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List of abbreviations

(high-density polyethene)
HDPE.....19
(polypropylene random copolymer)
PPR.....8
Biodegradable organic matter
BOM.....9
biofilms in drinking water distribution networks (DWDS)
DWDS.....3
chlorinated polyvinylchloride
(CPVC).....3
disinfection by-products
DBP.....9
Exopolysaccharide
(EPS.....9
opportunistic microorganisms (OPs)
OP.....6
polyethene
(PE).....3
Reasoner's 2A agar
R2A.....18
sodium chloride
(NaCl).....18
stainless steel
SS.....8
Trypton Soy Agar
TSA.....18
tryptone soy broth
(TSB).....18
unplasticized polyvinyl chloride
PVC.....3

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1. INTRODUCTION

Safe drinking water as defined by the WHO guidelines does not represent any significant risk to health over a lifetime of consumption, including different sensitivities that may occur between life stages. Water is necessary for life; hence every effort should be made to obtain as safe-to-drink water as is practically possible. For all typical domestic needs, such as drinking, cooking, and personal hygiene, safe drinking water is necessary.

A comprehensive approach to risk management and risk assessment of a drinking water supply boosts trust in the water's safety. To prevent contamination of drinking water or to decrease it to levels not harmful to health, securing the microbiological safety of drinking water sources is based on the deployment of numerous barriers from the catchment to the consumer.

Protection of water resources, appropriate selection, use of a sequence of treatment procedures, and management of distribution systems (piped or otherwise) to preserve and safeguard treated water quality are all measures that can be taken to increase safety.

The quality of microbial water frequently varies quickly and over a large range. Peaks in pathogen concentration that last only a short time can significantly raise the risk of disease and lead to waterborne disease outbreaks.

Furthermore, many people may have been exposed by the time microbiological contamination is found. For these reasons, even when end-product testing is frequently used to assess the microbiological safety of drinking water, it cannot be relied upon completely.

To ensure that drinking water is safe and of high quality, piping distribution systems are just as crucial as the actual treatment process. Water entering the distribution system must be safe for microorganisms and, ideally, stable for biological processes. To prevent post-treatment contamination of the water as it is delivered to the consumer, the distribution system itself must offer a safe barrier. While residual disinfection will offer some protection against recontamination, it also has the potential to conceal the presence of such contamination. On a global level, mismanagement in the house, however, is probably the biggest source of fouling (WHO & OECD, 2003).

Water quality within a building's piped distribution system is influenced by a variety of circumstances, and this can lead to microbial or chemical contamination of drinking

water. Plumbing systems that are poorly built may cause water to stagnate and create the ideal conditions for microbial development.

Poor management of these systems has led to disease and illness outbreaks. Drinking water distribution systems in buildings can be a source of contamination. Buildings' drinking-water networks can be connected to point-of-use equipment or water-based devices, and they are separated into hot and cold-water networks.

In drinking water distribution networks, bacteria thrive, especially when a residual disinfectant is not present. Most of them grow on meals as well as on particles, surfaces in touch with water, and surfaces where there is a supply of carbon from outside.

They can include a wide range of opportunistic pathogen microorganisms, including *Aeromonas, Flavobacterium, Serratia, Klebsiella,* and *Pseudomonas,* but there is no evidence that any of these organisms are linked to gastrointestinal infections brought on by drinking water consumption in the general population(WHO 2003).

Microorganisms from *Pseudomonas aeruginosa* can also flourish in distribution and plumbing pipes. They can spread a variety of illnesses, but healthy people are rarely seriously harmed by them. They have caused skin infections in hot tubs and spas, and they are a serious problem in hospital conditions where the organism can colonize injured places like burns and surgical wounds, the respiratory tracts of vulnerable persons, and physically damaged eyes.

This research employed pipe surfaces from a simulation system to culture the bacteria at various temperatures and chlorine treatments and analyze its biofilm-forming capabilities. Evidence from studies conducted in settings that closely resemble real-world conditions is needed to determine how temperature and chlorine treatments affect the growth of biofilms in drinking water distribution systems and, subsequently, how this affects the water quality (Douterelo et al., 2016).

2. GOAL OF THE THESIS

Pseudomonas spp. are Gram-negative, rod-shaped, oxidase-positive, non-spore-forming bacteria that are environmentally widespread, with some being opportunistic pathogens.

Pseudomonas aeruginosa is frequently found in faeces, soil, water, and sewage but cannot be used as a gauge of faecal contamination because it can also thrive in enriched aquatic environments and on the surface of organic materials in contact with water in addition to being frequently found in faeces and sewage.

A rise in water temperature or low flow rates in the distribution system, as well as complaints about taste, odor, and turbidity, are frequently linked to its presence, which is crucial in determining the general cleanliness of water distribution systems. It may also cause a decline in bacteriological quality.

The opportunistic bacterium *Pseudomonas aeruginosa* frequently causes surface infections after encountering seriously contaminated water (WHO & OECD, 2003). Several pipe materials have been recommended for water-drinking distribution pipework, among them include, galvanized steel or iron, copper tubing, polybutylene, chlorinated polyvinylchloride (CPVC), unplasticized polyvinyl chloride (PVC), polyethene (PE) etc.

One of the most important elements affecting the quantity and rate of bacterial growth in biofilms is pipe material. Under identical conditions, polyvinyl chloride (PVC) surfaces contained more total and cultivable cells than stainless steel surfaces because of the increased surface roughness of PVC, which was advantageous for the attachment of microbes and nutrients (Zhao et al., 2014).

More individuals are becoming concerned about the safety of their drinking water as living standards rise. Drinking water is often disinfected before leaving waterworks, and disinfectant residuals are utilised to reduce the number of biofilms in drinking water distribution networks (DWDS). However, numerous studies have indicated that even with some disinfection residuals, the quantity of microorganisms—typically bacteria-based remains high.

Researchers are unable to directly observe biofilm behaviour in real DWDS, communities of microorganisms, and disinfectant inhibitory mechanisms on biofilms due to the complexity, diversity of hydraulic parameters, and difficulties of sampling the real

pipes. To cultivate and study biofilm communities in laboratories, model systems that simulate real pipes have become the method of choice for scientists (Zhao et al., 2014). The goal of this work is to identify how varying environmental conditions can affect the production of biofilms on Polyvinyl chloride DWDS surface. To achieve this goal, the following tasks are needed to be fulfilled.

i. To find out the biofilm formation ability of two *Pseudomonas aeruginosa* isolate and ATCC 9027 strains on Polyvinyl chloride water pipe surfaces at different temperature treatments.
ii. To determine the chlorine effects on biofilm formation on drinking water distribution Polyvinyl chloride pipe surfaces by the *Pseudomonas aeruginosa* strains.

3. LITERATURE REVIEW

To increase the quality of treated water, a lot of studies have been done on this topic. Water distribution systems bring well-treated drinking water to consumer faucets. To prevent contamination and the growth of microorganisms, these drinking water distribution systems should serve as protective barriers and need to be operated and maintained (Liu et al., 2013).

Drinking water contains a complex organic content as well as an incredibly diversified but poorly characterized microbiota. The distribution systems receive treated drinking water that is loaded with physical particles, microorganisms, and nutrients (Liu et al., 2013).

The interface between water and pipe wall is a primary location for the collection of cells and organic waste as well as for bacterial growth in most water consumption networks (Bate et al., 2003). As a result of multiplication, bacteria separate off the pipe walls and/or are removed from them through shearing or erosion before being transported into the circulating water (Rittmann and Vernon, 1984).

The watery bacterial biomass (also called a biofilm) that grows on the pipes in biologically unstable networks serves as the origin of a complex trophic chain that includes free-protozoans and others (Wang et al., 2008).

3.1. Biofilms

Biofilms are cellular and noncellular matrices that have accumulated on solid surfaces and are physiologically active (Bakke et al., 1984). Most of the microbial biomass in DWDS is not found in the water column; rather, it is found adhering to the inner surfaces of pipes in the form of biofilms. Biofilms benefit implanted microorganisms by facilitating the sharing of nutrients and metabolic products as well as enhancing tolerance to environmental stress (Douterelo et al., 2018).

The formation and presence of biofilms in drinking water distribution systems have been reported frequently. The biofilms in distribution systems that receive both chlorinated and non-disinfected water have been found to include a wide range of diverse heterotrophic bacteria, some of which may be harmful (Momba et al., 2018).

The features of DWDS vary greatly to infrastructures (i.e., system materials and design), operational conditions (i.e., hydraulic conditions and disinfection) and water composition (i.e., physio-chemical, and microbial components). Only the operating circumstances could be easily controlled for controlling biofilm out of all these components. In essence, biofilm development and structural characteristics (composition and physical shape) are determined by many influences, both external and internal.

Biofilm would be impacted by DWDS environmental elements in complicated, interactive ways, and the metabolic activity of the microorganisms within it would alter the environment. As biofilms are ubiquitous and their thorough elimination is impossible, a better understanding of the interactions between biofilm and the surrounding environment, in particular, the operational conditions is necessary to ensure high water quality.

There are many areas in distribution systems where microbial activity can be detected, including water, pipe surfaces, and various deposits that have accumulated in pipes and storage tanks. The term "biofouling" is sometimes used to describe such common surface pipe colonisation (Flemming, 2002).

Biofilms can hasten the decomposition of disinfectants, encourage pipe corrosion, and harbour opportunistic microorganisms (OPs). The properties of the biofilms that grow in drinking water distribution systems may be significantly influenced by the material of the pipes.

The analysis of ecosystems, the design and operation of heat exchangers and pipelines subject to fouling, the design and operation of wastewater treatment plants, and the determination of the viability of biofilm reactors for biotechnological applications would all benefit from being able to predict the rate and extent of biofilm processes.

The structure of the biofilm found in real drinking water networks is unknown and has not yet been extensively documented because it is difficult to analyse such a very small amount of biomass without disrupting it. The complexity of this process is influenced by a variety of elements, including debris, corrosion products, mineral deposits, the development of corrosion tubercles that open new surfaces or niches for microbial colonisation, and others (Batte et al., 2003).

3.2. Influencing factors for biofilm formation in Drinking water distribution systems

Although treated drinking water may be free of faecal indicator organisms and detectable enteric pathogens, which reduces the risk of enteric illness, drinking water entering the distribution system may still have free-living amoebae and environmental strains of different bacterial species, also known as heterotrophic bacteria. Amoebae and heterotrophs will colonise a distribution system and create biofilms in the presence of favourable conditions (WHO & OECD, 2003).

Based on the numerous research on drinking water distribution systems and the unique characteristics of each phase in the distribution system, bulk water, suspended solids, pipe wall biofilm, and loose deposits are the phases in drinking water distribution systems that may be summed up. Bulk water is envisioned as a transmission channel for particles, germs, and nutrients throughout the distribution system (Liu et al., 2013).

A pipe wall biofilm develops on the inner surface of the pipe as the water main's bulk water passes through it. The particles can move through the network as colloids and suspended solids, or they can build up and settle as loose deposits on the bottom of the pipes. Differentiating between suspended solids and colloids can be challenging in real life. Most colloids will pass the filter when samples are collected by filtration greater than 0.5 µm (Sarin et. al., 2004).

These particles in distribution systems provide places for bacteria to adhere to and build biofilms, much like the pipe wall does (Bengtsson and Øvreås, 2010). The particles include organic and inorganic nutrients that could serve as bacterial adsorbents (Gregory 2006). These suspended particles are a big source of worry because they could shield bacteria from disinfection (Sarin et. al.,2004) from chlorine and ultraviolet light (Liu et al., 2013).

Water tainting, flavor and odor changes, corrosion, the creation of scale, and even pipe obstruction are all mostly caused by biofilms. The research on biofilms is difficult because it is challenging to access the inside surfaces of pipes within operating networks (Douterelo et al., 2016).

3.2.1. The effect of pipe surfaces on biofilm formations

The microbial density in DWDSs can be impacted by a variety of pipe material properties, including material roughness, surface energy, biological affinity, and hydrophobicity. According to Bimakr (2015), the pipe surface can have an impact on the biofilm populations, which could result in the presence and persistence of microbial infections. Biofilms were shown to flourish more on cement surfaces than PVC surfaces, according to Niquette et al., 2000) research.

The pipe material can have a big impact on microbial richness and variety. Regarding cast iron pipes, liberated iron may encourage the growth of iron bacteria, and corrosion scales may encourage the development and expansion of biofilm (Liu et al., 2013).

Zhou and coworkers (2009) claimed that due to the rough surface of stainless steel or ductile iron pipes, which may create more favourable conditions for biofilm formation, iron or steel pipes had a far higher potential for biofilm formation than polyethene pipes. However, it was shown that plastics PVC and polyethene (PE) stimulated biofilm formation more effectively than mild steel (Tsvetanova, 2006).

The research of Liu and co-workers (2014) found that the pipe materials (PVC and cast iron) may significantly affect the microbial population, and their ability to form biofilms. Although diverse but distinct species of bacteria that originate in bulk water may preferentially colonize the surfaces of PVC and cast-iron products, potential bacterial and eukaryotic pathogens appeared in biofilms established over both materials, and they concluded that it is challenging to decide which pipe material (PVC or cast iron) is suitable to employ as a significant plumbing component in a water delivery system.

Five bacterial strains were subjected to different pipe surfaces and had diverse levels of biomass. The PPR (polypropylene random copolymer) pipe had the highest biofilm biomass, which was followed by copper and SS (stainless steel) pipe. Stainless steel and copper pipes consistently produced less biofilm biomass than PPR pipes did. To encourage microbial development, a plastic pipe may leak nutrients. Copper ions may prevent bacterial growth when copper pipes corrode because they are released together with the corrosion (Shan et al., 2023).

3.2.2. Temperature effect on biofilms formation

According to Lund and Ormerod (1995), the water temperature had an impact on cell yield, on the lag phase between attachment and growth, as well as on the growth rate. It was discovered that the lag phase's duration was particularly significant. Before favourable conditions for the formation of biofilms are once again present, the attached bacteria may have been washed from the pipe wall into the water and have exited the distribution system.

The availability of nutrients, water flow rate, and temperature all affect the biofilm organisms' ability to grow and flourish. While biofilm growth and biofouling occur year-round in warmer seas, they are more noticeable only in the warmer months in temperate waters.

Abdallah and co-workers (2015)found that despite the growth temperature and surface type, *P. aeruginosa and S. aureus* biofilms included mostly living cells, as shown by the viability count. The outcomes also proved that neither the surface type nor the growth temperature had an impact on the biomass of the biofilm. On the contrary other studies showed that the water's BOM (Biodegradable organic matter) content and temperature both have a significant impact on biofilm formation. When compared to biofilm developed at the maximum temperature (18 °C), it took longer for the biofilm formed at the lowest temperature (6 °C) to stabilise (Ndiongue et al., 2005).

3.2.3 Chlorine treatment effect on biofilms formation

Disinfection is a crucial process that is used in water treatment plants to meet rising water demands and maintain biological stability in water distribution systems. The effectiveness of chlorination and chloramination as key methods for disinfecting water, as well as their simplicity of use and affordable prices, serve as strong justifications. To inactivate microorganisms at treatment facilities and to prevent microbial growth during distribution by depending on the disinfection residuals, disinfectants like chlorine or chloramine are frequently administered.

Wang and co-workers (2012) found that protein-based EPS from *Ps. putida* produced more disinfection by-products (DBP) when chlorine was added than polysaccharide-based exopolysaccharide (EPS) from *Ps. aeruginosa* biofilm. Further study is needed to examine the relationships between disinfection and EPS inside DWDS because single-strain biofilm rather than mixed culture biofilm was addressed in these investigations.

Additionally, biofilm appears to be more resistant to disinfection than planktonic organisms are (Hageskal et al., 2012), posing a higher hazard to water quality.

To prevent the growth of germs in drinking water distribution systems, disinfectants are frequently used. However, little is known about how disinfection affects the microbial community in drinking water. Disinfectants are frequently used to reduce the number of bacteria in DWDS while retaining a residual disinfectant (Mi et al., 2015).

Chlorine is present in most disinfected drinking water at concentrations of 0.2–1 mg/litre (WHO 2003). Due to the presence of organic waste and nutrients in DWDS, disinfectant treatment cannot prevent bacteria regrowth even at high dosages. A low disinfectant dosage increased the bacterial diversity in the DWDS biofilm, but a high dosage decreased it. Disinfectant type and dosage may have an impact on the bacterial community structure in DWDS (Lu et al., 2013).

According to the research of Shan and co-workers (2023), even in environments with high chlorine concentrations (>0.6 mg/L), some chlorine-resistant bacteria, including *Mycobacterium*, *Pseudomonas*, *Sphingobium, Staphylococcus, Acinetobacter, Bacillus, Acidovorax*, and *Sphingomonas,* can still form biofilms and thrive. *P. aeruginosa* is resistant to chlorine and other disinfectants used in water treatment, in addition to the fact that it thrives on a wide range of chemical substances (Falkinham et al., 2015).

In contrast, Lund and Ormerd (1995) found that the production of biofilms was not a possibility in chlorinated water. It must have been enough for the exceptionally low residual free chlorine concentration to be present in the water for the duration of the experiment to prevent bacterial attachment and biofilm formation. This, it appears, also prevented the deposition of organic matter from the water onto the pipe surface.

However, this study suggested that in fresh distribution systems with plastic pipes, keeping a low concentration of residual chlorine from the first day of (Lund and Ormerod, 1995).

Biofilms, where active bacteria make up around 95% of all bacteria, include most of the bacterial biomass. In DWDSs, biofilms can develop that are resistant to disinfectants, allowing bacteria to regenerate and causing issues with colour, turbidity, odour, corrosion, greater pathogen concentrations, and outbreaks of water-borne illnesses. Most drinking water disinfection techniques rely on disinfecting bulk water samples, but this

is insufficient to stop the microbial contamination brought on by biofilms in a DWDS (Zhu et al., 2021).

Additionally, it was noted that changes in the microbial community in drinking water correlated with the types of disinfectants (Wang et. al., 2012). *Cyanobacteria, Methylobacteriaceae*, and *Sphingngmonadaceae* predominated in chlorinated water, while Methylophilaceae, Methylococcaceae, and Pseudomonadaceae were more prevalent (Wang et. al., 2012). The different bacterial groups and possible pathogenic bacterial groups found in disinfected DWDS raise more questions regarding the safety of drinking water.

The chlorination experiments were run for 60 minutes at various residual chlorine concentrations (0.3, 0.6, 1, 2, 4, and 10 mg/L). The biofilm biomass removal rate of the six groups was significantly affected by residual chlorine. The six groups all had a greater reduction in biofilm biomass as the residual chlorine concentration rose, and this reduction was considerably greater when the residual chlorine concentration was greater than 1 mg/L (Zhu et al., 2021).

Potgieter and co-workers (2018) used samples from a drinking water distribution system that employs a sequential disinfection technique to evaluate the geographical and temporal microbial community dynamics. Their findings indicated a variation between samples taken at various times and locations, and the disinfection method had an impact on the composition of the microbial population.

According to Mi and co-workers (2015) low disinfectant dosage increased, whereas high dosage decreased, the variety of bacteria in the DWDS biofilm. The type and dosage of disinfectants may have an impact on the bacterial community structure in DWDS. Under chloramination and chlorination, respectively, Firmicutes and Proteobacteria were prevalent. Proteobacteria includes many taxonomic classes such as gammaproteobacterial e.g., *Pseudomonas spp.*

Due to their low cost and negligible risk to human health, chlorine and chloramine are the two primary disinfectants used in modern water treatment facilities. Chloramine is comparatively less active than chlorine and creates fewer disinfection by-products (DBPs). However, biofilm shows resilience to chloramine, and nitrifiers may reduce the concentration of disinfectant residue (Zhang and Edwards, 2009a).

3.3. *Pseudomonas aeruginosa* in biofilms

Ps. aeruginosa is a ubiquitous rod shaped bacterium commonly associated with soil and water. It has minimal nutritional requirements, which enables it to survive in many different environments; it is especially known for having a characteristically large genome with vast metabolic capabilities (Klockgether et al., 2011), It also serves as a model organism for biofilm formation (Masák et al., 2014).

Pseudomonas aeruginosa is an opportunistic pathogen to people who have immunosuppressive, chronic illnesses, or infections, it is a major cause of illness and death. It is highly prone to forming multicellular biofilms and is resistant to antibiotics (Diggle and Whiteley, 2020).

According to French pharmacist Carle Gessard (1882), in his study 'on the blue and green colouration bandages'; *Pseudomonas aeruginosa* produces several pigments in culture (blue-green colonies or whitish colonies on agar, Figure 1-2).

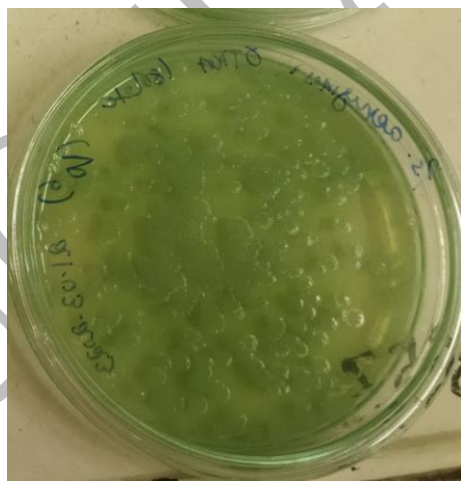


Figure 1: Pseudomonas aeruginosa isolate from tap water

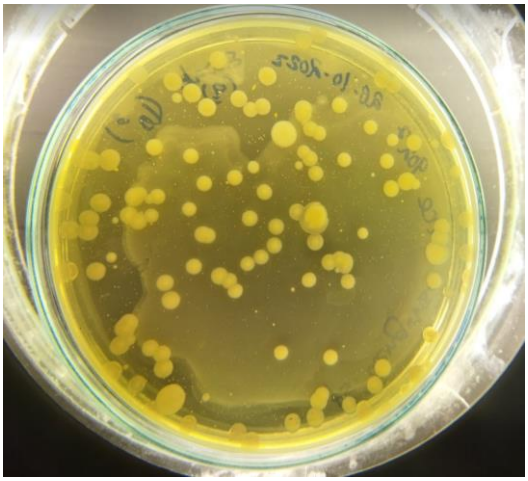


Figure 2: Pseudomonas aeruginosa ATCC 9027

It can survive between 4 and 42 ºC but has an ideal growth temperature of 37 ºC. The capacity of *Pseudomonas aeruginosa* to produce biofilms is due to its ability to synthesize three crucial polysaccharides, namely alginate, PSL, and PEL. Adhesins like lectins also assist in this process by recognizing certain sugars on the surface of host cells and other nearby bacterial cells (Diggle and Whiteley, 2020).

3.3.1. Microbiological importance of *Pseudomonas aeruginosa* in DWDS

Ps. aeruginosa is often found in a wide range of aquatic habitats, including tap water; it is known for being susceptible to multiple drug resistance, and its infections are exceedingly challenging to treat (Vaz-Moreira et al., 2012).

Numerous research that has been published has proven that tap water is a source of *P. aeruginosa* illness (Crivaro et al., 2009). However, according to a surveillance study on hospitalized patients, the majority were colonised before admission and *Ps. aeruginosa* in tap water was discovered to be the source of infection in 1 of 14 patients based on the identification of isolates from both water and patients (Colley et al., 2008).

Application of chlorine on *Pseudomonas aeruginosa* was found to cause injury to the cells which in turn influenced its resistance to antibiotics, a research study is however

needed to access the extent of chorine damage to *Pseudomonas* biofilms in drinking water (Figure 3.).

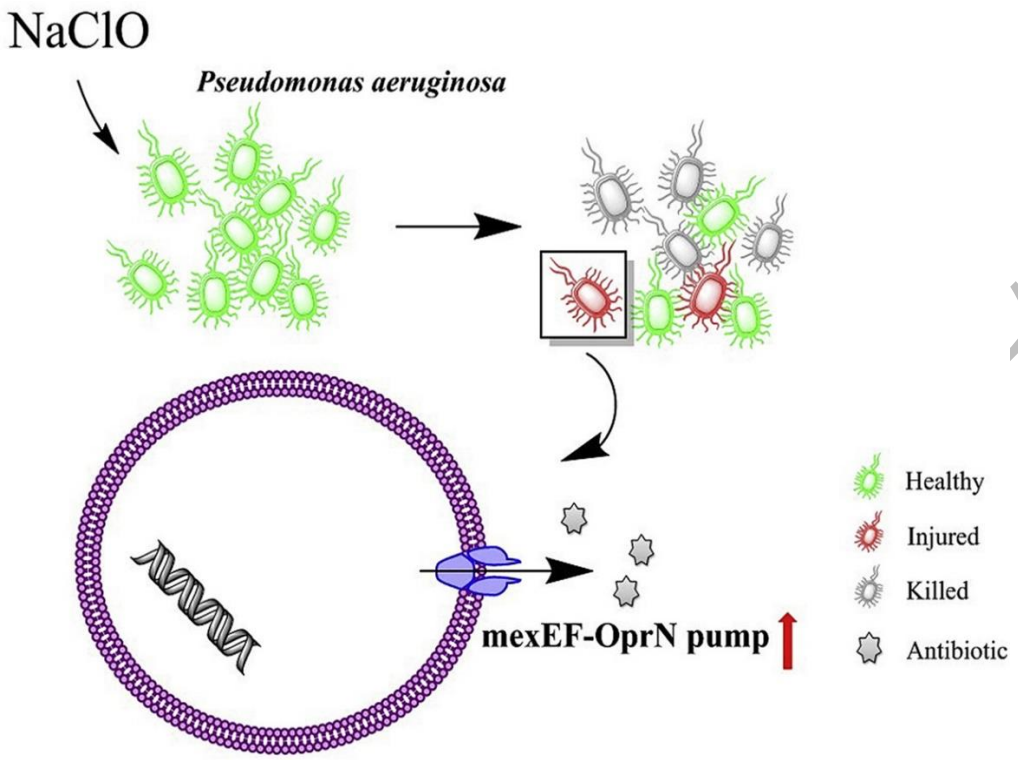


Figure 3: Chlorine effects on Pseudomonas aeruginosa cells (Hou et al., 2019)

3.3.2. *Pseudomonas aeruginosa* biofilm development cycle

The growth of *Pseudomonas aeruginosa* biofilms occurs in five stages. The first stage is the free attachment of planktonic cells to a surface. The next stage is the development of microcolonies, and the last stage is seeding dispersal, in which swimming cells from microcolonies leave their current surface to occupy a new one (Ma et al., 2009).

- Attachment: This stage often begins when biological material enters the pipeline. The pipe surface serves as a water-solid interface to promote spontaneous adsorption and the creation of a conditioning layer or film. The first stage of microbiological adhesion will then take place, mostly aided by conditioning (Shi, 2018).
- Microcolonies development: The main colonising bacteria proliferate and secrete EPS, which helps them establish stronger connections that adhere the compacted cell matrix to the pipe surface. The EPS encourages further colonisation through physical constraint and electrical contact. Bacterial growth

and proliferation result in a thicker and denser structure over time (Ramos-Martínez, 2016).

- Seeding dispersal: At the dispersion stage of biofilm growth Ma and co-workers (2009) stained microcolonies with lectin. In the core of ageing microcolonies, many swimming bacteria, which indicates active dispersion, were observed which meant the fully developed biofilms move to new locations after maturity.

The other stages include slime formation and steady-state stage, where the former occurs at the beginning of the micro-colonies' formation and the latter as the mature biofilms are ready for seeding dispersal.

3.4. Microbiological importance of Biofilms in DWDS

The presence of biofilms in DWDS might have several effects:

- Water quality deterioration

  Changes in taste, color, and odor are signs that there are biofilms present in the water. Fungi found in biofilms are, in fact, the primary source of this final issue. This is because many of these species' metabolic byproducts can alter the taste and odor of the treated water, which has an immediate impact on consumers (Ramos-Martínez, 2016).

- Infections

  Biofilms from opportunistic pathogens may cause infections in the vulnerable group or they may as well contain pathogenic bacteria. Even below detection thresholds, pathogens in water can unintentionally cling to biofilm, which serves as their environmental reservoir and could be a source of water pollution (Wingender and Flemming, 2011).

- Disinfectant decay

  Distribution system reactions between the flowing water mass and the pipe wall cause disinfectants to be consumed in the systems. Deposits, corrosion by-products, bacteria, organic contaminants, ammonium, and metal compounds (including ferrous and manganese ions) are some of the components of water that interact with and consume residual disinfectants (Al-Jasser, 2007).

- Harboring other microorganisms

  Biofilms in DWDS may provide nutrients for the growth of other microorganisms such as protozoa, fungi etc. (Chowdhury, 2012).

3.5. The biofilms investigation simulation model

Due to the restricted access to the pipes during routine operations, collecting biofilm samples from drinking water distribution systems can be difficult (Kitajima et al., 2021). Most data on drinking water distribution system biofilms have been acquired in small-scale laboratory reactors or by accessing a small number of chosen microorganisms under controlled settings, which do not accurately reflect the dynamics of various populations inside real networks.

At The University of Sheffield in the UK, a pilot-scale drinking water distribution facility was created since it was challenging to get in-situ biofilm samples from DWDS. Following 28 days of collection from the facility, samples of both biofilm and water were taken, and their microbial diversity and components were examined using the next-generation sequencing method 454 pyrosequencing (Douterelo et al., 2013).

Different forces were applied to test the strength of biofilms' attachment on the surfaces, following flushing. The amount of *Gamma proteobacteria* produced under low-variable flow conditions tended to decline, whereas the amount of *Pseudomonas* produced by biofilms cultured under steady state increased (Douterelo et al., 2013). Even while there was no obvious trend in the microbial community alterations, these data highlight the complexity and unpredictability of the microbial community within DWDS.

There is a possibility of biofilm mobilisation by external factors, which could result in a reduction in water quality, as supported by the positive association between planktonic cells and flow rates (Sekar et al., 2012). However, not all the biofilm components could be eliminated by the normal shear force in DWDS. In addition, Paul and coworkers (2012) found that biofilm cohesive strength increased after exposure to detaching shear force. This might be because the biofilm layer that remains after detachment would be further compressed by external shear stress, increasing their resilience to high external forces (Paul et al., 2012).

3.6. The research gap

After a vigorous review of the previous studies on biofilms and their mobility in drinking water distribution systems, several gaps were identified as follows:

- Environmental factors interact with biofilm, including its structure, composition, and community, according to prior studies. Hydraulic influence and determine biofilm properties by regulating material exchange and detachment forces, albeit no conclusive answer or method has been found. The microbial community varied between investigations, nevertheless, possibly since earlier research was done in either real systems or lab facilities under various operational settings. A thorough analysis of how biofilms react to environmental elements, particularly the connection between the microbial community and different temperatures, needs more research. This type of research is anticipated to offer practical biofilm management techniques, aiding in the maintenance of water quality across different climatic seasons.

- The characteristics of the biofilm EPS (extracellular polymeric substance) matrix impacting biofilm stability and increasing disinfection resistance ability have been examined to prevent the growth of biofilm. Few of this research, however, used individual bacterial species to examine EPS responses to environmental factors, making it impossible to determine the true disinfectant resistance ability of species such as *Pseudomonas aeruginosa*. This should be considered in future studies.

- Different studies explored Biofilms in general, with Proteobacteria such as *Legionella* heavily featuring and this leaves out *Pseudomonas aeruginosa* which is an opportunistic pathogen so interesting to study, in this case.

In the end, understanding how biofilms are influenced by temperature and different chlorine levels is crucial to improving biofilm management and suggesting efficient biofilm control solutions. If *Pseudomonas* biofilm-forming activity is favored by different temperature rates, it will be interesting to relate that to the different temperature levels experienced across different seasons i.e., winter, spring, summer, and autumn. This can be achieved through more and more simulation designs of DWDS to reflect an ideal situation, monitoring the temperatures and sampling from the real pipes. This will offer fresh perspectives on *Pseudomonas* microbial importance in drinking water quality and a different suggestion for its regulation within DWDS for the protection of the vulnerable group in society.

4. MATERIALS AND METHODS

4.1. Materials

4.1.1 Culture media

4.1.1.1. TSA agar

For the preparation of the Tryptic Soy Agar, 30 g of tryptone soy broth (TSB) (Biolab) powder and 15 g of bacteriological agar (Biolab) were measured into a holding bottle. 1000 ml of distilled water was added to the mixture and autoclaved at 121 ºC for 15 minutes.

4.1.1.2. R2A broth

Reasoner's 2A agar is a culture media which was developed to study potable water-invading microorganisms. To prepare it 3.0 g of R2A broth powder (Biolab) was measured into a holding bottle. 1000 ml of distilled water was added to the powder autoclaved at 121 ºC for 15 minutes.

4.1.1.3. Diluents

To carry out the decimal dilutions, 9 ml diluent containing test tubes was prepared. 1 g of peptone powder and 8.5 g sodium chloride (NaCl) was measured into a glass bottle using. 1000 ml of distilled water was added. Diluents were prepared by filling test tubes with 9 ml of peptone water using a Jencons zipette dispenser. The diluents were then autoclaved at 121 ºC for 15 minutes.

4.1.2 Cultures

Experiments were carried out with two strains of *Pseudomonas aeruginosa*:

- *Pseudomonas aeruginosa* (ATCC 9027),
- *Pseudomonas aeruginosa* strain isolated and identified previously from tap water.

The cultures were maintained on TSA agar slants and refrigerated to preserve the strains as stock cultures for the subsequent experiments.

4.1.2.1. Preparation of fresh cultures

From the stock cultures sterile agar slants have been inoculated and incubated at 30 °C for 24 hours.

4.2. Methods

4.2.1. Inoculating the pipe surfaces

i. Clean pipe surfaces (high-density polyethene – HDPE) were well-cut into approximately equal surface areas of 13.5 cm^2. They were sterilized using an alcoholic surface disinfectant and left to air dry inside the laminar flow chamber.

ii. 70 ml of R2A broth was filled into square plastic Petri dishes (Medium) and inoculated with *Pseudomonas aeruginosa* suspension to reach a 10^6 cell/ml initial cell count by using a McFarland Densitometer. The exact concentration of the suspension was determined also by applying traditional culturing methods.

iii. Using sterile forceps, the sterile-dry pipe surfaces were placed inside the R2A broth-*Pseudomonas aeruginosa* mixture with the concave side of the surfaces fully immersed into the solution (modelling the inner side of the pipe).

iv. They were left to stand for 1 hour inside the laminar flow chamber, for the bacterial cells to attach to the pipe surfaces.

v. After 1 hour, each of the inoculated pipe surfaces was rinsed with 10 ml of sterile and all were transferred (except 3 from each strain) into the large Petri dishes containing 140 ml of sterile R2A broth and then incubated.

4.2.2. Effect of different storage conditions on biofilm formation

4.2.2.1. Temperature treatment at 30 ºC.

18 sterile pipe surfaces were inoculated with the ATCC strain (as Set 1), and another 18 sterile pipe surfaces were inoculated with the *Pseudomonas aeruginosa* isolate strain (as Set 2) as described in 4.2.1.

After 1 hour of incubation, all the pipe surfaces each after being rinsed with 10 ml sterile distilled water, 15-15 pipe surfaces were transferred into sterile R2A broth and incubated at 30 °C for 7 days.

The sampling was done on the initial day (after the 1 hour of incubation) and later at intervals of 24 hours, 48 hours, and 7 days.

4.2.2.2. Temperature treatment at 8 ºC.

21 sterile pipe surfaces were inoculated with the ATCC strain (as Set 1), and another 21 sterile pipe surfaces were inoculated with the *Pseudomonas aeruginosa* isolate strain (as Set 2) as described in 4.2.1.

After 1 hour of incubation, all the surfaces have been rinsed with 10 ml sterile distilled water and 18-18 pipe surfaces were transferred into sterile R2A broth and incubated at 8 °C for 14 days.

Sampling was done on the initial day (after the 1 hour of incubation) and later at intervals of 24 hours, 48 hours, 7 days, 10 days, and 14 days respectively.

4.2.2.3. Temperature treatment at 15 ºC.

The procedure at 8 ºC was repeated.

4.2.2.4. Chlorine treatment

21 sterile pipe surfaces were inoculated with the ATCC strain (as Set 1), and another 21 sterile pipe surfaces were inoculated with the *Pseudomonas aeruginosa* isolate strain (as Set 2) as described in 4.2.1.

Both set 1 and 2 were transferred into 4 different big sterile glass Petri dishes each containing approximately 140 ml *of 0.1 mg/l chlorinated R2A broth* or 140 ml of *1.0 mg/l chlorinated R2A broth*.

Sampling was done on the initial day (after the 1 hour of incubation) and later at intervals of 24 hours, 48 hours, 7 days, 10 days, and 14 days respectively.

4.2.3. Sampling

At the start of every treatment, 1 hour after inoculation of the 70 ml of the R2A broth, 3 pipe surfaces from each strain set were swabbed using sterile cotton swabs and dipped into 9 ml diluents labelled swab 1, swab 2 and swab 3 respectively. This was recorded as Day (0) of sampling.

Steps of sampling

i. From the storage point of the inoculated pipe surfaces, 3 pipe surfaces from each of the bacterial strain sets were sampled.
ii. Using sterile forceps, each pipe surface was placed into a labelled sterile Petri dish and the rest of the pipe surfaces were returned to the storage unit for the next sampling days.
iii. The picked pipe surfaces were rinsed with 5 ml sterile water on both sides of the pipe, to wash away the suspended matter and unattached cells and leave the biofilm attached to the pipe surface free of matter or debris.

iv. Using sterile cotton swabs, each pipe surface was swabbed for approximately 1-2 minutes holding the swab at a distance from the tip in contact with the swabbing area, to avoid contamination.

v. Using sterile pair of scissors, the tip of the loaded cotton swab was precisely cut below the holding point, into a 9 ml diluent. Each strain per sampling day had 3 parallels.

vi. The two sets of strains produce 6 parallels per sampling day. The six parallels were then vortexed for 30 seconds to transfer the biofilm cells into the diluent.

vii. From the second day of sampling from each treatment, the six parallels were serially diluted, and the appropriate dilutions were pour-plated into sterile Petri dishes using a sterile TSA medium. Plates were then incubated at 30 ºC for 48 hours.

viii. After 48 hours, the incubated plates were enumerated for the total plate count.

4.2.4. Methods of evaluation

The data were analyzed using Microsoft Excel and presented in bar charts that show the biofilm growth against time.

The significant testing of the null hypothesis that *there is no difference between the two bacterial strains*, was performed using the t-test on the two data sets of the two strains.

5. RESULTS AND DISCUSSION

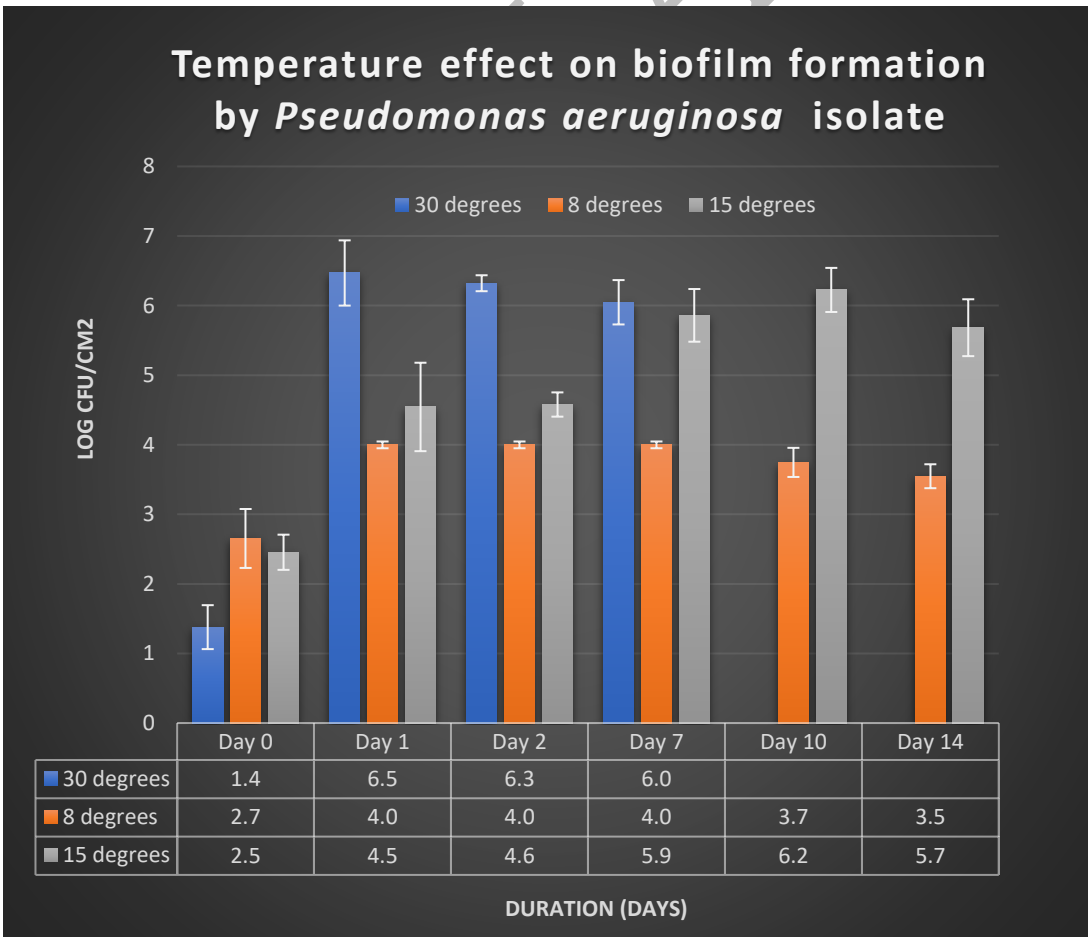
This chapter presents the study findings on the effect of temperature and chlorine on the biofilm-forming ability of *Pseudomonas aeruginosa* isolate and ATCC 9027 strains.

Temperature treatments of 30 ºC and 8 ºC were selected for this study to simulate summer and winter temperatures respectively while, 15 ºC, was selected to simulate Spring/Autumn temperatures in respect to Hungary where the *Pseudomonas aeruginosa* was isolated from tap water and study carried out in the same country.

The chlorine treatments of 0.1mg/L and 1mg/L concentrations were applied in this study per the WHO guidelines on drinking water disinfection with chlorine concentrations not exceeding 5mg/L.

5.1. Effect of temperature treatment on biofilm formation in DWDS by *Pseudomonas aeruginosa.*

5.1.1. Temperature effect on *Pseudomonas aeruginosa* isolate



	Day 0	Day 1	Day 2	Day 7	Day 10	Day 14
30 degrees	1.4	6.5	6.3	6.0		
8 degrees	2.7	4.0	4.0	4.0	3.7	3.5
15 degrees	2.5	4.5	4.6	5.9	6.2	5.7

Figure 4:Temperature effect on the biofilm formation by Pseudomonas aeruginosa isolate.

After 24 hours of storage time, biofilms by *Pseudomonas aeruginosa* isolate increased by 5.1, 1.3 and 2-$\log_{10}$ cycles at 30 ºC, 8 ºC and 15 ºC respectively.

After 48 hours, there was no significant difference between (day 1 and day 2) however, after 7 days, there was further 2-$\log_{10}$ cycles growth as compared to 48 hours i.e., 4-$\log_{10}$ cycles growth, compared to after 24 hours growth. At 10 days, there was no significant growth difference compared to 7 days. After 14 days of storage, the growth reduced by 1-$\log_{10}$ cycle, compared to 10 days, although it was still 3-$\log_{10}$ cycles higher than in 24 hours.

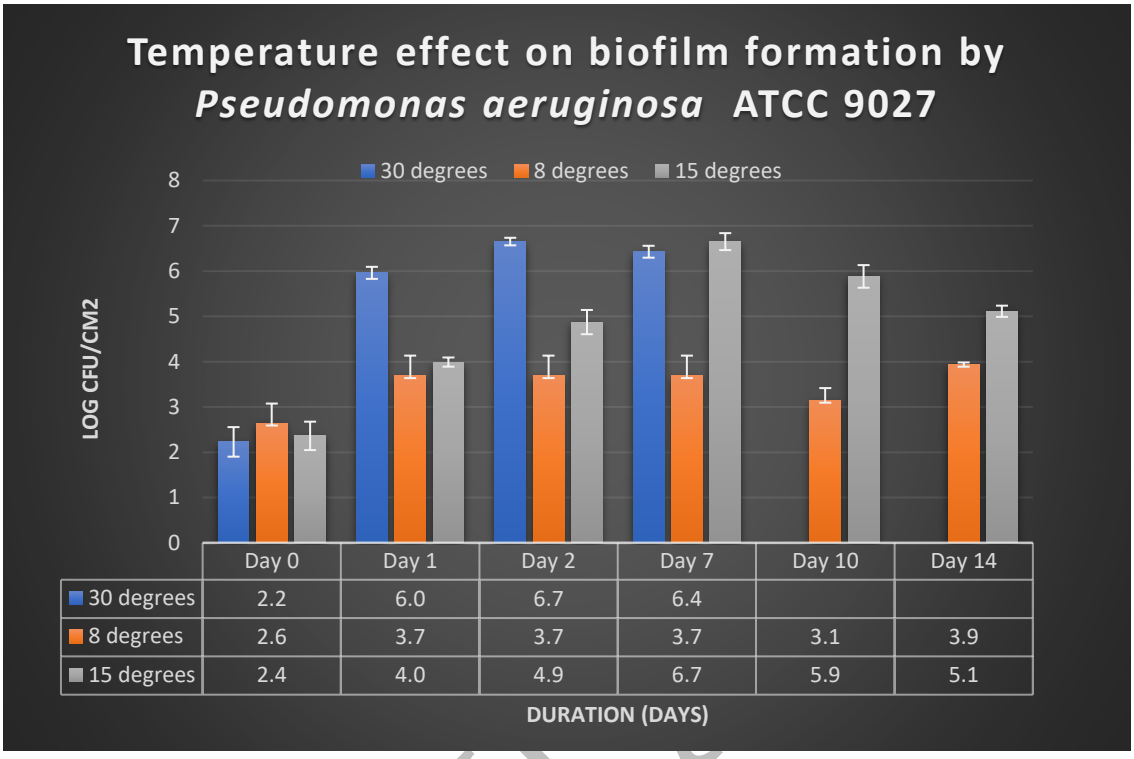
After 14 days there were 1.2-$\log_{10}$ cycles and 3.2-$\log_{10}$ cycles increase at 8 ºC and 15 ºC respectively. However, it was 0.8-$\log_{10}$ cycles and 0.5-$\log_{10}$ cycles decrease compared to 15 ºC and 8 ºC recorded highest growths (10 days) and (24 hours) respectively.

In Fig.4, it is evident that temperature had a clear and direct effect on the growth of *Pseudomonas aeruginosa* isolate biofilms, with the higher the temperature (optimum growth temperature of *Pseudomonas aeruginosa* is 37 ºC), the higher the growth of the biofilm.

However, it is important to note that the time factor also seemed to play a part with respect to temperature, for instance, at 15 ºC, the biofilms recorded a gradual growth during the first 48 hours, and after 7 days there was a 2- $\log_{10}$ cycles increase that maintained after 10 days and 14 days respectively.

The prolific growth after 24 hours at 30 ºC, shows that *Pseudomonas aeruginosa* isolate biofilms grow faster at high temperatures within a short time despite their initial cell number.

5.1.2. Temperature effect on biofilm formation of *Pseudomonas aeruginosa* ATCC 9027 strain.



	Day 0	Day 1	Day 2	Day 7	Day 10	Day 14
30 degrees	2.2	6.0	6.7	6.4		
8 degrees	2.6	3.7	3.7	3.7	3.1	3.9
15 degrees	2.4	4.0	4.9	6.7	5.9	5.1

Figure 5:Temperature effect on biofilm formation by Pseudomonas aeruginosa ATCC 9027 strains.

In Figure 5, at 30 ºC, 8 ºC and 15 ºC, after 24 hours there was a 3.8- $\log_{10}$ cycle increase at 30 ºC, and 1- $\log_{10}$ cycle increase at 8ºC, and 1.6- $\log_{10}$ cycles at 15 ºC.

After 48 hours, incubation temperature of 30 ºC had a 4.2- $\log_{10}$ cycles increase while at 8 ºC and 15 ºC there was a 1- $\log_{10}$ cycle increase and 2.5- $\log_{10}$ cycles increase respectively. The 2- $\log_{10}$ cycles increase was steady for the ATCC biofilms at 8 ºC for up to 7 days. After 10 days and 14 days, there was a slight decrease (0.6- $\log_{10}$ cycles) and a slight increase (0.2- $\log_{10}$ cycles) from the last steady growth at 7 days. However, this was 0.5- $\log_{10}$ cycles and 1.3- $\log_{10}$ cycles increase compared to the initial growth after 1 hour.

After 7 days there was a slight non-significant difference between the ATCC 9027 biofilms formed at 30 ºC and 15 ºC. This was the highest biofilm formation of the ATCC 9027 strain at 15 ºC which was the same as the highest at 30 ºC recorded after 48 hours, (24 hours) earlier at a higher temperature.

The highest biofilm formation at 8 ºC, was recorded after 14 days at a slight increase that had been maintained across 24, 48 and 7 days of storage. At 10 days, the growth of the biofilm at 8 ºC was suppressed further.

After 10 days and 14 days, the ATCC 9027 biofilms at 15 ºC showed a slight growth decrease from the highest growth recorded at 7 days, however, this was 2.5- $\log_{10}$ cycles and 1.8- $\log_{10}$ cycles increase from the initial biofilm growth recorded after 1 hour of inoculation.

From Figure 2, it is evident that at 15 ºC, the ATCC 9027 biofilms showed a gradual growth increase with respect to the increase in the days of storage, at 30 ºC, there was a faster higher growth after 24 hours to near maximum which came after 48 hours and significantly maintained even after 7 days.

At 8 ºC, the growth was suppressed regardless of the storage time. With even the highest initial biofilm count after 1 hour of inoculation, a low-temperature growth suppression effect is evident as the biofilms only grew by a 1- $\log_{10}$ cycle, compared to 2.7- $\log_{10}$ cycles and 1.6- $\log_{10}$ cycles increase at 30 ºC and 15 ºC respectively all after 24 hours storage.

5.1.3. Temperature effect on biofilm formation by the individual strains (*Pseudomonas aeruginosa* isolate and ATCC 9027) statistical evaluation.

Null hypothesis 1: there are no significant differences between the two strains in biofilm formation at 30 ºC, 8 ºC and 15 ºC respectively.

According to Table 1, the calculated p-values were above the significance level of (0.05) and therefore, the null hypothesis is accepted. This proved that the biofilm-forming abilities of *Pseudomonas aeruginosa* isolate and ATCC 9027 showed no significant differences under the influence of different temperatures.

Table 1: T-test statistical analysis of temperature effects on Pseudomonas aeruginosa isolate and ATCC 9027 strains.

			Temperature					
		30 ºC		**8 ºC**			**15 ºC**	
	isolate	**ATCC 9027**		**isolate**	**ATCC 9027**		**isolate**	**ATCC 9027**
Day 0	1.38	2.23	Day 0	2.65	2.64	Day 0	2.45	2.36
Day 1	6.47	5.96	Day 1	4.00	3.69	Day 1	4.54	4.00
Day 2	6.32	6.65	Day 2	4.00	3.69	Day 2	4.58	4.87
Day 7	6.05	6.43	Day 7	4.00	3.69	Day 7	5.86	6.65
			Day 10	3.75	3.14	Day 10	6.25	5.88
			Day 14	3.55	3.94	Day 14	5.68	5.11
	p-value	0.42		**p-value**	0.22		**p-value**	0.74

Null hypothesis II: there is no significant difference between the 8 ºC and 15ºC effects on the *Pseudomonas aeruginosa* biofilms.

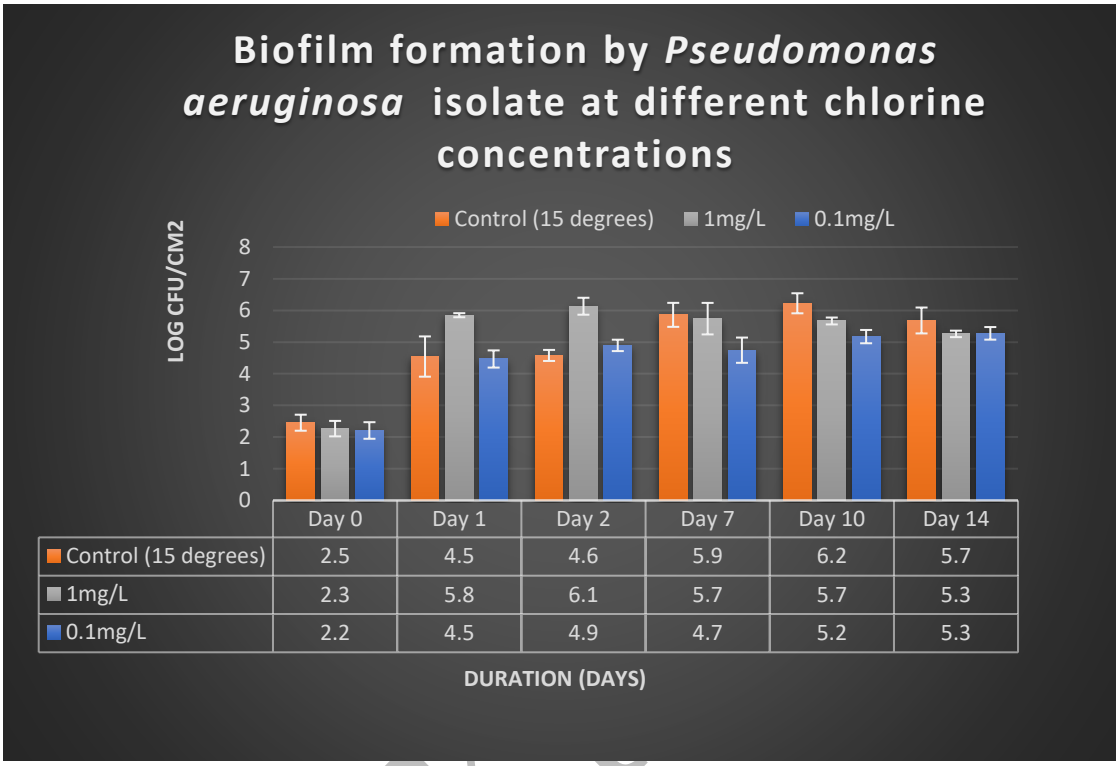
According to Table 2, the p-value of 0.04 and 0.05 is less/equal to the significance level of 0.05 and thus we reject the null hypothesis and agree that there was significant difference between the temperature effects on the concentration of biofilms formed by *Pseudomonas aeruginosa* strains both at 8 ºC and 15 ºC respectively.

Table 2:Temperature effects at 8 ºC and 15ºC on biofilms.

ATCC 9027		***Ps. aeruginosa* isolate**	
8 ºC	**15ºC**	**8 ºC**	**15ºC**
2.64	2.36	2.65	2.45
3.69	3.99	4.00	4.54
3.69	4.87	4.00	4.58
3.69	6.65	4.00	5.86
3.14	5.88	3.75	6.22
3.94	5.11	3.55	5.68
p-value	0.05	p-value	0.04

5.2. Effect of Chlorine treatment on biofilm formation in DWDS by *Pseudomonas aeruginosa*

5.2.1. Chlorine effect on *Pseudomonas aeruginosa* isolate



	Day 0	Day 1	Day 2	Day 7	Day 10	Day 14
Control (15 degrees)	2.5	4.5	4.6	5.9	6.2	5.7
1mg/L	2.3	5.8	6.1	5.7	5.7	5.3
0.1mg/L	2.2	4.5	4.9	4.7	5.2	5.3

Figure 6:Chlorine effect on Pseudomonas aeruginosa isolate.

According to Figure 6, biofilm formation as recorded at 15 ºC temperature treatment, was used as the control experiment for chlorine treatments of 0.1 mg/L concentrations and 1 mg/L respectively.

Irrespective of the initial suspension concentrations as shown in (Figure 10), the *Pseudomonas aeruginosa* isolate biofilms that formed after the 1 hour of inoculation showed no significant difference on either the control or chlorine treated R2A broth, for the control, 1 mg/L and 0.1mg/L respectively.

After 24 hours, there was a 2-$\log_{10}$ cycles growth increase of the biofilms in control and 0.1mg/L and a 4-$\log_{10}$ cycle increase in 1 mg/L. The 0.1mg/L chlorine concentration had minimal to significantly no effect on the biofilm formation while 1 mg/L had no reduction effect at all, instead they had an approximately 2-$\log_{10}$ cycles growth increase.

After 48 hours, 0.1 mg/L chlorine, the biofilm formation recorded a slight though non-significant growth compared to control while 1mg/L had no reduction effect at all, instead they had an approximately 2-$\log_{10}$ cycles growth increase. According to studies, chlorine

has been found to hinder biofilm growth. Zhang and Hu (2013) found that a dosage of 210 mg/L of chlorine slowed the formation of biofilms by 86.3%, but it had no effect on previously formed biofilms.

After 7 days, 0.1 mg/L chlorine concentration reduced the biofilm formation approximately by 1.5-$\log_{10}$ cycles, from under control, while 1 mg/L showed a growth inhibition effect but was not significantly effective.

After 10 days, 0.1 mg/L chlorine concentration reduced the biofilm formation approximately by 1-$\log_{10}$ cycle from under the control to, while 1 mg/L showed a growth inhibition effect but was not significantly effective.

After 14 days, both 0.1 mg/L and 1mg/L showed an almost equal growth inhibition effect respectively but were not significantly effective against the control.

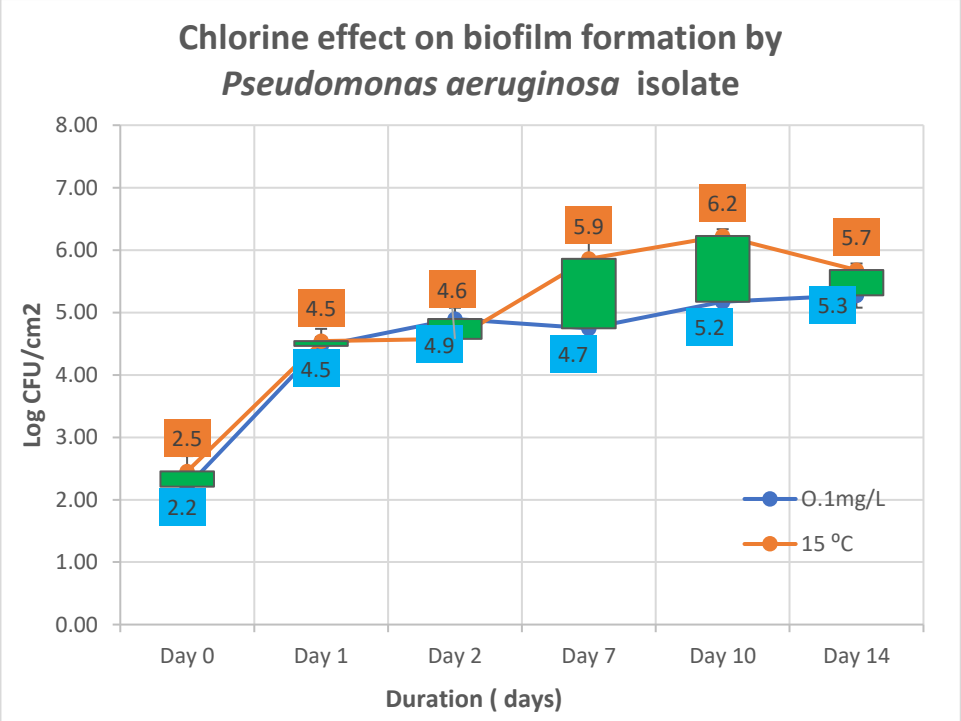


Figure 7: Chlorine (0.1mg/l) effect on Pseudomonas aeruginosa isolate strain

Figure 7 shows the different chlorine effects between the 0.1mg/l chlorine concentration on *Pseudomonas aeruginosa* isolate as illustrated by the up bars (green). We can statistically say that 0.1mg/L was more effective than 1mg/L (Fig.8) by a 2-$\log_{10}$ cycles reduction order. Especially after 10 days when the control had the highest biofilm growth, 0.1mg/L chorine concentration recorded a 2-$\log_{10}$ cycles growth decrease.

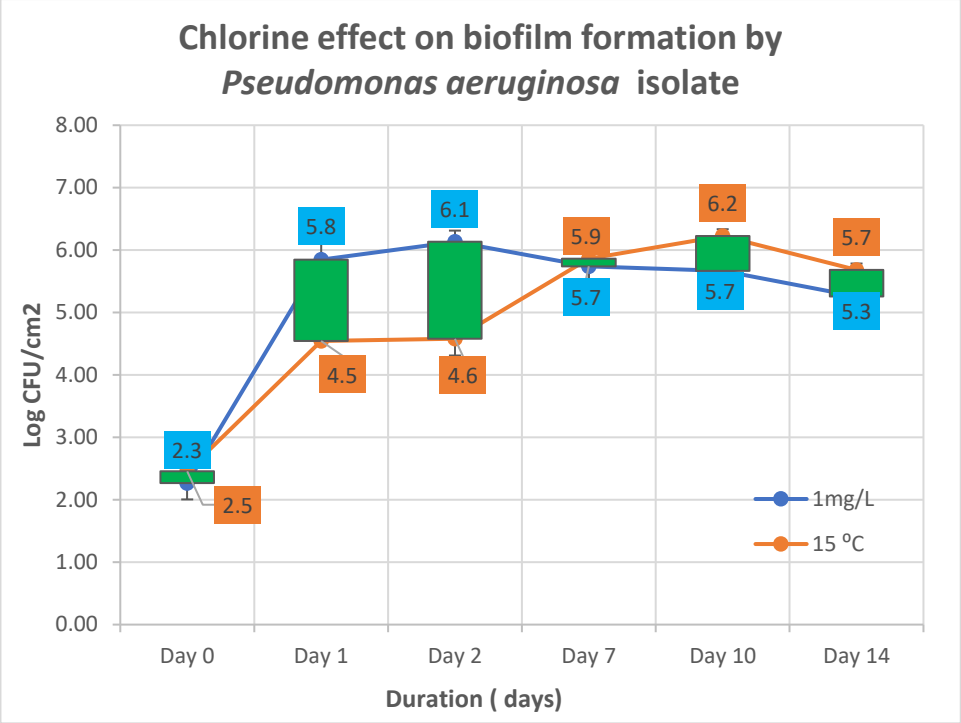
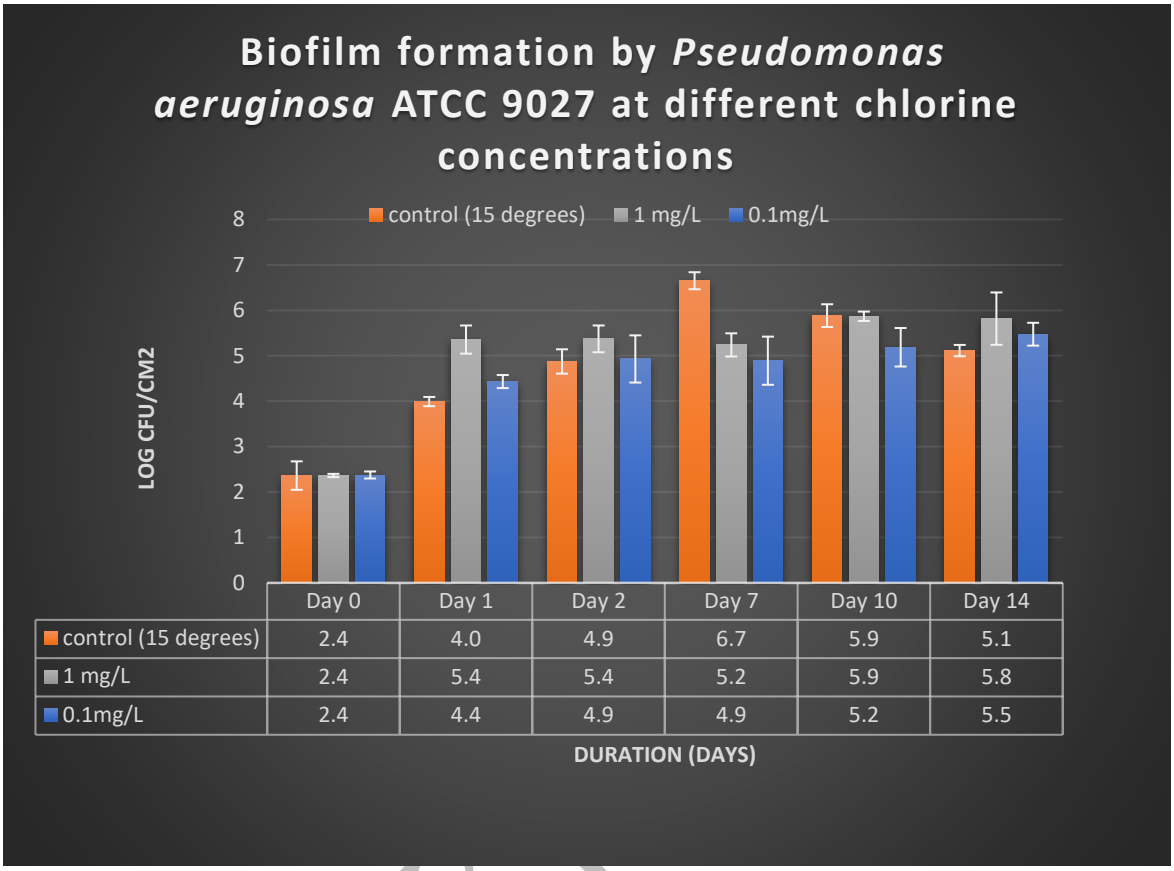


Figure 8: Chlorine (1mg/l) effect on Pseudomonas aeruginosa isolate

The chlorine treatment had no inhibition effect on the biofilm's growth after 24 hours of storage. After 48 hours, 0.1mg/l showed 0.3 -$\log_{10}$ cycles which were non-significant. The effectiveness of chlorine towards biofilm growth suppression was recorded after 10 days and 14 days for 1mg/l concentration and after 7 days through to 14 days for the 0.1 mg/l concentration, as shown in Figure 7 and Figure 8 above.

5.2.2. Chlorine effect on *Pseudomonas aeruginosa* ATCC 9027



	Day 0	Day 1	Day 2	Day 7	Day 10	Day 14
control (15 degrees)	2.4	4.0	4.9	6.7	5.9	5.1
1 mg/L	2.4	5.4	5.4	5.2	5.9	5.8
0.1mg/L	2.4	4.4	4.9	4.9	5.2	5.5

Figure 9: Chlorine effect on biofilm formation by Pseudomonas aeruginosa ATCC 9027.

As in Figure 9, after 24 hours, the initial growth (after 1 hour of inoculation, control, 0.1mg/L-and 1mg/L biofilm growth was 2 -$\log_{10}$ cycles for the control and in 0.1mg/L and 3-$\log_{10}$ cycles in 1mg/L. Both the 0.1mg/L and 1mg/L chlorine concentrations showed no reduction effect on the biofilm formation ability of the ATCC 9027 *Pseudomonas aeruginosa* strain. Instead, compared to the control sample growth of at 1mg/L there was 1-$\log_{10}$ cycles growth increase of the biofilms and a slight increase under 0.1mg/L chlorine concentration of approximately 0.5-$\log_{10}$ cycles.

After 48 hours, the biofilms had 2.5-$\log_{10}$ cycles increase in the control 3-$\log_{10}$ cycles increase in 1mg/L and a 3.5-$\log_{10}$ cycles increase in 0.1mg/L. Thus, no reduction effect was recorded from the two chlorine concentrations.

After 7 days, the control recorded a prolific growth of 4-$\log_{10}$ cycles increase of 0.1mg/L and 1.0mg/L both recorded 2.5-$\log_{10}$ cycles increase and 3-$\log_{10}$ cycles increase respectively. With respect to the control sample growth, the 0.1mg/L had a 2.5-$\log_{10}$ cycles growth inhibition effect and 1mg/L had a 1.5-$\log_{10}$ cycles growth inhibition effect.

After 10 days, about the initial growth (1 hour of inoculation), there was a 3-$\log_{10}$ cycles growth increase in control 1mg/L and 0.1mg/L respectively. With respect to the control, the chlorine concentrations showed a slight but non-significant growth reduction effect.

After the 14 days, there was a 3-$\log_{10}$ cycles growth increase across the control 0.1mg/L and 1mg/L) respectively. At this point, there was no reduction effect by either of the chlorine concentrations.

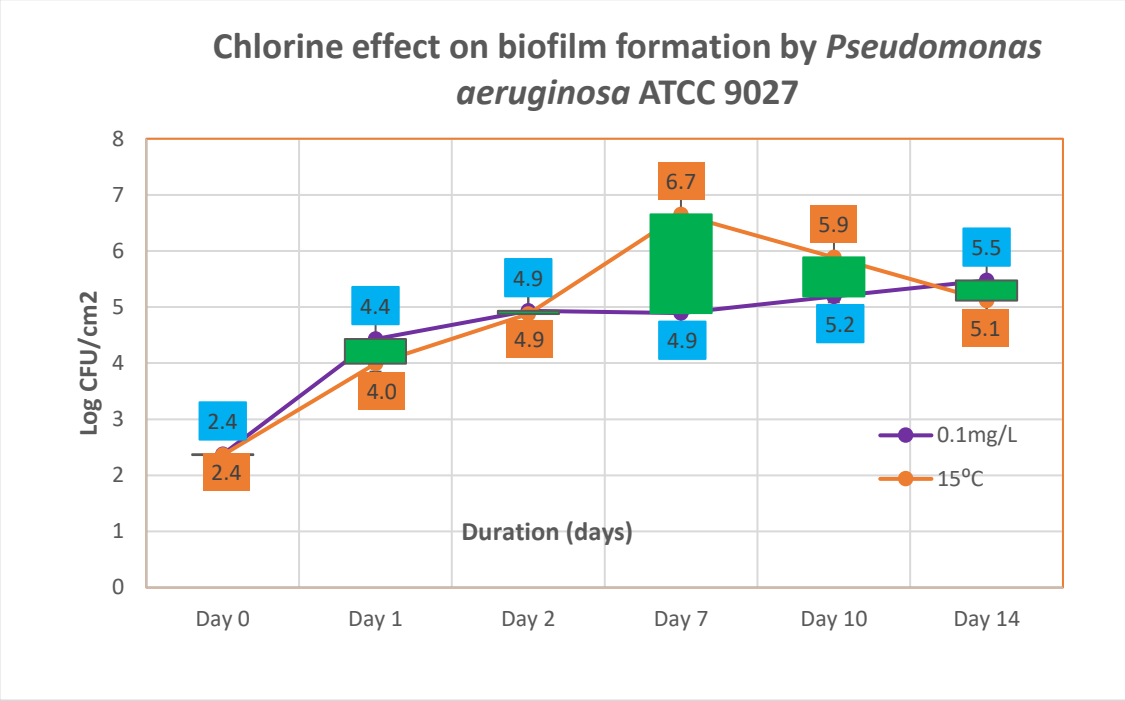


Figure 10: Chlorine (0.1mg/l) effect on Pseudomonas aeruginosa ATCC 9027

Figure 10 shows there is a significant difference between 0.1mg/L and 1mg/L (Fig.11) chlorine concentrations effect on the biofilm-forming ability of *Pseudomonas aeruginosa* ATCC 9027 strain. 0.1mg/L (Fig.10) is by averagely 1-$\log_{10}$ cycle more effective than 1mg/L as indicated by the up bars in (Fig.11).

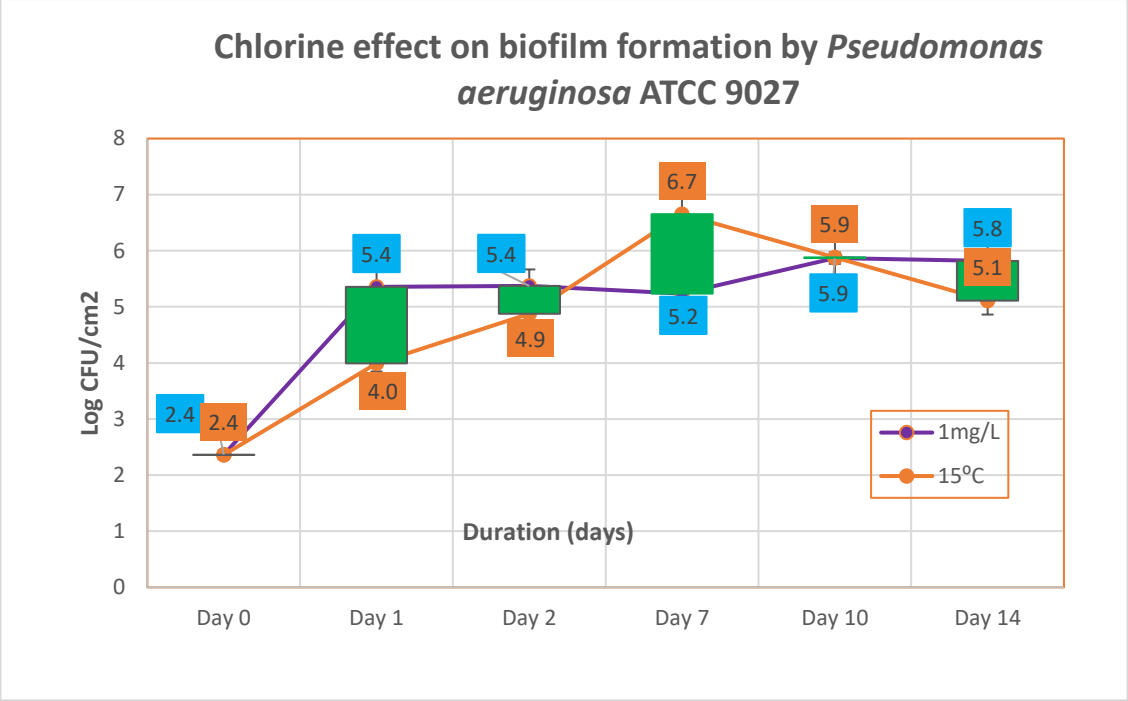


Figure 11: Chlorine (1mg/L) effect on ATCC 9027

Both chlorine treatments had no inhibition effect on ATCC 9027 biofilm growth after 24 hours of storage. The 0.1mg/l chlorine effect is visible after 10 days of storage. This could be in support of Xue and Seo (2013), findings that Alginate EPS synthesis substantially enhanced the size, surface charge, and resistance to chlorine disinfection of detached clusters. The redistributed biofilms of alginate-producing strains had larger total biomass, thicker biofilms, and more complex structural traits (both with and without chlorine conditions.

5.2.3. Comparison of the biofilm formation at 0.1 mg/L and 1 mg/L chlorine concentrations respectively.

Null Hypothesis: There is no significant difference in chlorine effects on the two *Pseudomonas aeruginosa* strains at (P-value =0.05).

According to Table 3, the P-values for *Pseudomonas aeruginosa* isolate and ATCC 9027 are 0.0373 and 0.02, respectively. Thus, they are below the level of significance (P-value=0.05) hence, we reject the Null hypothesis and conclude that there is a significant chlorine effect on the biofilm-forming ability of both strains.

Table 3: Chlorine effect on biofilm formation by Pseudomonas aeruginosa isolate and ATCC 9027 strains respectively.

		***Ps. aeruginosa* isolate**		**ATCC 9027**	
	0.1mg/L	**1mg/L**		**0.1mg/L**	**1mg/L**
Day 0	2.2093	2.2673	Day 0	2.3755	2.3648
Day 1	4.4665	5.8488	Day 1	4.4321	5.3568
Day 2	4.8956	6.1320	Day 2	4.9299	5.3724
Day 7	4.7440	5.7402	Day 7	4.8912	5.2391
Day 10	5.1731	5.6663	Day 10	5.1873	5.8691
Day 14	5.2758	5.2585	Day 14	5.4746	5.8172
	P-value	0.0373		P-value	0.02

5.3. Correlation between the initial cell count of the original suspension and biofilm formed after 1-hour of inoculation.

Irrespective of different initial concentrations of the original bacterial strain suspensions (Figure 12), after 1 hour of inoculation in a sterile R2A broth, both the control and the chlorine-treated samples recorded averagely the same number of biofilms.

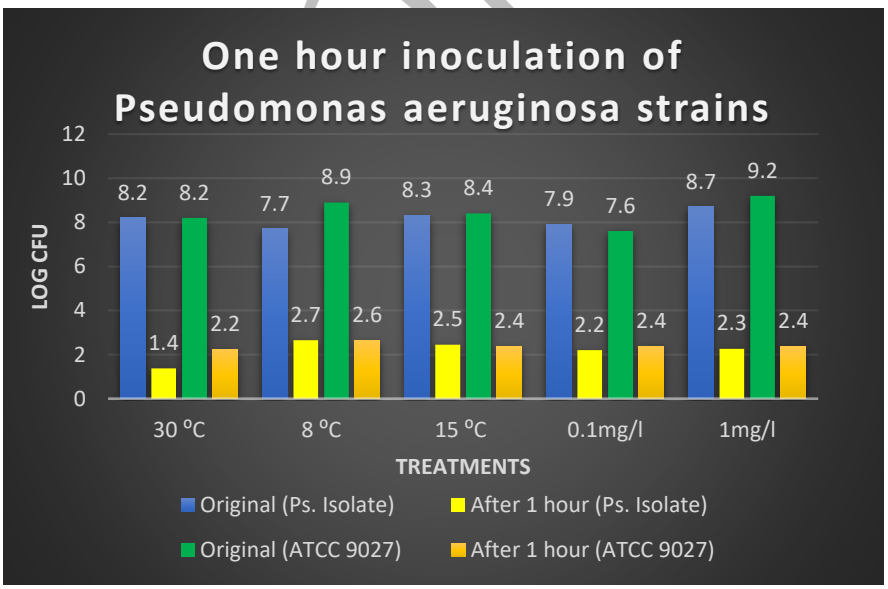


Figure 12: Pseudomonas aeruginosa cell attachment after one hour of inoculation.

10 suspensions were prepared for each strain with 5 original suspensions each. As indicated in Chapter 3, a new fresh 24-hour culture was used for a single treatment. As

in Table 4; the average initial concentration across all the 10 suspensions is 8.3 Log CFU/ml while the average cell attached after one-hour inoculation is 2.3 log CFU/cm^2.

Table 4: Average between initial concentration and after one hour sampling

			Ps. aeruginosa isolate				ATCC 9027			
		temperature	Chlorine			temperature	Chlorine			
	30 ºC	**8 ºC**	**15 ºC**	**0.1mg /l**	**1mg /l**	**30 ºC**	**8 ºC**	**15 ºC**	**0.1mg /l**	**1mg /l**	**average**
original (Log N)	1.2	7.7	8.3	7.9	8.7	8.2	8.9	8.4	7.6	9.2	**8.3**
after I hour (Log 10)	1.4	2.7	2.5	2.2	2.3	2.2	2.6	2.4	2.4	2.4	**2.3**

5.3.1. Chlorine effect on *Pseudomonas aeruginosa* during inoculation.

As in Figure 12, chlorine had no instant effect on the *Pseudomonas aeruginosa* during inoculation. Taking 15 ºC inoculation which occurred in a sterile R2A broth with no chlorine as the control, the ATCC 9027 strain recorded a 2.4 growth which was the same for 0.1mg/l and 1mg/l respectively although, the latter had initial concentrations of 7.6 and 9.2 respectively.

The lack of chlorine effect on *Pseudomonas aeruginosa* biofilms stretches to over 24 hours of storage as earlier indicated in 5.2.1 and 5.2.2 sub-chapters respectively. In a study by Xue and Seo (2013) chlorine prevented bacterial attachment, yet all strains tested were able to survive and immobilize themselves later, creating functional new biofilms.

6. SUMMARY

Biofilm formation in drinking water distribution systems is a study that aimed to find out the effects of temperature and chlorine on the ability of an opportunistic pathogen and very viable in water (*Pseudomonas aeruginosa*). Two strains of *Pseudomonas aeruginosa (*isolate from tap water and ATCC 9027) were investigated as test microorganisms. Well-cut HDPE pipe surfaces were used as the growth surfaces to simulate pipes used for distribution in drinking water systems.

At the beginning of every treatment, sterile pipe surfaces were submerged into the inoculated R2A broth and incubated for 1 hour. After I hour, the loose cells were washed off, 3 pipe surfaces were swabbed using sterile cotton swabs and the rest transferred into fresh sterile R2A broth. Biofilm formation was observed under different incubation temperatures: 30 ºC, 8ºC and 15 ºC for a period of 14 days. They were also subjected to growth at 0.1mg/l and 1mg/l chlorine concentrations respectively with a constant temperature at 15 ºC which was used as the control experiment.

From the results and discussion of this study, key observations included,

- Both the *Pseudomonas aeruginosa* isolate and the ATCC 9027 strain were significantly influenced by the temperature treatment. Both observed accelerated development at high temperatures (>30 ºC), progressive growth at medium temperatures (15 ºC), and reduced biofilm growth at low temperatures (8 ºC).
- Both the *Pseudomonas aeruginosa* isolate and the ATCC 9027 strains were less likely to form biofilms when chlorine was present at a concentration of 0.1 mg/L as opposed to 1 mg/L, where biofilm formation was high.
- For the first 24 hours of storage, *Pseudomonas aeruginosa* isolate and ATCC 9027 strains were not affected by chlorine concentrations of 0.1 mg/l or 1 mg/l.
- In contrast to its suppression of the *Pseudomonas aeruginosa* isolate biofilms, which was seen after 7, 10, and 14 days, 1mg/l only demonstrated a biofilm drop about the control for the ATCC 9027 biofilms after only 10 days. The isolate was therefore more responsive to 1mg/l than the ATCC 9027 strain.

The key experiences from this study include but are not limited to,

- The results were utilized as the benchmarks for all treatments for the remaining storage period after an hour of counting the amount of biofilm growth.

- ❖ It was assumed that during inoculation, biofilms adhered uniformly to all the pipe surfaces.

Key recommendations include,

i. To validate or affirm the prolific formation of biofilms across both strains, additional research and a repetition of the 1 mg/L chlorine concentration is required.

ii. All inoculated pipes within one hour can be swabbed, incubated, and counted; however, further research is needed to determine whether the initial concentration of the original suspension has an impact in this type of investigation. This will help with the notion that inoculation results reflect a uniform growth.

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8.DECLARATION

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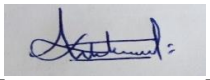

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9.STATEMENT ON CONSULTATION PRACTICES

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