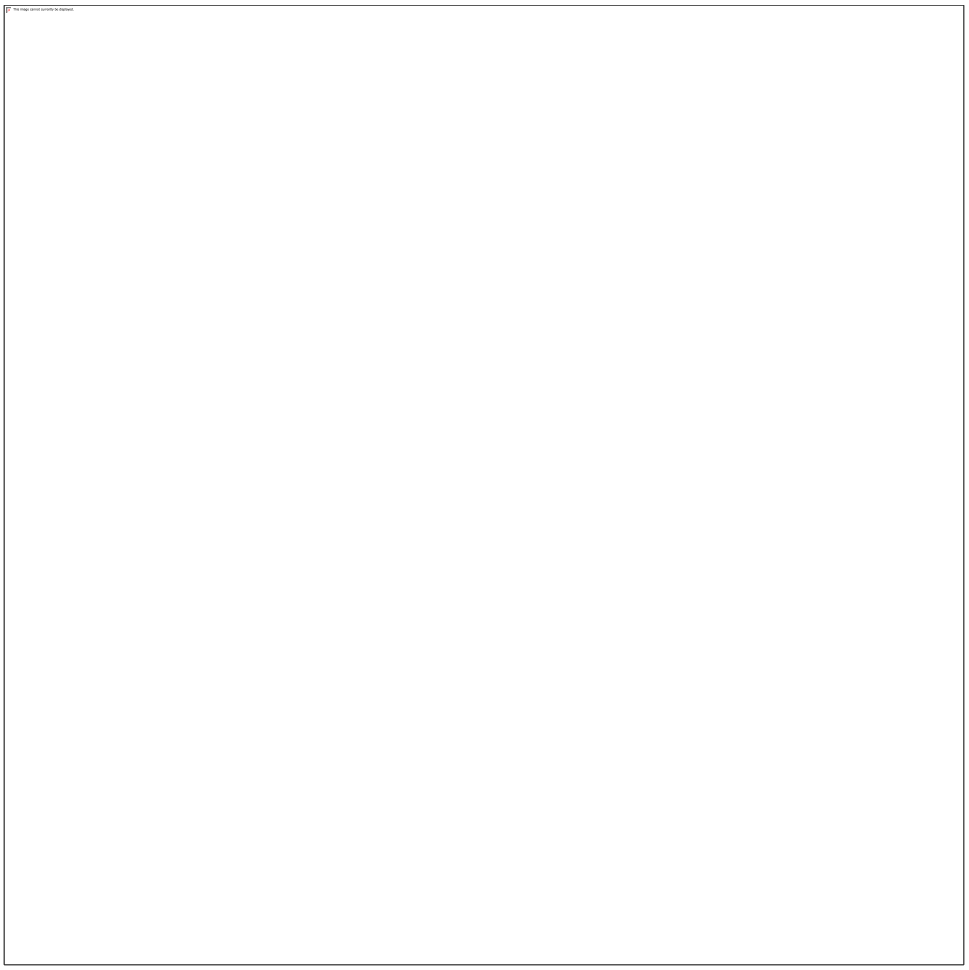

Isolation And Characterisation of Bacteria and Fungi from Biofilm Build Up in Pipes in a Water Treatment Plant in Harare.

By

Ian Fortune Dandajena

B213577B

A research project submitted in partial fulfilment of the requirements for the Bachelor of Science Honours Degree in Biotechnology.

June 2025

Approval form

The undersigned certify that they have read the dissertation titled 'Isolation and Characterization of Bacteria and Fungi from Biofilm Build Up in Pipes in a Water Treatment Plant' and confirm that it is suitable for submission to the Biological Sciences Department, Faculty of Science and Engineering, for assessment.

Signature of student:  Date: 20/06/2025

Signature of Supervisor: Siamayuwa CES Date: 20/06/2025

Signature of Chairperson of Department:  Date: 15/08/25

Declaration

I, Ian Fortune Dandajena (B213577B) declare that this research herein is my own work and has not been plagiarized from another source(s) without acknowledgement of the concerned author(s) either electronically or otherwise

Signature:  Date: 20/06/2025

Supervisor

I, Clayton Siamayuwa, declare that I have supervised this thesis, and I am satisfied that it can be submitted to the Biological Sciences Department, Faculty of Science and Engineering, at Bindura University of Science Education.

Signature: Siamayuwa CES Date: 20/06/2025

Dedication

I dedicate this project to my mother Scholastic Run'anga, for her unwavering faith and support in my capabilities.

Acknowledgements

Firstly, I would like to thank my family for their financial and emotional support throughout the entire four years of my education. My gratitude also goes to Mr. Siamayuwa and Mr. Selome for their advice and guidance throughout the project, and to Bindura University of Science Education and Delta Beverages Coca-Cola for providing the resources to complete the research. Above all, I would like to thank God for helping me throughout this period of my studies.

List of abbreviations

ANOVA- Analysis of Variance

EPS – Extracellular Polymeric Substance

cfu- Colony Forming Units

Cm- centimetre

ml- millilitre

PDA- Potato Dextrose Agar

NA – Nutrient Agar

TNTC- Too Numerous to Count

WTP – Water Treatment Plant

BAC – Benzalkonium Chloride

SDS – Sodium Dodecyl Sulphate

List of figures

List of tables

List of appendices

Abstract

Biofilms are complex microbial communities that adhere to surfaces and are embedded in a protective extracellular matrix. In water treatment plants (WTPs), biofilms form on the internal surfaces of pipelines and contribute to reduced water quality, resistance to disinfection, and infrastructure damage. This study aimed to isolate and characterize bacteria and fungi from biofilm buildup in WTP pipelines and to evaluate potential chemical treatment methods for biofilm removal. Samples were cultured on Nutrient Agar, MacConkey agar, and Potato Dextrose Agar. Sugar fermentation and biochemical tests, including catalase, oxidase, and urease were used for presumptive identification at genus level.While these methods offer useful metabolic and physiological insights, they do not provide definitive species-level identification without molecular confirmation. The bacterial isolates included *Escherichia coli* and *Klebsiella spp.*, while fungal isolates inclulded *Candida albicans*, *Cryptococcus* spp., and moulds likely to be *Fusarium* and *Trichoderma spp*. Crystal violet (CV) assay was used to investigate the efficacy of four treatments, Sodium Dodecyl Sulphate (SDS), Benzalkonium Chloride (BAC), Tween 80, and Sodium Hypochlorite (NaOCl), each applied at 5% concentration for 5, 10, and 15 minutes. OD_{595} values showed that NaOCl and BAC achieved the greatest biofilm reduction, while Tween 80 was least effective. A two-way ANOVA confirmed statistically significant effects of both treatment type and exposure time on biofilm reduction ($p < 0.001$), including a significant interaction between the two variables. These results suggest that time and chemical agent both contribute significantly to biofilm disruption and that certain detergents may serve as viable alternatives to conventional chlorination. The study concludes that integrating effective chemical treatments and microbial profiling can enhance biofilm control strategies in water treatment infrastructure.

Table of Contents

Chapter 1

Introduction

1.0 Introduction

Water treatment plants (WTPs) are critical infrastructures for providing safe and clean water for industrial, domestic, and municipal use. However, the internal components of these systems including pipes, tanks, and filters, often become colonized by microbial communities known as biofilms. These biofilms comprise diverse groups of bacteria, fungi, and other microorganisms embedded within a matrix of extracellular polymeric substances which they make themselves (EPS)(Elumalai et al., 2024).

Biofilms pose significant challenges to water treatment efficiency and public health. They protect embedded microorganisms from disinfectants and other treatment processes, making eradication difficult(Bezuidenhout, 2014). Furthermore, they can harbour pathogenic organisms capable of causing waterborne diseases. The presence of biofilms also contributes to the corrosion of pipes and equipment, leading to increased operational costs(Bezuidenhout, 2014). In addition, biofilms may foster the development of antimicrobial resistance, complicating both microbial control and water treatment strategies. Despite the risks posed by biofilms, there is limited understanding of the microbial diversity and characteristics within these communities in WTPs. This gap hinders the development of effective and targeted biofilm management strategies.

1.1 Background of the Study

Microbial biofilms in water treatment systems represent a persistent problem due to their structural complexity and resistance to conventional treatment methods. These biofilms can develop on most surfaces that are in contact with water and they are composed of a matrix that offers mechanical stability and chemical protection to embedded microorganisms(Goraj et al., 2021). The microorganisms residing in biofilms exhibit physiological traits that differ significantly from their planktonic (free-floating) counterparts, including increased tolerance to antibiotics and biocides.

The persistence of biofilms in WTPs compromises the microbial quality of treated water, undermines disinfection procedures, and may result in the distribution of contaminated water.

Understanding the microbial constituents of these biofilms, especially the bacteria and fungi involved, can provide critical insights into how they persist and how they might be effectively targeted. This study aims to bridge the knowledge gap by isolating and characterizing the microbial species within biofilms found in WTP pipelines and evaluating potential approaches for their eradication.

1.2 Problem Statement

Biofilm formation within the pipelines of water treatment plants (WTPs) poses a significant and multifaceted challenge to public health and infrastructure integrity. These biofilms, composed primarily of bacteria and fungi, not only resist conventional treatment processes such as chlorination and UV exposure but also serve as reservoirs for pathogenic and antibiotic-resistant microorganisms. This persistence can compromise water quality, leading to potential outbreaks of waterborne diseases. Moreover, microbial biofilms contribute to the corrosion and degradation of pipe materials, increasing maintenance costs and operational disruptions. Despite these concerns, there remains a limited understanding of the specific microbial communities that constitute these biofilms, particularly in terms of their diversity, resistance mechanisms, and ecological roles. The lack of comprehensive microbial profiling impedes the development of effective biofilm control and eradication strategies tailored to the specific conditions of WTPs. Therefore, there is a pressing need to isolate, identify, and characterize these microbial populations to inform the design of more robust, targeted interventions for biofilm management in water treatment systems.

1.3 Aim of the Study

To investigate the diversity and characteristics of fungi and bacteria in biofilms formed within water treatment plant pipelines, and to evaluate the efficacy of selected chemical treatments for biofilm removal.

1.4 Objectives

1. To isolate bacteria and fungi from biofilms in WTP pipelines.
2. To characterize the isolated microorganisms using colony morphology, enzymatic activity, which reflect their physiological and biochemical traits.
3. To evaluate treatment strategies for the disruption and eradication of biofilms based on the microbial profile.

1.5 Research Questions

1. What is the diversity and nature of bacterial and fungal species present in biofilms within WTPs?
2. What are their key morphological, biochemical and physiological traits?
3. What treatment approaches are most effective in disrupting or eradicating these biofilms?

1.6 Justification

The study is expected to contribute valuable knowledge to the field of water microbiology by identifying the microbial constituents of biofilms in WTPs. Understanding the diversity and characteristics of these organisms can facilitate the development of more effective biofilm control methods. Since traditional water treatment processes often fail to eliminate biofilm-associated microorganisms, this research could inform more robust and targeted interventions. Identifying the microbial composition and testing potential biofilm removal agents could inform improved maintenance protocols and reduce the risk of resistant, pathogenic organisms reaching consumers. This is particularly important in resource-limited settings where routine infrastructure upgrades may not be feasible.

1.7 Hypothesis

- **Null Hypothesis (H_0):** Chemical treatments do not significantly reduce biofilm biomass in WTP pipelines compared to a control.
- **Alternative Hypothesis (H_1):** At least one chemical treatment significantly reduces biofilm biomass in WTP pipelines compared to a control.

1.8 Limitations

- Sampling limitations due to restricted access to all parts of the treatment system.
- Methodological limitations related to the sensitivity and specificity of microbial isolation and identification techniques.
- Limited access to advanced laboratory equipment and reagents due to financial constraints.
- Time constraints may limit the scope of sampling, analysis, and experimentation.

1.9 Delimitations

- The study will focus exclusively on bacteria and fungi, excluding viruses and protozoa.

- Research will be limited to one or a few selected water treatment facilities.
- Sampling will be restricted to specific stages of the water treatment process (e.g., post-sedimentation or distribution lines).
- Only biofilms formed on certain pipe materials or under standard operational conditions will be considered.
- The study will be geographically confined to a specific region and conducted during a defined period.
- Only selected microbial isolation and characterization techniques (e.g., culture-based methods, microscopy) will be used.
- The study will focus on specific aspects of biofilm development such as microbial diversity, resistance, and response to detergents.

Chapter 2

Literature Review

2.0 Introduction

Biofilms are dynamic microbial communities embedded in an EPS matrix that adhere to surfaces. In water treatment plants (WTPs), biofilms are a persistent problem, forming on internal pipe surfaces and other components of the distribution system. These microbial aggregates present significant public health, operational and environmental concerns because of their resistance to conventional treatment methods and their potential to harbour pathogenic microorganisms (Elumalai et al., 2024). The formation, structure, microbial diversity, and impact of biofilms have been the subject of growing research interest, particularly as the demand for safe drinking water increases in urbanizing and industrializing regions.

The goal of this chapter is to explore and critically analyse existing literature on the biology and ecology of biofilms in WTPs. The review begins with a detailed explanation of biofilm formation and structure, followed by an examination of microbial diversity in biofilms, the associated public health and operational risks, and existing strategies for biofilm characterization and control. This synthesis aims to clarify current scientific understanding while identifying gaps that justify the current study's focus on isolating and characterizing bacteria and fungi from WTP biofilms(Elumalai et al., 2024).

2.1 Biofilms: Definition, Formation, and Structure

Figure 1 Biofilm formation.

Biofilms are structured microbial consortia encased in EPS, enabling their attachment to surfaces and resistance to environmental stressors. The EPS matrix consists of nucleic acids, polysaccharides, lipids and proteins, which provide a protective barrier and facilitate nutrient exchange and communication between cells (Elumalai et al., 2024). The biofilm formation process is usually split into five stages: (1) initial reversible adhesion of free floating microorganisms to a surface; (2) irreversible attachment facilitated by EPS production; (3)formation of microcolonies; (4) maturation of the biofilm and (5) dispersal of cells to colonize new niches (Shukla et al, 2021)(Erdei-Tombor et al., 2024)

Biofilms that form in drinking water systems are influenced by numerous environmental and physicochemical parameters. Flow rate, temperature, pH, pipe material, and nutrient availability play critical roles in modulating the composition and thickness of the biofilm. Yin et al. (2023) demonstrated that interactions between EPS and corrosion products within pipes can shape bacterial communities, leading to spatial and compositional variation in biofilm structure. Notably, EPS also facilitates microbial cooperation and protection from disinfectants by creating barriers to biocides (Patra et al., 2022).

Filamentous fungi are particularly adept at integrating into biofilms due to their hyphal growth and ability to form dense networks, enhancing the biofilm’s mechanical stability (de Siqueira, 2011). These fungi, once integrated into the matrix, contribute to increased biomass and structural complexity. Their presence is often overlooked in microbial assessments of water systems, yet they can significantly alter the biofilm's function and resistance characteristics (Kauffmann–Lacroix et al., 2016).

Moreover, the genetic exchange within biofilms promotes microbial adaptability and resistance. Biofilms facilitate horizontal gene transfer (HGT), a mechanism that contributes to the spread of antimicrobial resistance genes among bacterial populations (Labella et al., 2021). Consequently, biofilms in WTPs pose an escalating challenge, particularly in systems that rely heavily on chlorination or UV treatment, both of which have limited efficacy against mature biofilms (Shukla et al., 2021).

Biofilms are, therefore, not mere microbial assemblages but highly organized ecosystems with complex biochemical and physical interactions. Their resilience and adaptability necessitate more

sophisticated monitoring and management approaches, especially in water treatment settings where microbial contamination must be stringently controlled.

2.2 Biofilm Formation in Water Treatment Systems

Figure 2 Pipe with biofilm inside

Water treatment plants are designed to eliminate microbial contaminants, yet ironically, their infrastructure often provides a conducive environment for biofilm development. Biofilms in WTPs form on the inner surfaces of pipes, reverse osmosis membranes, sedimentation tanks, and other water contact surfaces. The presence of organic matter, nutrients, fluctuating water flow, and pipe surface irregularities promotes microbial colonization and biofilm maturation (Shukla et al., 2021).

The choice of pipe material significantly influences biofilm development. Goraj et al. (2021) observed that biofilms in polyethylene pipes were more diverse than those in cast iron or stainless steel, suggesting that the physicochemical properties of pipe surfaces affect microbial adhesion and community structure. Smooth surfaces tend to retard microbial attachment, while rough and porous materials accelerate colonization by providing more surface area and protective niches.

Reverse osmosis (RO) systems, a common component in modern WTPs, are particularly vulnerable to biofouling. Labella et al. (2021) isolated multiple bacterial strains from RO membranes and identified a high prevalence of antibiotic resistance and biofilm-forming capacity

among these isolates. Their study underscored the importance of continuous surveillance and microbial profiling to ensure the microbiological integrity of treated water.

Quorum sensing, a form of microbial communication, is essential for the formation and growth of biofilms. Bacteria and fungi use signalling molecules such as acyl-homoserine lactones and oxylipin respectively, to facilitate interspecies interactions and coordinate communal behaviour, including EPS production and virulence expression (Elumalai et al., 2024). These interactions lead to the development of multispecies biofilms with increased resilience and metabolic cooperation, complicating efforts to eradicate them using standard disinfection strategies.

Furthermore, environmental biofilms in WTPs are influenced by incoming microbial loads from source water. Mudau et al. (2024) reported that microbial communities persist from natural water sources thorough treatment plants into distribution systems. Their findings suggest that inadequate pretreatment or ineffective filtration allows fungal and bacterial propagules to persist through the treatment chain and establish biofilms downstream.

Filamentous fungi, often neglected in microbial assessments, are increasingly recognized for their role in water treatment system biofilms. Siqueira et al. (2013) showed that filamentous fungi like *Aspergillus*, *Penicillium*, and *Fusarium* remain present in Brazilian water distribution systems. These fungi, through their robust hyphal structures, enhance the structural stability of biofilms and contribute to pipe corrosion and water quality deterioration.

The formation of biofilms in WTPs is a complex, multi-stage process that is stimulated environmental, microbial, and engineering factors. The persistence of biofilms despite conventional water treatment highlights the need for targeted control strategies and regular microbial monitoring to prevent infrastructure degradation and safeguard water quality.

2.3 Diversity in Water System Biofilms

The microbial communities within water treatment plant biofilms are often diverse and dynamic, comprising bacteria, fungi, algae, protozoa, and archaea. Among these, bacteria and fungi dominate due to their rapid adaptability and resistance to conventional treatment methods(Elumalai et al., 2024). A clear understanding of microbial diversity is the key to developing effective control and remediation methods.

Bacterial biofilms in WTPs are commonly composed of genera such as *Pseudomonas*, *Acinetobacter*, *Aeromonas*, *Escherichia*, and *Klebsiella*, many of which possess significant pathogenic potential (Labella et al., 2021). These bacteria exhibit robust biofilm-forming capabilities and often display antibiotic resistance, raising concerns about their potential role in waterborne disease outbreaks and the spread of antimicrobial resistance (AMR). Aboelseoud et al. (2021) isolated similar bacterial species from poultry water systems and found that many were resistant to multiple antibiotics, suggesting a public health risk through indirect human exposure.

The fungal component of biofilms has historically been overlooked, but recent studies have underscored their importance. Siqueira et al. (2013) and Kauffmann–Lacroix et al. (2016) both emphasized that filamentous fungi, including *Aspergillus*, *Penicillium*, *Cladosporium*, and *Fusarium*, are frequently isolated from water distribution systems. These fungi are resilient to disinfectants and can survive extreme conditions within pipelines. Their presence is not merely passive fungi can produce secondary metabolites, including mycotoxins and organic acids, which contribute to pipe corrosion and biofilm stability (Zhao et al., 2024).

Fungi can also affect organoleptic properties of water. Zhao et al. (2024) associated specific fungal strains with taste and odour problems in drinking water, particularly in the presence of chlorination by-products and organic substrates. This impact extends beyond aesthetics; unpleasant odours or tastes can undermine public trust in drinking water safety.

Interestingly, fungal-bacterial interactions further complicate the ecology of WTP biofilms. These interactions can be synergistic or antagonistic. In some cases, bacterial EPS provides a protective scaffold for fungal hyphae, enhancing their survival and integration into the biofilm matrix. In others, competitive exclusion or antagonistic metabolites may restrict fungal colonization (Elumalai et al., 2024).

Environmental factors, including temperature, pH, flow velocity, and nutrient availability, influence microbial community composition in biofilms. Goraj et al. (2021) found that pipe material also played a pivotal role, with plastic and metal surfaces supporting different microbial assemblages. This variation suggests that system design and material choice could be used strategically to influence biofilm composition.

The origin of microorganisms in biofilms is another critical issue. Mudau et al. (2024) tracked microbial populations from natural sources through treatment systems and into distribution networks. They reported that despite treatment, many microorganisms—including potential pathogens—persisted and recolonized distribution infrastructure, often forming robust biofilms downstream.

2.4 Health and Operational Risks Posed by Biofilms

Biofilms in water treatment plants (WTPs) are not simply passive microbial accumulations—they pose significant risks to public health and operational integrity. These microbial consortia protect embedded organisms from disinfectants and environmental stresses, creating reservoirs for persistent contamination and system degradation (Elumalai et al., 2024).

From a public health perspective, the presence of pathogenic bacteria and fungi within biofilms represents a continuous source of microbial contamination. Many of the bacterial species isolated from WTPs, such as *Escherichia coli*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae*, are known pathogens with resistance to common antibiotics (Labella et al., 2021; Aboelseoud et al., 2021). These bacteria can detach from biofilms and enter the treated water supply, posing risks of gastrointestinal infections, urinary tract infections, and other health complications, especially in immunocompromised individuals.

Fungi, though less frequently assessed, are equally concerning. Kauffmann–Lacroix et al. (2016) reported that filamentous fungi like *Fusarium* and *Aspergillus* can survive treatment processes and form biofilms in distribution systems. Inhalation or ingestion of their spores can lead to opportunistic infections such as aspergillosis or allergic responses. Zhao et al. (2024) emphasized that some fungal metabolites can also affect the taste and odour of drinking water, indirectly impacting public perception and trust in water safety.

Antimicrobial resistance (AMR) within biofilms exacerbates the health threat. Biofilm-associated bacteria often exhibit up to 1000-fold increased resistance to antibiotics compared to their planktonic counterparts (Patra et al., 2022). This is due to multiple factors, including restricted penetration of antimicrobials through the EPS matrix, altered metabolic states of cells, and horizontal gene transfer. Clark et al. (2025) highlighted that sewage-impacted environments,

including nearshore and wastewater systems, serve as hotspots for AMR gene proliferation. Such findings suggest that biofilms in WTPs could similarly harbour and disseminate resistant genes, posing a risk far beyond localized water systems.

Operationally, biofilms lead to major technical challenges. The accumulation of biomass within pipes increases friction and flow resistance, requiring more energy to maintain pressure and flow rates. Biofilms also contribute to the biocorrosion of infrastructure, particularly when composed of sulphate-reducing bacteria and acid-producing fungi (Akpan et al., 2013). Over time, this corrosion compromises pipe integrity and necessitates frequent repairs or replacement—raising maintenance costs and increasing the risk of system failure.

Further, biofilms on membranes used in reverse osmosis and ultrafiltration systems reduce filtration efficiency by clogging pores and creating uneven flow patterns. This fouling leads to decreased water quality and shortened membrane lifespan, demanding more frequent cleaning and replacement (Labella et al., 2021). In treatment tanks and clarifiers, biofilms can disrupt sedimentation processes by altering surface properties and promoting microbial blooms that interfere with water clarity and disinfection.

These challenges underscore the need for early detection, continuous monitoring, and proactive mitigation of biofilms within water systems. Traditional control methods—such as chlorination and UV treatment—are often insufficient. Biofilms exhibit strong resistance to oxidizing agents due to the protective properties of their EPS matrix and the presence of stress-tolerant microorganisms (Shukla et al., 2021). Consequently, operational strategies must evolve to incorporate novel antimicrobial agents, pipeline materials that discourage microbial adhesion, and biofilm-resistant designs.

2.5 Methods for Isolation and Characterization of Microorganisms in Biofilms

Understanding the microbial composition of biofilms in water treatment systems necessitates reliable methods for their isolation and characterization. Various cultured-based and culture-independent methods have been developed over time, each with its advantages and limitations.

Traditional microbiological techniques remain valuable for isolating viable microorganisms. These include swabbing pipe surfaces, using scrapers or brushes to remove biofilm layers, and

then culturing the collected samples on selective media. This approach enables enumeration and basic identification based on colony morphology, staining characteristics, and biochemical tests (Aboelseoud et al., 2021). While cost-effective and straightforward, culture-based methods underestimate microbial diversity because many environmental organisms are non-culturable under standard laboratory conditions.

To overcome these limitations, molecular approaches have become essential in characterizing biofilm communities. PCR, qPCR, and next-generation sequencing (NGS) have been used to directly detect and identify microbial DNA in biofilm samples. De Siqueira (2011) applied both microscopic and molecular methods to characterise filamentous fungi in drinking water distribution systems, showing that combining fluorescence in situ hybridisation (FISH) and PCR-based assays provides a more comprehensive view of fungal diversity. Similarly, Siqueira et al. (2013) showed that molecular tools were instrumental in detecting filamentous fungi like *Cladosporium* and *Penicillium* in Brazilian water systems, which were not recovered through culture methods.

Microscopy also plays a critical role. Scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM) enable detailed, high-resolution imaging of biofilm structure and allow for high-resolution imaging of biofilm architecture and microbial distribution within the EPS matrix. These techniques help to visualize the complexity of microbial communities and their interactions with pipe surfaces (Elumalai et al., 2024). Moreover, live/dead staining kits used with CLSM can differentiate between viable and non-viable cells, offering insights into the efficacy of antimicrobial treatments.

Biochemical assays targeting the EPS components—proteins, polysaccharides, and nucleic acids—are valuable for characterizing the structural and protective elements of biofilms. These macromolecules influence biofilm resilience and resistance to disinfectants. Elumalai et al. (2024) noted that EPS analysis could help determine the biofilm's potential to resist biocides, making it a useful marker for evaluating treatment efficacy.

Metagenomics, though more complex and resource-intensive, enables comprehensive profiling of microbial communities, including bacteria, archaea, and fungi. This approach does not rely on

culturing and can reveal the presence of rare, unculturable, or dormant species. Mudau et al. (2024) emphasized that metagenomic analysis is especially important for tracking microbial diversity from natural sources through water treatment plants and into distribution systems, capturing transitions in community composition that may influence biofilm formation.

Another innovative method includes the use of bioaerosol sampling and pretreatment to assess airborne microbes associated with biofilms. Gaetano et al. (2024) highlighted this method's relevance in wastewater plants, suggesting its potential applicability in WTPs where biofilm-derived microorganisms might become aerosolized and pose occupational hazards.

Antibiotic susceptibility testing is frequently employed following microbial isolation to determine the resistance profiles of biofilm-associated strains. This typically involves disk diffusion or broth microdilution techniques. Labella et al. (2021) and Aboelseoud et al. (2021) both used these methods to demonstrate that bacterial strains from WTP biofilms often exhibit multidrug resistance, reinforcing the need for robust monitoring protocols.

Despite the availability of advanced methods, challenges remain. Sample representativeness is a major concern; biofilm heterogeneity across pipe materials and treatment stages can lead to biased or incomplete data. Moreover, contamination during sampling or DNA extraction can skew molecular results (Erdei-Tombor et al., 2024). These issues underscore the importance of method standardization and the use of complementary approaches to capture an accurate picture of microbial communities.

2.6 Antimicrobial Resistance and Biofilm Resilience in Water Systems

A major concern about biofilms in WTP is their natural resistance to antimicrobial agents, such as common disinfectants and antibiotics. This resilience poses a significant challenge to maintaining water quality and ensuring safe delivery through distribution systems.

Biofilms exhibit a unique mode of antimicrobial resistance that differs from planktonic (free-floating) microbial cells. The EPS matrix serves as a physical and chemical shield, restricting the entry of biocides and antimicrobial (Elumalai et al., 2024). Moreover, the spatial heterogeneity within biofilms creates microenvironments that support slow-growing or dormant cells, often referred to as persister cells, which are particularly difficult to eliminate (Shukla et al., 2021).

Multiple studies have highlighted the antibiotic resistance profiles of bacteria isolated from water treatment systems. Labella et al. (2021) found that strains isolated from reverse osmosis units in drinking WTP exhibited high levels of resistance to beta-lactams, aminoglycosides, and fluoroquinolones. These resistant strains also exhibited strong biofilm forming abilities, highlighting the connection between biofilm formation and multidrug resistance. Similarly, Aboelseoud et al. (2021) identified *Pseudomonas spp.*, *E. coli*, and other bacterial strains in poultry farm water systems that were resistant to multiple antibiotics and capable of forming dense biofilms.

The development of resistance is not limited to bacteria. Fungal pathogens such as *Candida* spp. and filamentous fungi within biofilms have demonstrated reduced susceptibility to antifungal agents. Kauffmann–Lacroix et al. (2016) observed that fungal biofilms in water systems exhibited increased resistance to conventional antifungals, a phenomenon attributed to both biofilm-mediated protection and inherent resistance mechanisms in the fungal cells.

Environmental stress factors in water systems, like limited nutrients, temperature changes and leftover disinfectants can trigger adaptive responses that enhance resistance. For instance, Yin et al. (2023) found that interactions between extracellular polymeric substances and corrosion products in pipes could shape microbial communities and promote the selection of resistant strains. These interactions often lead to complex biofilm architectures that are difficult to eradicate with standard treatment protocols.

The widespread use of antibiotics and disinfectants, particularly in agricultural and industrial settings, also contributes to the selection pressure driving resistance. Clark et al. (2025) documented the detection of antimicrobial resistance genes in biofilms from nearshore Antarctic environments exposed to sewage and wastewater, suggesting that such genes can be transported and maintained in extreme environments. This raises concerns about the global dissemination of resistance through water networks.

Furthermore, horizontal gene transfer (HGT) in biofilms is key to spreading resistance traits. The close cell arrangement within the biofilm matrix promotes the exchange of plasmids, transposons, and other mobile genetic elements that carry resistance genes (Shukla et al., 2021). As a result,

even non-pathogenic environmental bacteria can act as reservoirs, transferring resistance genes to pathogenic strains.

Given these challenges, there is a growing interest in alternative strategies for biofilm control that do not rely solely on traditional antimicrobials. Patra et al. (2022) explored marine antimicrobial peptides (AMPs) as potential biofilm disruptors, citing their broad-spectrum activity and lower likelihood of inducing resistance. Their study emphasized that AMPs could interfere with biofilm integrity by disrupting cell membranes or signalling pathways essential for biofilm maintenance.

2.7 Impacts of Pipe Materials and Water Chemistry on Biofilm Development

The physical and chemical characteristics of water distribution systems, particularly the type of pipe material and the quality of water chemistry, are essential for biofilm development and shaping microbial community structure. These factors not only influence the adhesion of microorganisms but also affect their growth dynamics, nutrient access, and resistance potential.

Different pipe materials, such as PVC, copper, stainless steel, and cast iron, have been shown to support varying levels of biofilm accumulation. Goraj et al. (2021) conducted a comprehensive study demonstrating that metallic surfaces, particularly iron-based materials, tend to support higher microbial diversity and biomass compared to non-metallic surfaces like PVC. This is attributed to the rough surface topography and corrosion potential of metal pipes, which provide more niches for microbial attachment and offer iron as a micronutrient that can enhance bacterial growth.

Corrosion is a particularly important factor in this context. Corrosion products, such as iron and manganese oxides, interact with extracellular polymeric substances (EPS) in biofilms, creating stable environments for microbial proliferation. Yin et al. (2023) showed that corrosion products can bind to microbial EPS, leading to the formation of compact and resilient biofilms. Additionally, the reaction between corrosion by-products and disinfectants can reduce the efficacy of the latter, allowing biofilms to persist even under treatment conditions.

Water chemistry—specifically pH, nutrient concentration, hardness, and residual disinfectants—also significantly influences biofilm development. For example, elevated levels of organic carbon, nitrogen, and phosphates can serve as nutrients for microbial metabolism and biofilm growth

(Shukla et al., 2021). Conversely, low-nutrient (oligotrophic) conditions may select for slow-growing but highly resilient microbial communities capable of forming robust biofilms.

Disinfectant residuals such as chlorine or chloramine are used to suppress microbial growth in distribution systems, but their effectiveness is limited by several factors. First, biofilms can consume and neutralize disinfectants before they penetrate deeply enough to kill embedded cells. Second, the biofilm matrix can act as a physical barrier, shielding microorganisms from chemical exposure (Elumalai et al., 2024). Erdei-Tombor et al. (2024) noted that disinfectant efficiency is further reduced in aged or corroded pipes, where biofilms are more firmly established and less accessible.

Moreover, the chemical interactions between disinfectants and pipe materials can produce disinfection by-products (DBPs), some of which may promote the growth of certain microbial groups or alter the microbial community composition (Goraj et al., 2021). These shifts can favour opportunistic pathogens such as *Legionella pneumophila* or *Mycobacterium avium*, which are known to persist in biofilm-protected environments.

Biofilm formation also varies with temperature and flow conditions. Stagnant zones or low-flow regions promote the accumulation of organic matter and microbial biomass, especially in warm climates. Akpan et al. (2013) found that stagnant zones in pipelines showed elevated biofilm formation, which correlated with higher corrosion rates. Seasonal temperature fluctuations can also alter biofilm structure and activity, with warmer temperatures generally enhancing microbial growth and EPS production.

2.8 Methods for Isolation and Characterization of Biofilm Microorganisms

The isolation and characterization of microorganisms within biofilms from water treatment systems are critical for understanding their composition, behaviour, and resistance traits. Over the years, multiple methods have been developed, combining classical microbiological techniques with modern molecular and imaging approaches.

Traditional culture-based methods remain fundamental for isolating viable bacteria and fungi from biofilms. These methods typically involve the mechanical disruption of biofilm matrices followed by serial dilution and inoculation onto selective and non-selective growth media. Such approaches

allow for the identification and enumeration of cultivable species and enable further analysis, such as antibiotic susceptibility testing. Aboelseoud et al. (2021) employed these methods to isolate and test bacterial strains from biofilms in poultry farm water systems, finding significant resistance to multiple antibiotics, a clear indicator of the risks associated with untreated or poorly managed biofilms.

However, a limitation of culture-based techniques is their inability to detect non-culturable or fastidious organisms, which may comprise a significant portion of the biofilm microbiome. To address this, molecular techniques such as PCR, qPCR, and DNA sequencing (16S rRNA and ITS regions) have been widely adopted. These tools allow researchers to identify bacteria and fungi at the genus or species level without the need for culturing (de Siqueira, 2011). For instance, de Siqueira's doctoral research utilized these molecular tools alongside microscopy to profile filamentous fungal biofilms found in drinking water systems, revealing a diverse array of fungal species not previously identified by conventional methods.

Fluorescence microscopy and scanning electron microscopy (SEM) provide visual insights into the structural complexity of biofilms as well as the arrangement of the microbial cells within these biofilms. CLSM in particular, when used with fluorescent staining (e.g., DAPI, SYTO 9, propidium iodide), can differentiate between living and dead cells and assess the architecture and thickness of biofilms (Elumalai et al., 2024). These methods are crucial for understanding microbial colonization patterns and EPS matrix composition.

Emerging technologies, including metagenomic sequencing and high-throughput amplicon sequencing, offer comprehensive data on the microbial community composition and functional potential. Such tools can detect unculturable microbes and provide insights into genes linked to virulence, biofilm formation and antibiotic resistance (Clark et al., 2025). Labella et al. (2021) applied such methods in a reverse osmosis water treatment plant, uncovering antibiotic resistance genes and biofilm-forming capacity in isolated bacterial strains, demonstrating the robustness of molecular techniques in tracking resistance in situ.

In addition, biochemical assays like the crystal violet assay and XTT reduction assay are employed to quantify biofilm biomass and metabolic activity, respectively. These are particularly useful for

evaluating the effects of antimicrobial treatments or environmental conditions on biofilm development (Elumalai et al., 2024).

Beyond individual characterization, some researchers have used community fingerprinting techniques like Terminal Restriction Fragment Length Polymorphism (T-RFLP) and Denaturing Gradient Gel Electrophoresis (DGGE) to compare microbial communities across different samples or time points. These methods, although semi-quantitative, provide rapid comparative analyses of microbial diversity and have been applied in longitudinal studies of biofilm development (Siqueira et al., 2013).

2.9 Emerging Strategies for Biofilm Control in Water Systems

Controlling biofilm formation in water treatment systems is a persistent challenge, especially given the increasing prevalence of antimicrobial-resistant organisms and the structural complexity of biofilms. Recent research has focused on novel and integrative strategies for biofilm management that extend beyond conventional disinfection methods such as chlorination and UV treatment.

One emerging strategy involves the use of antimicrobial peptides (AMPs), particularly those derived from marine organisms. These compounds disrupt biofilm integrity by targeting microbial membranes and interfering with quorum sensing mechanisms that mediate biofilm formation. Patra et al. (2022) highlight the effectiveness of marine AMPs in inhibiting bacterial adhesion and biofilm maturation across diverse environments. Their application in water systems, though still in the experimental phase, shows promise as a targeted approach that reduces reliance on harsh chemical disinfectants.

Another hopeful approach involves using quorum sensing inhibitors (QSIs) and enzymes that break down the EPS making up the biofilm matrix. These include dispersin B, DNases, and proteases, which weaken the structural cohesion of biofilms, making embedded microbes more susceptible to antimicrobial agents (Elumalai et al., 2024). QSIs specifically prevent microbial communication needed for biofilm initiation and maintenance, thereby reducing biomass accumulation without promoting resistance.

Materials engineering has also advanced with the development of anti-biofouling surfaces. Coating pipeline interiors with nanoparticles or antimicrobial polymers can reduce microbial adhesion and

limit initial colonization (Olanbiwoninu & Popoola, 2023). Additionally, pipe materials themselves influence biofilm formation; for instance, Goraj et al. (2021) demonstrated that stainless steel and polyvinyl chloride (PVC) were associated with significantly lower microbial biomass compared to traditional iron or concrete pipes. This finding suggests that infrastructure design choices can directly impact biofilm development and persistence.

Furthermore, the incorporation of real-time monitoring systems using biosensors and online flow cytometry provides continuous data on microbial loads and biofilm development in water systems. This enables early detection and timely intervention before biofilms become problematic. Erdei-Tombor et al. (2024) describe how sensor technologies integrated with smart treatment controls can dynamically adjust disinfectant dosages and flow rates based on microbial activity, optimizing system performance.

Physical cleaning methods, such as pigging (using devices to scrape biofilms from pipes) and high-pressure flushing, continue to be employed, often in combination with chemical treatments. However, these methods are labour-intensive and may not reach all parts of the distribution network. Their efficacy can be enhanced when used alongside targeted biocidal agents or enzymatic cleaners that penetrate and degrade biofilm matrices (Shukla et al., 2021).

Phage therapy, though less common in water systems, has also shown potential. Bacteriophages specific to biofilm-forming bacteria can selectively infect and lyse cells within the biofilm, reducing biomass without harming beneficial or non-target microorganisms. While still under exploration, this approach represents a biologically precise alternative to broad-spectrum antimicrobials (Mudau et al., 2024).

2.10 Knowledge Gaps and Future Research Directions

Despite considerable advances in the understanding of biofilm dynamics in water treatment systems, critical gaps persist that hinder the implementation of effective, evidence-based biofilm control strategies. A key limitation is the incomplete characterization of microbial diversity within biofilms, particularly the roles of non-bacterial organisms such as fungi, archaea, and protozoa. While studies have largely focused on bacterial communities due to their association with public health risks and infrastructure corrosion, recent research highlights the underestimated impact of

filamentous fungi and yeast-like organisms in biofilm resilience and water quality degradation (Kauffmann-Lacroix et al., 2016; Zhao et al., 2024).

Fungal organisms not only contribute to the physical robustness of biofilms but may also interact synergistically with bacteria, enhancing resistance to antimicrobial agents (Siqueira et al., 2013). However, most current monitoring and characterization techniques—particularly culture-dependent methods—tend to underrepresent these taxa, necessitating the broader application of high-throughput sequencing and metagenomics approaches (de Siqueira, 2011; Elumalai et al., 2024).

Another significant gap lies in the understanding of biofilm formation across different pipe materials and treatment stages. While some studies have explored material influence (Goraj et al., 2021), there is limited longitudinal data capturing how microbial communities evolve over time in response to shifts in hydraulic conditions, chemical dosing, and nutrient availability. Furthermore, the role of EPS in protecting embedded cells from oxidizing disinfectants remains poorly quantified in operational settings, despite its central importance in biofilm resilience (Yin et al., 2023).

Moreover, existing antimicrobial treatments often fail to consider the metabolic heterogeneity within biofilms, including the existence of dormant or slow growing persister cells. These phenotypic variants can survive biocidal treatments and repopulate the biofilm after treatment ceases (Patra et al., 2022). Few studies have explicitly targeted these subpopulations or evaluated the potential for resistance development under prolonged sublethal exposure, which is particularly concerning given the global rise in antimicrobial resistance (Clark et al., 2025).

The interplay between biofilms and emerging micropollutants—such as pharmaceuticals, heavy metals, and nanomaterials—also warrants further investigation. These compounds may influence biofilm development and microbial composition or serve as selective pressures that foster resistance traits (Yin et al., 2023). A more integrative understanding of these interactions is essential for risk assessment and treatment design.

In terms of technology, the field lacks standardized, cost-effective tools for in situ biofilm monitoring in full-scale systems. Although some biosensors and flow cytometry tools exist, they

are often limited by cost, operational complexity, and scalability (Erdei-Tombor et al., 2024). Developing user-friendly, portable detection systems would significantly improve real-time assessment and management of biofilms in diverse settings.

There is also a need for more comprehensive evaluations of novel interventions, such as antimicrobial peptides, phage therapy, and enzymatic treatments. While laboratory trials have shown promise, few have been translated into pilot-scale or full-scale applications in drinking water systems (Mudau et al., 2024; Patra et al., 2022). Bridging this gap requires interdisciplinary collaboration between microbiologists, engineers, and public health officials, as well as supportive regulatory frameworks for testing and deployment.

Lastly, climate change and urbanization are likely to alter water quality parameters, flow regimes, and microbial ecology in distribution systems. Yet, there is limited predictive modelling on how these shifts will affect biofilm formation, pathogen proliferation, and treatment efficacy in the coming decades. Developing predictive, system-specific models that incorporate microbial, chemical, and hydraulic data is essential for proactive water system management (Gaetano et al., 2024).

CHAPTER 3

Materials and Methods

3.0 Study Area

The study was conducted at a beverage manufacturing company in Harare, Zimbabwe. This plant sources council and borehole water which is then processed through in-house water treatment systems.

3.1 Water Sample Collection

3.1.1 Sampling Point

The water samples to be used for the project were collected at a beverage producing company. Special attention was taken to ensure that no cross contamination occurred during collection of the water samples. Two sampling points were selected, raw water and treated water

3.1.2 Sample Collection

Water samples were collected in sterile 250 ml Duran bottles. Aseptic techniques were employed to avoid cross contamination

3.1.2 Sample Movement

Once collected from the WTP in Harare, the water samples were moved to the microbiology laboratory at Bindura University of Science Education for testing, special attention and caution was taken to ensure that no cross contamination occurred during the movement of the water samples

3.2 Isolation of Microorganisms

3.2.1 Preparation of growth Media

Different media was used in the cultivation of the microorganism. Nutrient Agar (NA) was used as a general-purpose medium for the cultivation of both bacteria and fungi. MacConkey Agar was used for the selective growth of gram-negative bacteria. Potato Dextrose Agar (PDA) was used for the cultivation of yeasts and moulds.

Potato Dextrose Agar

PDA is a media that is commonly used in the cultivation of yeast cells and fungi. PDA was prepared by following the manufacturers preparation instructions. 39g of PDA was carefully weighed and suspended in 1000 ml of distilled water. The mixture was boiled until the PDA had completely dissolved and then autoclaved at 121℃ for 15minutes. After autoclaving, the agar as left to cool to 50℃ and poured into petri dishes.

MacConkey Agar

MacConkey agar is used to isolate gram-negative enteric bacteria and to differentiate between lactose fermenters and non-fermenters. The agar was prepared following manufacturer's instructions. To prepare it, 49.53 g of dehydrated medium was mixed into 1000 ml of distilled water and heated to boiling until fully dissolved. The mixture was then sterilized by autoclaving at 121°C for 15 minutes. After cooling to about 50°C, the agar was poured into petri dishes

Nutrient Agar

Nutrient Agar is a simple culture medium frequently used to grow non-fastidious microorganisms. It was prepared following manufacturer's instructions. It was prepared by mixing 28 g of dehydrated powder into 1000 ml of distilled water and heating the mixture to boiling until fully dissolved. The medium was sterilised by autoclaving at 121°C for 15 minutes. After cooling to around 50°C, the agar was poured into petri dishes.

Nutrient Broth

Nutrient broth is a basic culture medium that is commonly used to culture most microorganisms. Nutrient broth was prepared following manufacturer's instructions, 13g of the nutrient broth powder was dissolved in 1000 ml of distilled water and then p sterilised by autoclaving at 121°C for 15 minutes.

3.2.2 Inoculation of samples

The water samples were inoculated using direct plating. Direct plating was used for the isolation of the microorganisms.

Spread Plate

For spread plate, 1 ml of the water sample was inoculated onto the different agar plates. This was done by firstly shaking the water sample to ensure homogeneity then 1 ml was carefully pipetted

using a micropipette onto the correct agar. The pipette tips were changed each cycle to avoid cross contamination.

3.2.3 Incubation of agar plates

Once all the plates had been inoculated, they were incubated for different periods of time and temperature depending on the medium used. PDA plates were incubated at 25-30℃ for 72-120 hours. NA was incubated at 35-37℃ for 24-48hours. The agar plates were checked daily to monitor the growth of the microorganisms.

3.2.4 Isolation of Pure cultures

Once the various microorganisms had grown, the next step was to isolate pure cultures of each of the different colonies that appeared on the agar plates.

PDA

For yeasts, the colonies appear as small, round, and creamy white colonies. The yeast colonies were isolated by picking single colonies from the plate using a sterile loop and streaking it onto a new plate. The plates were incubated at 30℃ for 48hours. The steps were repeated until pure cultures were obtained, which was confirmed by observing uniform colony morphology. The mould observed on the plated was carefully isolated by cutting a small piece of it using a sterile scalpel blade and placing it on new media.

NA

The different colonies that were observed were carefully subculture using the same methods used for the yeasts. After the pure culture had been obtained, they were then grown on selective media to try to identify the bacteria.

3.3.0 Characterisation of microorganisms

For the identification of the bacteria and fungi, traditional identification tests such as morphological, biochemical and physiological tests were used (Kurtzman et al., 2011). These tests offered valuable information that aided in the identification of the bacteria. The morphological tests included looking at the colonies to examine their shape, size and colour. The biochemical tests helped to identify the metabolic properties of the bacterial isolates. The physiological tests assessed the tolerance of the bacteria to different environmental factors.

3.3.1 Morphological Tests

The bacterial and fungal isolates on the agar plates were initially characterised based on the colony morphology observed. The test involved checking the characteristics like colony shape, size, texture, colour, elevation and opacity. All the observed characteristics were recorded. The colonies were also viewed under a microscope to observe cellular structure (Doggett, 2000).

3.3.2 Biochemical Tests

Carbohydrate fermentation test

The different sugar solutions were prepared according to(Dahal, 2023b) but with slight alterations due to the available materials. 1% sugar solutions were prepared for glucose and sucrose as the available sugars. The 1% sugar solutions were made by weighing 1g of the sugar and dissolving it in 100 ml of distilled water. The sugar solutions were then supplemented with 0.02g of phenol red and 1.3g of NB. 10 ml of the solution was then carefully measured and put into test tubes with inverted Durham tubes and were sterilised by autoclaving. After autoclaving, the test tubes were left to cool.

Fresh cultures of the isolates, less that 48hours old were used in this test. A sterile inoculating loop was used to pick a well-isolated colony of the sample bacteria and inoculated into the broth with the with the different sugars, glucose and sucrose(Dahal, 2023b). The test tubes were incubated at 35℃ for 24-48hours with monitoring at 24 hours for colour changes and presence of trapped air in the Durham tubes. A positive result was indicated by the media changing colour from reddish orange to yellow(Dahal, 2023b). A negative result was shown when the medium did not change colour and stayed reddish orange(Dahal, 2023b). Gas production was indicated by formation of an air bubble in the Durham tube.

Urease test

Urea broth was made by following the instructions by(Sapkota, 2022b) but with some slight alterations due to the available materials. The broth was preprepared by carefully and accurately measuring 1g of dextrose, 1.5g of peptone, 5g of sodium chloride, 20g of urea, 2g of monopotassium phosphate and 0.012g of phenol red indicator(Sapkota, 2022b). all the ingredients were dissolved in 1000 ml of sterile distilled water. The broth was then poured into test tubes with

inverted Durham tubes added, and then the tubes were sterilised by autoclaving at 121℃ to for 15minutess. The media was left to cool after autoclaving and used.

Isolates to be used for the urease test were first dissolved in sterile distilled water and mixed with using a vortex meter to create a homogenous mixture. 1 ml of the isolate in solution was added to 10 ml of urea broth and vortexed to ensure proper mixing and then incubated at 35c for 24-48hours. Appositive result was demonstrated by a colour change from yellow to pink and a negative result was demonstrated by no colour change(Sapkota, 2022b)

Catalase test

A hydrogen peroxide solution for the catalase test was prepared by diluting 6% hydrogen peroxide stock solution with sterile distilled water in a 1:1 ratio to make a 3% solution. The solution was used after preparation. The catalase test was done following the protocol by(Sapkota, 2022a). The slide method was used. A small amount of the isolate which was 18-24hours old was collected using a sterile inoculating loop and placed onto a microscope slide. A drop of 3% hydrogen peroxide was dropped onto the microorganism using a dropper and the formation of bubbles was observed.

Oxidase Test

The oxidase test was done following the protocol described by (Dahal, 2023a). a well isolated colony of the test isolate was picked using a sterile inoculating loop and smeared over Whatman number 1 filter paper strip. Two drops od Kovacs' oxidase reagent were dropped onto the bacterial smear. The colour change and time taken for the colour change to happen were noted for up to 60 seconds. A colour change to purple or deep blue within 60 seconds was recorded as a positive result while no such colour change within that time was recorded as a negative result(Dahal, 2023a).

3.4.1 Biofilm Treatment Plan

To address the persistence of biofilms in water treatment pipelines, a chemical treatment strategy was developed using detergents and disinfectants that are known to have antibiofilm properties. The detergents tested included Sodium Dodecyl Sulfate (SDS), Benzalkonium Chloride (BAC), and Tween 80. A 5% solution of sodium hypochlorite (NaOCl) was used as a positive control, whereas sterile distilled water was used as a negative control. Treatments were prepared at 5%

concentration and applied for exposure periods of 5, 10, and 15 minutes. Each condition was tested in triplicate.

The crystal violet assay was done following the protocol (*Crystal Violet Staining Protocol | Abcam*, n.d.) with some slight alterations due to available resources. Biofilms were developed by inoculating sterile glass microscope slides placed in Petri dishes containing nutrient broth supplemented with 1% glucose. Individual bacterial or fungal isolates were used to form single-species biofilms. Slides were incubated statically at 30°C for 72 hours to allow mature biofilm formation. Post incubation, slides were rinsed with sterile saline and submerged in the respective treatment solutions.

Following treatment, slides were stained with 0.1% crystal violet solution for 10 minutes, rinsed, air-dried, and then de-stained with 95% ethanol. The eluted stain was transferred to cuvettes, and the optical density was measured at OD_{595} nm using a spectrophotometer. Percentage reduction in biofilm mass was then calculated relative to the untreated control using the formula:

$$\%\, Reduction \;=\; \frac{[(OD\_control \;-\; OD\_treatment)}{[OD\_control]} \times 100$$

Chapter 4

Results

4.1 Morphological Data

Table 1 shows the morphological results of the isolates that were isolated from the water samples

Table 1: Colony Morphology of Bacterial and Yeast Isolates

Isolate	Colony Shape	Margin	Elevation	Colour	Surface Texture	Opacity
Coliform Raw Water 1	Circular	Entire	Raised	Cream/light tan	Smooth	Opaque
Coliform Raw Water 2	Circular	Entire	Mucoid	Pale/off-white	Shiny, viscous	Opaque
Coliform Raw Water 3	Irregular	Wavy	Raised	Light brown	Moist	Opaque
Coliform Raw Water 4	Circular	Entire	Flat	Pale yellow	Smooth	Opaque
Coliform Treated Water 1	Circular	Entire	Convex	Cream	Mucoid	Opaque
Yeast Raw Water 1	Round	Entire	Raised	White to cream	Buttery	Opaque
Yeast Raw Water 2	Circular	Smooth	Convex	Cream to pinkish	Mucoid	Translucent
Yeast Treated Water 1	Irregular round	Lobate	Raised	Pink-red	Smooth	Opaque

Yeast Treated Water 2	Circular	Entire	mucoid	Cream to pink	Slimy	Translucent

Table 2 below, show the morphological characteristics of the fungal isolates from the water samples

Table 2: Morphological Characteristics of Fungal Isolates

Isolate	Surface Colour	Texture	Margin	Elevation
Raw Water	White, cottony, dense	Woolly, fluffy	Entire	Raised
Treated Water	Red centre fading to pale pink to cream edge	Suede-like, velvety	Entire, fading	Slightly raised

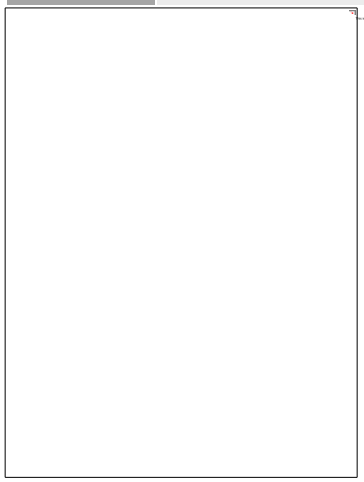

Figure 3: Raw Water Fungi

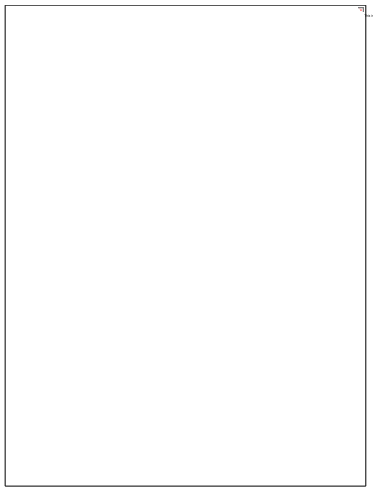

Figure 4: Treated Water Fungi

4.2 Biochemical Profiles

Table 3 shows the results of the sugar fermentation test on the isolates

Table 3: Results of Sugar Fermentation Test

Isolate	Glucose	Sucrose	Gas Production
Coliform Raw Water 1	+	+	+
Coliform Raw Water 2	+	+	–
Coliform Raw Water 3	+	+	+
Coliform Raw Water 4	+	+	+
Coliform Treated Water 1	+	+	–
Yeast Raw Water 1	+	–	–
Yeast Raw Water 2	–	–	–
Yeast Treated Water 1	–	–	–
Yeast Treated Water 2	–	–	–

Table 4 shows the combined results of the Catalase, urease and oxidase test results done on the isolates.

Table 4: Catalase, Urease and Oxidase Test Results

Isolate	Catalase	Urease	Oxidase
Coliform Raw Water 1	Positive (+)	Negative (–)	Negative (–)
Coliform Raw Water 2	Positive (+)	Positive (+)	Negative (–)
Coliform Raw Water 3	Positive (+)	Weak Positive (+/–)	Negative (–)
Coliform Raw Water 4	Positive (+)	Negative (–)	Negative (–)
Coliform Treated Water 1	Positive (+)	Positive (+)	Negative (–)
Yeast Raw Water 1	Negative (–)	Negative (–)	Not applicable
Yeast Raw Water 2	Negative (–)	Positive (+)	Not applicable
Yeast Treated Water 1	Negative (–)	Negative (–)	Not applicable
Yeast Treated Water 2	Negative (–)	Positive (+)	Not applicable

4.3 Microbial Identification

Based on the morphological and biochemical characteristics, the isolates were presumptively identified as shown in Table 5

Table 5: Presumptive Identification of Isolates

(NB: Species Level Identification is presumptive and based on the observed morphological and biochemical profiles.)

Isolate	Presumed ID
Coliform Raw Water 1	*Escherichia coli*
Coliform Raw Water 2	*Klebsiella spp*

Coliform Raw Water 3	*Enterobacter spp.*
Coliform Raw Water 4	*Escherichia coli*
Coliform Treated Water 1	*Klebsiella spp.*
Yeast Raw Water 1	*Candida albicans*
Yeast Raw Water 2	*Cryptococcus spp.*
Yeast Treated Water 1	*Rhodotorula spp.*
Yeast Treated Water 2	*Cryptococcus spp.*
Raw water Mould	*Fusarium spp.*
Treated Water Mould	*Fusarium spp. or pigmented Trichoderma spp*

4.4 Biofilm Removal

The tables below (table 6-8) show the mean OD_{595} values with standard deviation for the crystal violet assay at different times (5, 10 and 15 minutes.)

Table 6 : Mean OD595 values at 5 Minutes

Detergent	Mean and SD
BAC	0.25 ± 0.04
Tween	0.22 ± 0.03
SDS	0.28 ± 0.11
NaOCl	0.55 ± 0.03
Distilled Water	0.91 ± 0.01

Table 7: Mean OD595 values at 10 Minutes

Detergent	Mean and SD
BAC	0.16 ± 0.05
Tween	0.15 ± 0.03
SDS	0.2 ± 0.06

NaOCl	0.53 ± 0.04
Distilled Water	0.91 ± 0.01

Table 8: Mean OD595 Values at 15 Minutes

Detergent	Mean and SD
BAC	0.11 ± 0.03
Tween	0.08 ± 0.02
SDS	0.14 ± 0.04
NaOCl	0.47 ± 0.08
Distilled Water	0.91 ± 0.01

The graph below shows the percentage reduction of the biofilm by the different detergents used.

Figure 5: Biofilm Reduction

Statistical Analysis

A two-way ANOVA confirmed that both treatment type ($F=53.16$, $p<0.001$)) and exposure time ($F=2898.09$, $p<0.001$) significantly influenced biofilm reduction, and significant interaction effect ($F=54.99$, $p<0.001$).

Chapter 5

Discussion, Summary, Recommendations and Conclusion

This chapter focuses on interpreting the results from the project and linking them to the results that were found by other researchers

5.1 Discussion

5.1.1 Overview of findings

This study aimed to isolate and characterise bacteria and fungi from biofilm buildup in water treatment plant pipelines, and to assess the effectiveness of selected chemical agents at reducing the biofilm mass. The microbial isolates were characterised using biochemical tests. The results showed the presence of common biofilm forming organisms such as presumed *Escherichia coli, Klebsiella spp., Candida albicans, Cryptococcus spp. and Fusarium spp*. The treatment phase revealed that sodium hypochlorite (NaOCl) was the most effective biofilm reduction agent, but its corrosiveness raises operational concerns, followed by benzalkonium chloride (BAC), sodium dodecyl sulphate (SDS), and Tween 80. Biofilm biomass reduction increased with exposure time for all treatments. The following sections interpret these findings with context to existing biofilm management strategies and any operational constraints.

5.1.2 Interpretation of results

5.1.2.1 Microbial Identity and Biochemical Profiles

The isolates form the water samples were presumptively identified based on their biochemical properties. The biochemical characteristics were compared with those in literature. The coliforms RW1 and RW4 were presumptively identified as *E. coli*. *E. coli* is known to typically produce gas during the fermentation of glucose but does not always ferment sucrose (Yamada et al., 2011). Isolates RW1 and RW4 showed discolouration of MacConkey Agar further supporting their presumptive identification as *E. coli* The coliforms RW1 and TW1 were presumptively identified as from the *Klebsiella spp*. because they were both able to ferment both glucose and sucrose and they were urease positive, which are traits that are commonly used for the presumptive identification Chen et al., 2013). TW1 was also presumptively identified as *Klebsiella spp.* due to having mucoid colonies(Zhang et al., 2015).

The fungal isolates showed traits that were consistent with that of *Candida spp* (glucose and sucrose fermentation) and *Cryptococcus* (less fermentative, but positive for glucose fermentation under biofilm conditions) (Kelly & Kwon-Chung, 1992; Jain et al., 2009). The other biochemical profiles obtained from the catalase, urease and oxidase activity further supported the identification of the coliforms and yeasts. Oxidase-negative and catalase-positive results are characteristic of *Enterobacteriaceae*(Dahal, 2023a; Sapkota, 2022a). Urease activity was particularly relevant for differentiating *Klebsiella and Cryptococcus spp* from other enteric organisms(Sapkota, 2022b).

In addition to the bacterial and yeast isolates, filamentous fungi were also isolated from the water samples. There were two distinct colony types, one with a white cottony colonies that are characteristic of *Fusarium spp.* (Domsch et al., 1980). The mould found in treated in treated water had pink pigmented colonies which could suggest the identity to be either *Fusarium* or *Trichoderma spp.* both of which have been frequently isolated from industrial biofilms(Domsch et al., 1980)

5.1.2.2 Biofilm Treatment

The biochemical profiles that are described above not only enabled for the taxonomic classification of the isolates, but it also revealed functional traits that govern the biofilm persistence. The urease activity observed in the presumed *Klebsiella* elevates the local pH which then promotes the EPS cross linking which leads to enhanced detergent resistance and can also contribute to pipe corrosion via ammonia production (Akpan et al., 2013). the ability of some of the isolates to ferment glucose gives them the ability to sustain their growth in nutrient poor pipes.

Consistent with literature, NaOCl(5%) achieved the highest biofilm reduction(91% at 15minutes), due to its oxidative disruption of both microbial cells and the EPS matrix (Sharma et al., 2016). BAC, which is a quaternary ammonium compound followed closely (87% reduction) likely y destabilising lipid membranes and the EPS (Gilbert & Moore, 2005). SDS, which is less biocidal, can disrupt the liquid membranes reducing the biofilm mass moderately. Tween 80, which is a non-ionic surfactant, had the poorest performance(49%) of the tested detergents, which corresponds with the finding by Kim et al. (2014) that Tween 80 primarily facilitates the dispersion but not killing of microbes.

NaOCl which was used as the positive control for the biofilm reduction test showed an average of 91% biofilm removal at 15 minutes. This result matches the results by Patra et al. (2022) who

reported 90-95% reduction in *Pseudomonas* biofilms at 5% NaOCl. These findings contradict with those found by (Elumalai et al., 2024) who observed complete eradication. This divergence may be because of the different conditions and strain specific EPS composition.

BAC showed an 87% biofilm reduction and is known to be less corrosive than NaOCl. The BAC results were similar with those of (Goraj et al., 2021) who found that quaternary ammonium compounds (QACs) achieved greater that 85% reduction in PVC pipes without any material damage. The slow action of SDS mirrored the results by Shukla et al. (2021), who noted that anionic detergents need a prolonged exposure to solubilise the polysaccharide rich EPS. The weak performance of Tween 80 (49%) reduction was due to the fungal EPS barrier(Siqueira et al., 2011). The *Cryptococcus's* urease activity may have elevated the local pH causing the Tween 80 to hydrolyse into inactive fatty acids(Patra et al., 2022)

The data from the biofilm reduction revealed that not a single treatment was able to eradicate all of the biofilm components, hence there is need for a combinatorial approach. For bacteria dominated biofilms, the combination of BAC and a catalase inhibitor can prevent oxidative defence of microorganisms. For fungal-bacterial mixes, a combination of SDS and β-glucanase can degrade the fungal EPS while being able to solubilise bacterial matrices(Doggett, 2000). The supporting evidence for this combination is that (Huang et al., 2022) achieved 95% in similar consortia using that combo. The use of copper infused polymers can reduce the adhesion of *Cryptococcus* by 70%(Goraj et al., 2021). There is also the need to deploy ATP bioluminescence assays that are able to detect the regrowth of biofilms post treatment (Erdei-Tombor et al., 2024).

5.1.2.3 Statistical Analysis

To determine whether the treatment effects and exposure duration on biofilm reduction had a statistical significance, a two-way ANOVA with interaction was performed using the OD595 values obtained from the crystal violet assay. The factors that were analysed were the type of detergent used (BAC, SDS, Tween 80 and NaOCl) and the exposure time (5, 10 and 15 minutes). The ANOVA results showed a significant main effort for both the treatments. The interaction between the treatment and time was also statistically significant (p value <0.0010). The results suggested that the type of chemical used and the duration of the exposure both independently and interactively influence the extent of biofilm reduction. The ANOVA results implied that the efficacy of the detergents was not consistent across the time intervals, some treatments became

more effective with longer exposure (BAC, SDS, NaOCl), while others, such as Tween 80 showed moderate improvements.

5.2 Summary

This study investigated the microbial composition of biofilms that were collected from pipelines in a water treatment plant. The bacterial and fungal isolates were obtained using selective culture media and they were characterised using morphological and biochemical tests. Presumptive identification of the isolated microorganisms pointed to organisms like *E. coli*, *Klebsiella pneumoniae*, *Candida albicans*, and *Cryptococcus* spp. A biofilm treatment plan was designed to evaluate the efficacy of three chemical agents: SDS, BAC and Tween 80. Results using crystal violet assay showed that BAC was the most efficient followed by SDS with Tween 80 being the least effective. Biofilm reduction increased with longer exposure times across all treatments. The study highlights the resilience of mixed microbial communities in water pipelines and the need for multitargeted cleaning strategies.

5.3 Recommendations

This study presumptively identified the isolates based on morphological and biochemical profiles, but future studies should use molecular techniques like 16S rRNA sequencing for bacteria and ITS region sequencing for fungi to confirm microbial identities with higher accuracy. This would address the limitation of having to rely solely on phenotypic tests. Another recommendation for similar future studies is to incorporate viable cell count methods. Viable count assays like CFU/ml better assess the effectiveness of biofilm treatment as they help to distinguish between biofilm mass reduction and microbial killing. This study identifies NaOCl and BAC as potent biofilm control agents in WTP pipelines, but their long-term use may face challenges such as the corrosiveness of NaOCl and the resistance of BAC. For sustainable management, I would recommend combination therapies like pairing BAC with EPD-degrading enzymes to enhance penetration. For future studies, there would be need to expand the range of the treatment agents, exploring newer or synergistic agents such as enzymes, essential oils, biofilm dispersing peptides especially focusing on those that target fungal elements. Future work should also assess the financial and environmental impacts of the proposed chemical treatments to see whether its affordable and compatible with existing systems. Another recommendation would be the

development of preventative cleaning protocols that means that there would be routine dosing schedules

5.4 Conclusion

In conclusion, the study successfully isolated and characterised diverse bacterial and fungal species from biofilms in WTP pipelines. The study revealed the presence of potentially pathogenic organisms like *E. coli*, *K. pneumoniae*, *C. albicans*, and *Fusarium spp.* the evaluation of chemical treatments on the biofilms demonstrated that chemical like BAC and NaOCl were highly effective in the reduction of biofilm biomass, while chemicals like SDS and Tween 80 showed moderate efficacy. These findings aligned with existing literature on biofilm resistance, and it put an emphasis on the limitations of using single- agent treatments. The statistical significance of the exposure time and the treatment type showed the importance of prolonged and combinatorial approaches for proper biofilm management. Future research similar to this study should integrate molecular identification techniques, viable cell counts and environmentally sustainable agents to optimise biofilm control strategies. This study contributes to further understanding biofilm ecology in WTPs and provides a foundation for developing targeted interventions to safeguard infrastructure and water quality.

References

Aboelseoud, H., Ismael, E., Moustafa, G. Z., & Badawy, E. M. (2021). Hygienic studies on biofilms in drinking water systems in poultry farms: Isolation, molecular identification, and antibiotic sensitivity. *Journal of Animal Health and Production*, 9(4), 443–454.

Akpan, G. U., Abah, G., & Akpan, B. D. (2013). Correlation between microbial populations isolated from biofilms of oil pipelines and corrosion rates. *The International Journal of Engineering and Science*, 2(5), 39–45.

Bezuidenhout, C. (2014). Biofilm formation in surface and drinking water distribution systems in Mafikeng, South Africa. *South African Journal of Science*. https://doi.org/10.1590/SAJS.2014/20130306

Crystal violet staining protocol | Abcam. (n.d.). Retrieved June 12, 2025, from https://www.abcam.com/en-us/knowledge-center/cell-biology/crystal-violet-staining-protocol

Dahal, P. (2023a, March 31). *Oxidase Test- Principle, Procedure, Types, Results, Uses*. https://microbenotes.com/oxidase-test-principle-procedure-and-results/

Dahal, P. (2023b, April 13). *Carbohydrate Fermentation Test (Sugar Fermentation Test)*. https://microbenotes.com/carbohydrate-fermentation-test/

Doggett, M. S. (2000). Characterization of Fungal Biofilms within a Municipal Water Distribution System. *Applied and Environmental Microbiology*, *66*(3), 1249–1251. https://doi.org/10.1128/AEM.66.3.1249-1251.2000

Domsch, K. H., Gams, W., & Anderson, T.-H. (1980). *Compendium of soil fungi*. Academic Press.

Elumalai, P., Gao, X., Cui, J., Kumar, A. S., Dhandapani, P., Parthipan, P., Karthikeyan, O. P., Theerthagiri, J., Kheawhom, S., & Choi, M. Y. (2024). Biofilm formation, occurrence, microbial communication, impact and characterization methods in natural and anthropic systems: A review. *Environmental Chemistry Letters*, *22*(3), 1297–1326. https://doi.org/10.1007/s10311-024-01715-5

Erdei-Tombor, P., Kiskó, G., & Taczman-Brückner, A. (2024). Biofilm Formation in Water Distribution Systems. *Processes*, *12*(2), Article 2. https://doi.org/10.3390/pr12020280

Goraj, W., Pytlak, A., Kowalska, B., Kowalski, D., Grządziel, J., Szafranek-Nakonieczna, A., Gałązka, A., Stępniewska, Z., & Stępniewski, W. (2021). Influence of pipe material on biofilm microbial communities found in drinking water supply system. *Environmental Research*, *196*, 110433. https://doi.org/10.1016/j.envres.2020.110433

Huang, L., Jin, Y., Zhou, D., Liu, L., Huang, S., Zhao, Y., & Chen, Y. (2022). A Review of the Role of Extracellular Polymeric Substances (EPS) in Wastewater Treatment Systems. *International Journal of Environmental Research and Public Health*, *19*(19), 12191. https://doi.org/10.3390/ijerph191912191

Labella, A., Molero, R., Leiva-Rebollo, R., Pérez-Recuerda, R., & Borrego, J. J. (2021). Identification, resistance to antibiotics and biofilm formation of bacterial strains isolated from a reverse osmosis system of a drinking water treatment plant. *Science of The Total Environment*, 774, 145718.

Mudau, K. L., Kalu, C. M., Masindi, V., Maphangwa, K. W., & Tekere, M. (2024). Tracking bacteria and fungi diversity from natural water sources to drinking water treatment plants and distribution systems—A review. *Applied Ecology & Environmental Research*, 22(1).

Olanbiwoninu, A., & Popoola, B. M. (2023). Biofilms and their impact on the food industry. *Saudi Journal of Biological Sciences*, 30(2), 103523.

Patra, A., Das, J., Agrawal, N. R., Kushwaha, G. S., Ghosh, M., & Son, Y.-O. (2022). Marine Antimicrobial Peptides-Based Strategies for Tackling Bacterial Biofilm and Biofouling Challenges. *Molecules (Basel, Switzerland)*, *27*(21), 7546. https://doi.org/10.3390/molecules27217546

Sapkota, A. (2022a, January 10). *Catalase Test- Principle, Procedure, Types, Results, Uses*. https://microbenotes.com/catalase-test-principle-procedure-and-result-interpretation/

Sapkota, A. (2022b, January 23). *Urease Test- Principle, Media, Procedure, Result, Uses*. https://microbenotes.com/urease-test-principle-procedure-and-result/

Shukla, S. K., Khan, A., & Rao, T. S. (2021). Microbial fouling in water treatment plants. In T. S. Rao (Ed.), *Microbial and Natural Macromolecules* (pp. 589–622). Academic Press.

Siqueira, V. M., Oliveira, H. M. B., Santos, C., Paterson, R. R. M., Gusmão, N. B., & Lima, N. (2011). Filamentous Fungi in Drinking Water, Particularly in Relation to Biofilm Formation. *International Journal of Environmental Research and Public Health*, *8*(2), 456–469. https://doi.org/10.3390/ijerph8020456

Yin, H., Wang, H., Wang, M., & Shi, B. (2023). The interaction between extracellular polymeric substances and corrosion products in pipes shaped different bacterial communities and the effects of micropollutants. *Water Research*, 247, 120822.

Zhang, X., Li, X., Wang, M., Yue, H., Li, P., Liu, Y., Cao, W., Yao, D., Liu, L., Zhou, X., Zheng, R., & Bo, T. (2015). Outbreak of NDM-1-Producing *Klebsiella pneumoniae* Causing Neonatal Infection in a Teaching Hospital in Mainland China. *Antimicrobial Agents and Chemotherapy*, *59*(7), 4349–4351. https://doi.org/10.1128/AAC.03868-14

Zhao, H. X., He, H., Zeng, C., Zhang, T. Y., Hu, C. Y., Pan, R., ... & Xu, B. (2024). Overlooked role of fungi in drinking water taste and odor issues. *Environmental Science & Technology*, 58(40), 17817–17827.

Appendices

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Sample	195.8181	11	17.80	53.16	4.39755E-30	1.92
Columns	1940.815	2	970.41	2898.09	1.57707E-69	3.12
Interaction	405.0863	22	18.41	54.99	2.23334E-36	1.69
Within	24.10876	72	0.33			
Total	2565.828	107				

Appendix 1: Two-way ANOVA for biofilm reduction results.

		5 minutes	
Treatment	**Rep 1**	**Rep 2**	**Rep 3**
BAC	0.298	0.228	0.227
NaOCl	0.235	0.19	0.245
SDS	0.236	0.201	0.403
Tween	0.586	0.518	0.54
		10 minutes	
Treatment	**Rep 1**	**Rep 2**	**Rep 3**
BAC	0.207	0.11	0.15
NaOCl	0.18	0.134	0.134
SDS	0.224	0.245	0.14
Tween	0.488	0.57	0.529
		15 minutes	
Treatment	**Rep 1**	**Rep 2**	**Rep 3**
BAC	0.091	0.1	0.152
NaOCl	0.1	0.08	0.067
SDS	0.142	0.188	0.099
Tween	0.426	0.558	0.415

Appendix 1: OD595 Results