TREATMENT OF BIOFILMS AND OPPORTUNISTIC PATHOGENS IN SIMULATED DRINKING WATER DISTRIBUTION SYSTEMS USING UV LEDs

by

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DEDICATION PAGE

To my family, which has always supported me in every decision in favour of my future. Especially to my grandparents, Mario and Esther; to my parents, Esther and Gustavo; and my sisters, Diana and Eunice. Thanks for constantly checking on me and for all your love.

A mi familia, quien me ha apoyado siempre en cada decisión en favor de mi futuro. Especialmente a mis abuelitos, Mario y Esther; a mis padres, Esther y Gustavo; y a mis hermanas, Diana y Eunice. Gracias por siempre estar al pendiente de mí y por todo su amor.

To my hermanito, Arshdeep Singh, for becoming part of my family and always being there for me when I most needed it. I don't know what would have happened to me if you weren't with me. Thanks for all those Ice coffees, chai, and Wendy's meals. Thank you my hermano.

Mere hermanito Arshdeep Singh noo, mere parivaar da hissa banan layi te hamesha mere layi kharhan layi jadon mainu sabh ton vadd lorh see. Mainu nahi pata mainu kee ho jaanda je toon mere naal nayi hunda. Ohna sariaan ice coffee, chah te Wendy de khaane layi. Tera dhanvaad mere hermano.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Biofilm formation in drinking water distribution systems (DWDS) has important implications for drinking water treatment and protecting public health due to the potential proliferation of opportunistic pathogens (OPs). To inhibit the formation of biofilms and OPs in DWDS, Ultraviolet Light Emitting Diode (UV-LED) technologies show great promise due to their small footprint and versatility. This research aimed to expand the understanding of biofilm growth in DWDS and the application of UV LEDs to inactivate OPs and biofilms. Specifically, this thesis examined: (1) the inactivation of *Legionella pneumophila* monocultures and *Pseudomonas fluorescens* biofilms grown in CDC biofilm reactors on cast iron and stainless steel coupons, and (2) the inactivation of tap water natural microbiome biofilms (NMB) grown on biostud reactors. In both tasks, organisms were cultured and subsequently exposed to UV LEDs at 280 and 365 nm. Regardless of the culture, inactivation performance was greater with a wavelength of 280 nm; however, 365 nm could be further investigated for possible photolytic reactions. This study provides key information for addressing opportunistic pathogens in biofilms and further work should consider scaling.

LIST OF ABBREVIATIONS AND SYMBOLS USED

±	Plus or minus symbol
°C	Degree Celsius
µl	Micro Liters
µM	Micro moles
$\mu W/cm^2$	Microwatts per centimetre square
ATP	Adenosine Triphosphate
BCYE	Buffered Charcoal Yeast Extract
BSC	Biosafety cabinet
CDC	Centre for Disease Control
CFU	Colony-forming unit
CFU/cm^2	Colony-forming unit per centimetre square
CFU/ml	Colony-forming unit per millilitre
CI	Cast Iron
CSTR	Continuous stirred tank reactor.
DBPs	Disinfection-by-products
DNA	Desoxyribonucleic acid
DSA	Deposit and surface analysis
DWDS	Drinking water distribution systems
EPA	Environmental protection agency
EPS	Extracellular polymeric substances
GAC	Granular activated carbon
GCDWQ	Guidelines for Canadian Drinking Water Quality
h	Hour
HPC	Heterotrophic plate count
$k_{biofilms}$	Rate growth constant of biofilms ($days^{-1}$)
k_{max}	First-order inactivation rate constant ($cm^2\ mJ^{-1}$)
L	Litre
L. pneumophila	*Legionella pneumophila* (ATCC 33152)
LEB	Legionella Enrichment Broth
Ln	Natural logarithm
Log	Logarithm
Log_{10}	Logarithm base 10
LP UV	Low-pressure ultraviolet lamp
LPM	Litres per minute
LRV	Log Reduction Value
mg/L	Milligrams per litre
mJ/cm^2	Millijoules per centimetre square
mL	Millilitres

mm	Millimetres
nm	Nanometers
OPs	Opportunistic Pathogens
P. fluorescens	*Pseudomonas fluorescens* (ATCC 17569)
PBS	Phosphate-buffered saline
POE	Point of entry
POU	Point of use
PVC	Polyvinyl chloride
qPCR	Quantitative polymerase chain reaction
R2A	Reasoner's 2A
rpm	Revolutions per minute
SPC	Standard Plate Counts
SS	Stainless Steel
tATP	Total adenosine triphosphate
TSA	Tryptic soy agar
TSB	Tryptic soy broth
UV	Ultraviolet
UV LEDs	Ultraviolet light-emitting diodes
UVT%	Percentage of UV Transmittance at 254nm
VBNC	Viable but nonculturable
WHO	World Health Organization
MP UV	Medium-pressure ultraviolet lamps

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CHAPTER 1 INTRODUCTION

1.1. Biofilms as a current health risk

The presence of pathogens often compromises drinking water quality. The consumption of this type of water results in a wide range of diseases and health-related problems for susceptible people like young children, infants, the elderly, and immunocompromised people (Boyle et al., 1991; López et al., 2010; Chaves Simões & Simões, 2013; Health Canada, 2020). One of the current main concerns is biofilms, naturally immobilized cells deposited on a surface. They are ubiquitous, making it hard to develop efficient techniques for their control. Although biofilms have essential applications in the energy and chemical industry, they also pose severe concerns in the medical field as they cause around 75% of the microbial infections in a hospital scenario. Moreover, in food industries, biofilms are related to food spoilage (Muhammad et al., 2020); they are also a concern in the industrial setting regarding industrial equipment contamination, which can lead to potential loss of productivity and spreading of pathogens (Wilson et al., 2017). Nevertheless, recent cases of biofilm risks are related to the drinking water distribution systems (DWDS) because biofilms can host pathogenic microorganisms which produce a wide range of infectious diseases in humans (Romani et al., 2016; Shen, 2016; Coraça-Huber et al., 2020; Muhammad et al., 2020; Mazaheri et al., 2022).

1.2. Biofilms and Pathogens in Drinking Water Distribution Systems

Over the years, biofilms have developed the ability to attach to the walls of water pipes and create highly efficient and stable ecosystems, which are well adapted to survive prolonged periods of environmental stress (Declerck et al., 2009).

Within the distribution systems (Figure 1), the water flow carries a constant flux of nutrients, mixing, and substrate, which allow the microorganisms to deposit into the surface of the pipe and start adhering to it (Fish et al., 2016).

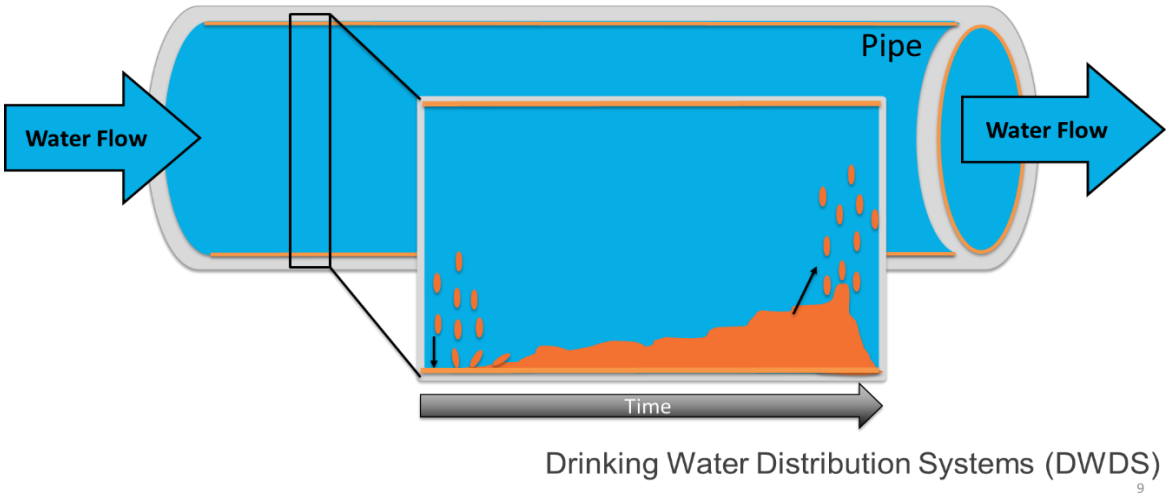

Figure 1. Biofilms in Drinking Water Distribution System

Over time, biofilms start forming, colonizing the surface of the pipe and causing several problems such as microbial-inducing corrosion, aesthetic issues (odour, colour and taste issues) and flow difficulties due to clogging; nonetheless, the biggest concern is health-related matters due to those biofilms provide habitat for waterborne pathogens (Fish et al., 2016; Fish & Boxall, 2018; Tsagkari & Sloan, 2018; Tang et al., 2021; Munoz et al., 2022). These pathogens often infect humans by getting into the drinking water distribution systems and getting attached to biofilms, which can provide them resistance to disinfectants (Boyle et al., 1991; Tang et al., 2021).

1.3. Current biofilm mitigation practices

The standard practice for drinking water disinfection is chemical addition, in which disinfectant substances such as chlorine (Cl_2), sodium hypochlorite (NaOCl) or monochloramines (NH_2Cl) are added. These substances produce a residual effect in the water, helping to mitigate microbial regrowth and contamination during distribution (Gagnon et al., 2008; Fish & Boxall, 2018). Despite using disinfectants in DWDS, the concentrations decrease along the service line due to residual decays, promoting bacterial activity and regrowth (Fish & Boxall, 2018). In addition, recent studies show that microorganisms in a biofilm matrix have been gaining more resistance to antimicrobials (Fish et al., 2016; Zhang et al., 2019; Munoz et al., 2022). Although chemical disinfection has been used for centuries (Shekhawat et al., 2021), these chemicals often react with the natural organic matter (NOM), producing disinfection-by-products (DBPs), which are toxic and cancerogenic to humans (Gule et al., 2016; MacIsaac, 2021; J. Li et al., 2023).

Alternate strategies for chemical disinfection include antifouling and antibacterial surfaces and coating development. These are superhydrophobic surfaces, thus eliminating adherence of biofilms on surfaces and introducing chemically bound antimicrobial agents on surfaces, like sodium silicate, which destroy microbes attempting to attach to the surfaces (Gule et al., 2016; Verderosa et al., 2019; Muhammad et al., 2020; Munoz et al., 2022). However, these treatments can have a very high initial cost and are susceptible to mechanical damage (Han et al., 2020).

1.4. UV LEDs as a biofilm-pathogen control

Conventional UV disinfection has been preferred over chemical disinfection because it does not change the water quality and does not form DBPs; it has also been proven effective

for inactivating bacteria, viruses and protozoa (Bolton & Cotton, 2022). The UV light penetrates the cell wall modifying the DNA structure (Figure 2), thereby preventing its reproduction.

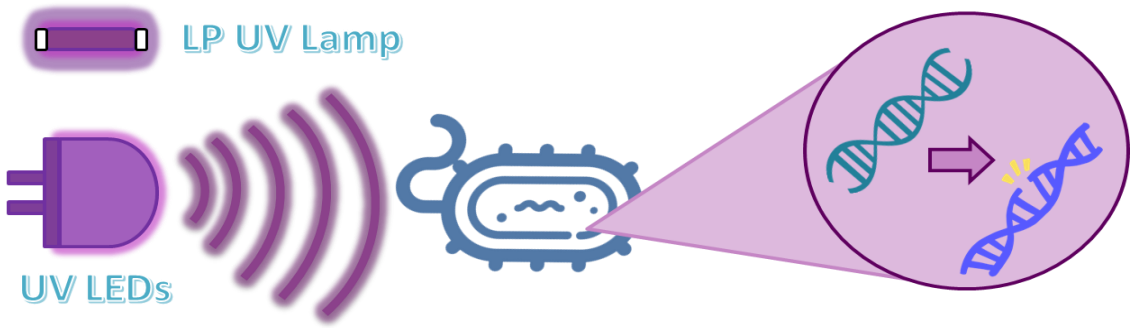

Figure 2. UV LEDs as a Biofilm-Pathogen control

Nonetheless, current UV devices are Low Pressure (LP) Lamps and Medium Pressure (MP) Lamps, which are mercury-based; so, breakage of these lamps represents an environmental hazard and requires special disposal conditions. Moreover, these lamps require high energy, leading to high operation costs and maintenance due to their relatively short life (Song et al., 2016). Over the past years, Ultraviolet light-emitting diodes (UV LEDs) have emerged as an alternative to mercury-free UV sources. LEDs have various advantages: they can operate at different wavelengths, and even multiple wavelengths; typically, conventional lamps use only 254 nm; they can also be used in compact or robust design, giving them design versatility; in addition, they have faster start-up time and lower voltages (Asif Khan, 2006; Muramoto et al., 2014; Chen et al., 2017; G. Q. Li et al., 2017; Kebbi et al., 2020; Wan et al., 2022). The need to use UV LEDs is due to the persistence of biofilms and pathogens, like *Cryptosporidium, Giardia, Pseudomonas or Legionella*, which are known to be resistant to disinfectants (AWWA, 2006). UV LEDs technology, have shown capacity to inactivate with significant efficiency pathogens and biofilms at relatively low fluences

(Rattanakul & Oguma, 2018; Gora et al., 2019; Galezzo & Susa, 2021; Ma et al., 2022; Wan et al., 2022). Despite its efficiency, more studies need to be done to understand biofilm formation in water distribution systems to address the following gaps: pathogen organisms, representative surfaces, UV wavelengths higher than 254 nm and different water matrices. In addition, wavelength adjustment also needs to be investigated, as recent information proposes that using multiple wavelengths could have synergistic effects, preventing microbial reactivation (Green et al., 2018; Linden et al., 2019; Galezzo & Susa, 2021; Wan et al., 2022).

1.5. Research Objectives

This dissertation aims to expand the understanding of biofilm growth in DWDS and the application of UV LEDs to inactivate biofilms and opportunistic pathogens, including *Legionella pneumophila* and *Pseudomonas fluorescens.* The main objectives of this thesis are summarized below:

The first objective of this research was to understand opportunistic pathogen biofilm growth and evaluate the inactivation efficiency with UV LEDs. Monocultures of pathogens were prepared, and CDC Reactors with cast iron and stainless steel coupons were used for biofilm culture. The organisms were treated with two UV wavelengths, and dose-response curves were created to assess the efficiency of the treatment using multiple quantification tools.

The second objective was to develop a method to enhance the growth of Natural Microbiome Biofilms (NMB) for later disinfection using UV LEDs. PVC coupons were placed in a biostud reactor to simulate new DWDS with plastic-like materials. Furthermore,

the impact of certain nutrients and temperature on biofilm growth was also investigated to determine the maximum growth over a short period.

1.6. Thesis Organization

This dissertation is organized into six chapters as follows:

- Chapter 1 provides a general introduction to the topics discussed in this dissertation and the primary goals of the research project.
- Chapter 2 presents background information related to this project's main topics and current relevant information. State-of-the-art articles that are relevant to the project are presented in this chapter.
- Chapter 3 explains the general materials and methods used in this research. More specific methodology and experimental conditions for each experiment are described in Chapter 4 and Chapter 5.
- Chapter 4 explains the the inactivation of *L. pneumophila* and *P. fluorescens* with UV LEDs. It also gives detailed information about testing, results, discussion and conclusion.
- Chapter 5 presents the experimentation for UV LED treatment for NMB. This chapter presents a detailed methodology concerning biofilm experimentation, detailed results, discussion and conclusions.
- Chapter 6 gives a conclusion summary of the experiment's results as well as recommendations for future work.

CHAPTER 2 BACKGROUND INFORMATION

2.1. Bacterial biofilm information

2.1.1. Morphology

Literature defines biofilms as complex communities of microorganisms of one or more species, adherent to a surface and encased in a self-produced polymeric substance matrix. Bacteria secrete compounds into the environment, known as extracellular polymeric substances (EPS), normally composed of exopolysaccharides, proteins and nucleic acids. (Coraça-Huber et al., 2020).

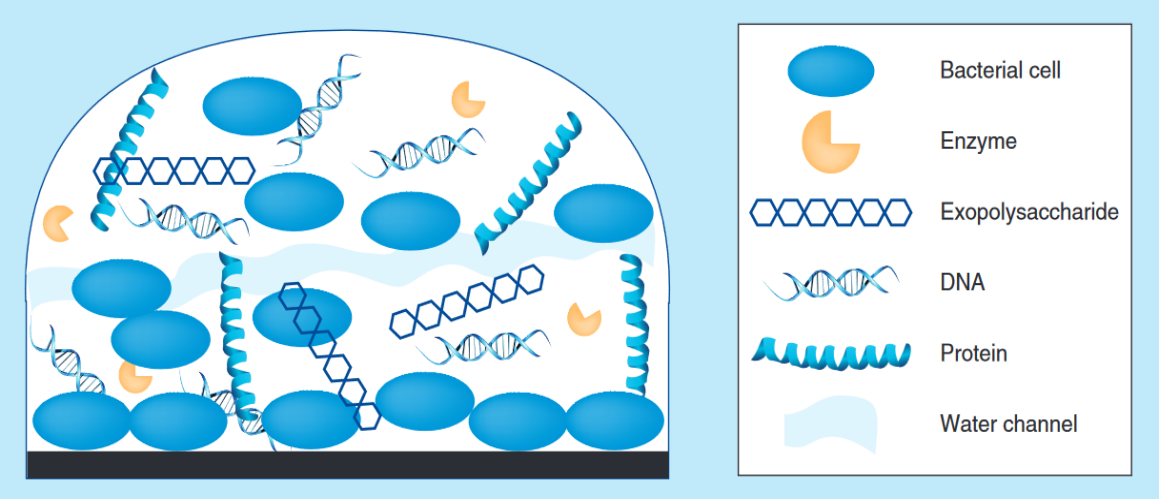

Figure 3. Biofilm structure (Rabin et al., 2015)

All biofilms have different structures (Figure 3) depending on the strain, quantity and quality of carbohydrates, nucleic acids and lipids (López et al., 2010). Carbohydrates such as mannose, galactose and glucose are the most abundant in biofilms. The structure also depends on environmental factors like nutrient concentration, pH and O_2 levels (Rabin et al., 2015).

2.1.2. Formation

Biofilm development and formation have very complex kinetics. However, literature mentions general cyclic mechanisms that most biofilms follow (See Figure 4): surface motility, polar attachment, lateral attachment, thickness growth, maturity and dispersion (Romeo, 2008).

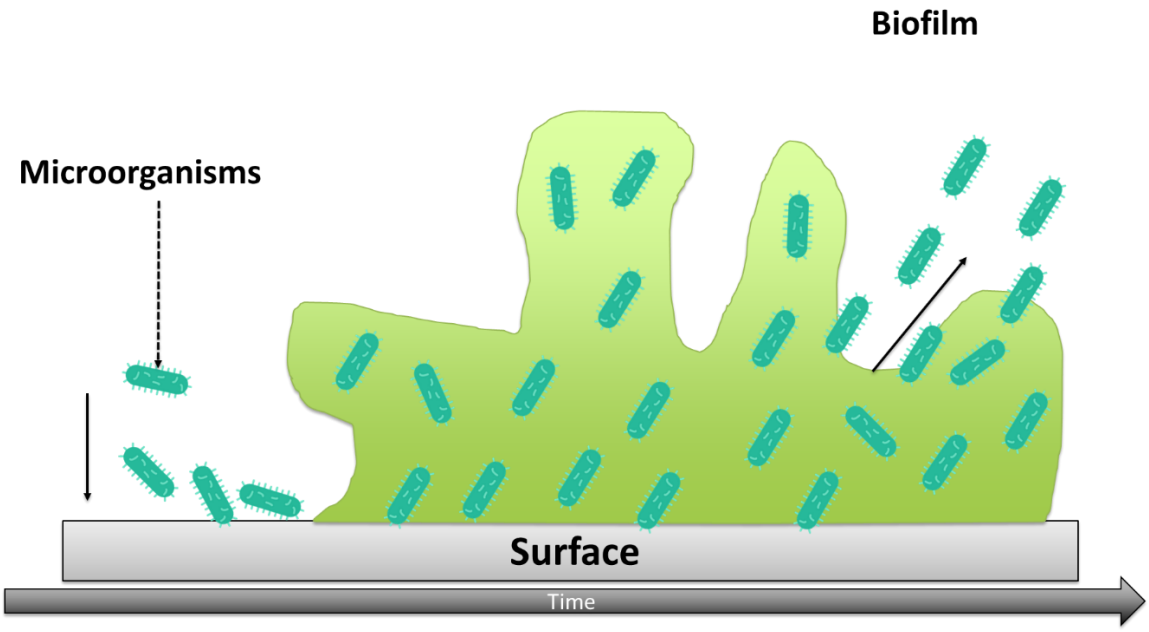

Figure 4. Biofilm formation

Surface motility refers to when planktonic (free-living) cells are deposited into a surface; then, the polar attachment occurs when cells start interacting with the environment by a pole (pili or flagellums) to ensure they have the optimal conditions for their reproduction. Later, cells start attaching to the surface, laterally producing adhesins to allow them to stick to the surface. During this time, cells begin producing EPS, resulting in a thicker matrix complex; cells start maturing and reproducing. In the end, new cells are released into the environment, colonizing more surfaces, finalizing the cycle and starting over (Armbruster

& Parsek, 2018). Cells released from biofilms often retain their disinfectant resistance and can develop more biofilms further down the distribution system. (Garvey et al., 2015).

Biofilm formation in DWDS depends on various environmental factors and water quality parameters such as temperature, pH, nutrient availability, substrate material, flow rate and surface morphology (Gule et al., 2016; Munoz, 2020). Studies show that bacteria are more likely to survive better on rough surfaces than on smooth surfaces. However, the literature indicates that certain strains colonize both types of surfaces equally (Chaves Simões & Simões, 2013; Mazaheri et al., 2022). Surfaces exposed to fluid environments (like water) tend to absorb proteins and nutrients, and depending on the organism, they colonize the surface more rapidly (Rumbaugh & Sauer, 2020). Hydrodynamics tends to accelerate biofilm growth overall in pipes. It is shown that different flow conditions can cause substantial changes to biofilm morphology and development; specifically, turbulent regimes can accelerate biofilm formation in pipes (Douterelo et al., 2014; Fish & Boxall, 2018; Tsagkari & Sloan, 2018).

2.2. Opportunistic Pathogens

Opportunistic pathogens (OPs) are a group of organisms that are ubiquitous in the environment and do not usually infect healthy hosts; however, they can cause severe infections in the susceptible and immunocompromised and those with underlying chronic diseases (Martínez, 2014; Health Canada, 2022).

Different types of microorganisms can be considered as OPs: Parasites (*Giardia, Cryptosporidium, Entamoeba*), Fungi (*Candida, Cryptococcus, Aspergillus*), Virus (*Herpes, CMV, HPV*) and Bacteria (*Salmonella, Pseudomonas, Legionella*). Sometimes,

multiple opportunistic pathogens can coexist within the same host and media (Gupta & Gupta, 2017).

When OPs become recurring in water bodies and reservoirs, they become a problem because they are capable of outbreaks associated with water exposure. These pathogens are better known as waterborne pathogens (AWWA, 2006).

2.2.1. Pseudomonas fluorescens

Pseudomonas spp. is a gram-negative, gamma proteobacteria ubiquitous in soil, water and plant surfaces. The strain of *Pseudomonas fluorescens* is not generally considered a human pathogen; however, reports show that it is present in low concentrations in the microbiota of several bodies of water and exists as saprophytes or in a commensal relationship with their hosts (Donnarumma et al., 2010; Scales et al., 2014; Ramos et al., 2015). Although that is significantly less virulent than *Pseudomonas aeruginosa*, *P. fluorescens* can cause bacteremia in humans; most of the reported cases of infections by this strain occurred in hospitals, often attributed to contamination of blood and medical equipment (Scales et al., 2014; Zekanović et al., 2022).

P. fluorescens, like other *Pseudomonas spp.,* can produce biofilms, which provides them resistance to harsh environmental conditions. The optimal growth temperature for *P. fluorescens* is between 25-30 °C; therefore, hot water environments are ideal for their reproduction (Montie, 1998). Isolation and quantification of *P. fluorescens* usually use plate counts of R2A or Tryptic soy broth, although molecular tools like qPCR are also available (Filloux & Ramos, 2014).

According to studies, this strain must be further studied at a molecular level to characterize the level of nosocomial infections inflicted in humans (Donnarumma et al., 2010);

moreover, this strain is more likely to host OPs like *Legionella pneumophila* (Stewart et al., 2012).

2.2.2. Legionella pneumophila

Legionella pneumophila is gram-negative coccobacilli, considered an OP, responsible for causing Legionnaire's disease or legionellosis, which is transmitted by inhalation of aerosols (Shen, 2016). These aerosols are produced in environments with steam and hot water, such as hospitals, saunas, hotels, cooling towers, cruise ships, etc. (Hoffman et al., 2007; National Research Council of Canada et al., 2018). Like this, *Legionella* enters the human lung, infects alveolar macrophages, and triggers severe pneumonia that can be fatal to immunocompromised people (Diederen, 2008). One of the main risks associated with *L. pneumophila* is that it is highly resistant to chlorine-based disinfection at concentrations between 0.2-0.6 mgCl/L (Kuchta et al., 1983; Cooper & Hanlon, 2010).

In the environment, *L. pneumophila* can survive within amoebas and biofilms. Often, *L. pneumophila* enters the DWDS and colonizes biofilms from other species formed inside (Abu Khweek & O. Amer, 2020). Biofilms develop three-dimensional structures containing water channels, allowing *L. pneumophila* to reside within them, producing adherence substances (Abdel-Nour et al., 2013; Abu Khweek & O. Amer, 2020). This allows *L. pneumophila* to obtain nutrients and energy, making their propagation easier; furthermore, the coexistence within biofilms makes *L. pneumophila* resistant to extreme pH temperatures and disinfectants (Shen, 2016).

The isolation and quantification of *L. pneumophila* are typically made through Buffered charcoal and yeast extract (BCYE) agar (Figure 5), as is the golden standard for its identification. This culture method usually takes up to seven days and uses a selective

media that provides the necessary nutrients for its development (Buchrieser & Hilbi, 2013). In recent years, more quantification methods have been developed to simplify the process of identifying *Legionella*: biochemical tests like Legiolert use an enzyme which colour-indicates the presence of *Legionella* with a culture time of seven days, but it does not require serial dilutions (McCuin et al., 2021); molecular tests like qPCR had been used to identify *Legionella* rapidly, with results available within 2 hours of sampling (Krøjgaard et al., 2011; Donohue, 2021).

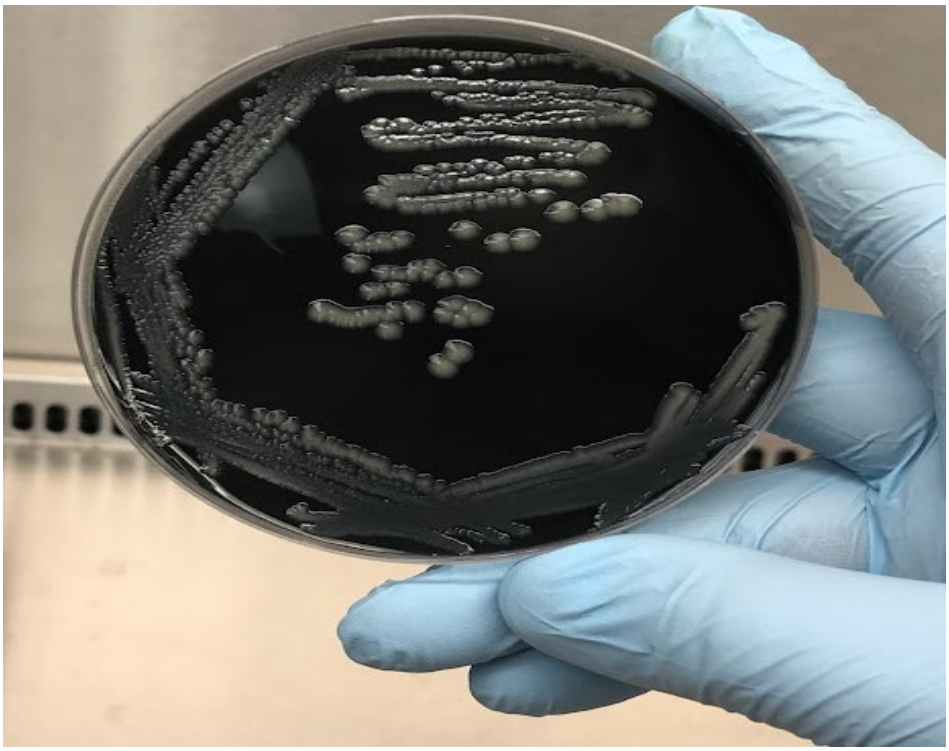

Figure 5. Example of Legionella pneumophila in BCYE Agar plate.

The first documented case of an outbreak of *L. pneumophila* was in a hospital in the 1980s in Oxford (Tobin et al., 1980), and the most recent was in the cooling towers of a company in Ontario, Canada in October of 2022 (HC Info, 2023). As this bacterium is still a current problem in DWDS, more treatments and technology should be developed for their control and prevention (Linden et al., 2019).

2.3. Microbial Testing Tools

Microbial testing tools in drinking water are essential to ensure the quality and safety of public health by regularly identifying and monitoring indicator microorganisms and waterborne pathogens (3M Canada, 2019).

2.3.1. Heterotrophic Plate Counts (HPC)

Heterotrophic Plate Count (HPC) is one of the main tests for detecting and monitoring microbial activity in drinking water. This test estimates the total concentration of an organism in a water sample. Heterotrophic microbiomes comprise bacteria, yeast and moulds. Therefore normally does not differentiate between bacterial types (Mendonca et al., 2020)

HPC test is not universal, as it depends on several conditions to achieve the desired result: temperature varies from 20-40 °C, incubation times from 1-7 days or weeks, and nutrient conditions differ by organism type (Bartram et al., 2003).

The spreading technique is one of the most common for HPC, which uses serial dilutions of the sample versed into agar plates (Sanders, 2012). Using a spreader, the sample is distributed over the agar's surface, resulting in discrete colonies spread across the agar surface; later, they are counted to obtain the concentration. A viable number of colonies in a standard agar plate is between 30-300 per plate. Most HPC test only detects 0.1-1% of the total population since most species do not grow properly on culture media (APHA et al., 2017).

The concentration of a sample is measured in a Colony-forming unit (CFU), depending on the sample can be either per mL (for liquid samples) or per cm^2 (for the surface area). This technique can quantify free-living organisms as well as biofilms. (Bartram et al., 2003).

The formula for calculating CFU is the following:

$$\frac{CFU}{cm^2} = \frac{(no.of\ colonies)}{sampled\ surface\ area} * dilution\ factor \quad \text{OR} \quad \frac{CFU}{mL} = \frac{(no.of\ colonies)}{volume\ of\ culture\ plate} * dilution\ factor$$

HPC can also be referred to as Standard plate count (SPC); the main difference is that HPC is commonly used with environmental samples, and SPC can be used with monocultures (or pure cultures: one single organism); however, the spreading technique and formulas are the same for both (Brown & Smith, 2015). In this dissertation, HPC and SPC are used interchangeably, understanding that in every context, unless otherwise noted, both terms refer to the spreading technique and calculations used the same way.

2.3.2. Adenosine Triphosphate (ATP)

Adenosine triphosphate (ATP) analysis is a test developed for monitoring and quantifying microbial activity in water samples. ATP is the primary energy carrier for cells; its quantification directly assesses cellular biomass's viability. ATP is a bioluminescence assay, meaning that the enzyme luciferase is introduced in the sample. Then, the light produced is measured using a luminometer; the light is directly proportional to the amount of ATP in the sample. The availability of results can be obtained relatively quickly and show great promise as a monitoring tool for microbial activity (Lee & Deininger, 2004; LUMINULTRA, 2019; Rauch et al., 2019).

The company LUMINULTRA® has developed two assays for ATP measurements: QGA (Quench-Gone Aqueous) for water samples and DSA (Deposit & Surface Analysis), which is better for biofilm measurement. The lowest detection limit is 0.1 pgATP/mL or cm^2 (LUMINULTRA, 2022, 2023).

To calculate Total ATP (tATP) in biofilms, which includes ATP from living cells (cATP) as well as ATP released from dead cells (dATP), the following formula is used:

C – Biofilm Collector calculations:

$$tATP\ \left(\frac{pgATP}{cm^2}\right) = \frac{RLU_{tATP} - Background\ RLU}{RLU_{ATP1}} * \frac{50{,}000\ (pgATP)}{A_{sample\ (cm^2)}}$$

Where:

- RLU = Relative light units
- RLU_{tATP}: RLU measured from the sample in the Luminometer.
- *Background RLU*: indicates no interference from external factors (must be < 20 RLU).
- RLU_{ATP1}: calibration from Luciferase enzyme (must be ≥ 5,000 RLU).
- $A_{sample(cm^2)}$: surface area of the collection device (coupon) in cm^2.

2.3.3. Quantitative Polymerase Chain Reaction (qPCR)

The quantitative polymerase chain reaction (qPCR) is an enzymatic reaction used to amplify fragments of nucleic acids (DNA). The assay is performed by a series of DNA denaturation steps, meaning heating the DNA strands at high temperatures (~95 °C) to separate them, then the primers target sequences for amplification thanks to the DNA polymerase enzyme. Cycles are repeated 25-40 times (Nolan et al., 2013).

This technique is broadly used in the clinical field as a confirmation test for diagnosing several diseases caused by pathogens. Nevertheless, in recent years it has been gaining more popularity as a powerful tool for quantifying pathogens in other fields like food technology, biotechnology and water treatment (Mérault et al., 2011; Donohue, 2021;

Harshitha & Arunraj, 2021). Thanks to the rapidness of the methodology, results can be available within 2 hours per sample (Krøjgaard et al., 2011).

2.3.4. Legiolert Test

Legiolert® is a test developed for identifying *L. pneumophila* in water samples; it uses the most probable number (MPN) technique for its quantification. It differs from traditional culture methods as the methodology is relatively more straightforward, rapid, and does not require serial dilutions (Rech et al., 2018). The test is based on an enzymatic reaction that colour-indicates the presence of this organism through the utilization of a substrate, which accelerates its reproduction. *L. pneumophila* uses this substrate to produce a brown colour within seven days of culture (IDEXX, 2022).

Recent studies show that using Legiolert as a quantification method for *L. pneumophila* is preferred over the traditional BCYE culture method due to its simplicity (Rech et al., 2018; Scaturro et al., 2020; McCuin et al., 2021). Moreover, the literature indicates that both methods do not show statistical significance in quantification (Scaturro et al., 2020). Furthermore, Legiolert overcomes the interferences of other microorganisms in potable water samples and presents advantages over the BCYE method. The detection limit of this test is 1 MPN/100 mL – 2272.6 MPN/100 mL (McCuin et al., 2021; Rech et al., 2018).

2.4. Ultraviolet Disinfection (UV) Technology

2.4.1. UV light

Ultraviolet (UV) light is the part of the electromagnetic spectrum that ranges from 100-400 nm. UV light is divided into four primary bands: V-UV (Vacuum UV) from 100-200 nm, UV-C from 200-280 nm, UV-B from 280-315 nm and UV-A from 315-400 nm (

Table 1). VUV is not transmitted through air, therefore, is not explored for germicidal effects (Kowalski, 2009).

Table 1. Primary bands of ultraviolet (UV) light (Kowalski, 2009).

Band	Wavelength (nm)	Type and classification		
UV-A	315-400	Non-germicidal (Near-UV, Blacklight)		
UV-B	280-315	Erythemal	Germicidal	Actinic
UV-C	200-280	Nucleotide disruption		
V-UV	100-200	Vacuum ultraviolet		

UV light < 315 nm is actinic, which can cause photochemical reactions. UV-A usually is not considered germicidal, but in human skin causes suntanning (Bolton & Cotton, 2022), although, in recent years, it has been argued that UV-A wavelengths can cause irreparable damage to DNA, preventing photoreactivation mechanisms; nevertheless, this is still being debated (Khan et al., 2018; Amar et al., 2021; Allahyari et al., 2022). UV-B range can cause sunburn and eventually skin cancer (erythemal). UV-C is entirely absorbed in the atmosphere. Thus, it does not impact humans directly. Nonetheless, current UV lamps for disinfection fall within the UV-C range; UV-C light is absorbed by RNA, DNA and proteins, causing cell mutations, cancer and apoptosis. Both ranges (UV-B and UV-C) are considered germicidal (Bolton & Cotton, 2008; Rauch, 2018; Soro et al., 2021).

2.4.2. Germicidal range

The germicidal range is between 200-315 nm for air, surface and water disinfection (Reed, 2010). The primary wavelength for UV disinfection is 254 nm (Chang et al., 2017).

When UV light (200-315 nm) passes through a microorganism, it is absorbed by the nucleotides (DNA and RNA) and disrupts its structure, thus preventing its reproduction. Therefore, the organisms can no longer infect, although the cells are still viable. This

disinfection mechanism is often called ‘inactivation’ because the microorganisms are not dead but not culturable (Bolton & Cotton, 2008).

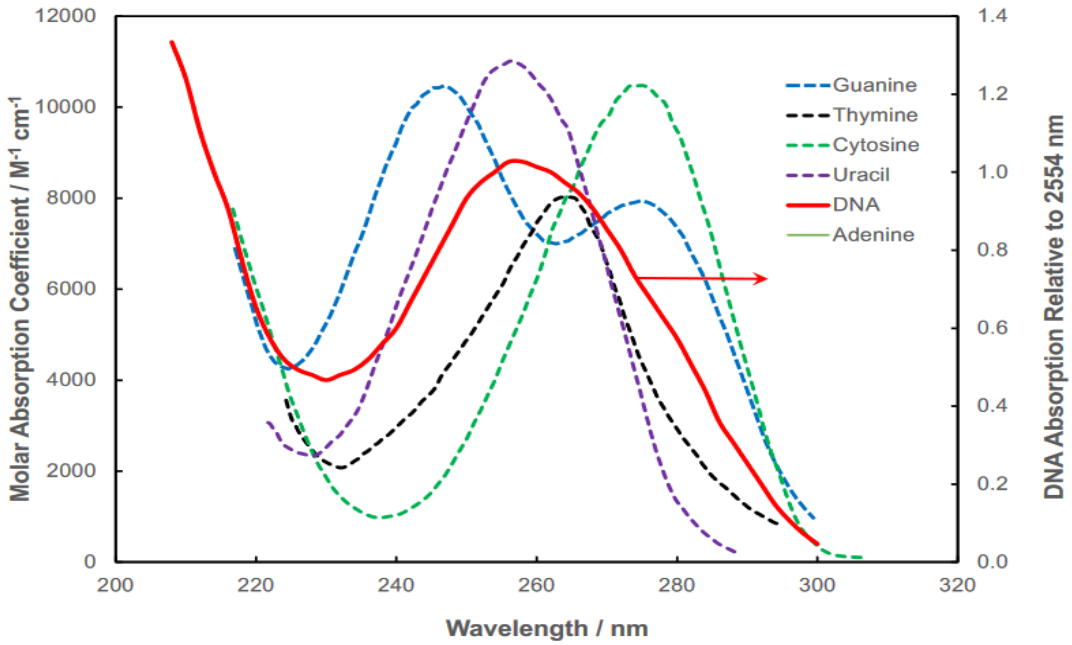

Figure 6. Absorption spectra of nucleotides and DNA (Bolton & Cotton, 2022).

DNA and RNA nucleotides absorb UV light between 200-300 nm (Figure 6), causing the formation of thymine dimers, which is a photochemical reaction of two adjacent thymine molecules from different DNA strands and creates a bond between them (thymine dimer). These dimers disrupt the structure of the DNA, creating more dimers, and the DNA can no longer replicate. This is the primary disinfection mechanism of UV disinfection (Bolton & Cotton, 2022).

UV is most effective for bacteria and protozoa (Beck et al., 2017), more recently for certain viruses (Rockey et al., 2020; Yoon et al., 2022), but slightly less effective on algae (Bolton & Cotton, 2008); this treatment is also effective against chlorine-resistant microorganisms,

like *L. pneumophila* (Cervero-Aragó et al., 2014; Rattanakul & Oguma, 2018; Allahyari et al., 2022).

To evaluate the inactivation of microorganisms (Table 2), the Log Reduction Value (LRV) is used as a form of expressing the percentage of disinfection of a technology (Schmidt et al., 2020).

Table 2. Log Reduction Values (LRV) percentages

LRV	Number of CFU remaining	Percentage reduction
0 LRV	1,000,000	0 %
1 LRV	100,000	90 %
2 LRV	10,000	99 %
3 LRV	1,000	99.9 %
4 LRV	100	99.99 %
5 LRV	10	99.999 %
6 LRV	1	99.9999 %

For LRV calculations, refer to section 3.9 of this dissertation.

2.4.3. UV LEDs

Ultraviolet Light-emitting diodes (UV LEDs) emit UV light from 200-400 nm (Beck et al., 2017). UV LEDs are a mercury-free alternative for multiple applications in the industry; in recent years, they have served as an alternative for the disinfection of air, surface and water (Song et al., 2016; G. Q. Li et al., 2017; Galezzo & Susa, 2021).

The structure of standard UV LEDs (Figure 7) is comprised of layers of Aluminum Nitride (AlN: Buffer Layer), aluminum gallium nitride (AlGaN: n-Layer + Active Layer + p-Layer) and gallium nitride (GaN: p-electrode) placed on a sapphire substrate (Muramoto et al., 2014). Electrons are captured in the active layer of the LED, where narrow quantum wells are created. Then, photons are released when these electrons are brought to a lower

energy state (Shatalov et al., 2014). Low wavelengths are achieved by increasing the aluminum in the layers, achieving wavelengths from 210-350 nm (Asif Khan, 2006); wavelengths >350 nm are achieved by introducing indium into the layers (Muramoto et al., 2014)

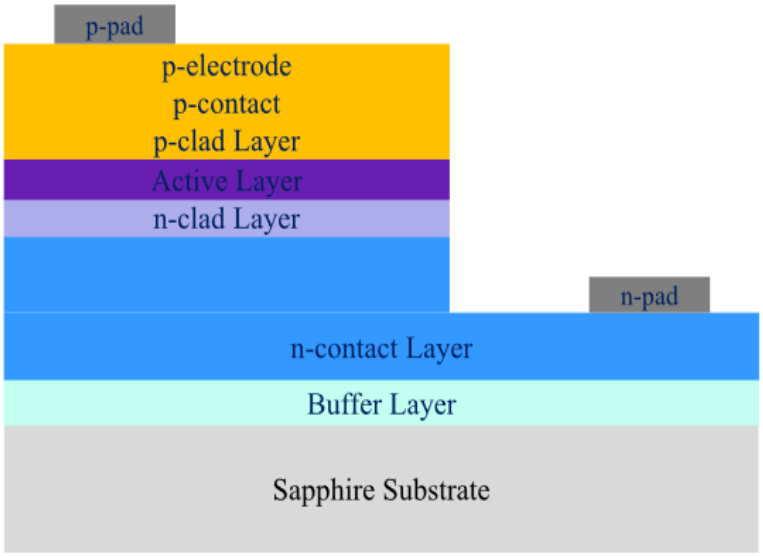

Figure 7. Structural diagram of a standard UV LED chip (Muramoto et al., 2014).

One of the significant challenges for UV LEDs is the low power output, generally due to low external quantum efficiencies (EQE) from UV LEDs (Ibrahim et al., 2014). Nevertheless, each year the property of materials leads manufacturers to develop better UV LEDs with better power outputs (DOWA HOLDINGS CO, 2017); for instance, a study conducted in China shows that arranges of the materials of the layer matrix performed better when using p-AlGaN, increasing power output to 55.7% and an EQE of 4.1%, obtaining better results than when using p-GaN; however more studies must be developed to improve the light shedding in wide-gap semiconductors (Wang et al., 2022).

2.4.4. Recent applications of UV LEDs for microbial inactivation

To date, multiple peer-reviewed recent applications of UV LEDs for microbial activation (pathogens and biofilms) have been published, using different wavelengths, organisms and arrangements. The following highlights recent studies on this matter.

Pathogens

The inactivation of *Escherichia coli*, *Listeria* and *Salmonella* by single and multiple-wavelength UV LEDs was studied by Green et al. (2018). The authors used 259, 268, 275 and 289 nm. They found that the highest disinfection occurred at 259 and 268 nm. Nonetheless, combining 259 and 289 nm synergistically affected the reduction of *E. coli* and *Listeria*.

Nyangaresi et al. (2019) studied the comparison of UV LED photolytic and UV LED/TiO_2 photocatalytic disinfection for *E. coli* in water. Disinfection was analyzed for 265, 275, 310 and 365 nm at an irradiance of 0.49 mW/cm^2 with 1.0g/L TiO_2. Results show that inactivation using 265 and 275 nm were the most efficient; however, when adding TiO_2, the inactivation improved significantly with 310 and 365 nm. Nevertheless, it was concluded that 275 nm presented the best performance in both arrangements with minor energy consumption. Therefore, 275 nm was appointed as a promising viable option for future research in water treatment.

A sequential utilization of the UV-A (365 nm) fluence rate for disinfection of water contaminated with *L. pneumophila* and *L. dumoffii* was performed by Allahyari et al., (2022). The authors used a commercially available UVA-LED with a dose of 17000 mJ/cm^2, obtaining, a 3 LRV for *L. pneumophila* and a 2.1 LRV for *L. dumoffii.* Results

show that this system is more economical than UV-C and UV-B systems; nevertheless, more tests are needed to validate this technology.

Lastly, the evaluation of disinfection efficacy of single UV-C and UV-A followed by UV-C LED irradiation on *E. coli*, *B. spizizenii* and MS2 bacteriophage in water were conducted by Nyangaresi et al. (2023). UV-C wavelengths were 267 and 278 nm, and UV-A was 368 nm. Conclusions state that both arrangements: single UV-C radiation and UV-A followed by UVC irradiation, showed higher inactivation efficiency. However, single 278 nm showed higher efficiency in terms of suppression of repair and energy consumption; therefore, it was suggested that single UV LEDs systems in water disinfection systems are a better option to reduce costs while having good inactivation levels, less energy consumption and repair suppression.

Biofilms

Gora et al. (2019) at Dalhousie University published a study on the biofilm inactivation of *P. aeruginosa* using UV LEDs. The study hypothesized that high doses of UV light would be required to penetrate and inactivate the biofilms. Silicone coupons were used in a CDC reactor to grow the biofilms, then treated at 255, 265 and 285 nm, and dose-response curves were generated. Results showed that an LRV of 1.3 at 265 nm was achieved. Likewise, biomass recovery was used with the ATP recovery method to contrast the results.

The inactivation of biofilm-bound bacterial cells using irradiation across UV-C wavelengths was studied by Ma et al. (2022). Inactivation of *P. aeruginosa* was performed at different wavelengths and devices: KrCl* excimer (222 nm), LP UV Lamp (254 nm) and three UV LEDs (260, 270, 282 nm). This study is also the first to measure UVT and the

Absorbance of biofilms using quartz coupons. Results show that 270 nm provided the highest disinfection performance. Biofilms presented resistance, obtaining only values from 1.5 to 2.5 LRV. Nonetheless, resuspended biofilms were susceptible, obtaining up to 6 LRV. This study improved fundamental knowledge and guidance for emerging UV LED technology for biofilm and pathogen control in water distribution systems.

2.4.5. Collimated beam studies

Most UV studies are performed using a collimated beam to determine the UV dose for different microorganisms. A collimated beam is an apparatus consisting of the output of the UV lamp directed into a horizontal surface using a cylindrical tube, referred to as the 'collimator'. Using collimated beams studies, dose-response curves can be developed to determine the inactivation rate of microorganisms and compare between UV treatment studies. Accurate determination of exposure times and wavelengths is necessary for microbial inactivation research. Bolton & Linden (2003) developed a standard protocol for UV dose determination in bench-scale experiments. This protocol was intended for UV LP or UV MP lamps but not for UV LEDs. The company Aquisense Technologies® adapted this protocol for the specific characteristics of the PearLab Beam™, which is the UV LED collimated beam used in this dissertation.

Table 3 are described key terms often used in UV studies.

Table 3. Key terms in UV studies

Term	Symbol	Units	Definition
Irradiance	E_e	mW cm^{-2}	Amount of light that hits a surface per unit area.
Fluence rate	H'_e	mW cm^{-2}	Amount of light that passes through a given volume of space per unit area.
Fluence	H_e	mJ cm^{-2}	Integration of the Fluence rate over a given time. Commonly referred to as 'UV Dose'.

For an accurate determination of the average fluence rate from a single irradiance measurement, five correction factors are considered. These factors help to have a more precise average fluence rate, given by the equation:

$$H'_{e,avg} = E_{e,0} * SF * PF * RF * DF * WF$$

The *sensor factor (SF)* accounts for the difference between the actual and displayed values; the calculation is based on LED emission and spectral responsivity. *Petri factor (PF)* accounts for the variance in irradiance across the sample plane. The calculation is based on irradiance measurements taken at small increments across the sample plane. It should be measured once for every experimental set up. *Reflector factor (RF)* is the portion of the radiation that is reflected as it moves between air and water. This factor is nearly consistent across the UV spectrum because air and water have consistent refractive indices. This value is fixed at 0.975. The *Divergence factor (DF)* considers the reduction in irradiance as the beam spreads between the sample's top and bottom planes. All UV sources exhibit beam divergence. The calculations are based on displacements, volume and diameter. *Water factor (WF)* asses the difference in irradiance from the sample plane to the bottom of the sample because all mediums absorb some amount of radiation as it travels through. The calculation is based on the absorption coefficient (UVT) and sample depth (Aquisense, 2021).

This calculation allows us to determine the exposure times (t) based on the average fluence rate ($H'_{e,avg}$) by the equation:

$$H_e\ (mJ\ cm^{-2}) = H'_{e,avg}(mW\ cm^{-2}) * t(s)$$

2.5. Regulation for UV Disinfection Systems for Drinking Water

Most UV systems are applied in treatment plants; therefore, most regulations focus on this application.

The Ultraviolet Disinfection Guidance Manual For The Final Long Term 2 Enhanced Surface Water Treatment Rule from the US EPA (2006) is a compilation of recommendations for UV disinfection in drinking water treatment plants using mercury-based lamps. The document also provides guidance on procedures, UV dose validation, and operational and water quality parameters when applying UV technology.

The certification NSF/ANSI 55-21: Ultraviolet Microbiological Water Treatment Systems helps manufacturers of UV disinfection devices (mostly POU and POE systems) to achieve the minimum requirements to verify that their products can disinfect contaminated water safely. This certification gives two classifications for UV devices, class A and class B. Class A systems must provide a fluence of 40 mJ/cm^2, designed to disinfect both *Cryptosporidium* and *Giardia*, along with bacteria and viruses, from contaminated water. Class B systems must provide a fluence of 16 mJ/cm^2 and are designed for the supplemental bactericidal treatment of average microbial load in drinking water. Moreover, the last revision (2021) updated testing systems with UV LEDs. This certification is not necessarily a regulation; however, customers and health officials can rely on this certification as a symbol of product quality (NSF International, 2021).

The American Water Works Association published the Ultraviolet Disinfection Systems for Drinking Water. This standard sets the minimum requirements for closed-vessels UV disinfection systems and equipment used for drinking water disinfection. Primarily, it targets pathogens like *Cryptosporidium, Giardia* and viruses. The standard can be

referenced for designing, installing, and commissioning UV disinfection equipment used in water treatment. Also, it is primarily focused on LP and MP mercury-based lamps and the parameters to consider for the UV system design (AWWA, 2022).

Furthermore, Health Canada (2022) has Guidelines for waterborne pathogens in drinking water. These guidelines aim to provide territories and provinces with guidance on waterborne pathogens not addressed in the Guidelines for Canadian Drinking Water Quality (GCDWQ). The guidelines propose that for UV treatments achieving a 4 LRV, different UV doses are required based on the literature. Waterborne pathogens like *Pseudomonas spp*. requires a relative 5 mJ/cm^2, and for *L. pneumophila,* a UV dose between 10-50 mJ/cm^2 is required. This document also emphasizes monitoring these microorganisms, which involves collaboration between water utilities that manage DWDS and those responsible for building plumbing systems.

To date, there are no specific regulations for UV LEDs devices, primarily because of the technology's relative novelty, which is not being used on a large scale, and because this technology still needs revision on the power output side and semiconductor technology (Ontiveros, 2019). The primary issue relies on the difference in the parameters between traditional UV technology (LM and MP lamps) and UV LED technology, especially regarding reactor design and wavelength adjustment. Although, each year, more data is being published that can lead to future regulations for this technology and better management strategies (Linden et al., 2019).

CHAPTER 3 MATERIALS AND METHODS

The common materials and methods across this project are described below. Methods specific to a chapter or experiment are further described in the appropriate section. All experiments were conducted at Dalhousie University in the Centre for Water Resources Studies microbiology laboratory. All experiments were done in duplicate.

3.1. Chemicals and Reagents

All reagents, growth media, and agars were prepared according to the manufacturer's instructions and autoclaved (AMSCO Lab 250, Steris Co, United Kingdom) at 121°C for 15 min for sterility. They were prepared using deionized water (DI) provided by a Milli-Q system (Reference A+, Millipore Corporation, MA, USA).

Tryptic soy broth (TSB) and Tryptic soy agar (TSA) were used as culture media (Becton Dickinson and Co., MD, USA) for *P. fluorescens*; R2A broth and agar (HiMedia Laboratories, PA, USA) were used for Natural Microbiome Biofilm (NMB) culture as they are broadly utilized for microbial monitoring of treated potable water and environmental samples. (Atlas, 2010). Legionella enrichment broth (LEB) was used to cultivate *L. pneumophila*. Complementary reagents like L-Cysteine and Iron pyrophosphate ($Fe_4(P_2O_7)_3$) were added (Sigma Aldrich, Darmstadt, Germany) to improve the recovery of *Legionella* (Sigma Aldrich, 2022).

Buffered yeast extract (BCYE) was used (Becton Dickinson and Co., MD, USA) for *Legionella* culture as it is the golden standard for *Legionella ssp.* growth and isolation (McCuin et al., 2021). Phosphate-buffered saline (PBS) was used for serial dilutions,

prepared in accordance with *Standard Methods for the Examination of Water and Wastewater* (APHA et al., 2017).

3.2. Opportunistic Pathogens Monocultures

3.2.1. Preparation and Inoculation Procedure

P. fluorescens (ATCC 17569) and *L. pneumophila (*ATCC 33152) were chosen to test the inactivation of opportunistic pathogens (Source: 10801 University Boulevard, MA, USA). The challenge microorganisms were selected for being waterborne pathogens, their ability to form biofilms (*P. fluorescens*), and their persistence in DWDS. Further details of the methods can be found in CHAPTER 4 of this dissertation.

For the preparation of monocultures, frozen stocks of the target microorganisms were preserved with 25% glycerol solution at -80°C until the day of the experiment. Frozen stocks were removed from the freezer and thawed inside the Biosafety Cabinet (BSC) (Thermo Scientific 1300 Series Class II Type A2). *P. fluorescens* was inoculated into a falcon tube containing 9 mL of sterile TSB (1:10 dilution) and placed in the incubator shaker (New Brunswick Scientific, Innova 40) at 27°C at 175 rpm for 24h. *L. pneumophila* was inoculated in a falcon tube containing 9 mL of sterile LEB and placed into the shaker incubator at 37°C at 175 rpm for 24h.

3.2.2. Cell Washing and Solution Preparation

The overnight culture of *P. fluorescens* was placed into the centrifuge (Thermo Scientific Multifuge X3R) at 5000 rpm for 5 minutes to form a pellet of cells. The supernatant was removed and replaced with sterile PBS, vortexed at max velocity for 1 minute, and placed in the centrifuge again. Cell washing was repeated three times.

In the case of *L. pneumophila*, the broth turned black overnight due to the oxidation of the iron present in the broth. To get a clear solution, a 1:10 subculture was prepared with 1 mL of overnight culture and 9 mL of LEB and incubated for four hours under the same conditions. Later, the procedure for cell washing was performed as with *P. fluorescens*.

Working solutions were prepared by making a 1:100 dilution of washed *L. pneumophila* OR *P. fluorescens* solution in sterile PBS, with an initial concentration of ~10^7 CFU/mL. A sample of this working solution was taken for UV-254 analysis (HACH, UV-VIS Spectrophotometer DR6000) to obtain the UVT%. This value was further used for fluence calculations.

3.3. Experimental Set-up for Biofilm Growth in CDC Reactors

For this experiment, the focus was to produce biofilms from OPs. In this case, *P. fluorescens* was chosen because it can produce biofilms. This specific strain is more likely to host *L. pneumophila.* (Stewart et al., 2012). The method used is a modified version of the Standard Operating Procedure *ASTM E2562-22: Standard Test Method for Quantification of Pseudomonas aeruginosa Biofilm Grown with High Shear and Continuous Flow using CDC Biofilm Reactor* (ASTM International, 2022). The modification relied on a water bath to increase the temperature (Figure 8).

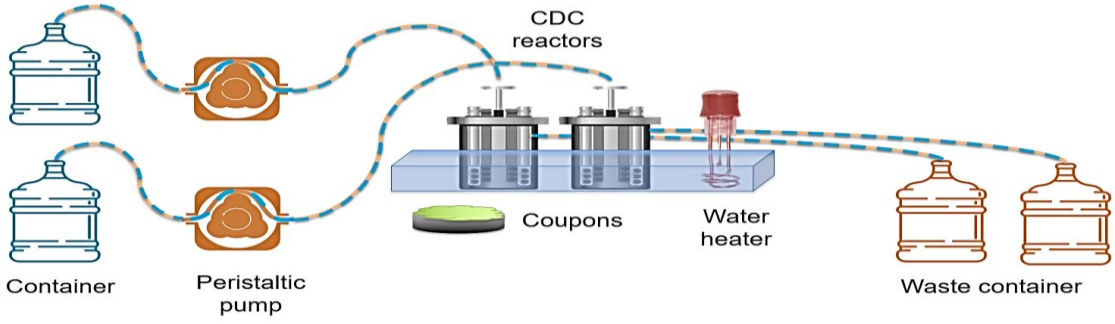

Figure 8. Experimental set-up for Biofilm growth in CDC reactors

3.3.1. Reactor's material preparation

CDC reactor components and tubing were hand-washed with soap and water to remove any residues. This experiment used two coupon materials: stainless steel (SS) and cast iron (CI) (Biosurface Technologies Corp., Bozeman, MT).

CI coupons were washed with a 10% nitric acid solution to remove rust, then rinsed with tap water and dried with a paper towel. SS coupons were washed with water and soap and dried with a paper towel. To completely dry, coupons were placed into an oven at 100°C and then put into the desiccator cabinet for 24h.

Once washed, CDC components (model CBR 90-1, BioSurface Technologies Corp., Bozeman, MT), coupons, and tubing were autoclaved at 121°C for 15 min. After autoclaving, coupons were placed in the desiccator cabinet for 24h to avoid that condensation produces rust in the coupons.

To enhance microbial growth, a feeding solution of 600 mL of TSB and 18L of dechlorinated tap water (see 3.4.2) was poured into a 20L carboy and autoclaved at 121°C for 15 min. This step was done before the experiment day to allow the solution to cool down for at least ten h.

3.3.2. Reactor's assembly

After autoclaving, all CDC components were assembled inside the BSC. Outlet tubing was clamped to avoid leakage throughout the experiment. Then, 495 mL of sterile dechlorinated tap water was poured with 5 ml of sterile TSB with a sterile funnel for a 1:100 dilution to prepare the reactor solution.

Later, coupons were placed in the rods, ensuring they were well tight to avoid the coupons from falling off; then, the rods were inserted in the reactor. Two reactors were prepared the same manner, one filled with SS coupons and the other with CI coupons.

3.3.3. Target microorganism inoculation

Inoculation of the reactors was made with 1 mL of an overnight culture of *P. fluorescens* by lifting 1 of the rods and dispensing the organism into the gap with a sterile pipette. The rod was reinserted once the inoculation was completed.

3.3.4. Benchtop set-up

The reactors were removed from the BSC and placed inside a water bath, ensuring that the stirring plates were aligned with the reactors.

The first step for biofilm formation was to put the reactor in batch mode. For this, the temperature was set at 27°C with an immersive water heater (KD Heater Co., Ltd., model KD-BH13K), and stirring was placed at the lowest rpm. The reactors were stirred at this setting for 24h, checking that the stirring mechanisms did not get stuck, which may occur at a low rpm.

After 24h, the reactor was set in CSTR flow mode by connecting the inlet and outlet tubing and letting the feeding solution flow through the reactor for another 24h. The feeding solution was pumped using a peristaltic pump (Cole-Parmer Inc, Anjou, QC, Canada) and opaque tubing (Masterflex Precision Tubing, Cole-Parmer Canada Inc., Anjou, QC, Canada) size 18. The peristaltic pump was calibrated to achieve a continuous flow rate of 10 mL/min and a Hydraulic Retention Time (HRT) of ~50 min. The outlet tubing from the CDC reactors was then opened to collect the waste into a biological waste carboy. See Figure 8 for reference.

3.4. Experimental Set-up for Biofilm Growth in Biostud Reactors

This experiment intended to grow Natural Microbiome Biofilms (NMB) from clean tap water using a Biostud reactor (Bio-inLine® Biofilm Reactor model IBR 500, BioSurface Technologies Corp., Bozeman, MT) containing a ½ inch canal that simulates piping conditions. Polyvinyl chloride (PVC) coupons (BioSurface Technologies, Bozeman, MT) were used for this reactor as a surface growth to simulate new piping in buildings. Microbial activity was monitored with ATP every third day, and disinfection quantification was made with HPC.

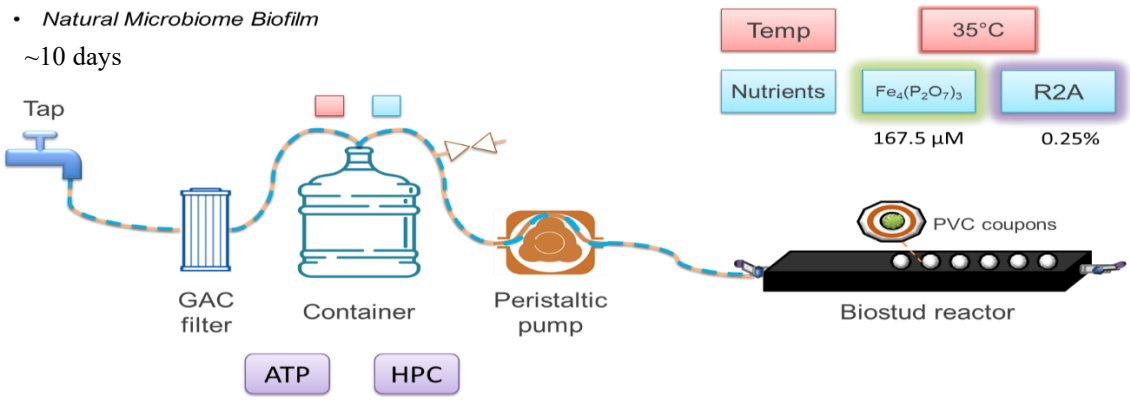

Figure 9. Experimental set-up of Biostud reactors

3.4.1. Reactors Assembly

The biostud reactor was unassembled for proper chemical cleaning with a solution of 10% Sodium hypochlorite, followed by cleaning with a 70% Ethanol solution. Opaque tubing (Masterflex Precision Tubing, Cole-Parmer Canada Inc., Anjou, QC, Canada) size 18 was washed with soap and let dry at room temperature.

PVC coupons were soaked in a solution of 1:1000 soap overnight, rinsed, and then kept in a solution of 70% Ethanol until they were required for experiment day.

The reactor was reassembled and tightened to avoid leakage. The coupons were left to dry in the open air for around 5 min before placing them in the coupon holders. Coupon holders were screwed into the reactor with the special coupon holder tool.

Tubing was connected to the reactors and pumps (Cole-Parmer Inc, Anjou, QC, Canada), and the outlet line was placed into the sink for drainage. The pumps were set for a flow rate of 10mL/min for an HRT of ~9.5 min. Inlet tubing was placed in 20 L water jugs, and the submergible water heater (KD Heater Co., Ltd., model KD-PD-AJS13K) was placed inside the jug and set to 35°C. See Figure 9 for reference.

3.4.2. Feed solution preparation

R2A Broth was prepared following the Manufacturer's instructions, autoclaved at 121°C for 15 min, then transferred into individual falcon tubes with the desired volume. A stock solution of Iron pyrophosphate was prepared by adding 74.521 g to 4 L of DI water for a final concentration of 0.025M. To fully dissolve the pellets, the solution was heated at around 35-40°C and then placed into a 4L capacity amber bottle to avoid oxidation.

Tap water used for this experiment was passed through a Granulated Activated Carbon (GAC) filter (PENTAIR, GAC-10, 9-3/4-Inch x 2-7/8-Inch) to remove residual chlorine. Then the reactors were filled with dechlorinated tap water, and nutrients were added at the desired concentration for NMB-enhanced growth. Several arrangements of temperature and nutrient concentration were performed to enhance NMB formation. Details are provided in CHAPTER 5 of this dissertation.

3.5. Sample Preparation and collection

Samples were taken for all experiments and quantified before and after UV treatment for later Log Reduction Values calculations (LRV).

3.5.1. Monoculture sample preparation

Samples of 18 mL from the working solution were versed into sterile 60mm x 35mm crystallizing dishes.

3.5.2. CDC reactor sample collection

Reactors were stopped after 48 h of biofilm culture of *P. fluorescens*. The outlet tubing was clamped, inlet and waste tubing were carefully removed, and reactors were placed inside the BSC for sampling. CDC rods were aseptically removed, then the coupons were placed into sterile 47 mm petri dishes and labelled.

3.5.3. Biostud reactor sample collection

Sampling was made near an ethanol Bunsen burner to maintain a sterile environment and avoid external contamination. The coupons were removed from the reactor using the coupon holder removal tool, placed into sterile 47 mm Petri dishes, and labelled.

3.6. Bench-scale UV disinfection Treatment.

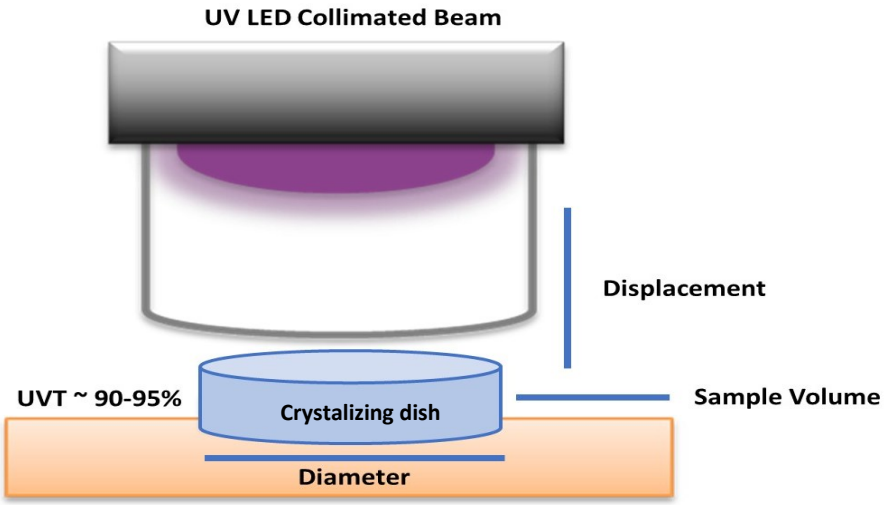

Figure 10. Input Experimental Conditions for Bench-Scale UV Disinfection Treatment

UV disinfection was performed using a UV LED collimated beam (PearlLab Beam™ 280/310/365, Aquisense Technologies). Further details regarding individual experimental designs and conditions can be found in each methods section of CHAPTER 4 and CHAPTER 5 of this dissertation.

Correction factors were calculated using the "PearlLab Beam Correction Factor spreadsheet'' provided by Aquisense Technologies (Aquisense, 2021) using input values from the experimental conditions (Figure 10). The centre point irradiance, E_0, was calculated using a UV radiometer (OCEAN OPTICS USB 4000), placing it in the center of the sample plane, and using the Spectrasuite™ software to calculate the Irradiance in $\mu W/cm^2$. The following experimental conditions were established depending on the experiment and input in the workbook: sample plane diameter, sample plane displacement, measurement spacing (set at 5 mm), sample volume, and UVT_LED peak (used UVT% value measured in 3.2.2).

Finally, the Target Fluences were input in the workbook, and it calculated the exposure time (in min or s). When exposure times were obtained, samples were placed under the UV light for treatment and later quantified. Further information on each sample treatment is described below. UV dose-response curves were obtained to evaluate the disinfection efficiency at different wavelengths and fluences.

3.6.1. Opportunistic Pathogens Treatment

A micro stir bar was used to mix the crystalizing dishes uniformly, and the stirring plate was set at low rpm (ensuring a vortex was not created). Then, dishes were placed into the sample plane, ensuring they were as centred as possible from the collimated beam.

3.6.2. Biofilm Treatment (CDC and Biostud)

Petri dishes with formed biofilm coupons were placed at the center of a rotating plate to ensure an average fluence across the irradiation plane. Because these were not liquid samples, all factors, except PF, were assumed to be 1. UVT was assumed to be 100% because of the absence of methods at the time for determining UV Transmittance in non-resuspended biofilms and with non-translucid materials (Ma et al., 2022).

3.7. Quantification methods of OPs

Treated samples were taken using sterile pipettes. A 500 µL aliquot of *P. fluorescens* sample was transferred into sterile microtubes for later serial dilution and standard plate counts (SPC). With *L. pneumophila,* samples were divided for the three quantification methods: 500 µL for SPC, 7.5 mL for qPCR, and 10 mL for the Legiolert test (18 mL total). The general methodology for each method is described below.

3.7.1. Standard Plate Counts

SPC were used for both experiments: OP Monocultures (3.2) and Biofilms (3.3). *P. fluorescens* biofilms from CDC reactors were recovered using the technique reported by Gora et al., (2019). The cotton swab is wet with the sterile PBS, then utilizing the swab pattern shown in Figure 11, then vortexed for 1 min to fully resuspend.

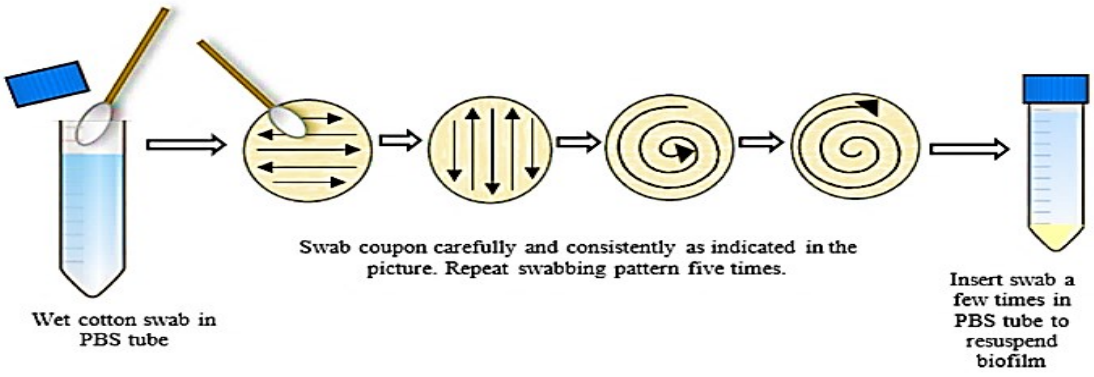

Figure 11. Biofilm recovery method (resuspension) adopted from Gora et al., 2019

Before plating, serial dilutions were done by taking a 50 µL aliquot from the sample and transferring it into 450 µL of sterile PBS with a sterile pipette and gently vortexed. This was counted as the first dilution (10^{-1}); later, an aliquot from the 10^{-1} tube was added into a new 450 µL sterile PBS tube to create the second dilution 10^{-2}. This procedure was repeated several times until the desired dilution was reached.

Once the dilutions were ready, 100 µL aliquot was inoculated in sterile Petri dishes (agar depending on the target microorganism: BCYE for *L. pneumophila* and TSA for *P. fluorescens*,) and spread (Figure 12) using the spreading technique (APHA et al., 2017; Rijal, 2022). Later, they were placed in the incubator at the optimal culture conditions of each target microorganism. Culture conditions for *P. fluorescens* are 27 °C for 24h and *L. pneumophila* 37°C for 2-7 days. Colonies were counted for later calculations after culture time.

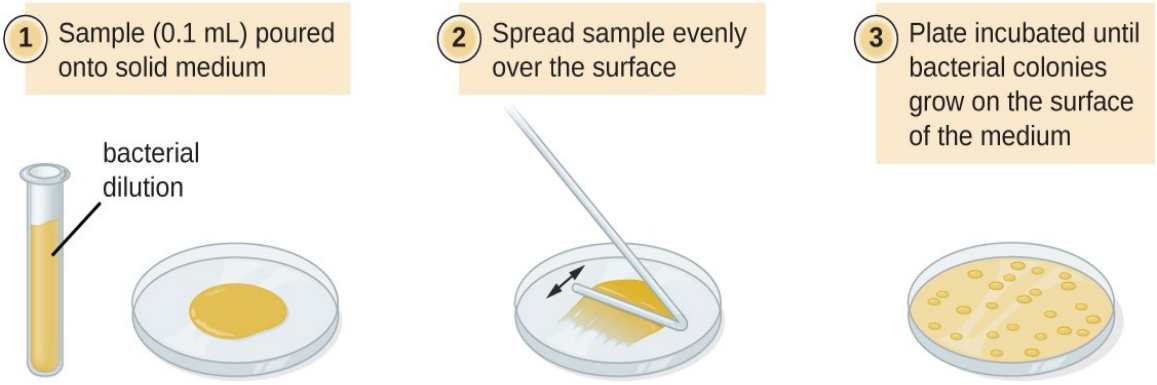

Figure 12. The spreading technique (Rijal, 2022).

3.7.2. Legiolert

The Legiolert test was performed (Figure 13) following the manufacturer's instructions (IDEXX, 2019). A 1:10 dilution of the sample was made with sterile PBS; then, Legiolert reagent was added, and the sample shook until dissolved. Immediately after, the sample

was transferred into a Legiolert tray and sealed using the Quanti-Tray Sealer PLUS. The trays were kept in the incubator for seven days at 37°C. A container with tap water was placed underneath the trays' rack to prevent the trays from drying out.

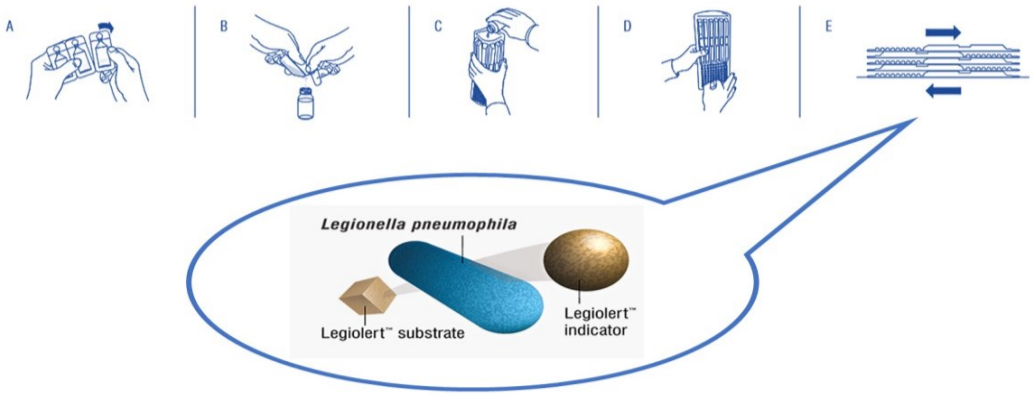

Figure 13. Legiolert procedure (IDEXX, 2019)

Positive wells were considered those which changed to a brown colour or appeared cloudy. For better reassurance, it was compared with a blank (reagent with sterile PBS, without sample). The MPN Generator Software™ was then used to determine the concentrations in MPN/100 mL (IDEXX, 2022).

3.7.3. qPCR

For this dissertation, qPCR analysis was made to quantify *L. pneumophila* using the test kit developed by LUMNULTRA®. The assay is divided into two stages: the first stage is a DNA extraction, in which the sample is lysed, and then the DNA is purified. The qPCR preparation requires adding the assay mix (primers and enzymes) and preparing a negative and a positive control in a laminar flow cabinet (Mystaire™ MYPCR32). Once the samples are ready, they are placed into a GeneCount™ Q-16 qPCR device.

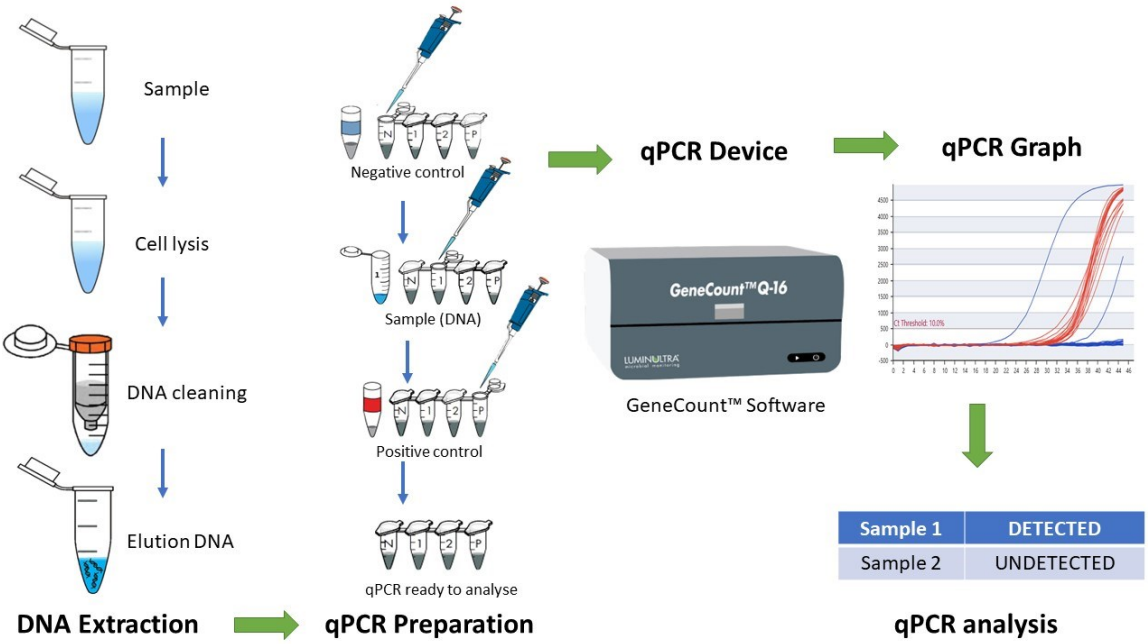

Figure 14. General methodology for qPCR analysis.

The GeneCount™ Q-16 Software for qPCR analysis gave the amplification results by cycles. If *L. pneumophila* was detected, the result appeared as DETECTED and gave the concentration in GU/mL (Gene Units per mL). If *L. pneumophila* was not detected, the result appeared as UNDETECTED. If dilutions were made, the appropriate dilution factor was applied to obtain the actual concentration. Depending on the sampled volume, the detection limit varies: for 50 mL of sample, the lowest detection concentration is 100 GU/mL (LUMINULTRA, 2021, 2022a). See Figure 14.

3.8. Quantification methods for Biostud reactor

3.8.1. Recovery ATP Methods

ATP quantification was performed for microbial activity monitoring for method development. The LuminUltra® Deposit and Surface Analysis (DSA) kit was used following the manufacturer's instructions (Figure 15), section C – Biofilm Collector (LUMINULTRA, 2022b).

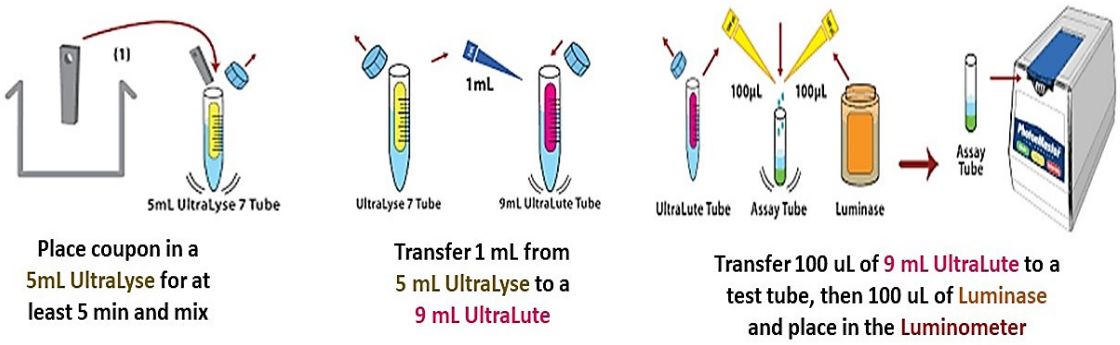

Figure 15. General methodology for ATP test (LUMINULTRA, 2022b).

3.8.2. HPC

HPC was performed with the samples from resuspended NMB. The procedure follows the principle described in 3.7.1 for resuspended biofilms, using R2A Agar instead.

3.9. Bacterial Log Inactivation Calculation

Log Reduction Value (LRV) was calculated to quantify the inactivation efficiency of treatments presented in this dissertation, using all quantification previously described.

LRV was calculated using Chick's law:

$$LRV = log_{10}\left(\frac{N_0}{N}\right)$$

Where:

- N_0: concentration of microorganisms before treatment (Control)
- N: Treated concentration
- The LRV for each quantification method was calculated using each experiment's control.

3.10. Statistical Methods

All calculations were obtained using Microsoft Excel. All linear regressions for *k* values and ANOVA were using the free statistical tool GInaFit v1.6, following the approach developed by Geeraerd et al., (2005). Even though the applicable methods for inactivation curves depend on the shape of the curve, this data it was only used Shape I (log-linear) because of the lack of shoulder (van der Waal, 2017).

CHAPTER 4 INACTIVATION OF *LEGIONELLA PNEUMOPHILA* AND *PSEUDOMONAS FLUORESCENS* WITH UV LEDS

4.1. Introduction

The microbiological quality of drinking water is often compromised by waterborne pathogens. These pathogens have gained resistance to traditional water disinfection technologies over the years, making it more challenging to deliver safe drinking water (Zhang et al., 2019). These pathogens pose high risks to human health, especially to immunocompromised and vulnerable people. One of the main current public health concerns is the formation of biofilms in Drinking Water Distribution Systems (DWDS). Biofilms are a conglomerate of microorganisms deposited on a surface and encapsulated in Extracellular Polymeric Substances (EPS), conferring a certain degree of protection against harsh environmental conditions, including standard disinfection techniques like chlorination (Vickery, 2019).

These biofilms can also host other pathogens like *Legionella pneumophila,* a waterborne pathogen resistant to chlorine that causes a severe pulmonary infection known as Legionnaire's disease (Murga et al., 2001; Abdel-Nour et al., 2013; Abu Khweek & O. Amer, 2020;). *Legionella* can persist within biofilms by producing a biofilm-like extracellular matrix, which helps them to get attached to the current biofilm and survive; colonization of biofilms depends on permissive species that do not produce enzymes and surfactants that are toxic to *Legionella*, preventing this microorganism from getting attached (Abdel-Nour et al., 2013). *P. fluorescens* has been reported as a common host for *L. pneumophila* (Stewart et al., 2012; Abu Khweek & O. Amer, 2020).

Disinfection with ultraviolet (UV) light for drinking water is a technology that has been on the market for many years due to its capacity to inactivate microorganisms without

producing disinfection by-products (DBPs) (Soro et al., 2021; Bolton & Cotton, 2022). However, current devices, like Low-Pressure (LP) UV lamps, pose certain disadvantages. Primarily, these lamps are mercury-based, potentially hazardous, and need particular waste disposal; they can be bulky, have high-energy consumption, and only work at 254nm. Due to the disadvantages of UV lamps, UV light Emitting-diodes (UV LEDs) have become more popular over the years as an alternative due that they do not contain mercury, have low energy consumption, and have the flexibility to fit in small systems. In recent years, more attention has been placed on applying UV LEDs as an alternative for biofilm-pathogen control in DWDS (Song et al., 2016; Chen et al., 2017; G. Q. Li et al., 2017; Rattanakul & Oguma, 2018; Wan et al., 2022).

Among the three types of UV light (UV-A, UV-B and UV-C), UV-C light has a high DNA adsorption, which is why it has been broadly used for inactivation studies (Song et al., 2016). Although UV-A radiation causes irreversible damage to cells by inducing photosensitizing reactions, this is the reason for investigating UV-A radiation's properties against photoreactivation mechanisms (Song et al., 2019; Allahyari et al., 2022).

This study aims to understand opportunistic pathogen biofilm growth cultivated in various media: i) monocultures in culture broth and ii) CDC Biofilm Reactors and create dose-response curves to evaluate the inactivation efficiency with UV LEDs. Therefore, this chapter explains individual experimental designs, results and discussions, and conclusions from both experiments.

4.2. Materials and Methods

4.2.1. Experimental Design and Set-up

This section explains the experimental design details for each experiment; however, the general methods for set-up and microbial quantification are outlined in CHAPTER 3.

4.2.1.1. Monocultures of opportunistic pathogens

Pseudomonas fluorescens and *Legionella pneumophila* were used as target organisms for treatment with UV LEDs by culturing overnight and cleaning the cells for a high UVT%. Experimental conditions are described in Table 4.

Table 4. Culture conditions for monocultures

Organism	Strain	Media	Temperature	Culture Time	Subculture	Initial concentration	
						Mean	SD
Pseudomona fluorescens	ATCC 17569	TSA	27°C	24 h	/	10^6	± 18.5
Legionella pneumophila	ATCC 33152	LEB	37°C	24 h	1:10 for 4 h	10^7	±17.8

TSA: Tryptic Soy Broth; LEB: Legionella Enrichment Broth; Initial concentration in CFU/mL

Cultures were cleaned by forming a pellet by centrifugation. The supernatant was replaced with sterile PBS, then vortexed at 3000 rpm (Figure 16); this process was repeated three times. The working solution was prepared using 2 mL of cleaned cells diluted in 200 mL sterile PBS (1:100 dilution). The UVT% was measured with spectrometry for further fluence calculations. Results ranged from 90-95% UVT. Samples were prepared by versing an aliquot of 18 mL of the working solution into crystallizing dishes (60 x 45 mm).

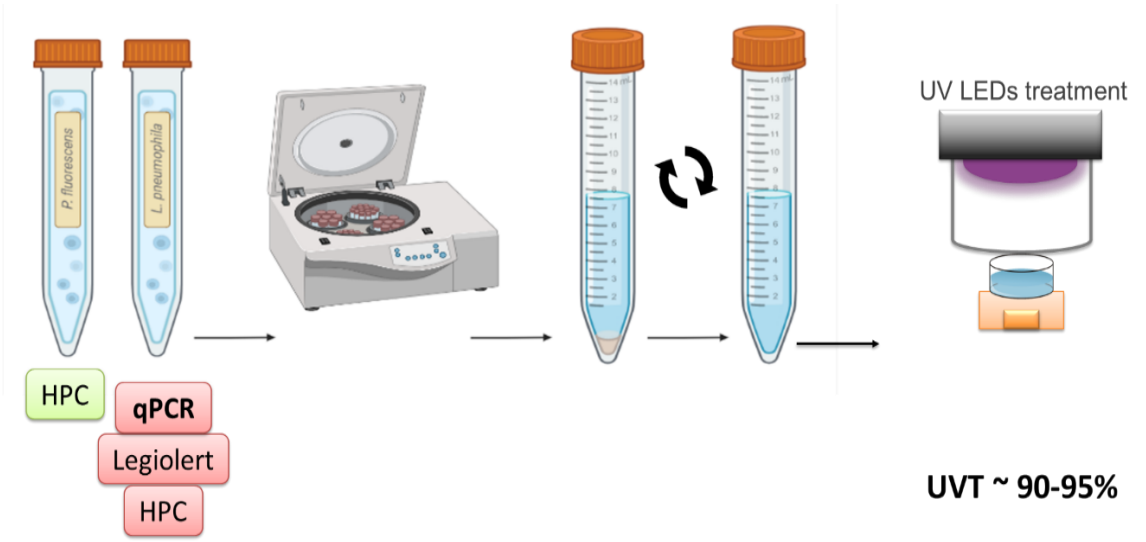

Figure 16. Methodology for Monoculture cell washing.

4.2.1.3. CDC Biofilm Reactors

This bench-scale experiment evaluates the efficiency of UV LEDs treatment to inactivate pathogen biofilms on different surfaces. The experimental design for each stage is described in Table 5 and Figure 17.

P. fluorescens was used as a biofilm producer using a CDC Biofilm and a modified version of ASTM E2562-22 *for Pseudomonas aeruginosa* biofilm growth. Two different metallic materials were chosen as a growth surface: cast iron (CI), as is one of the most frequently used materials for DWDS in North America (Ariaratnam et al., 2014), and stainless steel (SS), to simulate a general industrial equipment surface (Mazaheri et al., 2022). The reactors were connected in parallel, each with their own feeding solution, and immersed in a water bath at 27°C.

Table 5. Experimental Design Summary for CDC reactors

Step	Reactor Mode	Duration	Characteristics	CDC1 with cast stainless steel coupons	CDC2 with cast iron coupons
1	Batch mode	24 hours	Temperature	27 °C	27 °C
			Bacteria Inoculation	Inoculate *P. fluorescens*	Inoculate *P. fluorescens*
			Media	TSB media with tap water (Dil 1:100).	
			RPM	Lowest	Lowest
2	CSTR mode	24 hours	Temperature	27 °C	27 °C
			Media	TSB media with tap water (600 mL in 20L of tap water)	
			RPM	Lowest	Lowest
3	Coupon collection and analysis				

All media are sterile; Tap water is dechlorinated and sterile.

Samples were collected in sterile conditions and placed in 47 mm petri dishes for UV LED treatment.

Figure 17. Set-up of CDC reactors on the bench

4.2.1.4. UV LEDs Treatment

Table 6 and Table 7 provide a summary of experimental conditions for both experiments. The general methodology for UV LED in the collimated beam is described in CHAPTER 3.

Table 6. Experimental conditions for monocultures

Organism	Type	Wavelength (nm)	Fluences (mJ/cm^2)	Experimental values
Pseudomona fluorescens	Clear cells from monocultures	280	2, 4, 6, 8, 10, 12	Diameter: 60 mm Displacement: 90 mm Spacing: 5 mm Sample Volume: 18 mL UVT: 90-95%%
		365	20, 40, 60, 80, 100, 120	
Legionella pneumophila		280	2, 4, 6, 8, 10, 12	
		365	20, 40, 60, 80, 100, 120	

Table 7. Experimental conditions for CDC Reactors

Organism	Material	Wavelength (nm)	Fluences (mJ/cm^2)	Experimental values
Pseudomona fluorescens	Cast iron	280	20, 40, 60, 80, 100, 120	Diameter: 47 mm Displacement: 96 mm Spacing: 5 mm Sample Volume: 0 mL UVT: 100%
		365	200, 400, 600, 800, 1000, 1200	
	Stainless steel	280	20, 40, 60, 80, 100, 120	
		365	200, 400, 600, 800, 1000, 1200	

4.2.2. Quantification Methods

Treatment effectiveness was quantified using several methods. For monocultures, samples were analyzed using TSA for *P. fluorescens* using the spread technique for SPC.

For *L. pneumophila*, three quantification methods were used. Table 8 describes the quantification methods, a brief description of each method and the time of availability results is also included.

Table 8. Detection methods for L. pneumophila

Detection Method	Description	Availability of results	Units used in the method	Sample Volume used
Standard Plate Count (SPC) / HPC	BCYE agar containing 0.1% α-ketoglutarate and L-cysteine supplemented with dyes to aid identification of *Legionella pneumophila* colonies is the Golden standard for *L. pneumophila* detection.	~2-4 days 7 days (preferred)	CFU/mL	100 µL
Legiolert	It is based on a bacterial enzyme detection technology that signals the presence of *L. pneumophila*, which changes colour when it is detected.	7 days	MPN/ 100mL	10 mL
Quantitative Polymerase Chain Reaction (qPCR)	Determines the actual amount of PCR product present at a given cycle. Using a fluorescent report in the PCR reaction allows the measurement of DNA generation in the qPCR assay. Using specific primers for *L. pneumophila*, this assay has a high accuracy level.	~2h per run	GU/ml	7.5 mL

4.3. Results and Discussion

4.3.1. Inactivation of *P. fluorescens* monocultures

Figure 18 shows the inactivation of *P. fluorescens* monocultures after UV LEDs disinfection. Notably, inactivation is higher at 280 nm, achieving a ~4.5 LRV at 12 mJ/cm^2. The 365 nm wavelength only achieved LRV ≤ 2. Another study performed by Saha et al., (2014), where they used pure cultures of several pathogens including *P. fluorescens*, subsequently exposing the organisms at 254 nm (LP UV Lamp) and 22.5 mJ/cm^2 with and without mixing matrixes; the results show that the highest disinfection was with mixing achieving a 2 LRV. The authors stated that one of the probable causes for low LRV was the high absorbance of the fluid used (UVT= 3.16x10^{-3} %). Compared to this previous study, results are showing better performance at 280 nm, but lower values at 365 nm (280 nm LRV > 254 nm LRV > 365 nm LRV).

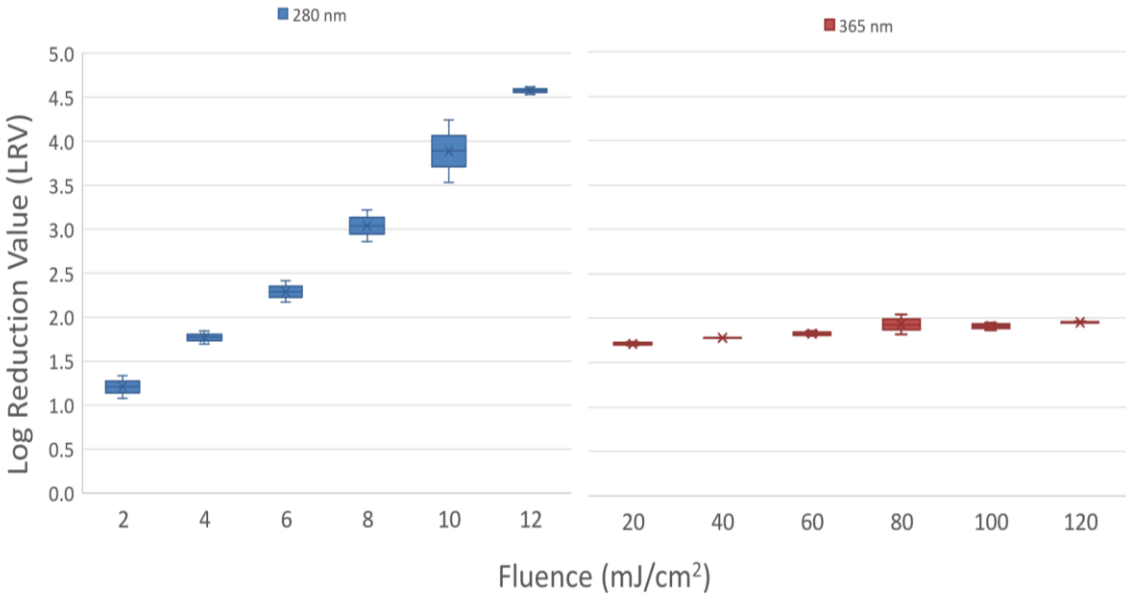

Figure 18. Inactivation of P. fluorescens after UV LEDs Treatment (Monoculture)

The k value (k_{max}) is the first-order inactivation rate constant, representing the speed of microbiological decrease per unit. This value was calculated using a log-linear regression to analyze the disinfection performance. k values are calculated using the following equation:

$$\frac{N}{N_0} = 10^{-kF}$$

Where F is the UV fluence at exposure time (Geeraerd et al., 2000).

Table 9. k values for P. fluorescens

P. fluorescens	**Material**	**Wavelength (nm)**	k_{max}	**± (SD)**
Monocultures	/	280	0.36	0.033
		365	0.002	0.0003
Biofilms	Stainless Steel	280	0.181	0.254
	Cast Iron		0.006	0.0004
	Stainless Steel	365	0.00025	0.0001
	Cast Iron		0.00033	0.0004

k values in cm^2 mJ^{-1}

Table 9 summarizes the *k* values obtained for *P. fluorescens*; in the case of 280 nm for monocultures, the *k* value is around 0.36 ± 0.033, which is similar to the values obtained for other strains of *Pseudomonas* (*k* = 0.42). They were also exposed at the same wavelength (Rattanakul & Oguma, 2018). Compared to other studies where *P. fluorescens* is used and treated only at 254nm (Saha et al., 2014; Zekanović et al., 2022), this study shows excellent performance in the UVC range (i.e., 280 nm) compared to the UVA range (i.e., 365 nm) where low disinfection was achieved even at high doses. Between both wavelengths, the rate of decrease at 280 nm was 180 times higher (0.36 vs 0.002).

4.3.2. Inactivation of *L. pneumophila* monocultures

A single-factor ANOVA was run for each method for initial quantification giving a P-value of 0.094 (P > 0.05; not statistically significant). This means that the three methods present the same accuracy in initial quantification; therefore, either is a reliable quantifying tool for *L. pneumophila*. The results are presented in Table 10.

Table 10. Results of initial quantification methods for L. pneumophila

Quantification method	***Legiolert***	***HPC***	***qPCR***
Units	MPN/mL	CFU/mL	GU/mL
Results	2.97E+06	8.10E+10	6.98E+07
	2.05E+06	7.50E+10	7.17E+07
	1.88E+06	8.80E+08	6.07E+07
	1.64E+06	7.10E+08	6.87E+07
Total Average	2.14E+06	3.94E+10	6.77E+07
ANOVA	P-value: 0.094		

Figure 19 shows that all three quantification methods achieved different LRVs. Quantification by HPC shows a progressive reduction for UV exposures at 280 nm,

reaching ~4 LRV at 20 mJ/cm^2. This result is similar to other studies of UV disinfection at 280 nm with *L. pneumophila,* where the quantification method also involved plate counts. (Cervero-Aragó et al., 2014; Rattanakul & Oguma, 2018; Allahyari et al., 2022). Regarding Legiolert, the maximum registered value was ~2 LRV at 20 mJ/cm^2, and for qPCR, ~1 LRV at 20 mJ/cm^2.

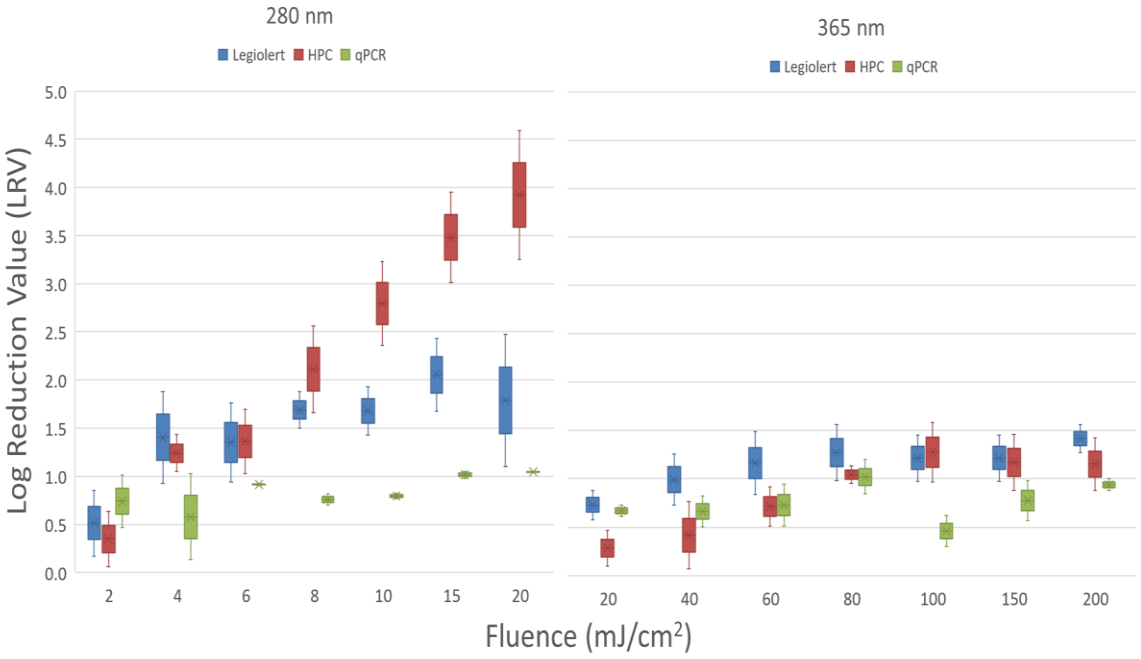

Figure 19. Inactivation of L. pneumophila after UV LEDs Treatment

According to several studies, *L.* pneumophila tends to enter a Viable but non-culturable (VBNC) state, which are the cells that are still alive but are damaged after certain treatments (Cervero-Aragó et al., 2014; L. Li et al., 2014; Dietersdorfer et al., 2018). VBNC cells do not usually appear in growth media but in colorimetric and fluorescent methods. Flow cytometry is one of the most common ways to validate cell viability and damage (Ducret et al., 2014; Dietersdorfer et al., 2018; Cervero-Aragó et al., 2019; Nisar et al., 2020). As the Legiolert test uses an enzyme to colour-indicate the presence of *L. pneumophila*

(IDEXX, 2022), this could lead to the quantification of VBNC cells, thus interpreting as low inactivation. Most qPCR tests only detect short genome sequences, which can lead to perceive an overestimation of viable organisms (Rockey et al., 2020). In this case, the qPCR only detects the presence of the bacterium by amplifying the part of the genome that indicates the genus and species of *L. pneumophila*. Therefore, quantifying the presence of that specific amplicon but does not indicate if the cell itself is damaged, dead, alive or viable. There are some modelling studies for quantifying genome degradation after UV treatment for viruses, but UV degradation quantification models for bacteria are not well yet reported in the literature (Rönnqvist et al., 2014; Chang et al., 2017; Rockey et al., 2020; Yoon et al. 2022). More studies, like the one conducted by Dusserre et al. (2008), in which they use qPCR, HPC, and flow cytometry to detect VBNC cells of *L. pneumophila*, should be developed to understand if this state would cause problems on DWDS and if it these specific quantification methods would be suitable for UV LED quantification performance. For 365 nm, as previously seen in other studies, the disinfection did not pass 1.5 LRV at high fluences. Nevertheless, a study conducted by Allahyari et al. (2022), in which disinfection of *L. pneumophila* was achieved at 1700 mJ/cm^2 obtaining ~3 LRV at 365 nm; therefore, it is established that for LRV > 3, it is required to use very high fluences (i.e. > 1000 mJ/cm^2) to inactivate *L. pneumophila* using only UVA wavelengths, which in terms of energy consumption it requires more power to achieve acceptable results.

Comparisons between 280 and 365 nm make it more perceptible to choose a suitable quantification method for UV LED studies. Using only 365 nm results as a reference would not have been perceptible for the difference between the three quantification methods.

4.3.3. Inactivation of *P. fluorescens* biofilms

Figure 20 shows notably a better performance inactivation for SS at 280 nm, reaching up to 4 LRV at 100 mJ/cm^2, in contrast with CI, which achieved disinfection of ~2.5 LRV at 100 mJ/cm^2. At 365 nm, even though the disinfection was overall below 1.4 LRV, the performance was slightly better for CI compared with SS, in which it was observed progressive disinfection reaching up to 1.3 LRV at 1000 mJ/cm^2 and below 0.5 LRV respectively; this means that UV at 365 nm may be transforming specific components present in the CI, which may induce indirect photolysis, enough to help to cellular degradation (Rockey et al., 2020). Nevertheless, more studies should be developed to understand this mechanism and indagate if, in this specific situation, this effect can be amplified for obtaining a higher LRV.

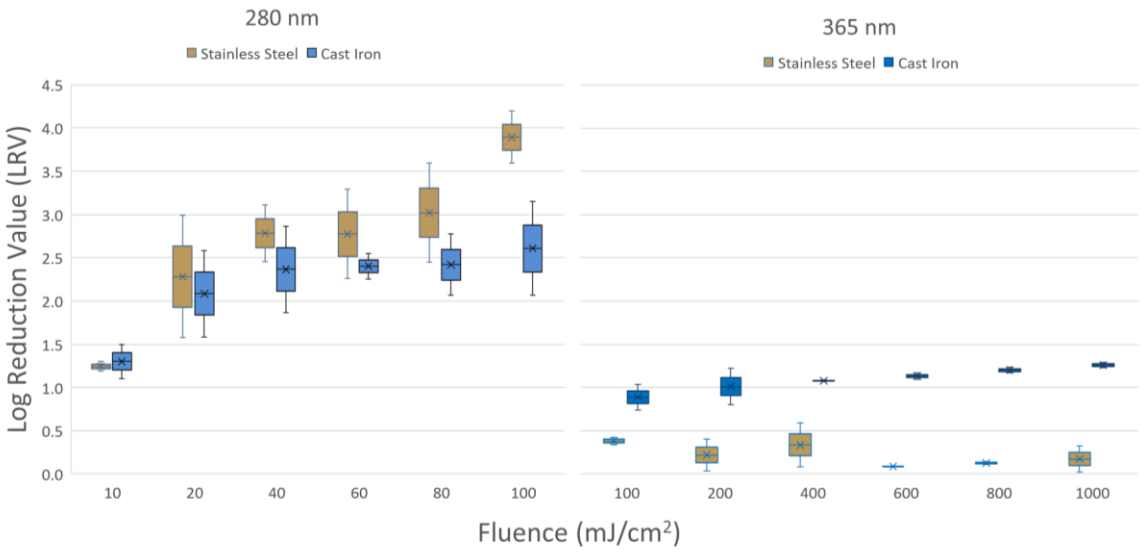

Figure 20. Inactivation of P. fluorescens after UV LEDs Treatment (Biofilms)

When comparing fluences for *P. fluorescens* inactivation, it was demonstrated that ten times more fluence was required to achieve similar results with biofilms. It is well established that bacterial communities in a biofilm form gain certain resistance to

treatments, making them more challenging to manage (Abdel-Nour et al., 2013; Rożej et al., 2015).

A closer look at the *k* values (Table 9) allows us to compare each treatment based on the inactivation constant of the same microorganism in different scenarios. At 280 nm, the rate constant of monocultures (0.36) was two times faster than SS biofilms (0.181) and 60 times faster than CI biofilms (0.006); at 365 nm, the rate constant of monocultures (0.002) was eight times faster than SS biofilms (0.00025) and six times faster than CI biofilms (0.00033). These results state a correlation based on the wavelength and growth surface.

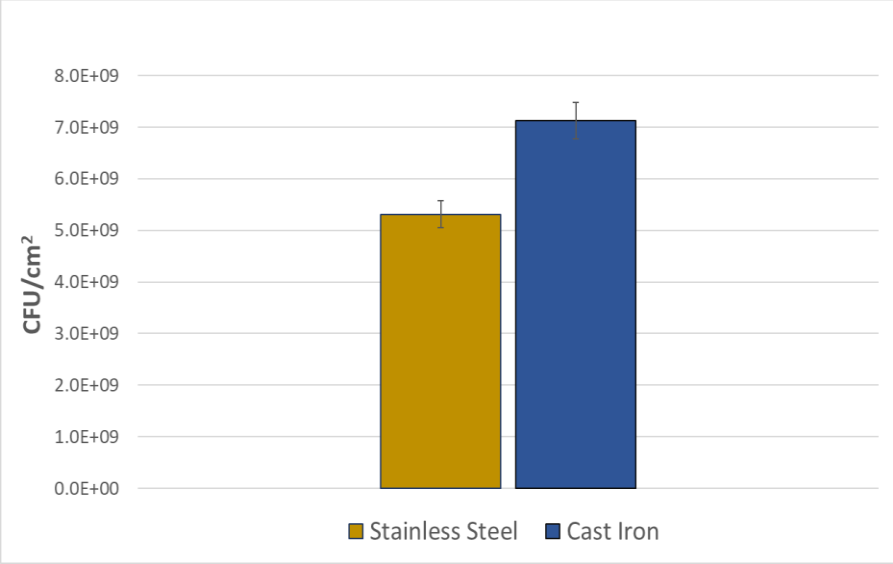

Figure 21. Biofilm growth per material - P. fluorescens

A one-way ANOVA was performed on the results of biofilm growth of *P. fluorescens* per material (Figure 21), obtaining a *P*-value of 0.84 ($P > 0.05$; not statistically significant). The results show that the difference between these two specific materials was not statistically significant to be a major factor to consider when interpreting the results of inactivation.

4.4. Conclusions

The objective of this chapter was to assess the efficiency of the inactivation of UV LEDs on OPs and biofilms of two primary organisms: i) *P. fluorescens* and ii) *L. pneumophila* and at two fluences, 280 and 365 nm. Two bench-scale experiments were implemented, one using washed cells from monocultures and the other using biofilms cultured on a CDC reactor with different growth surface materials. As such, these studies were evaluated and compared, and their independent results and conclusions are comprised in this chapter.

4.4.1. Inactivation of *P. fluorescens* monocultures

This experiment evaluated the inactivation performance of *P. fluorescens* monocultures using 280 and 365 nm. The main findings of this experiment include the following:

- UV LEDs were effective against *P. fluorescens* at 280 nm achieving up to 4.5 LRV at 12 mJ/cm^2. Inactivation at 365 nm only shows up to 2 LRV at 120 mJ/cm^2.
- *k* values for this experiment showed that at 280 nm, the inactivation constant (0.36) is similar to other strains of *Pseudomonas* that were exposed to the same wavelength. However, *k* values for 365 nm were 180 times lower than at 280 nm.

4.4.2. Inactivation of *L. pneumophila* monocultures

This experiment evaluated the inactivation performance of *L. pneumophila* monocultures using 280 and 365 nm. Three quantification methods were used to evaluate inactivation performance after UV LEDs treatment: plate counts, Legiolert and qPCR. The main findings of this experiment include the following:

- *L. pneumophila* recovery using the three different quantification methods was not significantly different (P =0.094).

- Results of 365 nm were not able to achieve LRV > 2. Nevertheless, when analyzing the results for treatment at 280 nm, the quantification methods showed different LRV values:
    - Plate counts achieved up to 4 LRV at 100 mJ/cm^2, similar to other studies with *Legionella*. Other studies also used standard plate counts as their quantification methods, as is the golden standard for *L. pneumophila* identification.
    - Legiolert only achieved up to 2 LRV at 100 mJ/cm^2. An overestimation of cells might be present due to VBNC cells that appear in colorimetric tests. However, more tests (like flow cytometry) should be done to confirm. Although Legiolert shows better performances in terms of efficiency in quantifying *L. pneumophila*.
    - qPCR achieved only 1 LRV at 100 mJ/cm^2; this is due to the qPCR primers that would only be detecting and quantifying the part of the genome indicating the genus and species but not the viability of the cells. Better modelling for qPCR quantification for genome degradation after UV treatment should be developed for bacteria. This test was the quickest in terms of the availability of results.

4.4.3. Inactivation of *P. fluorescens* biofilms

This experiment evaluated the inactivation performance of *P. fluorescens* biofilms cultured on CDC reactors using two different surface growth materials and treated with 280 and 365 nm. The main findings of this experiment include the following:

- Inactivation at 280 nm was notably better for SS, achieving a 4 LRV at 100 mJ/cm^2. CI biofilms only achieved a 2.5 LRV at the same fluence.
- At 365 nm, disinfection was slightly better for CI than SS, giving a 1.3 LRV at 1000 mJ/cm^2 and a 0.5 LRV, respectively.
    - According to similar studies, some compounds in CI may induce indirect photolysis enough to help cell degradation. However, more studies need to be addressed on this matter.
- It required ten times more fluence to achieve similar results for the biofilms of the same organism when compared with the monoculture experiment.
- *k* values for biofilm inactivation helped compare inactivation constant in different scenarios
    - For 280 nm, the rate constant of monocultures (0.36) was two times faster than with SS (0.181) and 60 times faster than with CI (0.006)
    - For 365 nm, the rate constant of monocultures (0.002) was eight times faster than with SS (0.00025) biofilms and six times faster than CI biofilms (0.00033).
- The difference in the growth of biofilms on SS and CI coupons was not significant (P = 0.84) to be considered as a factor to consider when interpreting the results of inactivation performance.

CHAPTER 5 UV LED TREATMENT FOR NATURAL MICROBIOME BIOFILMS

5.1. Introduction

Drinking water distribution systems (DWDS) are the hosts of biofilms, mainly composed of a conglomerate of microorganisms encapsulated in polymeric substances on a surface. These biofilms are the source of planktonic bacteria (Chaves Simões & Simões, 2013), which can lead to potential hazards regarding water microbiological quality such as odour, colour, taste, appearance, and, in severe cases, flow issues. These biofilms often enhance the proliferation of opportunistic pathogens, which can be a health hazard to humans. However, the drinking water autochthonous microbial ecosystem is not yet well understood due that includes premise of complex fungal, bacterial and protozoan diversity (Li et al., 2018). Therefore, studies with natural microbiome biofilms (NMB) are not well developed, primarily because they take a long time to form, and their growth is not uniform through time, making them challenging to study (Learbuch et al., 2021). Secondly, studies with tap water is not too frequent; typically, studies are made from Drinking Water Plants or Drinking wells samples and with metallic-type pipelines (Fish & Boxall, 2018; S. Liu et al., 2016; Munoz et al., 2022).

The use of plastic materials like polyvinyl chloride (PVC) is increasing for DWDS because they are easy to cut, handle, and resist corrosion (Rożej et al., 2015). NMB grown in plastic-type pipelines are often treated with sanitizers which can be chlorine-based or hydrogen peroxide-based. Nevertheless, although practical, these sanitizers can produce disinfection by-products (DBPs), potentially hazardous to human health (Maharjan et al., 2017).

UV LEDs as a biofilm control alternative have been gaining popularity over the years as a non-hazardous, innovative, and effective tool (Långmark et al., 2007; Jones et al., 2016;

Gora et al., 2019; Amar et al., 2021; Torkzadeh et al., 2021). However, studies on UV LED inactivation for NMB on plastic pipelines are not yet developed (Linden et al., 2019).

The objective of this Chapter was to develop a method to enhance the growth of NMB for later disinfection using UV LEDs. PVC coupons were used and placed in a biostud reactor to simulate new DWDS with plastic-like materials. Furthermore, the impact of certain nutrients and temperature on biofilm growth was also investigated to determine the maximum growth over a short period.

5.2. Materials and Methods

5.2.1. Experimental Design and Set-up

Table 11 summarizes the experimental set-up for the biostud reactors to grow NMB under different conditions. All general materials and methods for this experiment are outlined in CHAPTER 3.

Table 11. Summary of experimental growth conditions for NMB

Reactor	Coupon material	Temperature	Nutrient Concentration
A. Ambient	PVC	Room Temp	Dechlorinated tap water
B. Ambient + Nutrient		Room Temp	0.25% - R2A
C. 35 -Nutrient + High Iron dose		35 °C	0.25 % - R2A + 335 µM – Iron pyrophosphate
D. 35- Nutrient + Low Iron Dose		35 °C	0.25 % - R2A + 167.5 µM – Iron pyrophosphate

Iron pyrophosphate: $Fe_4(P_2O_7)_3$

Reactors were installed by inserting the coupons, connecting the tubing and preparing the feeding solution (Figure 22).

The first stage of the experiment was to compare the growth of NMB under different nutrient and temperature conditions. Iron pyrophosphate ($Fe_4(P_2O7)_3$) was chosen for being

a compound used as an iron supplement; according to several studies, iron is one of the main compounds that organisms need to carry out metabolic processes in order to develop better (Portier, 2014).

R2A broth is better known for culturing heterotrophic microorganisms from potable water samples (APHA et al., 2017), this broth was used to enhance microbial activity in the tap water, in an effort to reduce an extended culture period of NMB. Also, the temperature on the reactors was set to 35°C, the standard optimal temperature for the culture of microorganisms (Atlas & Snyder, 2013).

Figure 22. Biostud reactors (left) are connected in parallel with their feeding source (right). Experimental set-up.

For the first stage, all experiments were left initially at 28 days and done in duplicate, and ATP analysis monitored biofilm activity every third day. Growth curves were generated, and k_{biofilms} values were calculated to compare the growth rate between arrangements concentrations. k_{biofilms} values represent the exponential growth rate constant for a microorganism over time, in this case, biofilm growth. The exponential growth rate constant (k_{biofilms}) was calculated using the function for exponential growth:

$$y = A_x e^{kt}$$

Where:

- y = Exponential growth
- A_x = value at a given time
- e = Euler constant
- k = Exponential growth constant ($k_{biofilms}$) in days^{-1}
- t = time

Once the best nutrient combination (from Table 11) was chosen from the previous stage, biofilms were grown on the biostud reactors under the specified conditions.

The second stage evaluated the inactivation performance of NMB inactivation using UV LEDs. Coupons were carefully removed using the special coupon tool, placed in a 47 mm petri dish, and labelled. Once the correction factors and exposure times were calculated using the spreadsheet provided by Aquisense Technologies (as described in 3.6), the coupons were placed under a collimated beam in a rotating plate and treated at 280 nm and 365 nm. Table 12 shows the experimental conditions for the inactivation stage.

Table 12. Experimental conditions for NMB inactivation

Organism	Material	Wavelength (nm)	Fluences (mJ/cm^2)	Experimental values
NMB	Polyvinyl Chloride (PVC)	280	5, 10, 20, 40, 60, 80, 100	Diameter: 47 mm Displacement: 96 mm Spacing: 5 mm Sample Volume: 0 mL UVT: 100%
		365	50, 100, 200, 400, 600, 800, 1000	

NMB: Natural Microbiome Biofilms

After treatment, quantifications were made with Heterotrophic Plate counts (HPC) with R2A agar.

5.2.2. Quantification methods

5.2.2.1.ATP

ATP Deposits and Surface Analysis (DSA) quantification test (LUMINULTRA, 2022b) was used to monitor biofilm formation. Following the manufacturer's protocol for 'Biofilm Collector', coupons were carefully and aseptically removed every third day, then the entire coupon was deposited in a 5 mL UltraLyse collection tube and allowed rest on the bench for a minimum of five minutes for extraction. Later, it was vortexed at max speed for one minute to disperse the biofilm throughout the fluid. Then 1 mL of the UltraLyse Tube was transferred to the 9 mL UltraLute (Dilution) tube and mixed. Once the Luminometer was calibrated, the Relative Light Units (RLU) reading was taken and noted for later calculations, obtaining the tATP ($pgATP/cm^2$). This assay was done in duplicate.

5.2.2.2.HPC

Biofilms were recovered and resuspended using the recovery technique by Gora et al. (2019). Serial dilutions were done using sterile PBS and then plated on R2A agar using the spread technique inside the BSC to ensure sterility. This method is described in 3.7.1.

5.3. Result and Discussions

5.3.1. Growth of Natural Microbiome under different conditions

Results from the first stage of the experiment (Figure 23) show that the dechlorinated tap water could not produce a high enough quantity of biofilms over 28 days; other studies also found a minimal or null amount of biofilms after 28 days (Douterelo et al., 2014). However, it was noticeable that biofilm activity started the exponential phase around day three and a max growth around day nine for most of the other nutrient concentrations.

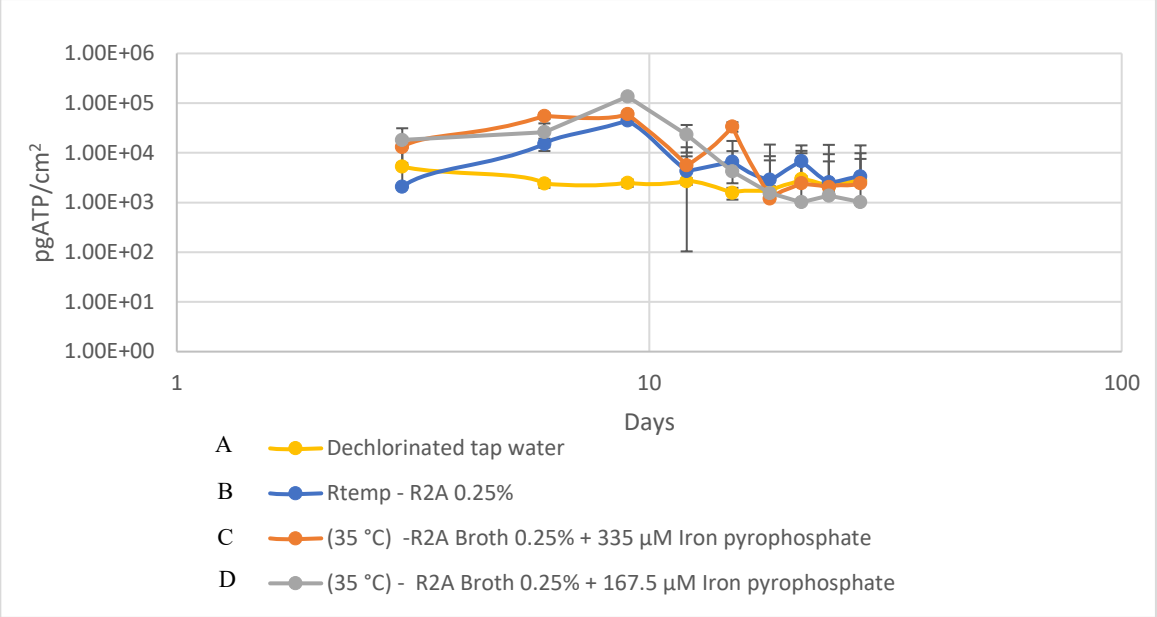

Figure 23. Growth of Natural Microbiome on Different Conditions

$k_{biofilms}$ values were calculated, obtaining the following results: A: -0.0843, B: 0.1210, C: 0.1694, D: 0.2238. It is shown that the A arrangement has a negative constant, which can be interpreted as a decay constant, meaning that microbial activity started to decrease over time. From arrangements B, C and D, the highest exponential growth rate constant is D: R2A Broth -0.25% + 167.5 µM at 35 °C with a max ATP recovery of ~135000 pg ATP/cm^2. This was the arrangement used for the second stage of the experiment, in which biofilms were grown in biostud reactors for later treatment with UV LEDs for inactivation evaluation performance.

The concentration of Iron pyrophosphate between C and D arrangements was half; nonetheless, the growth rate is notably different. This can be caused by an iron overload (for C arrangement), possibly resulting in a certain degree of iron toxicity, making biofilms stop or decelerate their growth (Braun, 1997).

5.3.2. Natural Microbiome Biofilm Inactivation

After UV LEDs treatment, results showed (Figure 24) better performance at 280 nm than at 365 nm, obtaining up to 2.3 LVR at 100 mJ/cm^2 and 0.8 LRV at 200 mJ/cm^2, respectively. The curves for each wavelength exposure range differed dramatically. For instance, the 280 nm curve shows a linear shape, a traditional first-order inactivation kinetics; on the other hand, there is a shoulder at 200 mJ/cm^2 for 365nm, which is the highest value with 0.8 LRV. This results in an inactivation curve without linearity; this curve shows a convex-tail shape, which assumes one initial significant subpopulation, that is more sensitive to stress and a minor that is less resistant (van der Waal, 2017). According to other studies, the resistance obtained by NMB can be suggested for the presence of mixed populations in addition to the biofilm and/or medium's protective effect (Coroller et al., 2006; Qi et al., 2016; Trubenová et al., 2022).

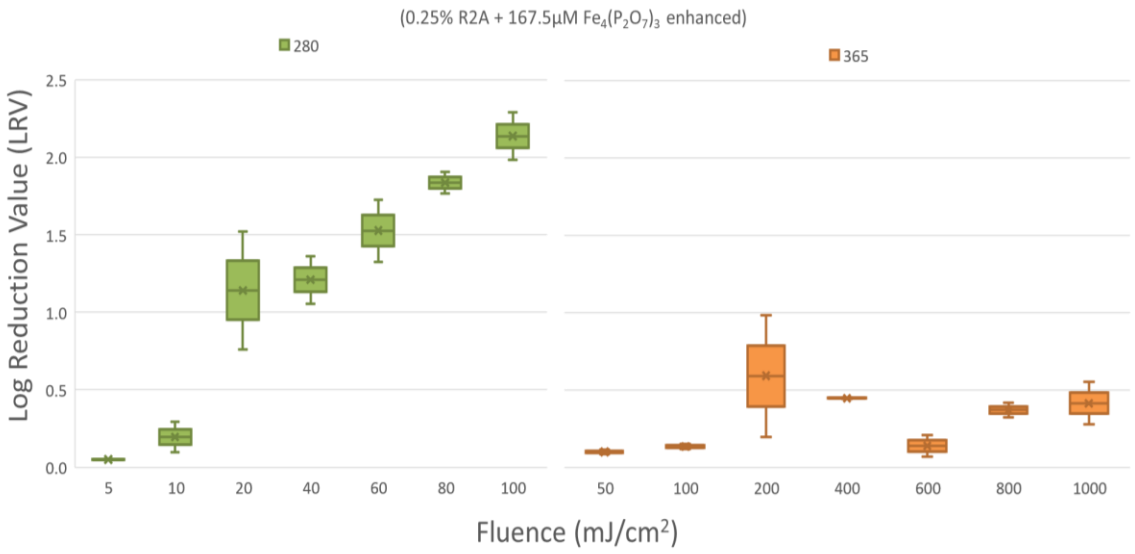

Figure 24. Natural Microbiome Biofilm Inactivation with UV LEDs Treatment

To determine if adding nutrients to the culture media impacted UV LED inactivation, meaning that the media absorbed part of the light imposed on the sample, wavelength scans were performed for two of the nutrients added: iron pyrophosphate and R2A broth.

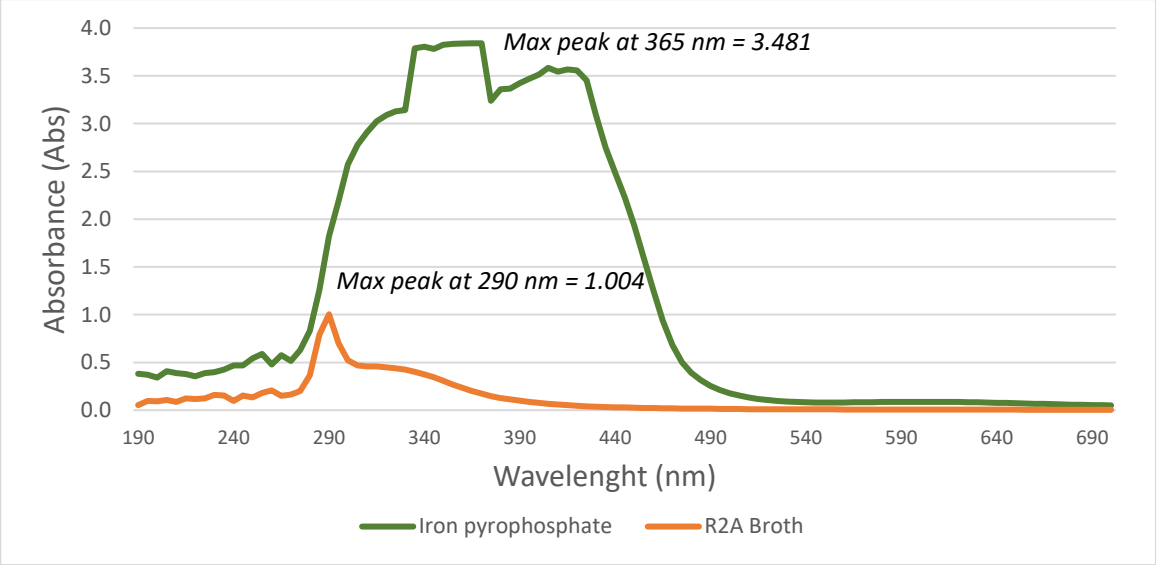

Figure 25. Wavelength scan of Iron pyrophosphate and R2A broth

Results of the wavelength scans (Figure 25) show that the absorbance of R2A broth is minimal, and the highest absorbance occurs at 290 nm, which means that the broth may not be affecting the inactivation performance; however, the highest absorbance of iron pyrophosphate occurs at 365 nm, meaning that for the 365 nm treatment, part of the light might be absorbed by the iron pyrophosphate and not the biofilm, leading to low LRV.

Factors that may limit the efficacy of UV disinfection, in this case, may arise from different sources, for instance, the inherent extra protection that biofilms provide to cells, the absorption of UV light by the compounds present in the media, certain species regrowth, and even, the provision of nutrients of surviving bacteria by UV-damaged cells (Kollu, 2014).

It is suggested for future research to investigate the factors that may limit UV disinfection efficacy on NMB by running a DNA sequencing to determine which autochthonous population is present and determine if there are groups that are more or less resistant to UV treatment; also to use other types of wavelengths or a combination of wavelength that can overcome the absorbance of UV light; moreover, to determine if UV-lysed cells may be a source of nutrients for surviving cells, gaining certain regrowth level.

5.4. Conclusions

The objective of this chapter was to develop a method for NMB growth enhancement for later UV LED disinfection. One bench scale conducted on two stages was performed on a biostud rector using PVC coupons to simulate a plastic surface.

The first stage intended to compare the growth of NMB under different conditions. The second stage used the best option from the previous stage for maximum growth for later inactivation with UV LEDs using 280 nm and 365 nm.

5.4.1. Growth of Natural Microbiome under different conditions

This stage evaluated the enhanced growth of NMB using both R2A and Iron pyrophosphate ($Fe_4(P_2O_7)_3$) as nutrients and at different temperatures. The main finding of this experiment includes the following:

- From the four nutrient arrangements, the best condition was D: 0.25 % - R2A + 167.5 μM – Iron pyrophosphate at 37 °C.
- Using monitoring by ATP, the maximum growth was ~140000 pg ATP/cm^2.
    - The ATP concentration between two of the arrangements with Iron pyrophosphate (335 and 167.7 μM) was significantly different even though

the iron pyrophosphate was the half; according to other studies, there may be an iron overload (335 μM), resulting in a toxicity degree.

- For a better comparison between conditions, $k_{biofikm}$ values (growth rate constant) were calculated, obtaining the following results:
    - A: -0.0843, this value is negative, which can be interpreted as a decay constant
    - B: 0.1210, the lowest constant value, consistent with the shape of biofilm growth curves
    - C: 0.1694, the second highest value, consistent with the shape of biofilm growth curves
    - D: 0.2238, the maximum constant value, consistent with the shape of biofilm growth curves

5.4.2. Natural Microbiome Biofilm Inactivation

This stage evaluated the inactivation performance of NMB using 280 and 365 nm using UV LEDs. The main findings of this experiment include the following:

- From the two wavelengths chosen, the best-performance disinfection occurred at 280 nm, obtaining the highest value of 2.3 LVR at 100 mJ/cm^2.
- It is noticeable that the inactivation curves presented different shapes.
    - The 280 nm curve shows a linear shape.
    - At 200 mJ/cm^2, the highest value was obtained for the 365 nm curve. This is the highest value obtained.
        - The results show curves with no linearity. The 365 nm shows a convex-tail shape.

- According to other studies suggested that for NMB, the presence of mixed populations can provide a certain degree of protection due to the resistance of specific bacterial subpopulations.
- Wavelength scans were analyzed for the nutrients to determine the culture media absorbed part of the light imposed on the sample.
    - R2A broth max absorbance was at 290 nm, which may not impact the inactivation treatment.
    - Iron pyrophosphate max absorbance was at 365 nm, which may have impacted the inactivation performance, possibly explaining low LRV.
- It is suggested that factors limiting UV disinfection on NMB are approached for future research:
    - DNA sequencing would help analyze populations that may have UV resistance.
    - Determine if other UV wavelengths or a combination may overcome nutrient absorption.
    - Determine if UV-lysed cells may be a source for surviving bacteria, leading to regrowth.

CHAPTER 6 CONCLUSIONS AND RECOMMENDATIONS

6.1. Conclusions

This research focused on expanding the understanding of biofilm formation in simulated DWDS and applying UV LEDs to inactivate opportunistic pathogens and biofilms. The first study (Chapter 4) was divided into two experiments to assess the inactivation performance of microorganisms in different matrixes. The first experiment used monocultures from *L. pneumophila* and *P. fluorescens*. The second experiment used CDC reactors to grow biofilms from *P. fluorescens* in CI and SS coupons*;* later, these organisms were treated with UV LEDs and created dose-response curves using multiple quantification tools to evaluate inactivation efficiency. The second study (Chapter 5) aimed to develop a method to enhance the growth of NMB on a biostud reactor using PVC coupons for later disinfection using UV LEDs. The method considered nutrients and temperature impact on biofilm formation. The main findings from Chapter 4 and Chapter 5 are summarized in Table 13 and Table 14, respectively.

Table 13. Main findings from Chapter 4

CHAPTER 4: Inactivation of *L. pneumophila* and *P. fluorescens* with UV LEDs.	
UV LED Treatment	**Main finding**

L. pneumophila monocultures	• Results from 365 nm did not show values >2 LRV.
	• Quantification methods show different results for 280 nm.
	  ○ Max disinfection at 100 mJ/cm2 with SPC shows up to 4 LRV, similar to other Legionella studies.
	  ○ Legiolert obtained up to 2 LRV, probably because the test is detecting VBNC cells.
	  ○ qPCR obtained only 1 LRV because primers only detect part of the genome that does not indicate possible cell damage.

P. fluorescens monocultures	• Results from 280 nm presented the best performance with 4.5 LRV at 12 mJ/cm^2. • Treatment with 365 nm presented low disinfection with 2 LRV at 120 mJ/cm^2.
P. fluorescens biofilms	• Inactivation at 280 nm was notably better for SS than with CI coupons at 100 mJ/cm^2 (4 LRV vs 2.5 LRV, respectively). • Treatment with 365 nm was slightly better for CI than for SS at 1000 mJ/cm^2 (1.3 LRV vs 0.5 LVR, respectively). o Other studies indicate a possible photolysis reaction, but more studies must be addressed. • It was required ten times more fluence to achieve similar results for biofilms compared with monocultures of *P. fluorescens*. • There was no statistical difference in the growth of both materials

Table 14. Main findings from Chapter 5

CHAPTER 5: UV LED Treatment for Natural Microbiome Biofilms (NMB)	
Item	**Main finding**

Growth of NMB under different conditions	• The best nutrient and temperature conditions was 0.25 % - R2A + 167.5 µM – Iron pyrophosphate at 37 °C.
	  o It allowed biofilm to have a maximum growth of ~140000 pg ATP/cm^2 in 10 days.
	  o The growth rate constant ($k_{biofikm}$) was calculated to be 0.2238

NMB inactivation with UV LEDs	• The best inactivation performance was at 280 nm, obtaining 2.3 LVR at a fluence of 100 mJ/cm^2. • The inactivation curves presented different shapes; 280 nm linear shape and 365 nm showed a convex-tail shape. • R2A broth does not show evidence of impact on the disinfection performance. • Iron pyrophosphate maximum absorbance was at 365 nm, indicating that it might have impacted disinfection, resulting in low LRV.

6.2. Recommendations for future studies

6.2.1. Recommendations for *L. pneumophila* studies

The results of this study suggest deepening the understanding of *L. pneumophila* quantification tools for UV LEDs treatment. For instance, overcoming the potential presence of VNBC cells to avoid misinterpretation of UV results is crucial. On the other hand, the qPCR test proved to be the best quantification tool as it can produce results relatively rapidly; however, it is essential to develop a qPCR-based technique to quantify cell damage for *L. pneumophila*. Current cultured-based methods can take up to seven days to obtain results, which is a long time for validating a UV technology in a scenario where it is necessary to take immediate corrective actions.

6.2.2. Recommendations for biofilm studies

It is recommended to implement molecular tests (e.g., 16S rDNA sequencing) for NMB, which can provide more information about the subpopulations present in the reactors. This can help to understand which types of organisms are more UV-sensitive at specific wavelengths. Therefore, this information might help design systems with targeted wavelengths to overcome UV resistance. Moreover, studies with more or a combination of wavelengths may help overcome UV light's nutrient absorption. According to other studies, UV-lysed cells may be a source for surviving bacteria, which may cause regrowth in the system; therefore, it is suggested to gather information on this matter for NMB studies to understand low LVR better.

6.2.3. Recommendations for UV LEDs studies

It is necessary to conduct more studies with UV LEDs in DWDS to help contribute to future applications of these devices within pipes. These systems could be installed in buildings to help protect people from potential pathogen exposures (Linden et al., 2019). Furthermore, the combination of treatments, like UV LEDs-Chlorine, might be a reliable approach for reducing the formation of DBPS but also having a residual effect in the water, which UV does not provide (L. Liu et al., 2019; J. Li et al., 2023). Exploring the possible synergistic effects of multiple wavelengths (sequencing or simultaneous) would be interesting. Recent studies show that it might help overcome the potential photoreactivation mechanisms that may occur when using single wavelengths (Beck et al., 2017; Green et al., 2018; Nyangaresi et al., 2023).

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