz J Microbiol, 2021).

2.1.3 Bacteriophage

Bacterial biofilms have been implicated as a source of infection and contamination in medical and industrial settings as well as in waterborne transmission of pathogens. (Naser et al., 2017).Bacteriophages are among the smallest but most abundant organisms on earth (~1031) (Suttle, 2005). For most phages, the tail mediates the anchoring of the phage to generally abundant bacterial outer membrane proteins that serve as specific receptors for their substrates.Bacteriophages, viruses that infect and replicate within bacteria, are perfectly adapted to infect biofilms. Owing to the co-evolution mechanism, phages are actively involved in biofilm formation, in two contradictory ways, as promoting or dispersing agents. Phages can be equipped with matrix-degrading enzymes that allow the effective infection of biofilm embedded cells. Bacteriophage is a kind of virus that invades bacteria, removes bacterial replication machineries, and injects its own nucleic acid to replicate inside the bacteria. After complete replication and multiplication, the new bacteriophages burst out of its host cell. (Drulis-Kawa & Maciejewska, 2021).Biofilms are surface-associated communities of bacteria encased in a matrix of complex heterogeneous extracellular polymeric substances composed of polysaccharides, proteins, nucleic acids, and lipids.(Naser et al., and Ahmed Abdullah, 2017) .

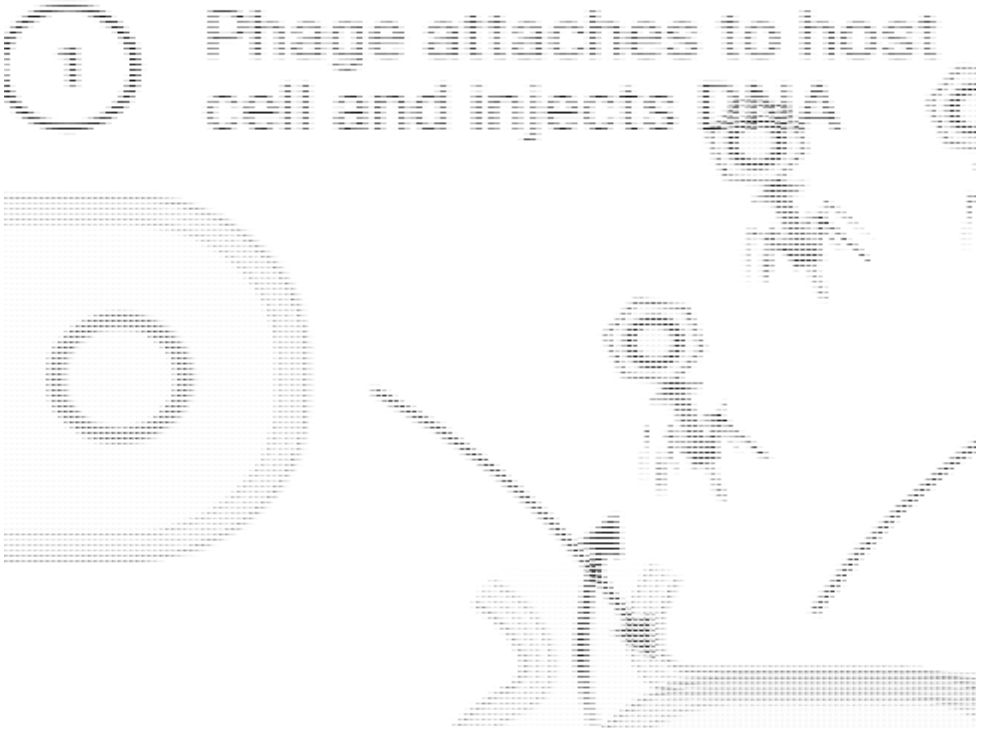

Figure 4: Common invasion process of bacteriophage in a bacterial cell

When the bacteriophage number increases the number of bacteria decreases and vice-versa. In Bangladesh between June to May the bacteriophage number declines resulting in inclined number of bacteria and also rise of number of patients affected with bacterial infection.The activity of the phage essentially increases the concentration of active pathogenic bacteria in water instead of decreasing it, Although an opposite scenario is observed between July to August. As bacteriophage number rises, the number of bacteria decreases (Naser et al., 2017).

In the time period from June to May as bacteriophage replicates inside the bacteria , they burst out from the bacteria and come out resulting in free DNA. This results in abundance of free DNA in the environment in that particular period of time. And it is assumed that,, these free bacterial DNA is responsible in biofilm formation of bacteria under this condition.

2.2 Disease caused by biofilm forming bacteria

2.2.1 *Vibrio cholerae*

Vibrio spp. are a group of common, Gram-negative, rod-shaped bacteria that are natural constituents of freshwater, estuarine and marine environments.Vibrio is a genus of ubiquitous bacteria found in a wide variety of aquatic and marine habitats; of the >100 described Vibrio spp., ~12 cause infections in humans. *Vibrio cholerae* can cause cholera, a severe diarrhoeal disease that can be quickly fatal if untreated and is typically transmitted via contaminated water and person-to-person contact.(Baker-Austin et al., 2018). Cholera is a waterborne disease, and the occurrence of epidemics coincides with increased prevalence of the causative V. cholerae strain in the aquatic environment . (Johirul et al., 2006). The pathogen V. cholerae causes potentially fatal diarrheal maladies in people. There are numerous *V.cholerae* serogroups. Two of them, O1 and O139, are known to lead to serious illness (Faruque et al., 2005).

Cholera pathogenesis follows a certain pathway. The pathogen enters the human host by contaminated water. After reaching and harboring the target organ (small intestine), Vibrio cholerae begins expressing virulence factors, like cholera toxin.

The cholera toxin is made up of the two subunits CtxA and CtxB, and the CtxB pentameric subunit is what binds to the ganglioside GM1 on the cell's plasma membrane. The cell then takes up the GM1-bound cholera toxin and transports the complex inside of it to the endoplasmic reticulum (ER). The CtxA and CtxB subunits separate from one another there. Once ADP ribosylation factor 6 (ARF6) activates the enzyme's allosteric activation upon release from the ER into the cytoplasm (Baker-Austin et al., 2018).

The G protein-coupled receptor is catalyzed by the ARF6-CtxA complex, which further initiates adenylyl cyclase. This causes the cystic fibrosis trans-membrane receptor to be phosphorylated (P), which raises the levels of cAMP in the cell (CFTR). Watery diarrhea is the end result, which is caused by an ion and water efflux into the small intestinal lumen (Baker-Austin et al., 2018)

2.2.2 Escherichia coli

Few microorganisms are as versatile as *Escherichia coli* . An important member of the normal intestinal microflora of humans and other mammals, *E.coli* has also been widely exploited as a cloning host in recombinant DNA technology. But *E.coli* is more than just a laboratory workhorse or harmless intestinal inhabitant; it can also be a highly versatile, and frequently deadly, pathogen. Several different *E.coli* strains cause diverse intestinal and extraintestinal diseases by means of virulence factors that affect a wide range of cellular processes.(Kaper, Nataro, & Mobley, 2004).

E.coli is a gram-negative and rod-shaped bacterium that belongs to the Gammaproteobacteria class and is categorized as a member of the Enterobacteriaceae family. *E.coli* can multiply in about 20 minutes when given the right conditions for growth (Jang et al., 2017).Although *E.coli* is widely employed as a fecal indicator bacteria (FIB) for evaluating water quality, its survival and proliferation in the environment raise issues about its suitability as a fecal indicator bacteria. Understanding the ecology of this bacteria is also crucial to preventing infection and the transmission of this pathogen to food, soil, and water since some *E.coli* strains and serotypes can lead to human disorders (Anderson, Whitlock, & Harwood, 2005).Usually, *E.coli* and its human host coexist in good health and with mutual benefit for decades. These commensal *E.coli* strains rarely cause disease except in immunocompromised hosts or where the normal gastrointestinal barriers are breached. Several extremely adapted *E.coli* clones that have developed certain virulence traits, which provide them a greater capacity for niche adaptation and enable them to cause a variety of diseases (Kaper, Nataro, & Mobley, 2004). Infection with one of the following pathotypes can cause enteric/diarrheal illness, urinary tract infections (UTIs), and sepsis/meningitis, three main clinical syndromes (Kaper et al., 2004).

E.coli must endure the low-pH environment of the animal or human stomach to reach the intestinal system; many *E.coli* strains are acid-resistant. The population structure of *E.coli* can be impacted by host intestinal circumstances. Based on the host-animal and dietary, *E.coli*

phylogenetic groupings are relatively abundant (Jang et al., 2017). *E.coli* is known to survive in natural habitats due to the formation of biofilms on surfaces in aquatic environments, such as sediments. The bacteria are guarded by biofilms from detrimental environmental factors including UV radiation, desiccation, protozoan predators, and chemicals like antibiotics and cleaners (McDougald, Rice, Barraud, Steinberg, & Kjelleberg, 2012).

2.3 Cholera Biofilm and epidemics

Epidemics of cholera caused by toxigenic *Vibrio cholerae* belonging to the O1 or O139 serogroups are a major public health problem in many developing countries of Asia, Africa, and Latin America. (Faruque et al., 2005). Cholera epidemics occur with seasonal regularity in the Ganges delta region of Bangladesh and India. Epidemics usually occur twice during a year, with the highest number of cases just after the monsoon during September to December. A somewhat smaller peak of cholera cases also is observed during the spring, between March and May. Although *V. cholerae* is a human pathogen, these bacteria constitute part of the normal aquatic flora in estuarine environments, and water is clearly a vehicle for transmission of *V. cholerae*. Although the seasonality of cholera in Bangladesh and elsewhere has been temporally associated with numerous physical and biological parameters. (Naser et al., 2005). These associations do not directly cause epidemics, nor do they end them. More than a century of public health experience has shown that toxigenic O1 and O139 *V. cholerae* cells cause cholera epidemics and that the elimination of these cells from drinking water ends cholera epidemics. The parameters that directly modulate the level of viable cells belonging to the pathogenic clones of *V. cholerae* O1 and O139 in the Ganges delta aquatic environment remain unknown. Furthermore, the fact that pathogenic strains of *V. cholerae* are clonally distinct from environmental, nonpathogenic *V. cholerae* strains. (Faruque et al., 2005). In March 2022, a cholera outbreak striked Dhaka city as around 1200 cholera patients from different areas were admitted in the hospitals, mainly the ones run by the International Centre for Diarrhoeal Disease Research, Bangladesh (icddr,b). This year, the number of patients was higher than normal, as the hospital authority claimed each day nearly a thousand of patients visited complaining about diarrhea and cholera ("Dhaka Wasa must

answer for cholera outbreak", 2022).During cholera epidemics, the presence of biofilm fragments containing aggregates of *V. cholerae was* isolated from cholera stool. Although these cells were initially found to be infective and culturable, they soon lost their infectivity, indicating a temporal constraint. Thus, these cells can only intensify the cholera epidemics in areas with poor sewage treatment facilities (Alam et al., 2007).

Throughout the year in Bangladesh, *V. cholerae* O1 remains in the aquatic environment as nonculturable coccoid cells in biofilms that can be detected using fluorescent antibody-based studies. The cells derived from these biofilms could be made culturable even after a year of dormancy, accounting for the annual cycle and epidemics of cholera. The resuscitation of *V. cholerae* in nature is usually expected to be caused by the fluctuations in temperature, nutrient levels, and the zooplankton (host of *V. cholerae*) blooms during summer. Surprisingly, the resuscitation was only observed when the cells were bound in biofilms, not in nonculturable microcosms (Alam et al., 2007). During epidemics, water samples showed the presence of single cells and biofilm-bound V. cholerae O1 in culturable state, however the rest of the year, these cells remained in a nonculturable state as a reservoir for the recurrent annual epidemics (Sultana et al., 2018).

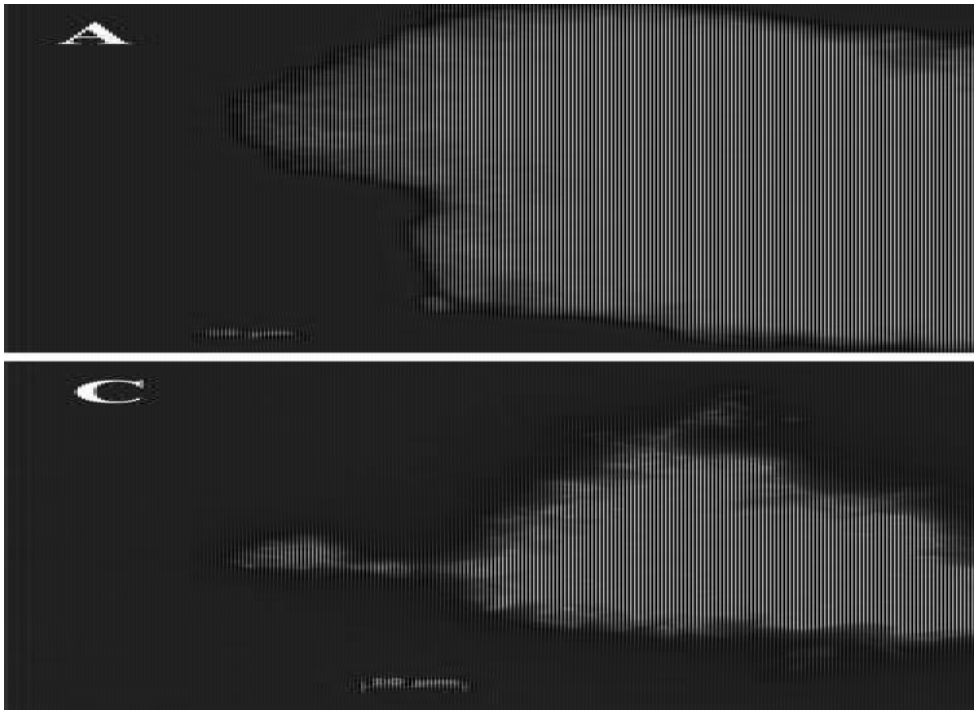

Figure 5:Direct fluorescent monoclonal antibody (DFA) detection of *V. cholerae* O1 in aquatic ecosystem of the Bay of Bengal shows biofilms of *V. cholerae* O1 during winter and monsoon months- A and C, and free-living V. cholerae O1 cells during spring and fall months- B and D (Sultana et al., 2018)

2.4 ELISA

In our conducted experiment , the optical density (OD) of the biofilm that had been formed because of sample water and autoclaved distilled water was measured by enzyme Linked Immunosorbent Assay (ELISA). Micro ELISA autoreader technique is an effective way of measuring the OD of a biofilm (Mosharraf et al., 2020).

ELISA is commonly used in almost every immunology and microbiology lab to measure the OD of biofilms.It depends on the principle of antigen- antibody interaction. This interaction can then be quantified using ELISA Auto reader machines by measuring the OD. Substances like peptides, proteins, antibodies, and hormones can be identified and measured using ELISA. ELISA has many other names and derivations like EIA, RIA, ELISPOT (Lequin, 2005) etc. Although, there are three main types of ELISA that are used in every immunology and microbiology lab. They are -

- Direct ELISA
- Indirect ELISA
- Sandwich ELISA

But in our experiment none of these methods were used. Only the OD measuring property of ELISA, auto reader was used to get the OD of the biofilms formed inside microtiter ELISA plates.

2.5 Crystal Violet Stain and Dissolving Crystal Violet Stain with Glacial Acetic Acid

The use of biofilms as model systems for investigating bacterial inter and intra-species interactions creates a need for suitable tools that enable high-throughput screening of the adhesive capabilities of the contributing species and their synergistic effects. Even though multiple methods have been developed for studies of such interactions there are still many limitations regarding reproducibility and resolution (Azeredo et al., 2017).

Among these methods, crystal violet (CV) staining of biofilms in microplate wells and pegs (Christensen et al., 1985; Ceri et al., 1999; Stepanović et al., 2000) is one of the most extensively used platforms for high through-put quantification of biofilm biomass

(Djordjevic et al., 2002; Extremina et al., 2011; Merritt et al., 2011; Røder et al., 2015; Doll et al., 2016). Crystal violet is used as an active component, primary stain, of Gram stain for differentiation of Gram-negative versus Gram-positive bacteria.Crystal violet binds negatively charged molecules and thus stains both bacteria and the surrounding biofilm matrix. (Merck, 2017).

CV in a cationic dye that can bind to the extracellular polymeric substances (EPS) and the microorganisms that make up the biofilm matrix and stain the entire biofilm, making it visible and quantifiable. To conduct the CV assay, the biofilm in each well of a 96-well plate was washed twice by gently removing 200 µL of the suspension from each well after completing the incubation of the biofilm. Then, 200 µL of sterile PBS was pipetted into the well and removed gently. The protocol was repeated twice to remove the non-adherent cells. The supernatant was discarded, and the plate was air-dried for 45 min. 200 µL of 0.1% (w/v) CV solution was added to each well and incubated for 20 min at 25 °C. The plate was washed gently twice using running distilled water, and 200 µL of 33% (v/v) acetic acid was added to de-stain the biofilm (Arzmi et al., and Zainal et al., 2023).

Chapter 3. Materials and Methods:

3.1 Organisms:

Three bacterial strains were chosen and tested under different settings for the investigation. Two of these were *Vibrio cholerae* stains, while the other one was *Escherichia coli.* These include:

1. *Vibrio Cholerae 1877*
2. *Vibrio Cholerae WT346*
3. *Escherichia coli 0157*

In this experiment, no bacteria that do not produce biofilms were utilized. These three bacterial strains are capable of producing biofilms. Because the goal of this study is to investigate how free DNA influences the development of biofilms. Therefore, organisms that do not produce biofilms are not included.

3.2 Bacterial Culture Media:

Luria Broth (LB) and LB Agar media were used in this experiment. All the organisms here are gram negative bacteria and LB is well suited for their growth. Moreover, Thiosulfate-citrate-bile salt-sucrose (TCBS) agar media was used to confirm whether *Vibrio Cholerae* strains were indeed *Vibrio Cholerae* . In addition, MacConkey agar (MAC) media was used for *E.coli 0157* strain. Because *E. coli O157* forms opaque, colorless colonies on the medium. MacConkey agar is both selective and differential, and it allows *E. coli O157* to grow and be easily distinguishable based on its ability to ferment lactose and produce acid, leading to characteristic colony pink/red colors. Other than that 0.8% LB Agar media was used as preservation media.

All of the cultures and media were obtained from the Life Science Laboratories at BRAC University. These cultures were revived, used and preserved by using standard protocols.

3.3 Biochemical Tests:

In order to verify the bacterial strains employed in this investigation, several biochemical tests were performed. The vibrio cholera strains were tested on Thiosulfate-citrate-bile salts-sucrose (TCBS) agar media to determine whether or not they were indeed vibrio. The*Vibrio Cholerae* strains were verified to be *Vibrio Cholerae* if the green TCBS agar turned yellow after streak plating them on the TCBS agar media plate and incubating it at 37°C for 24 hours. If the strains stayed green or any other color, they were considered to be something else. Two *Vibrio Cholerae* strains produced yellow instead of green colonies following TCBS plating.

In order to confirm the *E.coli 0157*, it was tested on MacConkey agar (MAC). On conventional lactose-containing media, *Escherichia coli* serotype O157:H7 rapidly ferments lactose and blends in with the majority of other *E. coli* strains. On the other hand, almost all isolates of serotype O157:H7 ferment D-sorbitol slowly or not at all, in contrast to about 80% of other *E.coli*. Because it replaces lactose in MacConkey agar with the carbohydrate sorbitol, sorbitol MacConkey Agar was created to capitalize on this property. It is the preferred medium for isolating *E.Coli O157:H7.* On the medium, *E. Coli O157* colonies are opaque and colorless. Due to its selective and differential properties, MacConkey agar enables *E. Coli O157* proliferates and is readily identifiable due to its capacity to digest lactose and generate acid, which results in the distinctive pink/red hues of the colonies. In order to maintain the appropriate bacterial strains throughout the study, these tests were conducted on a regular basis.

Figure 6: ***V.cholerae 1877*** **&** ***WT347*** **showing yellow colonies in TCBS agar plate.**

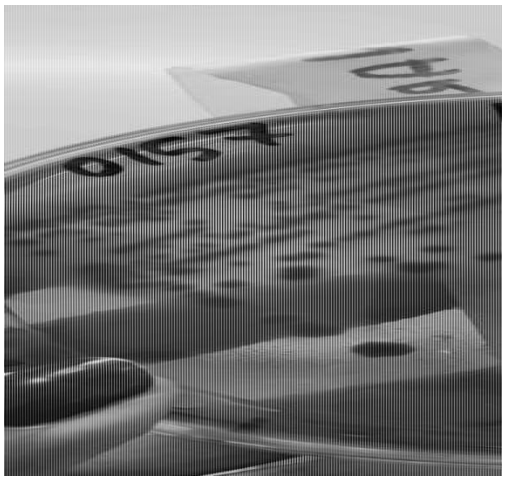

Figure 7: ***E.coli 0157*** **showing pink colonies in MAC agar plate.**

3.4 Sample Collection and Filtration:

Cholera and diarrhea is a waterborne disease caused by the bacterium Vibrio cholerae, which is spread through water or food contaminated with fecal matter. For this reason water was collected from specific lakes on specific days of each week from May to August for this study. Sample Water was collected in a 50ml Falcon tube. The sample water was then vortexed before filtration. Because vortexing mixes all particles and solutions evenly throughout the sample. For filtration, the sample water is poured into a beaker with a funnel set in it with whatman filter paper. Then a 22µm filter is then attached to the syringe to perform water filtration again. This setup helps to remove the tiny particles in the water.

Figure 8: Sample Water is kept in a 50 ml falcon tube which is preserved at 4 degrees Celsius

3.5 Overview of the Methods:

At first, sample water was collected from the specified lake (Gulshan Lake) and then water filtration was done using Whatman filter paper and 22μm filter. The strains were revived from the bacterial stocks. For young culture preparation, streaking of the respective strains (1877, WT346, 0157) in four quadrant methods in plates from stock in MAC and TCBS media. Then keep the culture plates for 24 hours in incubation to get single colonies. After 24 hours of incubation, add host (strain 1877, WT346, and 0157, from overnight culture fresh plate , each single 6 colonies) to the filtered water sample of 1 ml along with 4 ml of LB in the sterilized falcon tube. Moreover, for control added host (strain 1877, WT346, and 0157, from overnight culture fresh plate, each single 6 colonies) to the autoclaved distilled water of 1 ml along with 4 ml of LB in another sterilized falcon tubes separately for all the respective strains. Then, all the falcon tubes (the filtered water and the autoclave distilled water) were incubated in the shaker for 1.5-3 hours until it reached opaque turbidity and specific value at OD600. After that, took the young culture (both of filtered water and autoclave distilled water) of 500 microlitre in sterilized glass vials. Then, Kept the glass vials (both of filtered water and autoclave distilled water) without any movement for 36 hours. After 36 hours, wash the glass vials (both of filtered water and autoclave distilled water) for all with a PBS buffer of 600 microlitre for 2 times to remove the weak biofilms from the surface of vials. Then Stain with 500 microlitre of Crystal Violet for 5 hours, then wash with 600 microlitre of PBS buffer for 2 times and keep the vials inverted overnight. Then we noticed the ring around the vials, which indicates the formation of biofilm. Then 700 microliters of glacial acetic acid was left for 30 minutes to destine the biofilm rings. After 30 mins, slightly shaking the vials, the stain gets dissolved into the solution giving a blue solution. 200μL of this solution was then transferred into the appropriately labeled wells of a non-autoclavable microtiter plate and the OD was then measured using a Multi scanEX ELISA Machine at 620 nm absorbance. In addition, the changes in the thickness of the biofilm over the period was also observed by staining and imaging.

3.6 Revival of Bacterial Culture:

The laboratory stock of bacteria that had been kept in T1N1 medium was used to revive the strains. Stock cultures were revived by subculturing them on LB agar plates using the streak plate method. From these plates, single colonies were isolated after a 24-hours incubation period at 37°C.

3.7 Making Young Culture and Biofilm:

To facilitate the formation of biofilms, we had to make a young culture. For making a young culture, we put 4 ml of LB in a sterilized falcon tube and added 6 single colonies of a host bacteria (strain 1877,346 and 0157) and added 1 ml of filtered sample water. It was then placed in a shaker incubator for 1.5-3 hours in 37^{o}C until it reaches to opaque turbidity. For control this same procedure should be followed. But in this case, 1 ml of autoclave-distilled water should be used instead of sample water. After that, we divided 500 µl of the young culture among three sterilized glass vials. These glass vials (both of filtered sample water and control) were placed in a clean environment without any movement for 36 hours respectively in order to form biofilm. We took the vials and rinsed them with a PBS buffer of 600 microlitre for 2 times to remove the weak biofilms from the surface of the vials. After that, we applied 500 microlitre of crystal violet and waited 5 hours. We washed those vials again with a PBS buffer for 2 times and kept the vials inverted overnight. Then we noticed the ring around the vials, which indicates the formation of biofilm. If the young culture is left undisturbed for 36 hours a good biofilm forms that can be seen well on the surface.

3.8 Biofilm Staining and Washing:

The glass vials were gently washed with 600 microlitre of PBS buffer, dried, and stained with 500 microlitre of crystal violet dye for 5 hours. Then again wash with 600 microlitre of PBS buffer for 2 times to remove excess dye and keep the vials inverted overnight.

Figure 9: After using crystal violet, the changes in the biofilm ring show. The blue rings inside the glass vials are biofilm rings that were stained with crystal violet dye overnight and then washed with a PBS buffer.

3.9 Dissolving Stained Biofilm Rings:

The stained biofilm rings were first washed with a PBS buffer to remove excess dye. Then, glacial acetic acid was added to the rings, which immediately dissolved the stains. Vials stained with biofilm rings were kept for 30 min and transferred to microtiter plates to measure the OD of

biofilm rings. The obtained OD showed clear changes in the biofilm of the sample water and distilled water in the outcome.

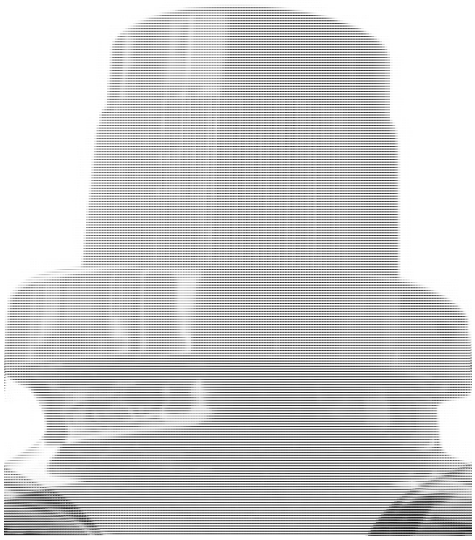

Figure 10: 700 microliters of glacial acetic acid used to dissolve stained biofilm rings.

3.10 Detection of Biofilm :

The stains on the biofilm rings formed in the vials were dissolved using glacial acetic acid. 200µL of the dissolved stain were then transferred into the 96-well microtiter plate. The reading was measured using Multi scanEX ELISA Machine at 620 nm wavelength.

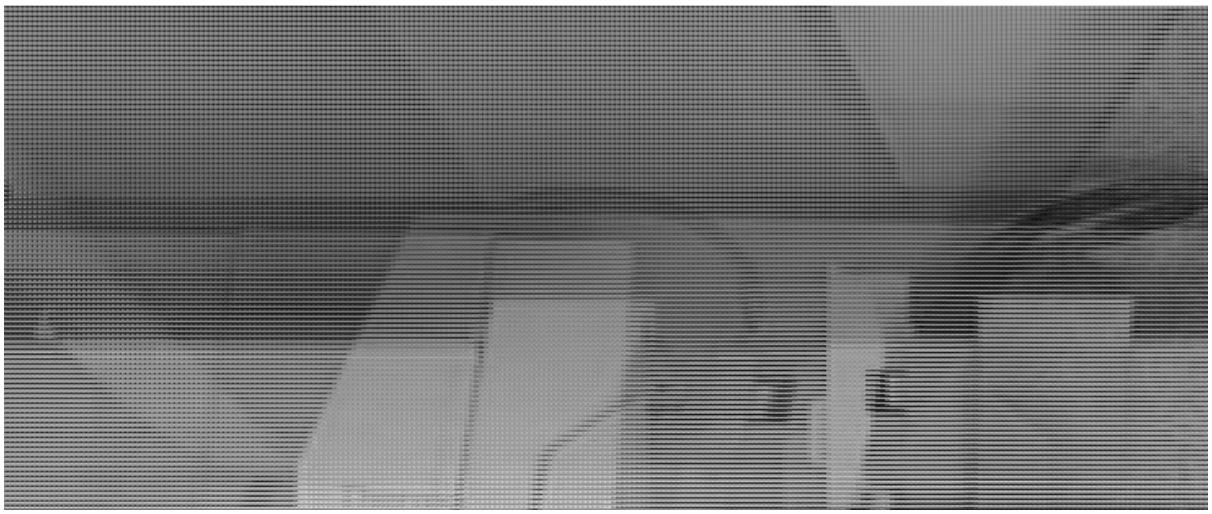

Figure 11: Multi scanEX ELISA Machine by Thermo Scientific

3.11 Statistical Analysis :

The statistical analysis was conducted utilizing Microsoft Excel (MS office version 2007). To assess the statistical differences between two groups, an independent sample T-test was employed, assuming equal variances.

Chapter 4. Results :

4.1 Sample Water Collection Date :

Sample no	**Date**
Sample no 1	16 May, 2023
Sample no 2	23 May,2023
Sample no 3	30 May, 2023
Sample no 4	6 June, 2023
Sample no 5	13 June, 2023
Sample no 6	20 June, 2023
Sample no 7	27 June, 2023
Sample no 8	4 July, 2023
Sample no 9	11 July, 2023
Sample no 10	18 July, 2023
Sample no 11	25 July, 2023
Sample no 12	1 August, 2023
Sample no 13	8 August, 2023
Sample no 14	15 August, 2023

Table 1 : Collected Date of the Sample Water.

4.2 OD of biofilm formed in ELISA plates :

Sample no	Strain	ELISA reading
Sample no 1	*Vibrio* 1877	0.177
	Vibrio 346	0.135
	E.coli 0157	0.195666667
Control (ADW) for Sample 1	*Vibrio* 1877	0.147
	Vibrio 346	0.112333333
	E.coli 0157	0.157333333
Sample no 2	*Vibrio* 1877	0.157666667
	Vibrio 346	0.139
	E.coli 0157	0.171666667
Control (ADW) for Sample 2	*Vibrio* 1877	0.1576666670
	Vibrio 346	0.112333333
	E.coli 0157	0.157333333
Sample 3	*Vibrio* 1877	0.178333333
	Vibrio 346	0.1326666667
	E.coli 0157	0.176666667

Control (ADW) for Sample 3	*Vibrio* 1877	0.1576666670
	Vibrio 346	0.112333333
	E.coli 0157	0.157333333
Sample 4	*Vibrio* 1877	0.2743333333
	Vibrio 346	0.301
	E.coli 0157	0.2193333333
Control (ADW) for Sample 4	*Vibrio* 1877	0.124
	Vibrio 346	0.29
	E.coli 0157	0.416
Sample 5	*Vibrio* 1877	0.3006666667
	Vibrio 346	0.2823333333
	E.coli 0157	0.28
Control (ADW) for Sample 5	*Vibrio* 1877	0.124
	Vibrio 346	0.29
	E.coli 0157	0.416
Sample 6	*Vibrio* 1877	0.1553333333
	Vibrio 346	0.1646666667
	E.coli 0157	0.168

Control (ADW) for Sample 6	*Vibrio* 1877	0.152
	Vibrio 346	0.189
	E.coli 0157	0.171
Sample 7	*Vibrio* 1877	0.1486666667
	Vibrio 346	0.1613333333
	E.coli 0157	0.122
Control(ADW) for Sample 7	*Vibrio* 1877	0.152
	Vibrio 346	0.189
	E.coli 0157	c
Sample 8	*Vibrio* 1877	0.182333
	Vibrio 346	0.188667
	E.coli 0157	0.157
Control(ADW) for Sample 8	*Vibrio* 1877	0.150333
	Vibrio 346	0.112333
	E.coli 0157	0.205
Sample 9	*Vibrio* 1877	0.153333
	Vibrio 346	0.133
	E.coli 0157	0.143
Control(ADW) for Sample 9	*Vibrio* 1877	0.150333

	Vibrio 346	0.112333
	E.coli 0157	0.205
Sample 10	*Vibrio* 1877	0.185
	Vibrio 346	0.125333
	E.coli 0157	0.233667
Control(ADW) for Sample 10	*Vibrio* 1877	0.150333
	Vibrio 346	0.112333
	E.coli 0157	0.205
Sample 11	*Vibrio* 1877	0.256333333
	Vibrio 346	0.150666667
	E.coli 0157	0.139
Control(ADW) for Sample 11	*Vibrio* 1877	0.150333333
	Vibrio 346	0.150666667
	E.coli 0157	0.205
Sample 12	*Vibrio* 1877	0.141666667
	Vibrio 346	0.151
	E.coli 0157	0.136666667
Control (ADW) for Sample 12	*Vibrio* 1877	0.153333333
	Vibrio 346	0.17

	E.coli 0157	0.132333333
Sample 13	*Vibrio* 1877	0.143
	Vibrio 346	0.198333333
	E.coli 0157	0.174333333
Control(ADW) for Sample 13	*Vibrio* 1877	0.153333333
	Vibrio 346	0.17
	E.coli 0157	0.132333333
Sample 14	*Vibrio* 1877	0.130666667
	Vibrio 346	0.1416666667
	E.coli 0157	0. 266666667
Control(ADW) for Sample 14	*Vibrio* 1877	0.153333333
	Vibrio 346	0.17
	E.coli 0157	0.132333333

Table 2 : OD reading of sample water and their control(ADW).

4.3 Graphs and Regression Analysis :

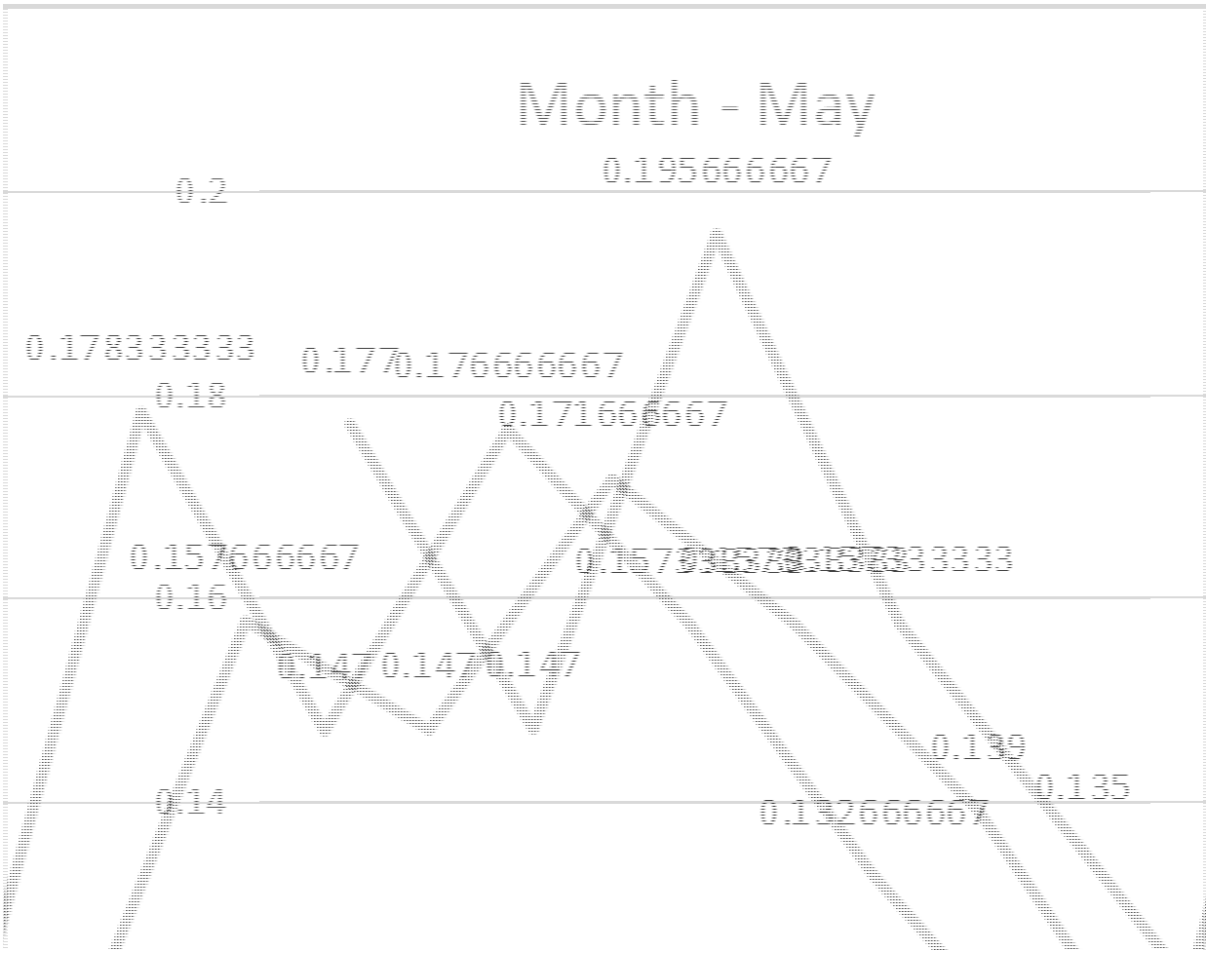

Figure 12: Graphical analysis of May sample and their controls.The graphical analysis of bacterial concentrations in the May water sample across three samples and their corresponding controls reveals distinct patterns and trends. In the first set of bars representing Sample 1 and Control 1, variations in the heights of bars for *Vibrio cholerae 1877*, *Vibrio cholerae WT346*, and *E.coli 0157* indicate potential shifts in bacterial concentrations. Similar observations can be made for Sample 2 and Control 2, allowing for a comparison of trends across replicates. Sample 3, although exhibiting a missing value in the *E.coli* 0157 control, provides additional insights into potential experimental variations. The color-coded lines facilitate an easy visual comparison, highlighting any consistent increases or decreases in bacterial concentrations. Notably, differences in the responses of

***Vibrio cholerae 1877*, *Vibrio cholerae WT346*, and *E.coli 0157* to the experimental conditions can be identified, aiding researchers in understanding the specific effects on different bacteria types.**

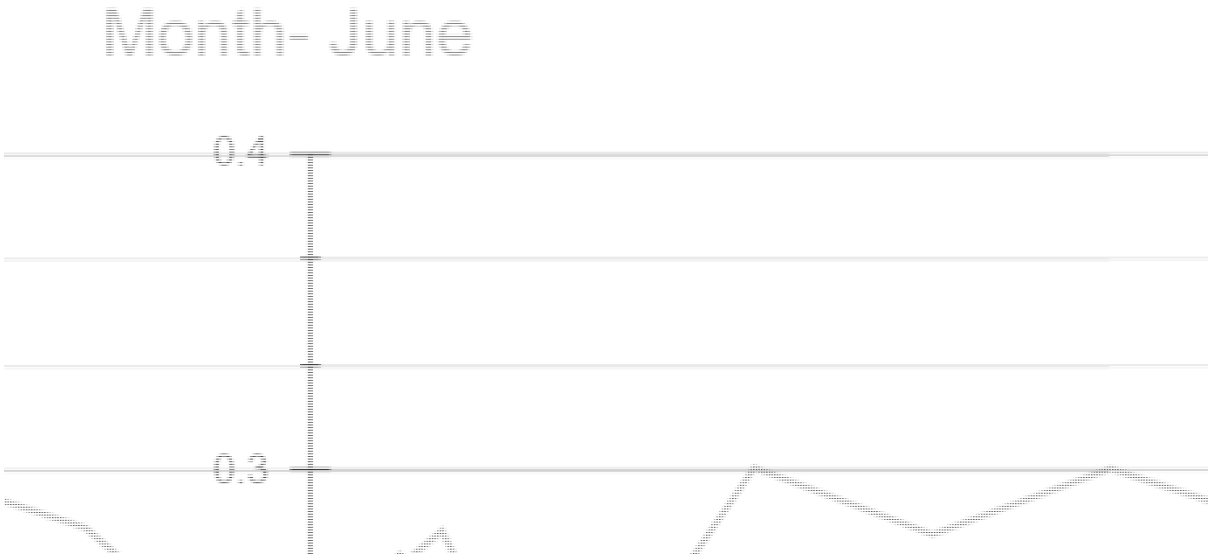

Figure 13: Graphical view of June sample and their control. The additional data from Samples 4 to 7 and their corresponding controls provide further insights into the variations in bacterial concentrations. In Sample 4, notably higher values are observed for *Vibrio cholerae 1877* and *Vibrio cholerae WT346* compared to their respective controls, indicating a potential increase in these bacterial types. Sample 5, on the other hand, exhibits relatively consistent values with its controls, suggesting a stable response of *Vibrio cholerae 1877*, *Vibrio cholerae WT346*, and *E.coli 0157* to the experimental conditions. Sample 6 displays a decrease in values for all bacteria types compared to the controls, indicating a potential inhibitory effect on bacterial growth. Finally, Sample 7 showcases lower values for *Vibrio cholerae 1877* and *Vibrio cholerae WT346*, but a slightly elevated value for *E.coli 0157*, pointing to a differential response among the bacteria types. The consistent use of color-coded lines in the graphical representation would aid in visually comparing the trends across all samples, further enhancing the interpretability of the dataset.

Figure 14: Graphical analysis of July samples and their controls (ADW).

In the analysis of Samples 8 to 11 and their respective controls, distinct patterns emerge in bacterial concentrations. Sample 8 reveals relatively consistent values for *Vibrio cholerae 1877*, *Vibrio cholerae WT346*, and *E.coli 0157* compared to their controls, indicating a stable response to the experimental conditions. Sample 9 exhibits a decrease in *Vibrio cholerae 1877* and *Vibrio cholerae WT346*, suggesting a potential inhibitory effect on these bacteria, while *E.coli 0157* remains relatively stable. Conversely, Sample 10 displays an increase in *Vibrio cholerae 1877* and *E.coli 0157* values, coupled with a decrease in *Vibrio cholerae WT346*, implying a complex response that may stimulate the growth of specific bacteria. Sample 11 introduces a notable increase in *Vibrio cholerae 1877*, accompanied by varying degrees of decrease in *Vibrio cholerae WT346* and *E.coli 0157*, reflecting a nuanced and unique response among the different bacteria types. A comprehensive graphical representation incorporating all samples would provide a visual summary of these trends, facilitating a holistic comparison of bacterial responses throughout the dataset. As always, the interpretation is contingent on the experimental context, and further statistical analyses may enhance the depth of understanding.

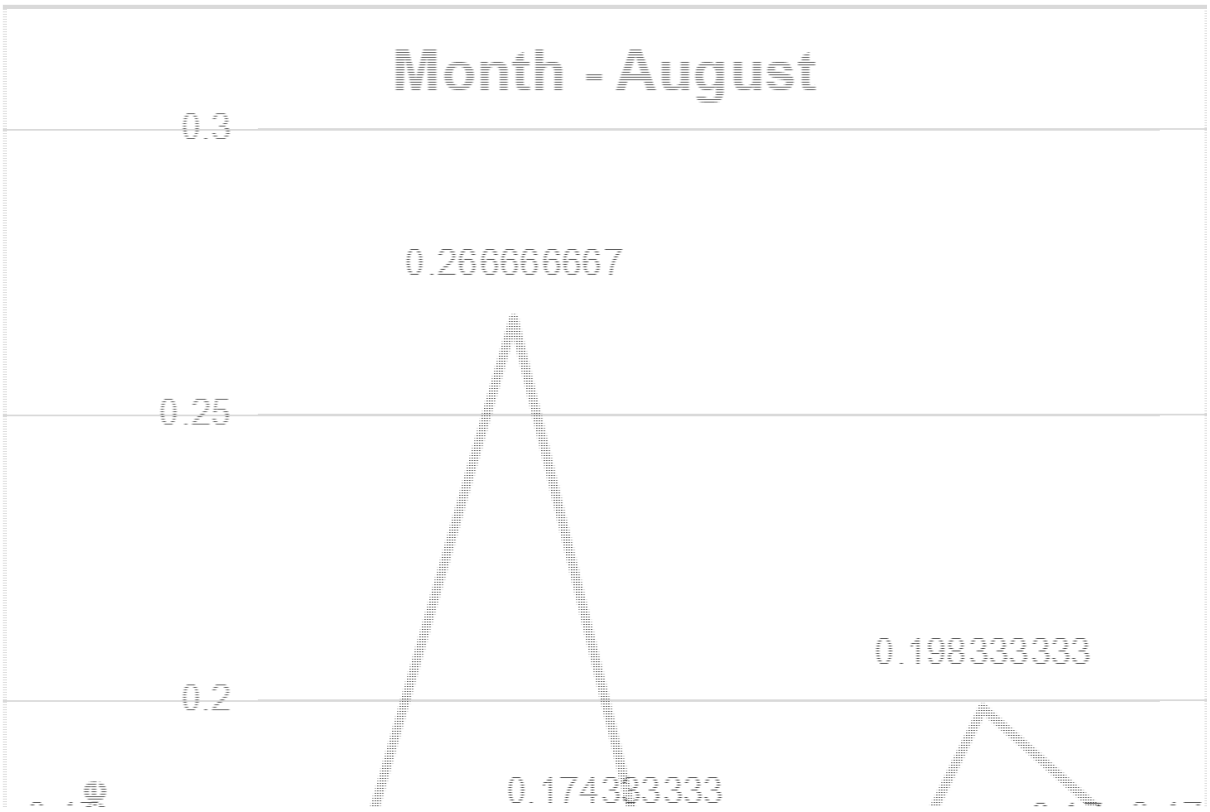

Figure 15: Graphical analysis of August samples and their controls (ADW). In the examination of Samples 12 to 14 and their corresponding controls, distinctive trends in bacterial concentrations come to light. Sample 12 exhibits values for *Vibrio cholerae 1877*, *Vibrio cholerae WT346*, and *E.coli 0157* that are slightly lower than their respective controls indicating a potential suppression of bacterial growth in the sample. In Sample 13, there is a moderate increase in *Vibrio cholerae 1877* and *E.coli 0157* values , while *Vibrio cholerae WT346* shows a more substantial increase, suggesting a potential stimulatory effect on these bacteria. Sample 14, on the other hand, displays lower values for *Vibrio cholerae 1877* and *Vibrio cholerae WT346* but a considerably elevated value for *E.coli 0157* . This disparity suggests a differential response among the bacterial types, potentially favoring the growth of *E.coli 0157* in this particular sample. A visual representation incorporating all samples would provide a comprehensive overview of these nuanced trends, aiding in the holistic comparison of bacterial responses throughout the dataset.

4.4 Average Absorbances for Strain *Vibrio cholerae 1877* :

May 1st 15 Days	May last 15 Days	June 1st 15 Days	June last 15 Days	July 1st 15 Days	July last 15 Days	August 1st 15 Days	August last 15 Days
0.167	0.178	0.2875	0.152	0.167	0.220	0.1423	0.130

Table 3: Average Absorbance of strain *Vibrio cholerae 1877* for all samples

Figure 16: Graphical analysis of Strain *Vibrio cholerae 1877* for all samples.The average absorbances for *Vibrio cholerae* strain 1877 exhibit notable variations over the course of four consecutive 15-day intervals spanning from May to August. In the initial half of May,

the absorbance value stands at 0.167, representing a relatively low level of bacterial presence. However, in the latter half of May, there is a discernible increase to 0.178, indicating a potential uptick in Vibrio cholerae concentration. Moving into June, the absorbance further rises to 0.2875 during the first 15 days, signifying a substantial surge in bacterial content.

Nevertheless, in the latter half of June, there is a noticeable decrease to 0.152, suggesting a possible decline in Vibrio cholerae abundance. July sees a fluctuation in absorbance values, with the initial 15 days registering 0.167 and the latter 15 days recording 0.220. This fluctuating trend persists into August, with absorbance values of 0.1423 in the first half and 0.130 in the latter half. These variations in absorbance values across the different time intervals underscore the dynamic nature of Vibrio cholerae strain 1877 concentration in the sampled environment. The observed fluctuations may be indicative of environmental factors influencing bacterial proliferation, highlighting the need for further investigation into the ecological dynamics of Vibrio cholerae in this context.

4.5 Average Absorbance for Strain *Vibrio cholerae WT346* :

May 1st 15 Days	**May last 15 Days**	**June 1st 15 Days**	**June last 15 Days**	**July 1st 15 Days**	**July last 15 Days**	**August 1st 15 Days**	**August last 15 Days**
0.137	0.132	0.291	0.163	0.160	0.137	0.174	0.141

Table 4: Average Absorbance of strain *Vibrio cholerae WT346* for all samples

Figure 17: Graphical analysis of Strain *Vibrio cholerae WT346* for all samples. Comparison of the average absorbances for *V.cholerae* strain WT346 over the four 15-day intervals from May to August reveals a distinct pattern of variation. In the initial half of May, the absorbance is recorded at 0.137, indicating a relatively moderate presence of the bacterial strain. This value slightly decreased in the latter half of May to 0.132, suggesting a subtle decline in *V.cholerae* concentration. As June commences, there is a sharp increase in absorbance to 0.291 during the first 15 days, signaling a significant surge in bacterial content. However, in the latter half of June, the absorbance drops to 0.163, suggesting a potential decrease in *V.cholerae* abundance. July exhibits consistent absorbance values of 0.160 and 0.137, for the first and last 15 days respectively, indicating a relatively stable bacterial concentration during this month. Moving into August, there is a notable increase in absorbance during the first 15 days (0.174), followed by a slight decrease in the latter half (0.141). This fluctuating trend in absorbance values underscores the dynamic nature of *V.cholerae* strain WT346 concentration in the sampled environment. The observed variations may be reflective of complex interactions between the bacterial strain and environmental factors, necessitating further investigation into the ecological dynamics of *V.cholerae* in this particular context.

4.6 Average Absorbances for Strain *E.coli 0157* :

May 1st 15 Days	May last 15 Days	June 1st 15 Days	June last 15 Days	July 1st 15 Days	July last 15 Days	August 1st 15 Days	August last 15 Days
0.183	0.176	0.249	0.145	0.15	0.186	0.155	0.266

Table 5: Average Absorbances of strain *E.coli 0157* for all samples

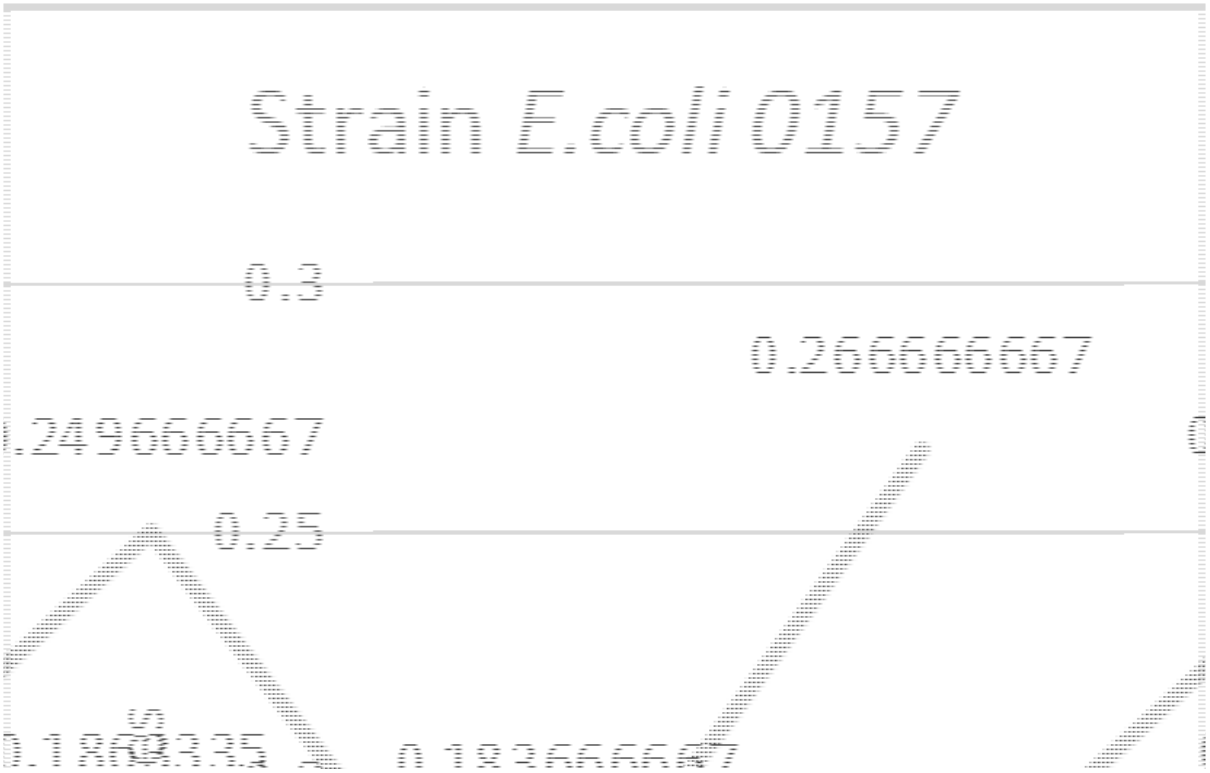

Figure 18: Graphical analysis of Strain *E.coli 0157* for all samples. Examining the average absorbances for *E.coli* O157 across the four consecutive 15-day intervals from May to August provides insights into the dynamics of bacterial presence in the sampled environment. In the initial half of May, the absorbance value stands at 0.183, indicating a notable level of *E.coli* O157 concentration. However, this concentration slightly decreases in

the latter half of May to 0.176, suggesting a potential decline in bacterial abundance. As June unfolds, there is a further decrease in absorbance to 0.249 during the first 15 days, signifying a potential reduction in *E.coli* O157 presence. In contrast, the latter half of June sees a slight increase in absorbance to 0.145, suggesting a fluctuation in bacterial concentration. Moving into July, there is a noticeable uptick in absorbance to 0.15 during the initial 15 days, followed by a more substantial increase to 0.186 in the latter half of the month. August continues this upward trend with an absorbance value of 0.155 in the first 15 days and a peak value of 0.266 in the last 15 days, indicating a significant surge in *E.coli* O157 concentration. The observed variations underscore the dynamic nature of *E.coli* O157 in the sampled environment, emphasizing the need for a comprehensive understanding of the ecological factors influencing its proliferation and persistence in this context.

The highest peak in absorbance for all provided strains of *Vibrio cholerae* and*E.coli* samples is observed in the first 15 days of June. In this month, dynamic fluctuations in bacterial concentrations are evident, with *Vibrio cholerae* strains 1877 and *WT346* experiencing significant surges in absorbance during the initial 15 days, followed by potential declines in the latter half. Conversely, *E. coli* O157 shows a notable reduction in absorbance during the first 15 days followed by a slight increase, emphasizing the intricate and dynamic nature of bacterial presence during this period.

Chapter 5. Discussion :

In this study the effect of free DNA on the survival of Bacterial biofilm was investigated. In developing countries like Bangladesh, seasonal epidemics like cholerae and diarrhea increase in summer in comparison to other seasons. Previously investigated, sunlight, temperature, irradiation, chemical treatment, bacteriophage infection can be possible reasons for durability of bacterial biofilm. Thus, this study investigated whether free DNA can also give endurance to the bacterial biofilm or not.

5.1 Key findings :

Bacteria form biofilms as part of their survival mechanisms, and biofilms are thus ubiquitous in nature. Recognition of the fact that bacterial biofilm may play a role in the pathogenesis of disease has led to an increased focus on identifying diseases that may be biofilm-related.(Drulis-Kawa & Maciejewska, 2021). Although free planktonic bacteria cells are responsible for the increased infectious diseases, biofilm itself can cause various diseases as well. In several instances, it has been observed that while the same bacteria do not normally cause disease when they are free-living, they do so when embedded in a biofilm. Many nosocomial infections from indwelling medical devices, including enteropathogenic infections, biliary tract infections, mouth infections, ocular infections, and others, have been linked to biofilm (Kolpen et al., 2022; Vestby, Grønseth, Simm, & Nesse, 2020).Numerous research point to a close relationship between biofilm dynamics and disease etiology.

Bacteriophages, viruses that infect and replicate within bacteria, are perfectly adapted to infect biofilms.Bacteriophages, because of the co-evolution mechanism, are actively involved in biofilm development in two opposing ways, as dispersing or promoting agents. Phages may contain matrix-degrading enzymes that enable the efficient infection of cells trapped in biofilms. In this situation, phages are a natural and practical weapon against microbial biofilms.

Prophages, on the other hand, control phage-mediated cell lysis and bacterial DNA release, which is a crucial mechanism for preserving the biofilm matrix (Drulis-Kawa & Maciejewska, 2021). Thus, bacteriophage is crucial for the development of the biofilm life cycle as this causes the assemble process of the free DNA in the biofilm matrix by doing cell lysis. The formation of a liquid crystalline structure in the biofilm matrix would be helped by the concurrent rise in DNA and phage concentrations (Secor et al., 2015).

Finally , as we assumed that the cause of these free DNA are the bacteriophages , through destroying bacteria. Eventually, more biofilms rise due to the rise of the bacteriophages as more phages attack more bacteria, releasing DNA in the process which is helping in biofilm development. As, from May to June the amount of bacteriophages increases the quantity of free DNA will simultaneously increase in the environment. The number of phages that kill bacteria and generate free DNA in the environment increases in May to June. Bacteria that survive during this period are induced to create biofilms by these free DNAs. In the above graph (from the result part) also there is positive induction from May to June that clearly indicates that the increment of free DNA is the cause of biofilm ring formation that is the root cause of cholera and diarrhea. Besides, from July to August there is negative induction as mentioned in the above graph (from result graph) which means the amount of free DNA is gradually decreasing in that period of time. This also means the bacteriophage decreases and there might be less biofilm formation.

We worked with 3 bacterial strains for the experiment. These are *Vibrio cholerae* 1877, *Vibrio cholerae* WT346 and E.coli 0157. *Vibrio cholerae* 1877 and *Vibrio cholerae* WT346 were grown in TCBS and *E.coli* 0157 was grown in MAC media respectively, *Vibrio cholerae* strains thrived, generating both robust growth and numerous single colonies. Following 36 hours of incubation, *E.coli* 0157 displayed the most substantial biofilm ring, outperforming the *Vibrio cholerae* strains. *Vibrio cholerae* strain 1877 also exhibited notable biofilm formation, emphasizing the strain-specific nature of biofilm development. Perfect turbidity was achieved between 1.5-3 hours, and subsequent biofilm formation was accomplished by placing young cultures in sterilized glass vials for 36 hours. Crystal violet staining revealed distinct blue biofilm rings, and OD measurements at 620 nm highlighted significant differences between sampled water and distilled water during summer months. There was a major difference in the OD measurements between the sample water and distilled water from the last two weeks of May to June. Whereas,

The difference of the OD measurements between those were slowly decreased in the month of July to August. From this also it can be assumed that the rise and fall of free DNA is the cause behind the differences of OD measurements between distilled and sample water. Across the May, June, and August samples, graphical analyses underscored variations in bacterial concentrations and unique responses to experimental conditions. Notably, different strains exhibited distinct trends, suggesting the importance of considering strain-specific behaviors in biofilm formation. These findings contribute valuable insights into the dynamics of bacterial biofilm formation, emphasizing the interplay between bacterial strains, growth conditions, and environmental factors.

5.2 Limitations:

In this experiment we used three strains of bacteria to determine the effect of free DNA in the water that could give durability to biofilms. Moreover, only two strains of *Vibrio cholerae* have been used in the experiment and one strain of *E.coli*. Thus,if more strains of other bacteria and strains of *Vibrio cholerae* would have been used, a more generalized result could have been determined. In addition, in the first few days of conducting the experiment the strains were first grown in LB Agar medium which would give minimum growth to the bacteria but no single colony was found because of that. Nevertheless, after using TCBS and mac media to grow the bacterial strain for young culture many single colonies were found. Besides, in the case of strain WT346 of *Vibrio cholerae* e sometimes there would have been few single colonies after incubation which could have been cause for contamination in the time of streaking. Also the source of our sample was Gulshan Lake, Dhaka, Bangladesh. So, samples from different sources and different times should be used to conduct this research in future.

In addition, in the experiment months of May, June, July and August had been considered to know the effect of free DNA that could cause survival of biofilm and such reasons for seasonal

epidemics. If more water samples would have been collected from the initial months of May then a more organized result could have been concluded.

In Bangladesh, cholera outbreaks occur in two different seasons. This study was conducted from May to August. That is the effect of free DNA for biofilm formation was not included in this experiment. So, study is not conducted for a few months prior to May and later of August. If the data for those months are also included, a broader picture could be derived.

5.3 Future Prospect of the Research:

To carry out this study, data were collected from the summer and rainy season. For the proper results of the study and to collect one year data, winter data should be collected. Additionally, the experiment has to be continued for at least one more year to derive a conclusive result. Comparable outcomes across all possible configurations can provide stronger evidence in favor of our research's objective. Further investigation could provide additional details regarding biofilm if the hypothesis is validated. Designing several anti-biofilm treatments will be made easier by this work. It would be preferable if more seasonal pathogenic agent strains could be incorporated into the study. Both clinical and natural sources for the strains should be used. Potential sample sources include the natural reservoirs of these diseases. To further assess the data, clinical samples should also be used. Thus it will be understood what effect free bacterial DNA obtained by cutting down the bacteria by using phage has upon them

5.4 Future research:

This study looks into the effect of free DNA on biofilm. Sunlight, temperature, radiation, chemical treatment, besides bacteriophage infection leaves scope for further research to find out whether any other factors affect biofilms. Furthermore, whether this mechanism works at all in

winter and if not why the mechanism does not work in winter can be further investigated. This study primarily focuses on the effect of bacteriophage and free DNA in summer and rainy season on the biofilm produced by *Vibrio cholerae* . The effect of free DNA on biofilm produced by other seasonal pathogens can also be further investigated.

Chapter 6. Conclusion :

To sum it up, the study was conducted to analyze the effects of free DNA on bacterial biofilm produced by bacteria causing seasonal epidemics. In summer, frequent cholera and diarrhea outbreaks occur. Increasing free DNA could be a major factor resuscitating biofilm in summer causing these seasonal epidemics. Free DNA can enhance biofilm formation in bacteria by serving as a matrix that stabilizes the structure. It acts as a scaffold for microbial adhesion and provides stability to the biofilm, promoting bacterial aggregation. In our study, the results suggested that the surge in cholera and diarrhea cases observed during the summer months appears to be linked to heightened biofilm formation facilitated by free DNA. The biofilm rings in our studies depict the difference in the result. Thus the result indicates that the amount of free DNA in water increases by killing the bacteria during summer season (May and June) and that free DNA will induce the survivor to go to the biofilm. As a result the amount of biofilm increases, resulting in more cholera and diarrheal diseases during this time. But towards July and August, the amount of biofilm gradually decreases and the amount of free DNA in the water also decreases and then the number of cholera and diarrhea patients also decreases. Deciphering the diverse activities and coexistence of bacteria and phage in the environment is necessary to comprehend the prognosis of numerous diseases. The biofilm is already being studied by researchers from all around the world with success. Our knowledge of the effects of bacteriophages and free DNA on the environment, including humans, will expand as a result of this study. More seasonal data, though, are needed to firmly validate this claim.

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