Application of Cold Plasma for Inactivation of Waterborne Pathogens

Submitted in complete fulfilment for the degree of Master of Applied Science in the Department of Biotechnology and Food Science, Durban University of Technology, Durban, South Africa

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Reference Declaration in Respect of a Master's Dissertation

I, Mrs Amelia Rampersad (Student No. 19401807), Dr Kevin Reddy and Professor Feroz Mahomed Swalaha do hereby declare that in respect of the following dissertation:

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Author’s Declaration

This study presents original work by the author. It has not been submitted in any form to another academic institution. Where use was made of the work of others, it has been duly acknowledged in the text. The research described in this dissertation was carried out in the Department of Biotechnology and Food Science, Faculty of Applied Sciences, Durban University of Technology, South Africa, under the supervision of **Prof. Feroz Mahomed Swalaha** and **Dr Kevin Poobalan Reddy**.

Students signature

ABSTRACT

The inactivation of harmful pathogenic microorganisms during water treatment is essential for ensuring safe and clean water for human consumption. Current treatment technologies exhibit limitations in effectively eliminating pathogenic contaminants, necessitating the exploration of advanced disinfection technologies. This study investigates the application of atmospheric cold plasma (ACP) as a novel disinfection method for water contaminated with various pathogens, optimising key treatment parameters such as electrode distance and gas type (oxygen, argon and air) at voltages of 9.56 kV- 13.53 kV over treatment durations of 0.5-2.5 minutes.

The study assessed ACP's efficacy against chlorine-resistant and non-chlorine-resistant bacteria, examining direct ACP treatment and effects after 24 h storage post-treatment. Bacterial suspensions at initial concentrations of 1×10^7 CFU/mL were exposed to varying treatment conditions, with bacterial inactivation analysed via colony counts and statistical analyses (two-way ANOVA with Tukey's post-hoc). The results indicated significant log reductions in bacterial populations, with ACP achieving up to 4-log reductions, particularly against chlorine-resistant, Gram-negative bacteria. Among the gases tested, argon plasma had the highest bacterial inactivation rates, outperforming oxygen and air plasma, particularly against chlorine–resistant bacteria.

Environmental water samples treated with ACP showed 90-100% bacterial inactivation, corresponding to log reductions of 3-5 logs, consistent across both direct and 24 h storage post-treatment samples. Argon and oxygen plasma showed high efficacy, with oxygen plasma having the highest inactivation in the uMhlanga Lagoon sample. The sustained antibacterial effect of ACP after 24 h storage post-treatment was attributed to its disruption of bacterial cellular functions, effectively inhibiting regrowth and ensuring long-term water safety.

These findings confirm the potential of ACP as a highly effective and adaptable disinfection technology for water purification, particularly in targeting bacteria with carrying resistance profiles. While this study highlights the efficacy of ACP in inactivating pathogenic bacteria, further research is needed to ensure the safety of treated water for human consumption and to evaluate its effectiveness in removing chemical contaminants. This study demonstrates that ACP is a highly effective disinfection technology for water treatment, with consistent success in inactivating a wide range of pathogenic bacteria.

DEDICATION

In humble reverence, I dedicate this thesis to Lord Shiva, the auspicious deity who embodies the principles of strength, transformation and cosmic energy. Lord Shiva, the great Yogi and source of inspiration has been my guiding force throughout this academic journey. His divine blessings have granted me the courage to overcome challenges, persevere in times of difficulty and ultimately achieve my scholarly goals. I express my deepest gratitude to Lord Shiva for being the spiritual anchor and guiding light on this profound voyage of knowledge and self-discovery.

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LIST OF ABBREVIATIONS & ACRONYMS

ACP	– Atmospheric cold plasma
APPJ	– Atmospheric pressure plasma jet
BA	– Blood agar
BOD	– Biochemical oxygen demand
BOM	– Biodegradable organic matter
CFU	– Colony forming unit
COP	– Cold oxygen plasma
CP	– Cold plasma
CRB	– Chlorine-resistant bacteria
DALYs	– Disability-adjusted life years
DBD	– Dielectric barrier discharge
DBPs	– Disinfectant by-products
DO	– Dissolved oxygen
DT	– Direct treatment
DWTCs	– Drinking water treatment chemicals
E	– Electric field
E_{max}	– Dielectric breakdown
EMF	– Electric magnetic field
Ev	– Electron volt
MF	– Microfiltration
MST	– Microbial source tracking
NA	– Nutrient agar
NB	– Nutrient broth
NGS	– Next generation sequencing
NICD	– National Institute for Communicable Diseases
NM	– Nanometre
ns	– not significant (no significant differences)
NTP	– Non-thermal plasma
PBS	– Phosphate-buffered saline

PEFs	– Pulsed electric fields
PPE	– Personal protective equipment
PT	– 24 h storage post-treatment
qPCR	– Quantitative polymerase chain reaction
RF	– Radio Frequency
RNS	– Reactive nitrogen species
RO	– Reverse osmosis
ROS	– Reactive oxygen species
SS	– Saline solution
Te	– Electron temperature
Ti	– Species temperature
TP	–Thermal plasma
UF	– Ultrafiltration
ΔV	– Potential difference

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CHAPTER 1: INTRODUCTION

Potable water is an essential life-supporting resource, vital for the survival of all living organisms. The quality of water is often assessed based on the concentration and state (dissolved or particulate) of natural and inorganic substances, along with specific physical properties (United Nations, 2021). Globally, a significant portion of the population lacks access to potable water, with 771 million individuals facing this challenge and 1.7 billion needing access to improved sanitation (World Health Organization, 2022). Inadequate access to clean water has led many to intentionally use water contaminated with pathogens or unacceptable levels of contaminants (Ramírez-Castillo *et al.,* 2015).

The consumption of contaminated water can result in extensive, acute and chronic diseases causing infections or fatalities (Iaccarino, 2019). According to a study by the World Health Organization, unsafe drinking water, sanitation and hygiene practices in South Africa were responsible for over 7000 deaths and more than 640,000 disability-adjusted life years (DALYs) in children under the age of five. Over 500,000 deaths in Africa and Southeast Asia were attributed to unsafe drinking water, sanitation and hygiene (World Health Organization, 2023). On a global scale, about 7.2 million Americans get sick and approximately 2.2 million people die (3.2% deaths) every year because of diarrhoea, gastrointestinal diseases and methodical aids due to hazardous water, poor sanitation and poor hygienic conditions (Centers for Disease Control and Prevention, 2023; Ramírez-Castillo *et al.,* 2015). To overcome the aforementioned challenges, the United Nations (UN) through Sustainable Development Goal 6 (SDG6), aims to make safe drinking water universally accessible by 2030 (United Nations, 2021).

Chlorine is extensively utilised as a disinfectant for treating water against microbial contamination primarily due to its effectiveness in eradicating a broad

spectrum of microorganisms and being a powerful oxidant (Galeano *et al.,* 2019; Tsagkari & Sloan, 2023). However, not all microorganisms are eliminated by this method resulting in the survival of chlorine-resistant bacteria (Ding *et al.,* 2019; Tsagkari & Sloan, 2023). A recent study indicated that atmospheric cold plasma (ACP) application at high voltages induced a higher percentage of injury in chlorine-resistant *Salmonella enterica* cells than in non-chlorine-resistant cells (Kazemzadeh *et al.,* 2022). A study using UV irradiation proved effective initially to inactivate chlorine-resistant bacteria by damaging their DNA and preventing further growth (Cai *et al.,* 2022). However, this method was a temporary solution since the bacteria were reactivated through DNA photoreactivation (Li *et al.,* 2019; Cai *et al.,* 2022).

Water-borne diseases are prevalent in developing regions of the world, and drinking water supplies are not always safe and cannot be assured since each treatment process has its drawbacks (Ferreira *et al.,* 2021). As a result of these challenges, sustainable and cost-effective technologies are necessary. The treatment processes need to operate sustainably and be easy to maintain (Zaman, 2014). In addition to traditional water sterilisation techniques like the use of chlorine and thermal disinfection, atmospheric cold plasma can be used to inactivate pathogens in water (Pūle, 2016).

Cold plasma technology is an emerging solution for water treatment with the ability to oxidise contaminants and disinfect water (Gururani *et al.,* 2021).
Atmospheric cold plasma may be used for waterborne pathogen inactivation and can provide an alternative to traditional tertiary water disinfection methods like thermal disinfection and chlorine use (Pūle, 2016). Atmospheric cold plasma, specifically corona discharge systems which effectively eliminate a variety of microorganisms, efficiently disinfect water (Korachi *et al.,* 2009). Reactive species produced during plasma discharge, such as oxygen-based reactive species, atomic oxygen and ozone, play a crucial role in microbial cell death by interacting directly with microbial membranes (Korachi & Aslan, 2013). Atmospheric cold plasma stands out for its ability to leave no toxic residues,

ease of use, low energy consumption and operation at ambient temperatures, making it a promising solution for water treatment (Thakulla & Fisher, 2023; Gururani *et al.,* 2021).

Bacterial inactivation using ACP is influenced by various factors, including initial microbial concentration, ACP device type, exposure distance, treatment time, operating gas composition and flowrate, as well as the nature of the microorganism and growth media (Das *et al.,* 2022). Dielectric barrier discharge (DBD) is a commonly used ACP technology for water treatment due to its stability, uniformity and limited arc formation and behaviour (Ojha *et al.,* 2021). Another category of cold plasma discharge employed for water disinfection is atmospheric pressure plasma jets (Gururani *et al.,* 2021). Chandana *et al.* (2018) assessed the effectiveness of non-thermal atmospheric pressure plasma jets in diminishing bacterial load in a liquid solution. They found that this non-thermal plasma technology could serve as an alternative for sterilising heat-sensitive and vacuum-sensitive living tissues (Gururani *et al.,* 2021). Understanding and optimising these factors are essential for harnessing the full potential of ACP in bacterial inactivation.

The application of chlorine treatment dates to the early 20th century, marking a significant advancement in public health practices (Costa *et al.,* 2023). It was not possible to create plasma on sensitive materials such as food, water, and human tissue for disinfection until the mid-20th century because until then plasma could only be created at relatively high temperatures in a vacuum. However, technological advancements in the last decade have enabled the production of heat or cold plasma under atmospheric conditions, providing numerous benefits. These factors have propelled new antimicrobial techniques to the forefront of emerging technology (Korachi and Aslan, 2013). Atmospheric cold plasma has numerous benefits, but it has not been fully developed as a wastewater treatment technique due to a lack of information regarding capital investment, proficient application and operating costs, thus necessitating additional research to facilitate its commercialization, as this is a potential

solution to water crises and to meet the demand for potable water (Gururani *et al.,* 2021).

In this study, the use of atmospheric cold plasma for the treatment of waterborne pathogens was tested to determine the efficacy of water disinfection. This study aimed to optimise the use of atmospheric cold plasma to inactivate waterborne pathogens to ensure the safety of drinking water. The hypotheses and null hypotheses for each of the objectives are as follows:

Objective 1 – To optimise atmospheric cold plasma production by verification of the dielectric constants using various gases, voltages and distances between electrodes.

- Hypothesis: The dielectric constants of gases, voltages and electrode distances will significantly influence the efficiency of ACP production.
- Null Hypothesis: There is no significant influence of the dielectric constants of gases, voltages and electrode distances on the efficiency of ACP production.

Objective 2 - To determine the optimum parameters for the inactivation of chlorine-resistant and non-chlorine-resistant bacterial pathogen loads in water using atmospheric cold plasma.

- Hypothesis: There are specific optimal parameters (gases, voltages and treatment times) that maximise the inactivation of chlorine-resistant and non-chlorine-resistant bacterial pathogens in water using ACP.
- Null Hypothesis: There are no specific optimal parameters that significantly affect the inactivation of chlorine-resistant and non-chlorine-resistant bacterial pathogens in water using ACP.

Objective 3 – To determine the kinetics and extended effects of microbial inactivation after direct cold plasma treatment and after 24 h storage post-treatment.

- Hypothesis: Atmospheric cold plasma treatment will result in significant bacterial inactivation, and this effect will be sustained during the 24 h storage post-treatment period, particularly for chlorine-resistant bacteria.
- Null Hypothesis: Atmospheric cold plasma treatment will have no significant effect on bacterial inactivation after the 24 h storage post-treatment period.

Objective 4 – To apply the optimal process parameters to disinfect river and natural water resources.

- Hypothesis: The application of optimal ACP treatment parameters will significantly reduce bacterial contamination in river and natural water resources, ensuring their disinfection.
- Null Hypothesis: The application of optimal ACP treatment parameters will have no significant effect on the bacterial contamination levels in river and natural water resources.

CHAPTER 2: LITERATURE REVIEW

2.1 Introduction

Matter is classified into three states: solid, liquid, and gas with plasma sometimes added as a fourth state, though the first three are more commonly explored and understood (Fitzpatrick, 2022). Plasma is an ionised gas consisting of light electrons and heavy ion particles, where positive particles and free electrons are nearly equal, resulting in a near-neutral net charge (Fitzpatrick, 2022). The term “plasma” was originally defined as a state with equal numbers of ions and electrons. Over time, the definition has evolved to encompass a state where a significant proportion of atoms and molecules are ionised. Plasma exists over a wide range of temperatures and pressures, appearing naturally in phenomena such as the aurora borealis, stars and lightning (Fitzpatrick, 2022). Artificially, plasma is utilised in technologies such as fluorescent and neon lights and plasma TVs.

Unlike gases, plasma is an excellent conductor of electricity and is influenced by magnetic fields (Šimončicová *et al.,* 2019). In water treatment, plasma can break molecular bonds, enhance oxidation and initiate chemical reactions through free radicals (Šimončicová *et al.,* 2019). Atmospheric cold plasma, a type of non-thermal plasma, has shown effectiveness in disinfecting water by eliminating bacteria, fungi and yeasts (Korachi *et al.,* 2009). Research indicates that plasma corona discharge systems can effectively sterilise water targeting a broad spectrum of microorganisms including bacteria such as *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Streptococcus mutans*, yeast (*Candida albicans*), fungi (*Aspergillus niger*), and green algae (Korachi *et al.,* 2009). Technological advancements enable the production of cold plasma under atmospheric conditions, making it a viable solution for antimicrobial applications (Korachi & Aslan, 2013). Despite its potential, the use of ACP for wastewater treatment requires further research to

address capital investment, proficient application and operating costs (Gururani, *et al.,* 2021).

Atmospheric cold plasma presents a promising and innovative approach for water disinfection, offering a non-thermal method that is both effective and environmentally friendly. Its ability to eliminate a wide range of microorganisms, including bacteria, fungi and algae positions ACP as a potential alternative to traditional water treatment methods. However, while ACP shows great promise, challenges related to its practical application, such as capital investment, operating costs and system optimisation must be addressed. Continued research and technological advancements are crucial to improving the efficiency, scalability and economic feasibility of ACP for widespread use in water disinfection, ensuring its viability as a sustainable solution for public health.

This chapter presents a comprehensive literature review that explores the current state of knowledge regarding ACP technology and its application in water disinfection. The literature review will discuss the principles and mechanisms of plasma generation, the various gases used in plasma treatment, and the factors influencing the effectiveness of plasma inactivation of waterborne pathogens. It will also highlight previous studies on the use of ACP for disinfection, focusing on its potential for both immediate and sustained microbial inactivation. This review aims to establish a scientific foundation for the experimental work conducted in this study and to identify gaps in current research, particularly regarding the optimisation of ACP parameters for water treatment.

2.2 Waterborne pathogens

The term “water quality” refers to the overall condition of water in terms of its suitability for specific uses or processes, including drinking, irrigation and industrial applications. This includes various physical, chemical and biological

characteristics to determine if the water meets the required standards for its intended use (Omer, 2019). It encompasses factors such as the concentration and state (dissolved or particulate) of organic or inorganic materials and specific physical characteristics. Monitoring water quality involves *in situ* measurements and laboratory analyses of water samples, either on-site or in a laboratory (Omer, 2019). Unsafe water sources often contain elevated levels of harmful pathogenic microorganisms and contaminants, necessitating treatment to ensure the water is safe for consumption or other uses (Hasan & Muhammad, 2020).

Waterborne pathogens primarily enter drinking water through human or animal faeces and can cause gastrointestinal infections. Contaminated drinking water supplies, groundwater, and reservoirs can lead to disease outbreaks and fatalities if not properly treated (Health Canada, 2022). For instance, the *E. coli* O157: H7 outbreak in Walkerton, Ontario, Canada in May 2000, resulted in seven deaths and over 2300 illnesses (O'Connor, 2002). *Campylobacter* spp. contamination in drinking water supplies has been implicated in outbreaks of Campylobacteriosis, with reported cases ranging from a few to several thousand.

Diarrhoea, a common health consequence, poses the highest risk of waterborne pathogen-related deaths, particularly affecting children (Malebatja & Mokgatle, 2022). Without access to clean drinking water for rehydration, the impact of diarrhoea can be fatal. Diseases such as Cholera and Schistosomiasis, identified by the World Health Organization (WHO) as major contributors to the Global Water Health Challenge, further illustrate the severity of waterborne pathogens. Cholera alone is estimated to cause 1.3 million to 4.0 million cases and 21,000 to 143,000 deaths globally when left untreated (World Health Organization, 2017). Additionally, pathogens like *Legionella pneumophila* and *Mycobacterium* spp., *Yersinia* spp. and *Acinetobacter* spp. although primarily transmitted through inhalation or contact, are found in water sources (Health Canada, 2022; World Health Organization, 2017).

Contaminated water often contains elevated levels of organic and inorganic contaminants. Organic contaminants, originating from wildlife or plants, include carbon-based substances, while inorganic contaminants consist of materials such as sand, salt, iron, calcium salts and various minerals (Akhtar *et al.*, 2021). Carbon-based contaminants encompass pathogenic microorganisms such as bacteria, protozoa, viruses and chemical compounds. The extent of contamination in water, primarily sourced from natural reservoirs, is influenced by the sanitation conditions surrounding these water sources. Surface water sources are susceptible to contamination from debris upstream, consisting of both solid matter and pathogenic microorganisms. Conversely, underground water sources may have higher concentrations of inorganic chemicals and lower levels of pathogens (Belle *et al.,* 2023; Verlicchi & Grillini, 2020). Hazardous water sources harbour high levels of harmful pathogenic microorganisms and various contaminants, necessitating treatment to render the water safe for drinking or other activities (Some *et al.,* 2021; Zaman, 2014). Overall, microbial contaminants affect the disinfection of water by resisting treatment, promoting regrowth, and contributing to the formation of disinfection by-products. Effective water management requires understanding microbial contaminants and employing appropriate treatment methods to ensure water quality (Some *et al.,* 2021; Zaman, 2014).

2.2.1 Chlorine-resistant bacteria

Chlorine-resistant bacteria (CRB) are bacteria exhibiting high resistance to chlorine disinfection (above 0.5 mg/L for drinking water and above 4 mg/L for wastewater effluent) or thriving on residual chlorine (Zhang *et al.,* 2021; Luo *et al.,* 2021). The chlorine-resistant bacteria used in this study, isolated from water, include *Enterobacter aerogenes,* used as a benchmark for assessing disinfectant efficacy, given its association with human illness (Microchem, 2022); *Pantoea* sp., which can infect humans and plants but have a complex host-disease relationship (Soutar & Stavrinides, 2019); *Sphingomonas paucimobilis*, a Gram-negative bacterium known for its antibiotic resistance and

biofilm formation (Ionescu *et al.,* 2022); *Staphylococcus aureus,* a Gram-positive, coccus-shaped bacterium known for causing a wide range of clinical diseases (Taylor and Unakal, 2021) and *Staphylococcus haemolyticus,* an opportunistic pathogen notable for its high antibiotic-resistance and ability to form biofilms (Eltwisy *et al.,* 2022).

2.2.2 Disease caused by waterborne pathogens

Microorganisms are ubiquitous inhabitants of water, soil and sewage environments. Waterborne pathogens like *Aeromonas hydrophila*, *Campylobacter* spp., *C. jejuni*, *E. coli*, *H. pylori*, *L. monocytogenes*, *Salmonella* spp., and *Vibrio cholerae* are commonly transmitted through contaminated water or food (World Health Organization, 2017). Table 2.1 presents bacterial pathogens identified in drinking water systems and their associated diseases.

Table 2.1. Pathogenic bacteria in drinking water systems and their related diseases [a] (Ramírez-Castillo *et al.,* 2015).

Pathogens	**Associated Disease** [b]	**Health Significance** [c]	**Persistence in drinking water supplies** [d]
Bacteria			
Campylobacter spp., *C. jejuni*	Diarrhoea, gastroenteritis	High	Moderate
Yersinia enterocolitica	Diarrhoea, reactive arthritis	High	Long
Escherichia coli, particularly enterohaemorrhagic *E. coli* (EHEC), and others such as enteropathogenic (EPEC), enterotoxigenic (ETEC), and enteroinvasive (EIEC)	Acute diarrhoea, bloody diarrhoea, and gastroenteritis	High	Moderate

Pathogens	Associated Disease [b]	Health Significance [c]	Persistence in drinking water supplies [d]
Bacteria			
Burkholderia pseudomallei	Melioidosis	High	May multiply
Legionella pneumophila and related bacteria	Acute respiratory illness, pneumonia (legionellosis)	High	May multiply
Non-tuberculous mycobacteria	Pulmonary disease, skin infection	Low	May multiply
Pseudomonas aeruginosa	Infections in the lungs, urinary tract, and kidney. Can cause inflammation and sepsis	Moderate	May multiply
Salmonella enterica serotype Typhi	Typhoid fever, paratyphoid fever, and other serious salmonellosis	High	Moderate
Other salmonellae	Gastroenteritis, reactive arthritis	High	May multiply
Shigella spp.	Bacillary dysentery or shigellosis	High	Short
Vibrio cholera	Gastroenteritis, cholera	High	Short to long
Helicobacter pylori	Chronic gastritis, ulcer disease, and gastric cancer	Low	Moderate

[a]n Adapted from Table 7.1 in WHO Guidelines for drinking water quality (World Health Organization, 2015); [b] Data obtained from (World Health Organization, 2015, Cabral 2010, Straub and Chandler 2003, and Nygård 2008); [c] Health significance relates to the severity of impact, including association with outbreaks; [d] Detection period for the infective stage in water at 20°C: short - up to 1 week; moderate - 1 week to 1 month; long - over 1 month (Ramírez-Castillo *et al.,* 2015).

2.2.3 Detection methods for waterborne pathogens

Currently, there is no standardised approach for the comprehensive analysis of all pathogenic microorganisms in water (Straub and Chandler, 2003). The detection methods face challenges such as physical variations among pathogens, low pathogen concentration in large water volumes, the presence of sample inhibitors and the need for specific detection techniques (Ramírez-Castillo *et al.,* 2015; Straub and Chandler, 2003). Key requirements for reliable analysis include specificity, sensitivity, reproducibility, speed, automation and cost-effectiveness (Oon *et al.,* 2023).

Culture-dependent methods are common but have limitations such as low sensitivity and extended result times. Many pathogens, such as *E. coli*, *Helicobacter pylori*, and *Vibrio cholera*, are visible but not culturable, potentially leading to false negatives with culture-based methods (Ramírez-Castillo *et al.,* 2015). Indicator pathogens, used in both culture and molecular methods, monitor water quality and indicate pathogen presence (Motlagh & Yang, 2019; Richiardi *et al.*, 2023). *E. coli*, a widely used indicator, is cost-effective but does not account for all waterborne pathogens such as viruses and protozoa (Bridle, 2020).

Molecular techniques utilising deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) technologies such as quantitative polymerase chain reaction (qPCR) and next-generation sequencing (NGS) offer high specificity and phylogenetic insights into pathogens (Zhang *et al.,* 2021; Van Bemmel, 2021). These methods play a crucial role in assessing the microbiological quality of water, gauging pathogen removal efficacy in drinking and wastewater effluent and utilising microbial source tracking (MST) tools (Sresung *et al.,* 2023). These methods can link indicators to specific host sources and track pollution sources (Cabral, 2010; Sresung *et al.,* 2023).

2.3 Standards for the quality of potable water

Stringent guidelines regulate the quality of drinking water to protect public health and prevent waterborne diseases. Regulatory bodies such as the World Health Organization (WHO) and the South African Bureau of Standards (SABS) through the South African National Standard (SANS 241) provide guidelines that define the maximum allowable microbial concentrations in potable water. These standards are crucial for ensuring the safety of water for human consumption, as contaminated water can pose significant health risks (World Health Organization, 2022; De Bruin *et al.,* 2023).

The World Health Organization provides global standards for drinking water quality, setting acceptable limits for various microorganisms in water. According to World Health Organization guidelines (2022), drinking water should have no more than 0 colony-forming units (CFU) of *E. coli* or faecal coliforms per 100 mL of water. This guideline is a key benchmark for water safety, as the presence of *E. coli* indicates faecal contamination, which is strongly associated with the risk of waterborne diseases such as cholera, dysentery, and typhoid. Table 2.2 below summarises the WHO's recommended microbial limits for drinking water (World Health Organization, 2022).

Table 2.2. World Health Organisation guidelines for maximum microbial levels in drinking water (World Health Organization, 2022).

Microbial Indicator	Maximum Allowable Limit	Water Type
E.coli	0 CFU/100mL	Portable water
Faecal coliforms	0 CFU/100mL	Portable water
Total coliforms	0 CFU/100mL	Portable water

In South Africa, the SANS 241 Standard, as outlined by the SABS, regulates the microbial quality of drinking water to ensure public safety (De Bruin *et al.,* 2023). The limits for microbial indicators in potable water are the same as those set by the WHO. SANS 241 prescribes stringent requirements for E. coli, faecal coliforms, and total coliforms, ensuring water safety by enforcing a maximum of 0 CFU per 100 mL for these indicators in drinking water. Table 2.3 below summarises the microbial standards for potable water as per SANS 241.

Table 2.3. SANS 241 guidelines for maximum microbial levels in drinking water (De Bruin *et al.,* 2023).

Microbial Indicator	Maximum Allowable Limit	Water Type
E.coli	0 CFU/100mL	Portable water
Faecal coliforms	0 CFU/100mL	Portable water
Total coliforms	0 CFU/100mL	Portable water

In contrast, river water typically has higher microbial concentrations due to contamination from agricultural runoff, sewage, and other pollutants. Water quality for rivers is not subjected to the same stringent microbial limits as drinking water. However, when assessing water quality for recreational or drinking purposes, guidelines recommend certain thresholds for microbial content.

For recreational water, the presence of E. coli is used as an indicator of water contamination. The United States Environmental Protection Agency (EPA) and various other regulatory bodies recommend that recreational water should not exceed 200 CFU of *E. coli* per 100 mL. For river water intended for drinking, however, *E. coli* concentrations should ideally be less than 500 CFU per 100 mL before treatment. Table 2.4 below summarises these thresholds for river water.

Table 2.4. Recommended microbial thresholds for river water quality (United States Environmental Protection Agency (U.S.E.P.A.), 2021).

Microbial Indicator	Maximum Allowable Limit	Water Type
E.coli	200 CFU/100mL	Recreational water
E.coli	500 CFU/100mL	River water (pre-treatment)

Detecting all pathogens in water can be labour-intensive and expensive. Therefore, microbial indicators such as *E. coli* and faecal coliforms are widely used to assess the safety of water. These indicators are microorganisms whose presence suggests that water may be contaminated with harmful pathogens, such as bacteria, viruses or protozoa that pose health risks to humans (Richiardi *et al.,* 2023).

*Escherichia coli i*s particularly important as it is highly specific to human and animal faecal contamination. As a faecal coliform, *E. coli* is unique in that only pathogenic strains of this bacterium are associated with disease. Its presence signifies that the water is not safe for recreational purposes and poses a significant health risk (Health Canada, 2020). Other microbial indicators, such as enterococci and total coliforms, may also be used depending on the specific regulatory requirements and the intended use of water (e.g. recreational vs potable). However, *E. coli* remains the most commonly used indicator in the context of drinking water safety (Richiardi *et al.,* 2023).

2.4 Overview of cold plasma technology

Plasma has witnessed rapid development, gaining significant attention for its applications in fields such as biomedical devices, textile surface modification,

chemical removal from heat-sensitive materials, water disinfection, wound healing and food sterilisation (Korachi & Aslan, 2013). Plasma can be categorised into two main types based on the production method: thermal plasma (TP) and non-thermal plasma (NTP) as illustrated in Figure 2.1 (Šimončicová *et al.,* 2019). Thermal plasma or hot plasma is characterised by the equilibrium between electrons and gas temperatures, typically reaching several thousand Kelvin (Korachi & Aslan, 2013). The non-thermal plasma or cold plasma consists of gas particles at moderate temperatures that possess highly energetic electrons (Šimončicová *et al.,* 2019).

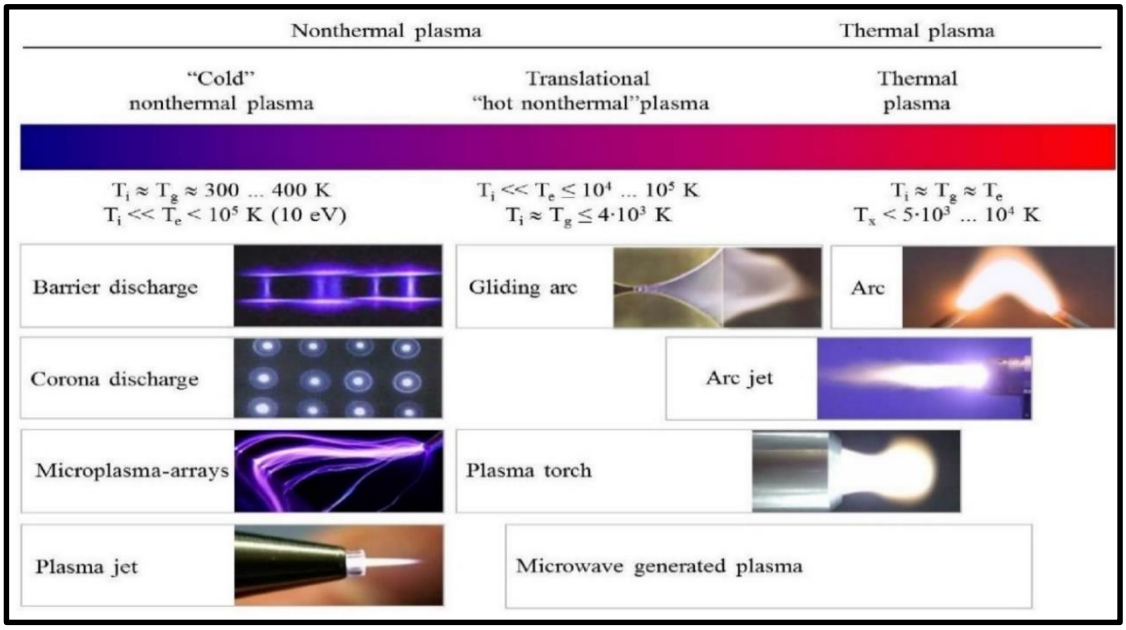


Figure 2.1: Division of different types of plasma by the temperature of plasma species, adapted from Weltmann & von Woedtke (Weltmann & von Woedtke, 2011).

2.4.1 Advantages and disadvantages of plasma

Cold plasma offers several advantages, such as low operational costs, short treatment times at low temperatures, nontoxic nature and, significant reductions in water usage during purification processes (Ziuzina *et al.,* 2013). It is versatile with applications across various products and industries, especially in decontamination and disinfection. Plasmas generated in ambient air are cost-

effective, consumable-free and can be used to disinfect items with externally located microorganisms. For e.g., non-thermal plasma is ideal for sanitizing or decontaminating surfaces before packaging or as part of the packaging process, without distorting or altering product shape (Korachi & Aslan, 2013). Dielectric barrier discharge is particularly advantageous due to its easy discharge ignition, versatility in different electrode geometries and ability to treat objects within sealed packaging. This makes it highly suitable for industrial applications (Ziuzina, 2015). Furthermore, non-thermal plasma stands out because it generates by-products that can treat water, control odours and address discolouration issues. A significant benefit is its lack of reliance on consumables, unlike chemical decontamination methods (De Vries *et al.,* 2015).

However, cold plasma technology faces certain limitations. It cannot eliminate physical contaminants and requires additional systems like filtration to complement the plasma treatment for the removal of larger organic particles such as sediments. Other challenges include technical difficulties in plasma generation, the complexity of equipment, and potential impacts on the sensory and nutritional qualities of treated food products. Moreover, the antimicrobial action of cold plasma varies based on the type of plasma generated, requiring further research for successful industrial-scale applications (Niemira, 2012).

2.4.2 Plasma by-products (reactive species)

Plasma by-products are reactive species such as ozone, free radicals and ions that are generated during plasma discharge, contributing to the disinfection and breakdown of contaminants. The production of non-thermal plasma involves electrical discharges in fluid or gas mixtures, resulting in various by-products. These by-products vary depending on the type of plasma discharge and the amount of input energy. They can include both desirable substances, such as reactive oxygen species (ROS) for disinfection and potentially harmful substances, necessitating careful consideration in different applications (De Vries *et al.,* 2015).

2.4.3 Thermal plasma

Thermal plasma is generated under conditions where local thermodynamic equilibrium is achieved, usually at atmospheric pressure (1 atm). In this state, there is a high frequency of collisions between electrons and larger particles/ions, resulting in thermodynamic equilibrium where the electron temperature (*Te)* converges with the temperature of the dominant particle (*Ti)*. These particle temperatures typically exceed 100 eV, characterizing thermal plasma (De Vries *et al.,* 2015). Thermal plasma is created when gas flows through a high electrical potential, leading to thermal ionisation and joule heating, often under high pressure (Rathore, *et al.,* 2020). Examples of thermal plasma include plasma cutters, welding torches, lightning bolts, and even the surface of the sun (Boulos *et al.,* 2023). However, due to its high temperatures, thermal plasma is unsuitable for water treatment, as it would lead to water boiling (De Vries *et al.,* 2015).

2.4.4 Non-thermal plasma

Non-thermal plasma, also known as "non-equilibrium plasma" or "cold plasma" is defined by the efficient acceleration of relatively light electrons in an electric field. These highly energetic electrons generate free radicals through various physical and chemical processes. These radicals react with contaminants, breaking them down into simpler by-products (Ruma *et al.,* 2016). Non-thermal plasma unlike thermal plasma has a particle temperature much lower than thermal plasma – around 2 eV compared to 100 eV for thermal plasma (Shenton & Stevens, 2001). Non-thermal plasma can be generated by various methods, including dielectric barrier discharge, atmospheric pressure discharge plasma, corona discharges,

pulsed arc discharge and plasma jet, as illustrated in Figure 2.2 (Nisha *et al.,* 2019).

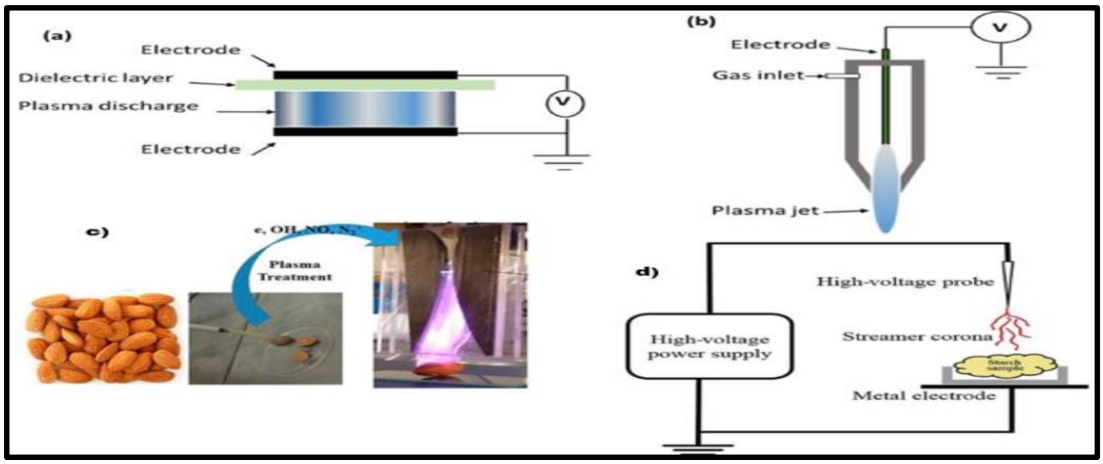


Figure 2.2: Methods of non-thermal plasma discharge a) Dielectric discharge, b) Jet discharge (Pankaj & Keener, 2017), c) Gliding arc discharge (Khalili *et al.,* 2018), d) Corona discharge (Wu *et al.,* 2018) (Nisha *et al.,* 2019).

One of the key applications of non-thermal plasma is in water treatment, where it can generate ozone and other reactive particles that are highly effective for pathogen inactivation. However, generating non-thermal plasma in liquid environments poses more challenges and is more energy-intensive compared to generating it in a gas, and limited research has been conducted in this area (De Vries *et al.,* 2015). Non-thermal plasma, used under low-pressure conditions with a low collision frequency generates reactive species while maintaining the surrounding gas or liquid at close to ambient temperature. This characteristic makes it ideal for a range of applications, including neon lights and plasma balls, where minimal thermal effects are desired. The ability of non-thermal plasma to generate low-density free radicals and reactive molecules without reaching thermochemical equilibrium is a key advantage in applications that involve sensitive materials, such as food and water treatment (Šimončicová *et al.,* 2019).

2.4.4.1 Atmospheric pressure plasma jet (APPJ)

Atmospheric pressure plasma jet stream generates non-thermal atmospheric pressure discharges, creating plasma jets influenced by the gas stream speed. The faster the gas stream, the more intense the plasma jets, as ionisation occurs within the gas flow. The plasma is generated using gases like helium, oxygen, and argon, fed through a nozzle to an electrode, which is powered by radio frequency (RF). This generates energetic electrons that collide with the feed gas, producing excited-state particles, atoms, free radicals and ion-electron pairs. Even as these particles rapidly recombine when exiting the plasma, the effluent still contains neutral metastable species and radicals useful for applications such as surface modification of polymers, microbial elimination, sterilisation of medical equipment, and other medical and industrial applications (Ruma *et al.,* 2016).

2.4.4.2 Corona discharge

Corona discharges occur when high-voltage electrical discharges pass through air gaps at atmospheric pressure, generating non-thermal plasma. The high-voltage electric field generates weak electrical discharges at the electrode edges, pulling charged particles from one electrode to another. This ionises oxygen molecules, forming ozone. If the voltage increases too much, the electric field can break down, transitioning the corona into a spark discharge. Corona discharges can be positive or negative, with negative coronas being more effective for ozone generation, producing seven to ten times more ozone than positive coronas (Zhu *et al.,* 2020; De Vries *et al.,* 2015).

2.4.4.3 Dielectric barrier discharge (DBD)

Dielectric barrier discharge, also known as barrier discharge or salient discharge, is a form of alternating current (AC) discharge that produces strong

thermodynamic, non-equilibrium plasma at atmospheric pressure. It involves two electrodes, at least one of which is coated with a dielectric material to prevent direct contact between the plasma and the electrodes (see Figure 2.3).

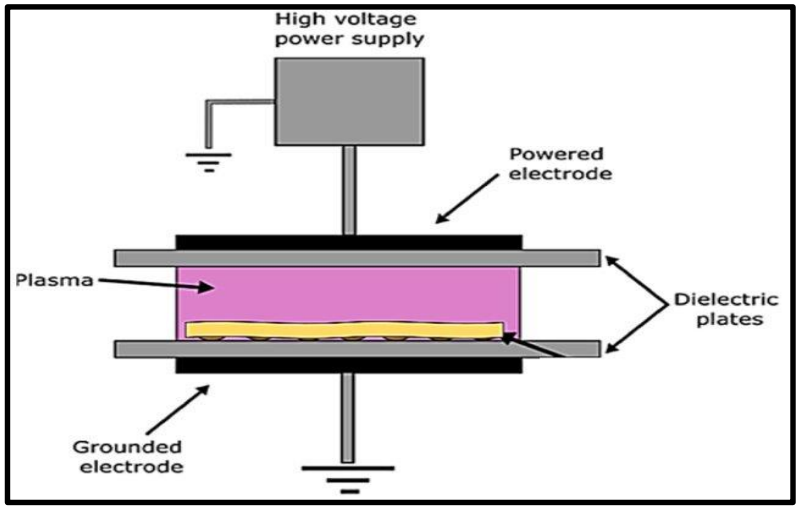


Figure 2.3: Diagram of a dielectric barrier discharge (Starič *et al.,*2020; Perera *et al.,* 2022).

This setup prevents sparks and electrode etching, allowing the generation of plasma through the collision of energetic electrons with reactant particles. Joule heating raises the temperature of electrons, producing non-thermal plasma. Applications of DBD include ozone production, sterilisation, contamination control, and material processing (Lu *et al.,* 2012; Ruma *et al.,* 2016).

2.4.4.4 Pulsed arc discharge

Pulsed arc discharge plasma has gained popularity due to its simplicity and efficiency, with applications spanning military, industrial and environmental fields. It involves a high-voltage, pulsating discharge between two electrodes, typically occurring at a frequency of 0.05 to 500 kHz. This technique generates plasma similarly to DBD, but instead of capacitive coupling, it uses an electrical arc to ionise the gas. The pulsed nature of the arc prevents electron overheating, ensuring the plasma remains non-thermal. Pulsed arc discharge

is effective for ozone generation and microbial inactivation (De Vries *et al.,* 2015; Liu *et al.,* 2022; Zambelli, 2023).

2.5 Mechanisms of atmospheric cold plasma for bacterial activities

Atmospheric cold plasma (ACP) has garnered attention as an advanced non-thermal technology for microbial inactivation and decontamination (Yin *et al.,* 2023). While the precise mechanism of ACP's bactericidal activity is yet to be fully understood and acknowledged, it is known that ACP is generated by electrical discharges between electrodes, producing ROS and reactive nitrogen species (RNS) that have strong bactericidal properties. These reactive species, including atomic oxygen, ozone, hydroxyl radicals, nitric oxide and peroxynitrite play a crucial role either independently or collaboratively in bacterial inactivation by disrupting cell walls and damaging DNA when using ACP (De Vries *et al.*, 2015; Ziuzina *et al.,* 2015; Yin *et al.,* 2023) as illustrated in Figure 2.4.

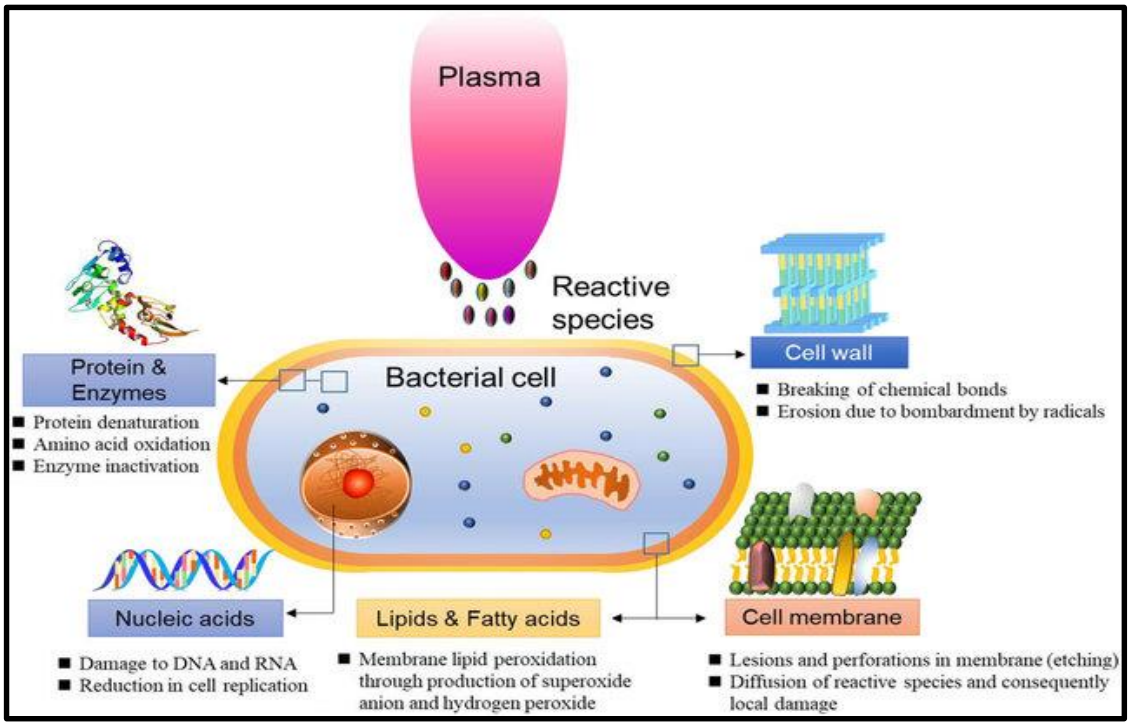


Figure 2.4: A schematic of the mechanics of cold plasma sterilisation of bacteria (adapted from Misra & Jo, 2017; Deng *et al.,* 2020).

The antimicrobial efficiency of ACP depends on key factors such as electrical power, gas composition and the system configuration. High voltage intensities and optimised gas mixtures enhance the inactivation of pathogens, making ACP highly effective for sterilizing food, medical devices and water without causing thermal damage (Korachi and Aslan, 2013; Ziuzina *et al.,* 2015). Atmospheric cold plasmas' advantage lies in its ability to sterilise heat-sensitive products at atmospheric pressure and low temperatures, which is not possible with thermal plasma. Various ACP methods, such as dielectric barrier discharge, corona discharges and radio frequency (RF) torches have been developed for bio-sterilisation applications (Korachi and Aslan, 2013; Yin *et al.,* 2023).

Plasma generation methods, including DBD, have gained attention due to their wide range of applications and extensive research support. Double barrier discharge is especially convenient in creating plasma under atmospheric conditions, where oxygen (O_2) and nitrogen (N) contribute significantly to the formation of reactive species. The presence of oxygen in the air leads to the generation of ROS, such as hydrogen peroxide, hydroxyl radicals, ozone, superoxide, singlet oxygen, and atomic oxygen (Sachdev *et al.,* 2021). Simultaneously, nitrogen in the atmosphere promotes the formation of RNS, resulting in compounds like peroxynitrite, nitric oxide and nitrite (Bradu *et al.,* 2020; Sachdev *et al.,* 2021). These reactive species play a role in plasma-mediated microbial inactivation. However, the effectiveness of these reactive species depends on several factors, including gas pressure, composition, temperature, moisture and plasma excitation properties (Dharini *et al.,* 2023; Sachdev *et al.,* 2021). Precise control of these parameters is critical for optimising plasma generation and microbial inactivation processes (Dharini *et al.,* 2023; Sachdev *et al.,* 2021). The efficiency of microbial inactivation is influenced by external factors like treatment duration, post-treatment storage time and the proximity of plasma discharges to the target surface. Research has highlighted the importance of these variables in achieving effective microbial inactivation (Ziuzina *et al.,* 2013).

2.6 Kinetics of microbial inactivation

The antimicrobial efficacy of ACP is influenced by various factors such as the power level used to generate the plasma, the gas mixture in the plasma emitter, exposure time, system design, flow rate, and pressure (Korachi and Aslan, 2013). Additional factors including microorganism concentration, device type, exposure distance, and gas composition impact the effectiveness of ACP for microbial inactivation (Das *et al.,* 2022). Inactivation kinetics are typically described using first-order reaction models, where microbial population reduction is often quantified using log reductions which reflect the percentage of bacteria killed (Microchem, 2015). A 1-log reduction corresponds to a 90% decrease in bacterial population count, and higher reductions such as 3-log or 6-log signify greater inactivation (Microchem, 2015).

Plasma-based technologies are particularly important for disinfection, especially in applications where low temperature and minimal material damage are essential. Atmospheric cold plasma has emerged as an effective method for water disinfection due to its ability to generate Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS), which possess potent antimicrobial properties (Ziuzina *et al.,* 2015; Barjasteh *et al.,* 2021). Plasma for disinfection was initially limited to vacuum systems; however, recent advances have made ACP generation feasible, making it an economically viable option for various fields, including medicine, dentistry, agriculture, and water treatment (Korachi and Aslan, 2013; Barjasteh *et al.,* 2021). This technology is particularly advantageous for heat-sensitive applications due to its non-thermal nature, causing minimal damage to materials (Scholtz *et al.,* 2021).

In an experimental setup for ACP, the design typically involves aluminium disc electrodes separated by a polypropylene container, which also serves as the dielectric barrier (Figure 2.5). There are two primary methods of treatment: direct treatment, where the sample is exposed to plasma but is not in direct contact with the electrodes, and indirect treatment, where the sample is exposed

to plasma but is not in direct contact with the electrodes. Direct plasma treatment is generally more effective for bacterial inactivation in water, while indirect plasma treatment is more effective for food products (Ziuzina *et al.,* 2013). Direct treatment mainly targets bacterial cell membranes, leading to cell disruption, after which plasma-related mechanisms cease, and biochemical processes take over (Dobrynin *et al.,* 2009; Rao *et al.,* 2023).

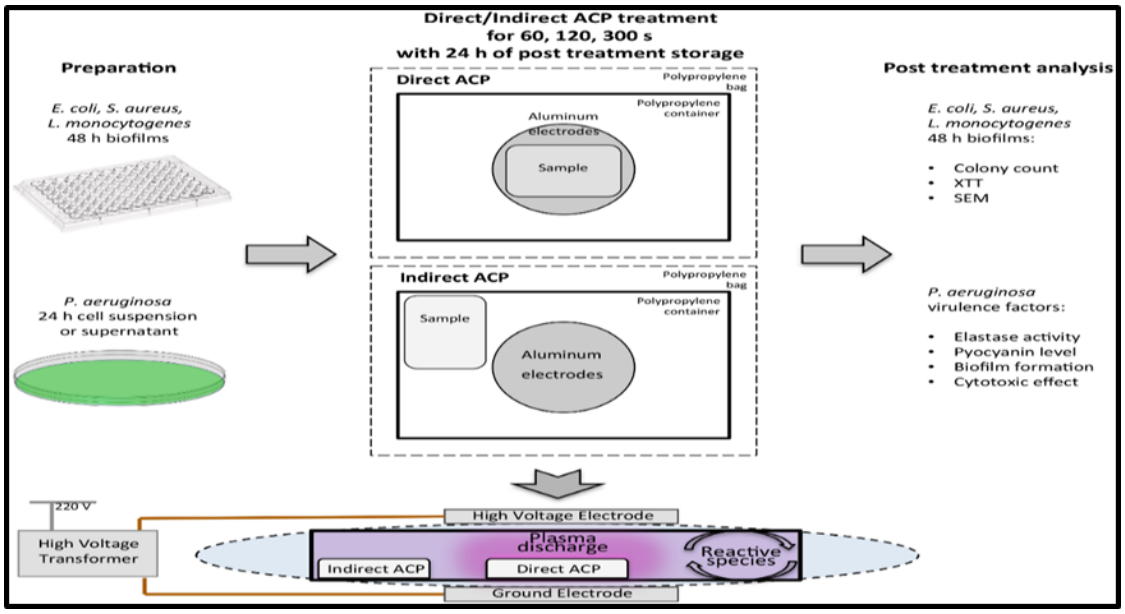


Figure 2.5: Experimental setup**.** Either a microtiter plate containing bacterial biofilms or a petri dish containing *P. aeruginosa* cells TSB suspension or cell-free supernatant were placed inside the plastic container between the electrodes for direct ACP treatment. For indirect ACP treatment, a separate container was used and samples were placed outside the plasma discharge (Ziuzina *et al.,* 2015).

Atmospheric cold plasma's antimicrobial action is a result of multiple mechanisms, including physical damage by high-energy electrons, oxidative stress from ROS and RNS, and possible chemical reactions that interfere with bacterial cellular functions (Scholtz *et al.,* 2021). However, quantifying the exact dose of plasma for effective antimicrobial actions remains challenging due to the complex interactions between plasma species and microbial cells (Scholtz *et al.,* 2021).

Plasma treatment also has an extended antimicrobial effect after treatment, which is influenced by factors such as post-treatment storage duration, where residual reactive species continue to act on the microorganisms (Ziuzina *et al.,* 2015; Čobanović *et al.,* 2023; Zver *et al.,* 2023). This residual effect contributes to sustained bacterial inactivation, making ACP a viable method for ensuring long-term microbial suppression in water.

2.7. Advantages and disadvantages of atmospheric cold plasma for water disinfection

Atmospheric cold plasma offers several advantages for water disinfection, including low-temperature operation, cost-effectiveness, energy efficiency and environmentally friendly by-products like ROS and RNS, which provide potent antimicrobial properties (Šimončicová *et al.,* 2019). These by-products, including ozone and hydrogen peroxide, provide a chemical-free alternative for disinfection, which can be applied across industries such as food processing, healthcare, and water treatment (Chen & Wirz, 2021). Atmospheric cold plasmas' ability to degrade organic compounds through oxidation further enhances its disinfection capacity (De Vries *et al.,* 2015; Mouele *et al.,* 2021). Moreover, the low operational costs, minimal environmental impact and rapid disinfection times make ACP a promising technology for various applications (Šimončicová *et al.,* 2019).

However, ACP also faces certain challenges. High initial investment costs for plasma equipment and difficulty in determining the optimal plasma dose for effective disinfection are significant obstacles (Šimončicová *et al.,* 2019). Variability in plasma penetration depth can affect the uniformity of disinfection, requiring careful system design and parameter optimisation. Additionally, while ACP is energy-efficient, some studies have noted that energy consumption can still be high in certain setups, though advances in technology are addressing this issue (Naicker *et al.,* 2023). Plasma ozonation may also produce undesirable by-products such as aldehyde and bromates, with formaldehyde

and bromates being of particular concern due to their potential carcinogenicity. Bromate formation is more likely in the presence of bromide ions, requiring careful pH control during treatment to minimise these by-products (Hossen *et al.,* 2023; United States Environmental Protection Agency (U.S.E.P.A.), 1999a; United States Environmental Protection Agency (U.S.E.P.A.), 1999b).

2.8 Conventional water treatment methods

Conventional water treatment methods such as membrane filtration, ozone technology, reverse osmosis and plasma treatment are commonly used in both domestic and industrial applications. However, these methods face challenges related to affordability, efficiency and environmental impact. Many of these treatments rely heavily on chemicals, leading to by-product generation and limited microbial contaminant removal (Singh *et al.,* 2023). In developing regions where waterborne illnesses are widespread, affordability and accessibility challenges are prominent. Additionally, the reliability of the drinking water supply poses a further concern (Treacy, 2019). Despite advancements in water treatment technologies, each method presents its unique set of advantages and disadvantages (Zaman*,* 2014).

2.8.1 Membrane filtration: Advantages and disadvantages

Membrane filtration is widely used for physical removal of solid particles and microorganisms. These filters are classified as microfiltration (MF) or ultrafiltration (UF) based on pore size. Microfiltration membranes with pores ranging from 0.03 to 10 µm, can filter out large contaminants but are ineffective against viruses that can cause diseases (Abdelrasoul & Doan, 2020). Ultrafiltration membranes, with smaller pores (0.001 to 0.03 µm), can remove viruses but are more prone to soiling and blockages. In both cases, membrane lifespan is limited by particle build-up and regular cleaning with chemicals is required, adding to operational cost (Ibrar *et al.,* 2019; De Vries *et al.,* 2015). In membrane filtration, approximately 15% of the treated water is wasted due to

contaminants accumulating in the system, and this wastewater needs careful disposal (De Vries *et al.,* 2015; Abdelrasoul & Doan, 2020). While membrane filters provide an effective solution for certain contaminants, their limitations in affordability, maintenance and waste generation remain significant challenges.

2.8.2 Reverse osmosis (RO): Advantages and disadvantages

Reverse osmosis is a highly efficient technology for removing a broad range of contaminants, including microorganisms and viruses. To maintain their effectiveness, it is crucial to backwash and clean RO membranes regularly, as they are prone to soiling. Proper maintenance can extend the life expectancy of a membrane to between three and five years (Abdelrasoul & Doan, 2020). Unlike membrane filtration, RO membranes have smaller pores and require external pressure for operation, forcing water through a selectively permeable membrane while larger contaminants are left behind (Cannon, 2020). The process produces potable water on one side of the membrane and concentrated waste on the other. The operational process of RO is illustrated in Figure 2.6.

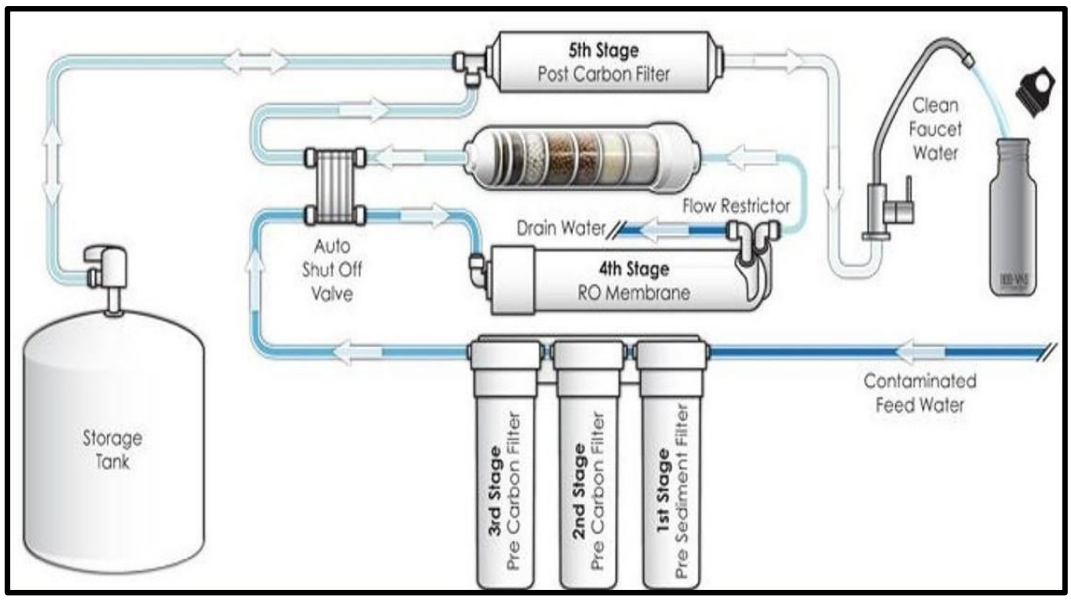


Figure 2.6: Operational process of reverse osmosis to disinfect water (Cannon, 2020)

Reverse osmosis membranes stand out as the most advanced and energy-

efficient, requiring only pressure to operate and do not involve chemical reagents, making them environmentally friendly. Notably, the process can be executed at room temperature and the equipment is compact, requires minimal space and is easy to operate (Vedantu, 2023). The primary drawbacks of RO are the inflexibility of the system and the elimination of essential trace elements from water. Additionally, RO generates a significant volume of concentrated waste and requires strict management to avoid membrane damage due to pressure inconsistencies (De Vries *et al.,* 2015; Vedantu, 2023).

2.8.3 Ozone treatment: Advantages and disadvantages

Ozone (O_3) is an effective water disinfectant produced by applying high-voltage energy to oxygen molecules. As a potent oxidiser, it breaks down microbial cells and various pollutants in water and wastewater (United States Environmental Protection Agency (U.S.E.P.A.), 1999a; Tripathi & Hussain, 2022). The use of ozone in water treatment provides several advantages, including its ability to quickly eliminate microorganisms without leaving harmful residuals (United States Environmental Protection Agency (U.S.E.P.A.), 1999a; United States Environmental Protection Agency (U.S.E.P.A.), 1999b). The generation of ozone in wastewater treatment plants, illustrated in Figure 2.7, typically involves applying a high-voltage alternating current and an oxygen-bearing gas across a dielectric discharge gap.

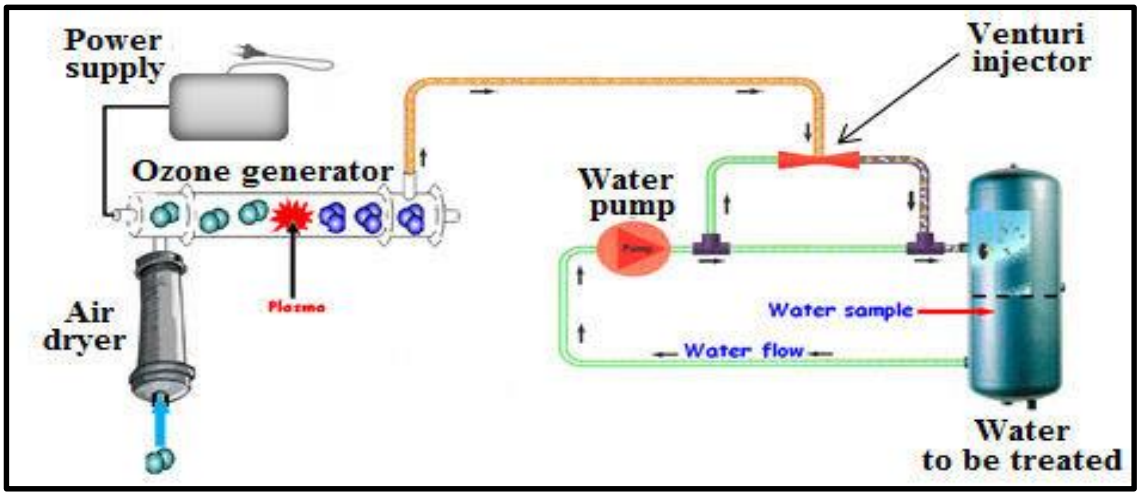


Figure 2.7: Descriptive representation of the water treatment process using ozone

(Hammadi *et al.,* 2016).

Ozone offers several advantages in water treatment. The ozonation process requires a shorter contact time compared to chlorine and effectively destroys bacteria and viruses. Its rapid decomposition results in no harmful residuals, and microorganisms do not regrow after treatment. Handling ozone involves less risk and the concentration of dissolved oxygen (DO) in the effluent increases eliminating the need for reaeration and potentially enhancing DO levels in the receiving stream. Despite its benefits, ozone treatment requires complex equipment and materials resistant to its highly reactive and corrosive nature. High levels of suspended solids or organic matter in water make ozone treatment less cost-effective. Ozone is extremely irritating and potentially toxic, requiring the management of off-gases to prevent worker exposure (United States Environmental Protection Agency (U.S.E.P.A.), 1999a; United States Environmental Protection Agency (U.S.E.P.A.), 1999b).

2.8.4 Chlorine: Advantages and disadvantages

Chlorination is a widely used water treatment method involving the addition of chlorine or chlorine compounds (hypochlorite solution (NaOCl), or calcium hypochlorite ($Ca(OCl)_2$). However, the persistence of chlorine in treated water can pose health risks if levels exceed the safe drinking limit of 4 ppm or mg/L (Centers for Disease Control and Prevention, 2020). Exceeding these limits can result in eye and nose irritation, stomach discomfort, and in severe cases, even anaemia. Another concern involves the sourcing, careful preparation, and practical implementation of the chlorination compound (United States Environmental Protection Agency (U.S.E.P.A.), 2021). Developed countries, like Australia, rigorously assess these risks, finding little difference in the dangers presented by untreated water-containing pathogens compared to water with elevated levels of residual disinfectant compounds (Mizozoe *et al.,* 2019). In developed nations, government oversight ensures that water supply companies adhere to acceptable standards, but in financially challenged

countries such governance is often lacking (Centers for Disease Control and Prevention, 2020).

Studies have shown that portable water distribution systems maintained at 0.5 to 1.0 mg/L of free chlorine can still recover large numbers of human pathogens contrary to chlorine's bactericidal properties (Costa *et al.,* 2023). In chlorinated drinking water, the majority of viable bacteria are attached to particles. Particles and surfaces are usually protected from sterilisation, so microorganisms that are entrapped or adhered to them are not inactivated (Geldreich, 2020). Geldreich, (1996) in his study found there was a higher level of resistance among bacteria from chlorinated systems compared with non-chlorinated systems. Biofilms (attached growth) on pipe walls are associated with bacterial regrowth in distribution systems (Tsagkari & Sloan, 2023). Potable water may develop biofilms when it contains biodegradable organic matter (BOM) and inorganic nutrients (Pick, 2019; Waqas *et al.,* 2023; Tsagkari & Sloan, 2023).

When chlorine is introduced into water, it undergoes chemical reactions that produce by-products such as hypochlorous acid and hypochlorite ions which act as potent disinfectants. These residues or disinfectant by-products (DBPs) may be harmful to humans and aquatic life (Albolafio *et al.,* 2022). These compounds act by disrupting the cellular structures of bacteria, viruses and other pathogens preventing their ability to reproduce and causing microbial death. The versatility and relatively low cost of chlorine make it a practical and commonly utilised method for large-scale water disinfection ensuring the delivery of safe drinking water to communities (Adefisoye & Olaniran, 2022; Misra & Jo, 2017; Venkobachar *et al.,* 1977).

In many cases, chlorine-resistant bacteria have been identified in post-chlorinated water, raising concerns about its efficacy in eliminating pathogens (Albany, 2021). Chlorine offers cost-effective water disinfection and residual protection against recontamination. However, it is less effective against protozoa, and biofilms can form in chlorinated systems enabling bacteria to re-

grow (Mizozoe *et al.,* 2019; Centers for Disease Control and Prevention, 2023; Tsagkari & Sloan, 2023). Moreover, chlorination by-products (DBPs) pose potential risks to human and aquatic health (Mizozoe *et al.,* 2019; Albolafio *et al.,* 2022; Centers for Disease Control and Prevention, 2023).

2.9 Process parameters for cold plasma efficacy

Cold plasma has emerged as a promising technology for wastewater treatment, with its efficacy dependent on several key process parameters (Das *et al.,* 2022). One of the critical factors is gas composition, as it directly influences the types and concentration of reactive species generated during the cold plasma process (Ziuzina, 2015). Different gases or gas mixtures yield varying ROS and RNS, which are essential for contaminant degradation and microbial inactivation

Additionally, the electric field strength applied is another pivotal factor, as it plays a significant role in determining the efficiency of cold plasma treatment. A stronger electric field enhances the production of reactive species which in turn increases microbial inactivation and improves overall treatment efficiency (Feizollahi *et al.,* 2021). Treatment time, another critical parameter, dictates the duration over which the cold plasma is applied and significantly influences the kinetics of contaminant removal. Longer treatment times generally allow for more extensive breakdown of contaminants and increased microbial inactivation (Das *et al.,* 2022).

Optimising cold plasma systems for wastewater treatment requires a detailed understanding of the interplay between gas composition, electric field strength and treatment time. These parameters must be carefully balanced to maximise the efficiency of contaminant removal while minimizing energy consumption and operational costs (Das *et al.,* 2022).

2.9.1 Gas composition and reactive species formation

The choice of gas composition in cold plasma systems is a critical determinant of reactive species formation, influencing the overall efficacy of contaminant degradation. Different gases introduce varying molecular structures, leading to the production of distinct reactive species during the plasma process. For instance, the use of oxygen-rich gases can enhance the formation of hydroxyl radicals known for their potent oxidizing capabilities. Conversely, nitrogen-based gases contribute to the generation of nitrogen radicals, introducing alternative pathways for wastewater treatment. Understanding the intricate relationship between gas composition and reactive species formation is essential for tailoring cold plasma systems to target specific contaminants effectively (Gururani *et al.,* 2021; Xu *et al.*, 2020).

2.9.2 Electric field strength and plasma characteristics

The electric field strength applied during cold plasma treatment significantly influences the characteristics of the plasma discharge and, consequently its efficiency in microbial inactivation. High electric field strengths contribute to increased electron energy and collision frequencies, promoting the dissociation of gas molecules and the subsequent production of reactive species. This phenomenon emphasises the importance of optimising electric field strength where researchers can adjust the plasma properties to enhance microbial inactivation, showcasing the intricacies of process parameter optimisation (Das *et al.,* 2023).

2.9.3 Treatment time and contaminant removal kinetics

The duration over which cold plasma is applied, commonly referred to as treatment time is a fundamental parameter that dictates the kinetics of contaminant removal. Longer treatment times generally result in increased exposure of contaminants to reactive species, leading to enhanced degradation

efficiency. However, optimal treatment times vary for different contaminants, and a nuanced understanding of these kinetics is vital for achieving wastewater treatment. Studies have indicated that while extended treatment times may be effective for certain contaminants, diminishing returns may occur beyond a certain duration. Balancing treatment time with energy consumption considerations is crucial for designing sustainable and economically viable cold plasma systems for wastewater treatment (Moldgy, 2019).

2.9.4 Interactions between process parameters

Understanding the complex interactions between different process parameters is crucial for optimising cold plasma systems. Gas composition, electric field strength and treatment time do not operate in isolation; rather they often interact in intricate ways. Specific gas compositions may exhibit synergistic effects when coupled with optimal electric field strengths resulting in enhanced reactive species generation. Additionally, treatment time can influence the overall effectiveness of these interactions. Investigating and comprehending these interdependencies is pivotal for designing robust and efficient cold plasma systems tailored for various wastewater compositions (Mouele *et al.,* 2021; Zeghioud *et al.,* 2020).

2.9.5 Environmental conditions and energy efficiency

While the focus on process parameters is integral to the effectiveness of cold plasma in wastewater treatment, it is equally important to consider the environmental impact and energy efficiency of the technology. The type of gas used can impact the overall carbon footprint of the process. Moreover, optimising treatment time and electric field strength not only influences efficiency but also plays a role in minimizing energy consumption. Striking a balance between effective contaminant degradation and sustainable energy-efficient operations is essential for the broader acceptance and implementation of cold plasma technology in wastewater treatment (Patange *et al.,* 2018).

2.9.6 Input gas composition-tailoring reactive species generation

The input gas composition is a fundamental process parameter in cold plasma wastewater treatment, influencing the types and concentrations of reactive species generated during the plasma discharge. The choice of gas significantly shapes the chemical environment within the plasma, dictating the nature of interactions between electrons, ions and neutral species. For instance, employing oxygen-rich gases can lead to the production of hydroxyl radicals (OH), known for their strong oxidizing capabilities. In contrast, nitrogen-based gases may yield nitrogen radicals, offering alternative pathways for microbial inactivation. Manipulating input gas composition allows for tailoring the plasma reactive species profile, enabling a more precise and effective approach to target specific contaminants in wastewater (Shen *et al., 2*019).

2.9.7 Input power and electric field strength-driving plasma generation

Input power, closely linked to electric field strength, is another critical parameter governing the efficiency of cold plasma in wastewater treatment. Higher input power leads to an intensified electric field, facilitating electron acceleration and collision frequencies with the plasma discharge. This elevated energy state promotes the dissociation of gas molecules and the subsequent generation of reactive species responsible for microbial inactivation. By carefully adjusting input power levels, the intensity of the electric field can be modulated, thereby influencing the nature and concentration of reactive species produced during cold plasma treatment (Aggelopoulos, 2022; Umair *et al.,* 2019).

2.9.8 Input frequency and treatment time-controlling plasma dynamics

The input frequency, often associated with the treatment time, represents another dimension of input as a process parameter in cold plasma systems. It refers to the frequency at which electrical pulses are applied to sustain the plasma discharge. The choice of input frequency can influence plasma

dynamics and treatment efficiency. Understanding how input frequency interacts with other process parameters, such as gas composition and electric field strength, is crucial for optimising treatment protocols. Additionally, treatment time, determined by the duration of applying the input power, governs the exposure of contaminants to reactive species, highlighting its significance in achieving desired treatment outcomes (Aggelopoulos, 2022; Gururani *et al.,* 2021).

2.10. Conclusion: Implications of the literature review for the current study

In the past decade, various disinfection and pathogen removal technologies have been extensively studied and applied in industries such as food safety, healthcare and water treatment. Traditional methods, including chlorine-based disinfection, ultraviolet (UB) radiation, ozonation, filtration and chemical agents have demonstrated effectiveness in bacterial reduction (Collivignarelli *et al.,* 2018). However, these approaches while effective present several challenges, such as the development of microbial resistance, environmental concerns and the degradation of heat-sensitive products. Moreover, thermal treatments like pasteurization can degrade heat-sensitive products, compromising their quality and nutritional value (Zhao *et al.,* 2019).

One significant issue is chemical resistance, where certain pathogens, including bacteria and viruses, develop tolerance to disinfectants like chlorine and hydrogen peroxide, reducing long-term efficacy. Additionally, chemical disinfectants may have harmful environmental consequences, particularly in water treatment, where residual chemicals can contaminate ecosystems. Recent studies have raised concerns about the presence of chloro-nitramide anion, a by-product of chloramine used in water disinfection, which has been detected in tap water and may pose health risks (Fairey *et al.,* 2024).

To overcome these limitations, there is a growing interest in non-chemical,

environmentally friendly disinfection technologies that do not produce harmful by-products and are suitable for sensitive materials. One such technology is ACP which addresses several critical gaps left by traditional methods. First, it provides broad-spectrum antimicrobial efficacy, including against pathogens resistant to conventional disinfectants. Second, ACP is non-toxic and chemical-free, offering a safer and more sustainable alternative with minimal environmental impact. Furthermore, its non-thermal nature ensures compatibility with heat-sensitive products, making it advantageous for industries where traditional methods are impractical or costly (López *et al.,* 2019).

In summary, while conventional disinfection technologies have proven valuable, they present limitations that ACP effectively mitigates. With its unique combination of broad-spectrum effectiveness, environmental safety and suitability for sensitive applications, ACP is a promising alternative that addresses the gaps left by conventional processes. This dissertation aims to explore the potential of ACP to fill these gaps, particularly focusing on its effectiveness, safety and environmental benefits compared to traditional disinfection methods. The findings of this study will help to further clarify ACPs' roles in disinfection applications and pave the way for widespread use in real-world scenarios, particularly in water treatment for public health.

CHAPTER 3: METHODOLOGY

3.1 Introduction

This study focused on the use of ACP for the treatment of water-borne pathogens to determine its efficacy in water disinfection. The primary aim was to optimise the use of ACP to inactivate waterborne pathogens, ensuring the safety of drinking water. The methodology outlined is designed to achieve this aim by addressing the four key objectives of the study. Each objective will be met through a combination of experimental design, data collection and analysis.

Objective 1: Optimise ACP production

The first objective aimed to optimise the production of ACP by verifying the dielectric constants using various gases, voltages and distances between electrodes. To achieve this, a series of experiments were conducted to assess the influence of the different gases (argon, oxygen and air), varying electrode voltages and the distance between the electrodes on the production of cold plasma. This involved measuring the dielectric properties under different conditions and recording the energy efficiency and stability of plasma generation. This objective was critical to ensuring that the production parameters were optimised for effective disinfection which was foundational to the success of the subsequent objectives.

Objective 2: Determine the optimum parameters for inactivation of pathogens

The second objective focused on determining the optimum parameters for inactivating chlorine-resistant and non-chlorine-resistant bacterial pathogen loads in water using ACP. This was achieved by exposing water samples containing different bacterial strains (both chlorine-resistant and non-chlorine-

resistant) to ACP under varying conditions of voltage, gas type and exposure time. The effectiveness of pathogen inactivation was assessed by comparing the bacterial load before and after treatment using microbiological assays. This objective was crucial for identifying the conditions under which ACP was most effective in disinfecting water, particularly against more resilient pathogens that are commonly found in water sources.

Objective 3: Assessment of the kinetics and extended effects of microbial inactivation

The third objective involved determining the microbial kinetics and extended effects of microbial inactivation after direct cold plasma treatment and following a 24 h storage post-treatment period. To accomplish this, water samples were treated with ACP, and pathogen levels were monitored at various time intervals, both immediately after treatment and after 24 h storage. This provided insight into the persistence of microbial inactivation and helped assess whether ACP treatment offered long-term disinfection or if the treated pathogens may regrow. The findings for this objective were essential for understanding the durability of ACP's effects, which was important for ensuring continued safety in stored water.

Objective 4: Application of optimal parameters to disinfect river and natural water resources

The final objective applied the optimal ACP process parameters to disinfect river and natural water resources. Using the conditions identified in objectives1-3, water samples from natural sources (such as rivers) were subjected to ACP treatment to test the real-world applicability of the optimised process. The effectiveness of the treatment was evaluated by measuring the reduction in microbial load and assessing the quality of the water post-treatment. This objective provided practical evidence of ACP's effectiveness in large scale water

disinfection, particularly in the context of natural water sources that may contain a wide range of pathogens.

Each of these objectives contributed directly to the overall aim of the study i.e. optimizing the use of ACP for the inactivation of water-borne pathogens and ensuring the safety of potable water. By systematically addressing each objective through controlled experiments, this methodology ensured that the study identified the optimal parameters for ACP treatment, assessed its effectiveness against various pathogens, and evaluated its potential for real-world application in natural water sources. Through this approach, the study provided valuable insights into the feasibility and practicality of using ACP as an effective water disinfection method.

The methodology employed in this study, along with the defined objectives to achieve the set aim, is illustrated in Figure 3.1. Initially, the plasma device underwent optimisation for cold plasma production through the verification of operating parameters, leading to the creation of plasma arcs upon exceeding the dielectric breakdown voltage. Optimisation of the plasma device for distance was then conducted based on the respective gases. Subsequently, the optimal parameters and microbial kinetics for inactivating chlorine-resistant and non-chlorine-resistant bacterial pathogens were determined, considering both direct treatment and 24 h storage post-treatment.

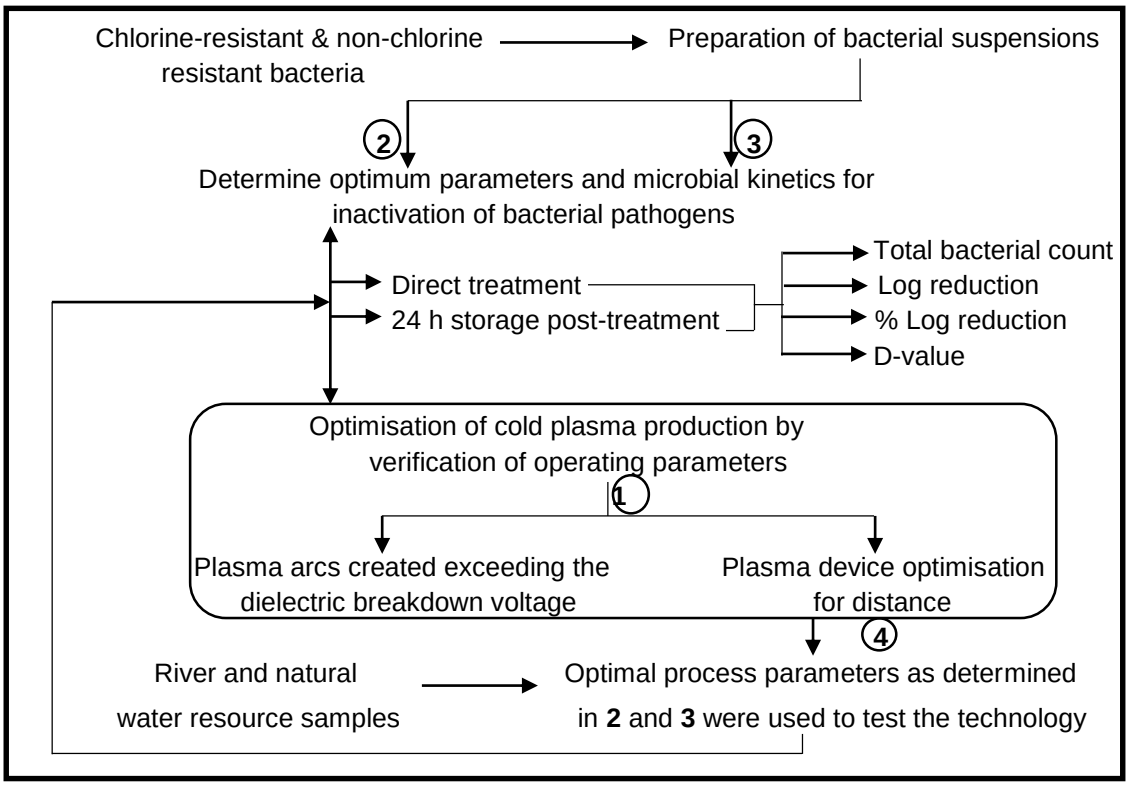


Figure 3.1: Brief overview of methodology with numbers 1-4 indicating the objectives undertaken to complete the aim and objectives of this study.

Various metrics, including total bacterial count using the pour plate method (survival growth), log reduction, percentage (%) log reduction (indicative of microbial inactivation), and the D-value (time taken to achieve a 1-log reduction or a 90% log reduction), were calculated. Following the determination of optimum process parameters, the technology was tested on samples obtained from river and natural water resources.

3.2 Optimisation of cold plasma production by verification of operating parameters

Figure 3.2. illustrates the experimental dielectric barrier discharge (DBD) ACP laboratory setup designed for the optimisation of cold plasma production. The setup consisted of a handheld plasma device constructed primarily from perspex, featuring dimensions of 75 mm in width, 50 mm in height, and 10 mm

in thickness. The device's width incorporated a central division separating the gas cavity from the electronics.

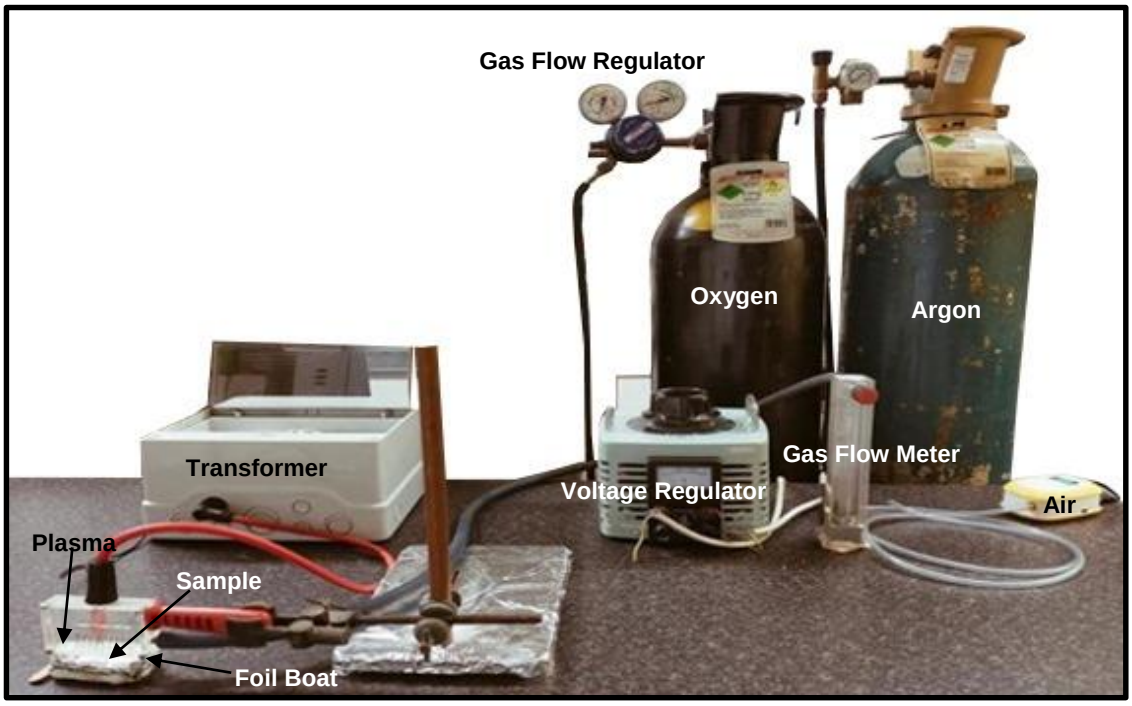


Figure 3.2: Experimental laboratory setup of cold plasma disinfection device showing various components including voltage and pressure regulation and the various gases used for plasma generation.

Nine plasma needles, each equipped with a ballast resistor, enabled the generation of multiple jet streams from a shared power source. To generate the output voltages of 9.56 kV, 10.82 kV and 13.53 kV for the study, a transformer was used as the primary power source in combination with a voltage regulator. The transformer stepped up the input voltage from a lower level to a higher value, providing the necessary electrical power for the cold plasma system. Once the transformer raised the voltage, the voltage regulator was then used to precisely control and adjust this voltage to the required levels of 9.56 kV, 10.82 kV and 13.53 kV, ensuring a stable and constant output. The combination of the transformer and the voltage regulator ensured that the voltage conditions were consistent and reliable throughout the experiment. The transformer provided the initial high voltage and the regulator fine-tuned and stabilised it, allowing the cold plasma system to operate efficiently at the desired voltages for

pathogen inactivation. The gases used namely oxygen (O_2), argon (Ar) and air, were controlled using a gas flow regulator and a flow meter. Following the methods demonstrated by Feizollahi *et al.,* (2021) for DBD ACP production, the device was optimised for ACP production.

Working with high voltages such as 9.56 kV, 10.82 kV and 13.53 kV, in the laboratory meant several safety systems were essential to protect both personnel and the equipment. To ensure safe operation, insulated wiring and components to prevent direct contact with high-voltage parts were used. The equipment was grounded, providing a safe path for electrical currents in case of malfunction. Additionally, circuit protection measures, such as fuses and circuit breakers were incorporated to disconnect the power during overloads or short circuit conditions. Safety relays and emergency shut-off mechanisms were also in place to detect faults and immediately cut off power if hazardous conditions arose. As part of the safety protocol, personnel protective equipment (PPE) was worn, including insulated gloves, safety boots, and arc flash protection. By implementing these safety systems, the risks were minimised associated with high voltage operations, ensuring the safety of personnel and the protection of the equipment throughout the experiment.

To optimise the production of atmospheric cold plasma, the dielectric constants were verified by experimenting with different gases, voltages and electrode distances. This was explored by assessing electric field strengths under different potential differences and determining the necessary plate separation distance (d), as illustrated in Figure 3.3 to generate a plasma arc for the gases: oxygen, argon, and air. This was conducted at a consistent flow rate of 5 ℓ/min and a gas pressure of 100 kPa, employing voltages of 9.56 kV, 10.82 kV, and 13.53 kV. The optimisation outcomes, particularly the distance required for a plasma arc, derived from the verification of dielectric constants across varied gases and voltages will serve as a basis for all subsequent testing.

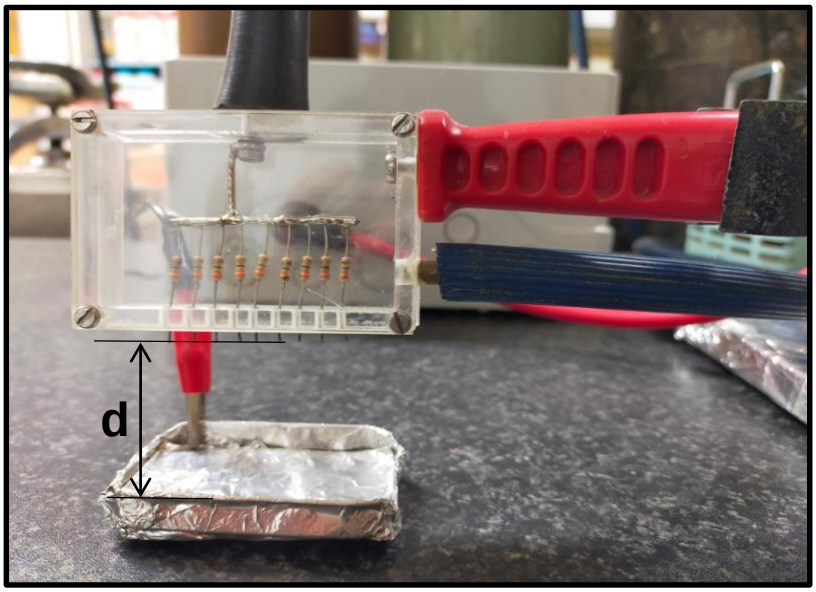


Figure 3.3: Handheld device depicting the distance (d) between the electrode and the ground plate.

A volume of 5 ml of distilled water was positioned on a sterile aluminium foil boat (approximately 5 cm) set on an aluminium tray between the electrodes to generate plasma at output voltages of 9.56 kV, 10.82 kV and 13.53 kV using oxygen, argon and air gases. The plasma needles were encapsulated in the gas chamber such that each gas formed a plasma arc. Distinct spacing between the grounding plate and the electrode was observed for the different gases and the working distance was documented when plasma was produced and an arc was generated.

The selection of gases such as oxygen (O_2), argon (Ar), and air for cold plasma production is based on their specific roles in enhancing effectiveness. Oxygen is chosen for its ability to generate reactive oxygen species (ROS), including ozone, which are essential for disinfection and pathogen inactivation (Zhang *et al.,* 2023). Argon, an inert gas, is preferred for its stability and ability to create uniform and reproducible plasma discharges, making it ideal for controlled experiments (Palumbo *et al.,* 2020). Air, a mixture of nitrogen and oxygen, is used for its cost-effectiveness and ability to produce a broad spectrum of

reactive species, enhancing its applicability for real-world disinfection applications (Lan *et al.,* 2023). Together these gases provide a balanced approach for optimising cold plasma systems for pathogen inactivation.

When a plasma arc was created for each voltage using oxygen, argon and air, it signified the presence of an electric field, indicating that the dielectric breakdown has been surpassed. The dielectric breakdown constants for the different gases are illustrated in Table 3.1. Plate separation distances (mm) were measured using a vernier calliper. The strength of the electric field (E) was calculated for various potential differences (ΔV) and their corresponding plate separation distance (d) needed to initiate plasma for oxygen, argon and air. This ensured the optimisation of the equipment for plasma production, marking the commencement of the remaining experimental study.

Table 3.1: Dielectric breakdown constants for various gases used in this study based on the Raether method (2022) (Flynn, 2021)

Gas	E_{max} **(**$V.m^{-1}$**)**
Air	2.94×10^{6}
Argon	1.09×10^{6}
Oxygen	3.03×10^{6}

3.3 Determination of optimum parameters for inactivation of bacterial pathogens using cold plasma

The determination of optimum parameters in this study was achieved by systematically varying key factors that influence the efficacy of cold plasma treatment for bacterial inactivation. These factors included the choice of gas (argon, oxygen and air), voltage levels and treatment duration. By conducting experiments with the different gas types the study assessed how each plasmas composition affected the inactivation of both chlorine-resistant and non-

chlorine-resistant bacteria. Voltage levels were also varied to identify the optimal energy input required for effective pathogen removal without causing undesirable side effects, such as excessive heating or damage to the water condition. Additionally, the study examined the impact of treatment duration by comparing direct cold plasma treatment (DT) with a 24 h storage post-treatment (PT) to evaluate if extended exposure to plasma-activated water led to further inactivation of bacteria. The combination of these parameters allowed the study to identify the most effective conditions for maximum bacterial inactivation while considering both DT and 24 h storage post-treatment thus determining the optimal parameters for cold plasma water disinfection.

3.3.1 Collection and storage of chlorine-resistant and non-chlorine-resistant bacteria

The selected bacterial strains for this study comprised both chlorine-resistant bacteria and non-chlorine-resistant bacteria. Among the chlorine-resistant bacterial indicators were *Staphylococcus aureus* (G1), *Enterococcus aerogenes* (E2), *Sphingomonas paucimobilis* (C1), *Staphylococcus haemolyticus* (J1), *Pantoea* sp. (F1), Isolate 1 (G2) and Isolate 2 (J3). These bacterial strains were sourced from uMngeni-uThukela Water Microbiology Laboratory in Pietermaritzburg. Standardised inoculum was prepared in Nutrient broth (NB) by isolating colonies from an 18-24 h culture grown on Nutrient agar (NA) and stored at 4°C.

The non-chlorine-resistant bacterial indicators included *Acinetobacter baumannii (ACIBA 01), Aeromonas hydrophila (AERHY 01), Campylobacter jejuni (CAMJE 01), Escherichia coli (ESCCO 41), Listeria monocytogenes (LISMA 04), Salmonella isangii* (SALIS 01) *and Shigella flexneri (SHIFL 01).* These clinical strains were sourced from the National Institute for Communicable Diseases (NICD). Standardised inocula were prepared by isolating colonies from 18-24 h cultures grown on 5% and 10% Blood agar (BA), and subsequently stored at 4°C. All non-chlorine-resistant bacterial strains were

grown on 5% Blood agar, except for *Campylobacter jejuni (CAMJE 01),* which was grown on 10% Blood agar.

The bacterial strains used in this study, including both chlorine-resistant and non-chlorine-resistant bacteria, were stored and handled under specific conditions to ensure their viability before collection and incubation. After subculturing, the bacterial strains were stored at 4°C to preserve their viability between subculturing and experimentation. For subculture preparation, colonies were isolated from 18-24 h cultures grown on specific agar media. Chlorine-resistant strains were cultured on Nutrient agar (NA), while non-chlorine-resistant strains were cultured on 5% or 10% Blood agar (BA), depending on the strain, with *Campylobacter jejuni* being specifically grown on 10% Blood agar. These storage and incubation conditions ensured the bacterial cultures remained stable, allowing for accurate and consistent subsequent experiments.

3.3.2 Sample collection procedure of water samples

The number of water samples was equal to 10, they were collected from various locations in the Durban and North surrounding areas, including rivers, estuaries, lagoons and dams as illustrated in Table 3.2. Overall, the combination of practical constraints (cost, site availability) and the methodological needs of the study led to the selection of 10 sampling sites and a single round of sampling, ensuring that the study remained feasible, focused and methodologically sound while still providing valuable insights into the effectiveness of ACP for water disinfection. The sampling process involved using ten 500 mL glass Schott bottles, which were sterilised at 121°C for 15 minutes and then allowed to cool. To obtain the samples, the bottles were submerged 300 mm below the water surface, with the neck of the bottle facing downward and tilted horizontally against the water flow, facilitating the filling of the bottle. The bottles were promptly capped, labelled and stored in a cooler box at temperatures ranging

from 5–10°C. All collected samples were transported to the laboratory and analysed within 6-12 hours of collection.

Table 3.2: List of sampling sites for water samples

Water Samples		
No.	**River Name**	**Symbol**
1	*Tinley Manor River*	R1
2	*Tongaat River*	R2
3	*Umhlatuzana River*	R3
4	*Salt Rock River*	R4
5	*Durban Harbour*	E1
6	*La Mercy Estuary*	E2
7	*uMhlanga Lagoon*	L1
8	*Blue Lagoon*	L2
9	*Inanda Dam*	D1
10	*Shongweni Dam*	D2

3.3.3 Sample analyses of chlorine-resistant and non-chlorine-resistant bacteria

In total, seven chlorine-resistant bacteria and seven non-chlorine-resistant bacteria as named in Section 3.3.1, underwent direct cold plasma treatment and were subsequently stored for 24 h post-treatment at room temperature (22°C - 25°C). All bacterial samples in solution were analysed after direct plasma treatment using oxygen, argon and air at varying durations of 0.5, 1.0, 1.5, 2.0 and 2.5 min, and output voltages of 9.56 kV, 10.82 kV and 13.53 kV, followed by a 24 h storage post-treatment (22°C - 25°C).

3.3.4 Preparation of chlorine-resistant and non-chlorine-resistant bacterial cell suspensions

The chlorine-resistant bacterial cultures were cultivated and sustained on Nutrient agar, while the non-chlorine-resistant bacterial cultures were

maintained on Blood agar, both stored in the refrigerator at 4°C. For the preparation of cell suspension, each chlorine-resistant bacterium was inoculated onto Nutrient agar and incubated at 37°C for 48 h. For non-chlorine-resistant bacteria, Blood agar for each bacterial culture was inoculated and incubated at their respective growth temperatures (37°C for 48 h for all the cultures except *Campylobacter jejuni* which was incubated anaerobically at 37°C for 48 h). An isolated colony from the chlorine-resistant culture was inoculated into 10 mL tubes containing saline solution. In a separate procedure, an isolated colony from the non-chlorine-resistant culture was inoculated into 10 mL tubes containing Tryptone Soy broth, followed by overnight incubation at 37°C. Serial dilutions of each bacterial culture (chlorine-resistant and non-chlorine-resistant) were conducted and plated on Nutrient agar for chlorine-resistant bacteria and on Blood agar for non-chlorine-resistant bacteria. For plasma treatment, all cells were adjusted to a concentration of 1.0×10^7 CFU/mL in saline solution (SS) or phosphate-buffered saline (PBS) following the method by Han *et al.,* (2016). Serial dilutions were employed again to achieve the final concentration. This involved taking 1 mL of the bacterial suspension into 9 mL of saline solution, resulting in a concentration of 1.0×10^7 CFU/mL. This last tube containing saline solution served as the working inoculum, having a final concentration of 1.0×10^7 CFU/mL (American Society for Microbiology, 2005).

3.3.5 Experimental assay for the optimisation of water treatment using atmospheric cold plasma

The experimental assay in this study was designed to systematically assess the efficacy of ACP for the inactivation of both chlorine-resistant and non-chlorine-resistant bacteria in water. The process involved exposing each bacterial type to direct cold plasma treatment, followed by a 24 h storage post-treatment. The direct cold plasma treatment aimed to immediately inactivate the bacteria through the generation of ROS and RNS during the plasma exposure. These reactive species are known to damage the cellular structure of bacteria, leading

to the breakdown of cell membranes, protein degradation and DNA damage, which ultimately renders the bacteria non-viable.

The inclusion of the 24 h storage post-treatment allowed the study to investigate whether the effects of cold plasma treatment extend beyond the immediate contact time and whether bacteria continued to be inactivated over time. This aspect of the experiment was particularly important in understanding the long-term efficacy of cold plasma as a water treatment method. In some cases, bacteria may not be immediately inactivated during the plasma exposure but may become inactive after a period of incubation, suggesting that post-treatment effects can contribute significantly to the overall disinfection process. This 24 h storage post-treatment provided an opportunity to assess the delayed impact of cold plasma on microbial inactivation, which is important for real-world applications where water may be stored before being used or consumed.

The experiment was conducted in duplicate for each bacterial type, ensuring that the results were reproducible and statistically reliable. By performing these assays with both chlorine-resistant and non-chlorine-resistant bacteria, the study was able to determine if the cold plasma treatment was effective across different bacterial strains which is crucial for evaluating the broad-spectrum disinfection potential of cold plasma. Moreover, this experimental setup allowed for the optimisation of key parameters such as exposure time, gas type, voltage levels and the effects of post-treatment storage, providing valuable insights into how cold plasma can be tailored to achieve maximum efficacy for water treatment.

The results from this experimental assay were critical for optimising the water treatment process using ACP, as they will identify the optimal conditions that produce the greatest microbial inactivation. By understanding how variables such as treatment duration, post-treatment effects, and bacterial resistance impact the efficacy of cold plasma, the study aimed to establish guidelines for implementing this technology in large-scale water disinfection applications.

Furthermore, this research could contribute to developing more efficient, sustainable and cost-effective methods for water treatment particularly in regions where excess to clean potable water is a significant concern.

3.3.5.1 Direct atmospheric cold plasma treatment

A volume of 5 ml of the prepared suspensions containing chlorine-resistant and non-chlorine-resistant bacteria, with a bacterial concentration of 1 x 10^7 CFU/mL, was placed on a sterile aluminium foil boat (approximately 5 cm) positioned on the aluminium tray. Although the plasma discharge was directed from the top down into the sample (as shown in the photos), the process was carried out exactly as described in the methodology with the sample exposed to plasma for durations of 0.5, 1.0, 1.5, 2.0 and 2.5 minutes. The output voltages were set at 9.56 kV, 10.82 kV and 13.53 kV, ensuring consistent exposure for the experiments. The placement of the sample was deliberately chosen for optimal plasma exposure, despite the differences in visual representation in the photos.

The positive controls included chlorine-resistant and non-chlorine-resistant bacteria with an initial bacterial concentration of 1 x 10^7 CFU/mL that were prepared but not subjected to any ACP treatment or 24 h storage post-treatment. The negative control comprised sterile distilled water that underwent direct cold plasma treatment.

3.3.5.2 Post-treatment analysis and total bacterial count

Following a 24 hour settling period at room temperature after the direct plasma treatment, total bacterial counts were determined using the quantitative plate colony count method to assess bacterial survival and growth for chlorine-resistant and non-chlorine-resistant bacteria. This analysis was conducted for all samples, including those subjected to direct treatment and those stored for

24 h post-treatment, by following established guidelines (American Society for Microbiology, 2005; Freese *et al.,* 2003).

The plate count method involved serial dilution of the samples using sterile phosphate-buffered saline (PBS) for non-chlorine-resistant bacteria and sterile saline solution (SS) for chlorine-resistant bacteria. Serial dilutions with a constant dilution factor of 10 were performed until the bacteria were adequately diluted for counting on Nutrient agar (chlorine-resistant bacteria) and Blood agar (non-chlorine-resistant bacteria). The dilutions were achieved by transferring 1 mL of suspension into 9 mL of PBS/SS, creating a dilution series from 10^{-1} to 10^{-7}. Aseptic techniques were followed to ensure accurate preparation, with each dilution being homogenised before the next transfer. A 0.1 mL aliquot from each dilution was mixed with 20 mL of molten selective agar (Nutrient agar/Blood agar) and allowed to solidify. The plates were inverted and incubated at 37°C for 48 h with *Campylobacter jejuni* being incubated anaerobically at 37°C for the same duration. After incubation, colony counts were determined to assess the bacterial survival post-treatment (American Society for Microbiology, 2005).

3.3.6 Measurement of log reduction, percent reduction & D-value

The measurement of log reduction as depicted in Table 3.3 was completed using the following formula:

Log Reduction = $\log_{10}(A/B)$ (**Eq. 1)**

Where A is the initial quantity of viable microorganisms before treatment and B is the number of viable microorganisms after treatment (Hamilton, 2010).

Table 3.3: Interpretation of the log reduction with a microbial reduction percentage (Biologicalprep, 2021)

Starting Number = 1 Million Bacteria		
Log Reduction	**% Reduction**	**Bacteria Remaining**
0	0%	1,000,000
1	90%	100,000
2	99%	10,000
3	99.9%	1,000
4	99.99%	100
5	99.999%	10

The measurement of percent reduction is done using the following formula:

Log Reduction = (A-B) * 100/A **(Eq. 2)**

Where A is the initial quantity of viable microorganisms before treatment and B is the number of viable microorganisms after treatment. The D-value which is the treatment time taken in minutes that will result in reducing microorganisms by one log cycle or by 90% was calculated using the formula:

$D = t / [\log_{10} (Q1) – \log_{10} (Q2)]$ **(Eq. 3)**

Where D is the D-value, t is the total time that has passed, Q1 is the initial quantity and Q2 is the final quantity.

3.4 Kinetics of microbial inactivation after direct cold plasma treatment and 24 h storage post-treatment

Data analysis from the experimental processes, as described in section 3.3.5, involved assessing the results of direct ACP treatment and 24 h storage post-treatment. The analysis focused on the inactivation effect (log reduction and % log reduction) and the time required to achieve a 1-log reduction (90% bacterial inactivation), which is commonly referred to as the D-value. These parameters were tested for both chlorine-resistant and non-chlorine-resistant bacteria under varying treatment conditions using oxygen, argon and air plasmas at voltages

of 9.56 kV, 10.82 kV and 13.53 kV, with exposure durations of 0.5, 1.0, 1.5, 2.0 and 2.5 minutes.

The inactivation kinetics were analysed by applying a first-order model, where the reduction in microbial population is proportional to the remaining population, with the results plotted as survival curves. The log reduction and D-values were used to assess the effectiveness of the plasma treatment over time, both during direct exposure and after 24 h storage, for the different gases tested.

3.5 Statistical analyses

3.5.1 Electric field strength for optimisation of plasma production

All analyses were performed in triplicate. Linear regression was used to get a line of best fit using GraphPad Prism v.8.0.2. When performing the linear regression to determine the line of best fit, certain constants, also known as parameters of coefficients were calculated. For a simple linear regression, the equation of the line of best fit was expressed as: $y = mx + b$

Where:

y = The dependent variable (the outcome or response variable).

x = The independent variable (the predictor variable).

m = The slope of the line (also called the regression of coefficient).

b = The y-intercept of the line (the value of y when x=0).

The R^2 or coefficient of determination (COD) measures the proportion of the variance in the dependent variable that is predictable from the independent variable. A higher R^2 value indicates a better fit of the regression line to the data. For a goodness of fit, the R^2 value is a measure of how closely the regression line (the predicted values) fits the actual data points. An R^2 of 0.9 indicates a very good fit, as the model explains the vast majority of the variability in the data. Only 10% of the variability is left unexplained by the model, which suggests that the model captures most of the underlying trend. Also, a R^2 of 0.9

suggests that there is a strong correlation between the independent and dependent variables.

3.5.2 Enumeration of chlorine-resistant and non-chlorine-resistant bacteria and water samples (See Table 4.3 & Table 4.4)

All analyses were performed in duplicate. A simple linear regression was used to analyse the results for the surviving bacterial counts of microorganisms in both chlorine-resistant and non-chlorine-resistant bacteria. Two-way ANOVA with Tukey's post-hoc comparison test was used to analyse the total bacterial counts, log reduction and percentage log reduction of chlorine-resistant and non-chlorine-resistant bacteria for the gases oxygen, argon and air for direct treatment and 24 h storage post-treatment after 2.50 min. For the water samples, two-way ANOVA with Dunnett's post-hoc comparison test was used to analyse the total bacterial counts, and Tukey's post-hoc comparison test was used to analyse the log reduction and percentage log reduction using GraphPad Prism v.8.0.2.

Different post-hoc analysis tests were used because of the two types of data being analysed, viz. bacterial counts and log reductions or percentage log reductions. These required different methods of statistical comparison, and therefore, each post-hoc test was suited to its specific situation.
Turkey's post-hoc test was typically used when comparisons needed to be made between all pairs of treatment groups (e.g. different gases, different exposure times, etc.). In this case, Turkey's test was applied to the log reduction and percentage log reduction of chlorine-resistant and non-chlorine-resistant bacteria, as the study compared multiple treatment groups (oxygen, argon and air) against each other. Turkey's test helped determine which specific groups differed significantly from one another by controlling the Type 1 error rate when making multiple comparisons.

Dunnett's post-hoc test was often used when comparing multiple experimental

groups to a single control group. In this case, the total bacterial counts from the water samples were compared to a baseline (or control) treatment group, so Dunnett's test was appropriate. It specifically compared each treatment group (e.g. different gases or exposure times) to the control without inflating the Type 1 error rate, which could have happened if all pairwise comparisons were made.

Turkey's test was more suited for comparing all treatment groups against each other in a balanced design, which is why it was applied to the log reduction and percentage log reduction data for the chlorine-resistant and non-chlorine-resistant bacteria. Dunnett's test was best when comparing multiple experimental groups to a single control, which is why it was applied to analyse the total bacterial counts from the water samples (where one group was treated as the control). In conclusion, these post-hoc tests were chosen based on the specific nature of the comparisons being made. Turkey's test was used for comprehensive pairwise comparisons of all groups, while Dunnett's test was chosen for comparisons of each treatment group to a control group. This allowed for more accurate, context-specific statistical analysis.

3.5.3 Data analysis of the water samples using the optimal parameters (See Annexure 1 & Annexure 2)

The enumeration of total bacterial counts in both the initial and final water samples was performed using the pour plate method. The samples underwent both direct treatment and 24 h storage post-treatment under optimal conditions (treatment time of 3 minutes at 13.53 kV using oxygen, argon and air plasma). To calculate the impact of bacterial inactivation, log reduction, percentage log reduction, and the D-value were determined after both direct treatment and 24 h storage post-treatment.

Statistical analysis was performed using the Two-way ANOVA and Tukey's post-hoc comparison test for bacterial counts, log reduction, and percentage log reduction of chlorine-resistant and non-chlorine-resistant bacteria exposed to

different gases (oxygen, argon and air). Dunnett's post-hoc comparison test was used for comparing bacterial counts across the different conditions, as appropriate. The D-value was calculated for all conditions to determine the time required to achieve a 1-log reduction of bacterial populations.

Serial dilutions were carried out with a constant 10-fold dilution factor, transferring 1 mL of bacterial suspension into 9 mL of sterile SS (for chlorine-resistant bacteria) or PBS (for non-chlorine-resistant bacteria), up to 10^{-8} dilutions. Two plates were prepared for each dilution to ensure consistency and reproducibility of colony counts. Experiments were conducted in duplicates to minimise random error and improve reliability, providing a more robust dataset for analysis. This approach is commonly used in microbiological research to reduce variability and ensure the reliability of the results.

CHAPTER 4: RESULTS

4.1 Electric field strengths and plasma arcs

4.1.1 Electric field strength, potential differences and plate separation distance relationship

The electric field strength, potential difference (ΔV) and plate separation distance (d) to produce a plasma arc are illustrated in Table 4.1. It is evident that for each potential difference and the corresponding separation distance, the electric field produced exceeded the dielectric breakdown voltage. As the potential difference increases, so does the plate separation distance.

Table 4.1: Electric field strength for various potential differences (ΔV), and their corresponding plate separation distance required to create an arc using oxygen, argon and air

	d (mm)	ΔV (kV)	E (V/m)	% (> E_{max})	Plasma Arcs
	3.00	9.56	3.19×10^6	5.3	Figure 4.1. a)
Oxygen	3.50	10.82	3.09×10^6	2.0	Figure 4.1. b)
	4.10	13.53	3.30×10^6	8.9	Figure 4.1. c)
	6.50	9.56	1.47×10^6	34.9	Figure 4.2. a)
Argon	8.24	10.82	1.31×10^6	20.2	Figure 4.2. b)
	9.50	13.53	1.42×10^6	30.3	Figure 4.2. c)
	1.75	9.56	5.46×10^6	85.7	Figure 4.3. a)
Air	2.75	10.82	3.93×10^6	33.7	Figure 4.3. b)
	3.50	13.53	3.87×10^6	31.6	Figure 4.3. c)

4.1.2 Visual evidence of the plasma arcs to confirm the dielectric breakdown

The following figures depict the plasma arcs formed for each of the varied voltages and gases used. Figures 4.1 (a-c), 4.2 (a-c) and 4.3 (a-c) provide visual evidence of the plasma arcs to confirm the dielectric breakdown. The plasma arcs created as a result of exceeding the dielectric breakdown voltage during the varying voltages using different gases also created different distances between the electrodes as depicted in Table 4.1.

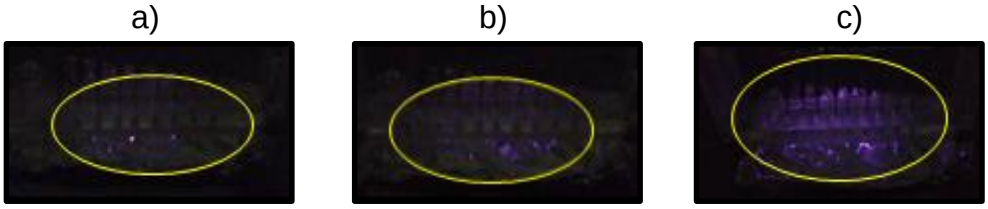


Figure 4.1: Visual plasma arcs for oxygen at a) 9.56 kV with a distance of 3 mm between the electrode and ground plate, b) 10.82 kV with a distance of 3.5 mm between the electrode and ground plate and c) 13.53 kV with a distance of 4.1 mm between the electrode and ground plate.

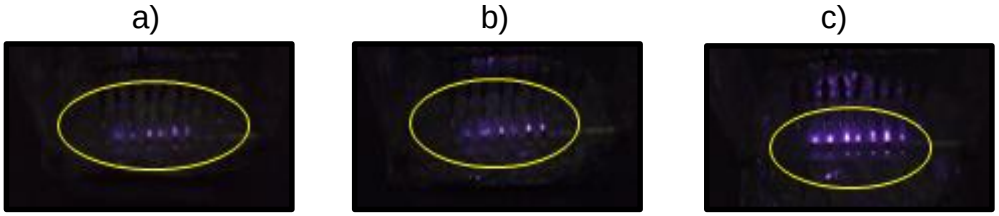


Figure 4.2: Visual plasma arcs for argon at a) 9.56 kV with a distance of 6.5 mm between the electrode and ground plate, b) 10.82 kV with a distance of 8.25 mm between the electrode and ground plate and c) 13.53 kV with a distance of 9.50 mm between the electrode and ground plate.

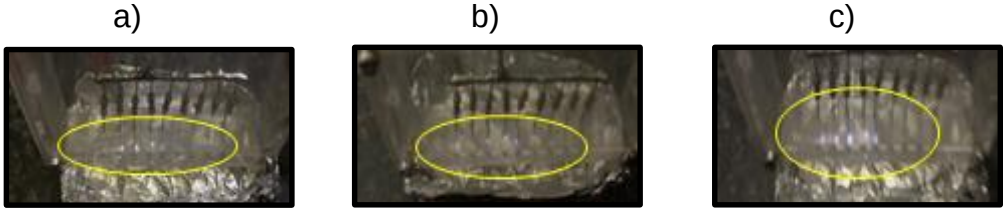


Figure 4.3: Visual plasma arcs for air at a) 9.56 kV with a distance of 1.75 mm between the electrode and ground plate, b) 10.82 kV with a distance of 2.75 mm between the electrode and ground plate and c) 13.53 kV with a distance of 3.50 mm between the electrode and ground plate.

4.1.3 Electric field strength for optimisation of plasma production

As illustrated in Figure 4.4 (a-c), an increase in the potential difference increased the plate separation distance to create a plasma arc using the different gases. For an electric field to be created and a plasma arc created, the dielectric field breakdown constant must be broken and clear evidence as depicted in Table 4.1 and visually captured for each of the gases are illustrated in Figures 4.1 (a-c), 4.2 (a-c) and 4.3 (a-c).

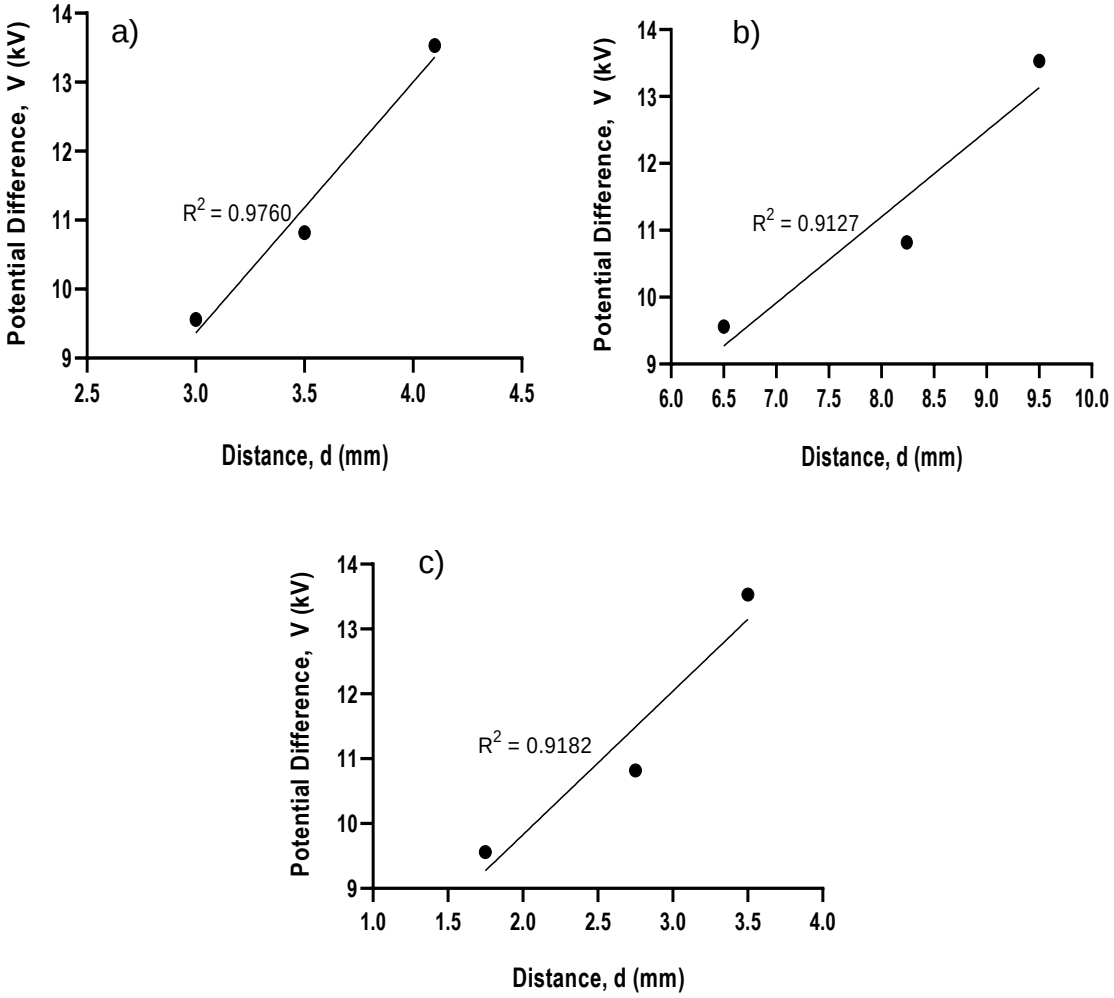


Figure 4.4: Potential difference ΔV (kV) versus distance d (mm) for 4.4. a) oxygen, b) argon and c) air. The slope of the line of best fit represents the average electric field strength.

4.2 Chlorine-resistant and non-chlorine-resistant bacteria

The chlorine-resistant and non-chlorine-resistant bacteria used in this study as listed in Table 4.2 were recognised with their Gram reaction and identified to strain level. The chlorine-resistant bacterial strains sourced from uMngeni-uThukela Water Microbiology Laboratory were identified to the strain level using a combination of biochemical and molecular identification methods. These methods included standard biochemical tests and in some cases, molecular techniques such as PCR or 16S rRNA sequencing. The strains were identified based on their Gram reaction and further biochemical characteristics as outlined in Table 4.2. However, Isolate 1 and Isolate 2 could not be identified to strain level despite several attempts by both the laboratory and during this study. Despite these efforts, the strains could only be classified based on their Gram reaction. As a result, it was concluded that no further identification would be pursued for Isolate 1 and Isolate 2 beyond the initial Gram reaction, and they were subsequently treated as unidentified bacterial isolates and named Isolate 1 and Isolate 2 for this study.

Table 4.2: List of chlorine-resistant and non-chlorine-resistant bacteria

Chlorine-resistant Bacteria		
No.	**Name & Identification**	**Type**
1	Isolate 1 (G2)	Gram-negative
2	*Staphylococcus aureus* (G1)	Gram-positive
3	*Enterobacter aerogenes* (E2)	Gram-negative
4	*Sphingomonas paucimobilis* (C1)	Gram-negative
5	*Staphylococcus haemolyticus* (J1)	Gram-positive
6	*Pantoea* sp. (F1)	Gram-negative
7	Isolate 2 *(*J3)	Gram-negative

Non-chlorine-resistant Bacteria		
1	*Acinetobacter baumannii* (ACIBA 01)	Gram-negative
2	*Aeromonas hydrophila* (AERHY 01)	Gram-negative
3	*Campylobacter jejuni* (CAMJE 01)	Gram-negative
4	*Escherichia coli* (ESCO 41)	Gram-negative
5	*Listeria monocytogenes* (LISMA 04)	Gram-positive
6	*Salmonella isangii* (SALIS 01)	Gram-negative
7	*Shigella flexneri* (SHIFL 01)	Gram-negative

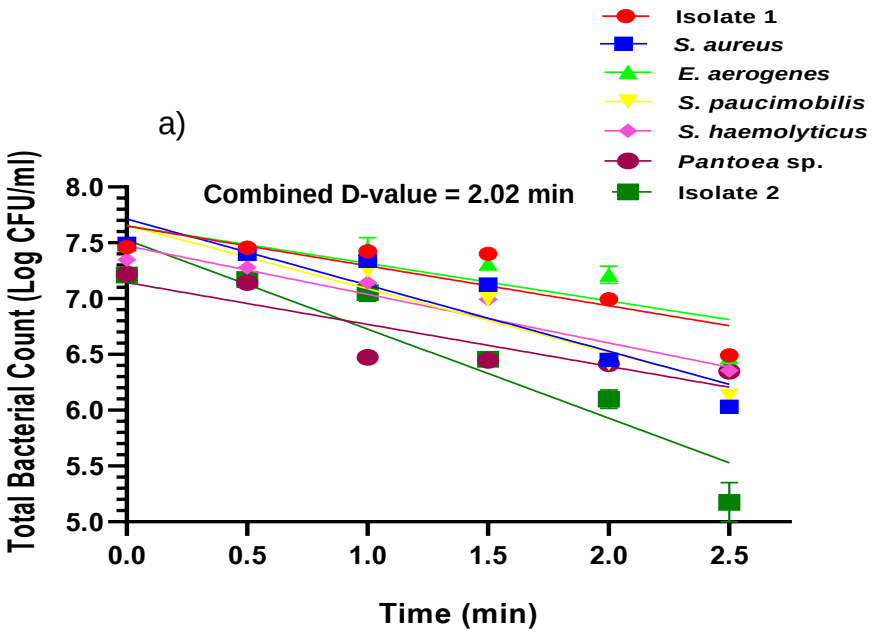
4.3 Enumeration of chlorine-resistant and non-chlorine-resistant bacteria

4.3.1 Enumeration of chlorine-resistant bacteria after direct treatment

The total bacterial counts (Log CFU/mL) obtained for all chlorine-resistant bacteria after direct plasma treatment at 13.53 kV for oxygen, argon and air plasma between 0-2.5 min is depicted in Figure 4.5 (a-c). The surviving bacterial counts for the chlorine-resistant bacteria for direct treatment ranged from 4.97 Log CFU/mL to 6.73 Log CFU/mL for oxygen, argon and air plasma after 2.5 min. The simple linear regression for oxygen and argon plasma respectively in Figure 4.5 (a and b) revealed that the difference between the slopes was not significantly different for the chlorine-resistant bacteria (oxygen: F = 2.409; DFn = 6; DFd = 28; P=0.0529 and argon: F = 0.7284; DFn = 6, DFd = 28; P=0.6306) and therefore was possible to calculate one slope for all the data (oxygen pooled slope =
-0.4948 and argon pooled slope = -0.5953). The differences between the elevations/intercepts were extremely significant for oxygen and argon plasma (oxygen: F = 6.171; DFn = 6; DFd = 34; P=0.0002 and argon: F = 2.448; DFn = 6; DFd = 34; P=0.0448). The linear regression for air in Figure 4.5 (c) revealed that the differences between the slopes were significant (F = 3.057; DFn = 6; DFd = 28; P=0.0199) and because the slopes differed so much, it was not possible to test whether the intercepts differed significantly.

In Figure 4.5 (a), using oxygen plasma, Isolate 2 exhibited the lowest surviving bacterial counts (5.05 Log CFU/mL) whereas Isolate 1 demonstrated the highest surviving bacterial counts (6.50 Log CFU/mL) after direct treatment in 2.5 minutes. Isolate 2 had the highest inactivation while Isolate 1 had the lowest inactivation to oxygen plasma after direct treatment. In Figure 4.5 (b), using argon plasma, Isolate 2 had the lowest surviving bacterial counts (4.97 Log CFU/mL) whilst *S. aureus* had the highest surviving bacterial counts (6.73 Log CFU/mL) after direct treatment in 2.5 minutes. Isolate 2 was the most inactivated while *S. aureus* was the least inactivated to argon plasma after direct treatment. In Figure 4.5 (c) using air plasma, *Pantoea* sp. had the lowest surviving bacterial counts (5.05 Log CFU/mL) whilst Isolate 1 had the highest surviving bacterial counts (6.52 Log CFU/mL) after direct treatment in 2.5 minutes. *Pantoea* sp. was the most inactivated while Isolate 1 was the least inactivated to air plasma after direct treatment. For the direct treatment at 13.53 kV, argon was the best gas with the lowest surviving bacterial counts and the lowest combined D-value of 1.68 min for chlorine-resistant bacteria after 2.5 minutes as seen in Figure 4.5 (b).

In Figures 4.5 (a-c) the inactivation coefficients such as the decimal reduction time are summarised in Table 4.3 and are not denoted on the graphs.



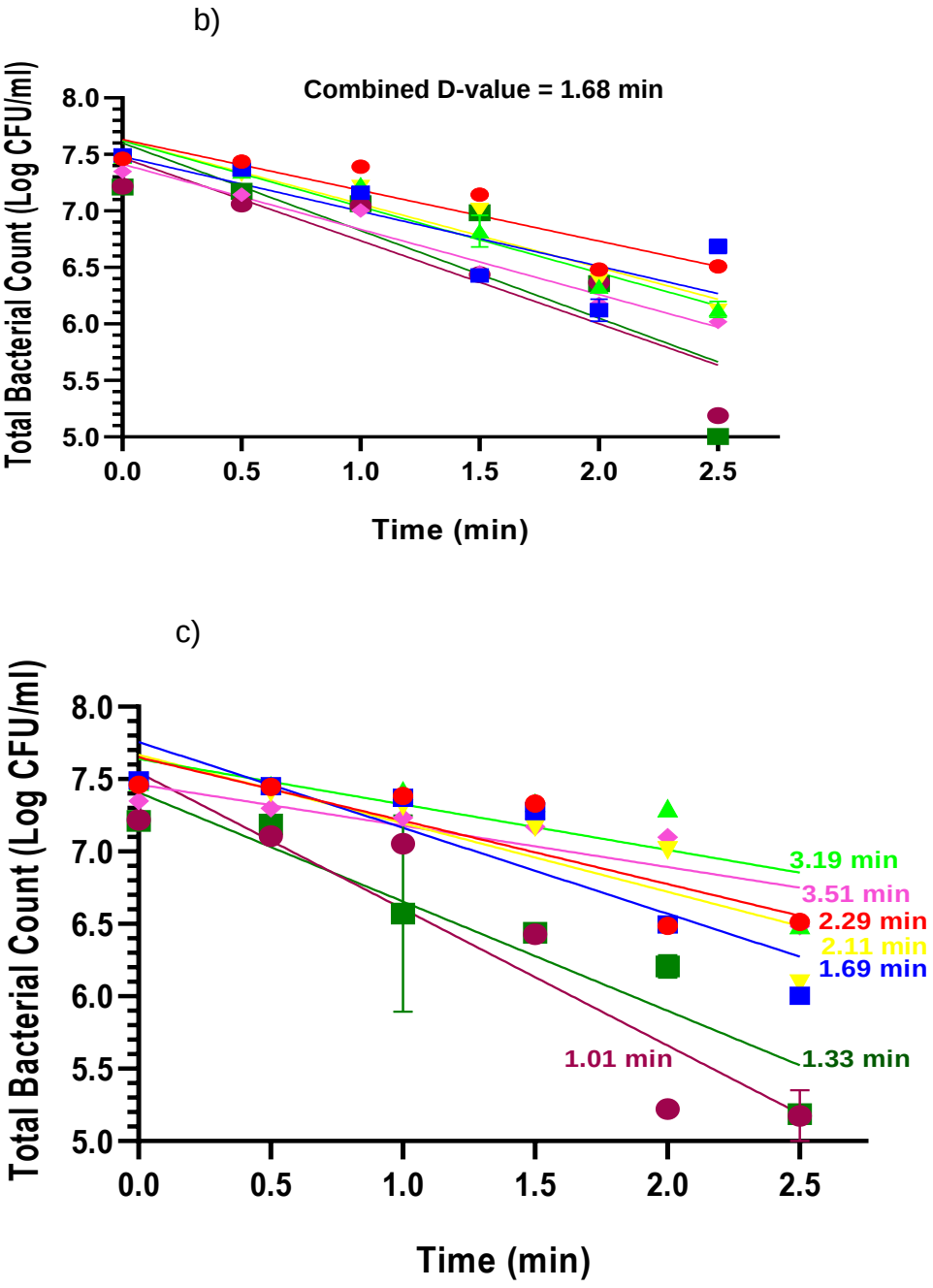


Figure 4.5: Total bacterial counts of chlorine-resistant bacteria at 13.53 kV after direct treatment using a) oxygen, b) argon and c) air after 2.5 min. The best fit straight line is represented with plotted values as mean ± standard error of the mean and n=2. The combined D-value (min) is the inverse of the pooled slope for argon and oxygen. For air, the differences between the slopes were significant and different therefore a pooled slope was not calculated instead individual D-values were calculated for each chlorine-resistant bacteria.

4.3.2 Enumeration of chlorine-resistant bacteria after 24 h storage post-treatment

The total bacterial counts (Log CFU/mL) obtained for all chlorine-resistant bacteria after 24 h storage post-treatment at 13.53 kV for oxygen, argon and air plasma after 0-2.5 min is depicted in Figure 4.6 (a-c). The surviving bacterial counts for the chlorine-resistant bacteria for 24 h storage post-treatment ranged from 4.37 Log CFU/mL to 6.94 Log CFU/mL for oxygen, argon and air plasma after 2.5 min. The simple linear regression for oxygen, argon and air plasma respectively in Figure 4.6 (a-c) revealed that the difference between the slopes was not significantly different for the chlorine-resistant bacteria after 24 h storage post-treatment (oxygen: F = 1.203.; DFn = 6; DFd = 28; P=0.3337, argon: F = 1.197; DFn = 6; DFd = 28; P=0.3365 and air F = 2.275; DFn = 6; DFd = 28; P=0.0650) and therefore was possible to calculate one slope for all the data (oxygen pooled slope = -0.6371, argon pooled slope = -0.8086 and air pooled slope = -0.6160). The differences between the elevations/intercepts were extremely significant for (argon: F = 4.881; DFn = 6; DFd = 34; P=0.0011 and air: F = 5.290; DFn = 6; DFd = 34; P=0.0006). The differences between the elevations for oxygen plasma were not significant (F = 1.683; DFn = 6; DFd = 34; P=0.1553). Since the Y-intercepts were not significantly different, it was possible to calculate one Y-intercept for all the data. The pooled intercept equalled 7.607.

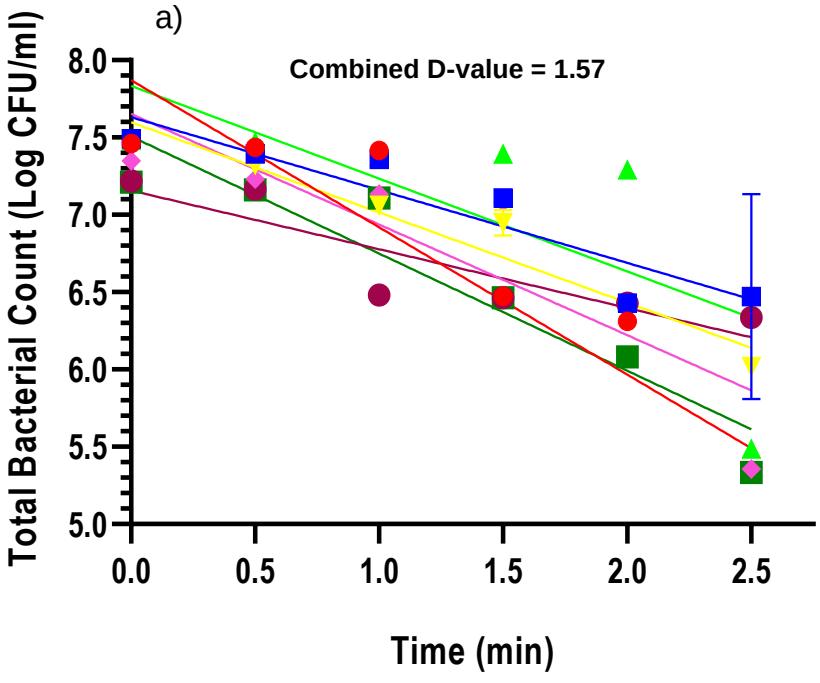
In Figure 4.6 (a), using oxygen plasma, Isolate1 had the lowest surviving bacterial counts (4.97 Log CFU/mL) whilst *S. aureus* had the highest surviving bacterial counts (6.94 Log CFU/mL) after 24 h storage post-treatment in 2.5 minutes. Isolate 1 had the highest bacterial inactivation while *S. aureus* had the lowest bacterial inactivation to oxygen plasma after 24 h storage post-treatment.

In Figure 4.6 (b), using argon plasma, *E. aerogenes* had the lowest surviving bacterial counts (4.37 Log CFU/mL) whilst *S. aureus* had the highest surviving bacterial counts (6.69 Log CFU/mL) after 24 h storage post-treatment in 2.5

minutes. *E. aerogenes* was the most inactivated while *S. aureus* was the least inactivated by argon plasma after 24 h storage post-treatment.

In Figure 4.6 (c), using air plasma, Isolate 2 had the lowest surviving bacterial counts (5.08 Log CFU/mL) whilst Isolate 1 and *E. aerogenes* had the highest surviving bacterial counts (6.52 Log CFU/mL) after 24 h storage post-treatment in 2.5 minutes. Isolate 2 was highly inactivated while Isolate 1 and *E. aerogenes* were least inactivated after 24 h storage post-treatment. During the 24 h storage post-treatment, argon had the highest effect with the lowest surviving bacterial counts and the lowest combined D-value of 1.24 min whilst air had the highest surviving bacterial counts with the least effect and the highest combined D-value of 1.62 min for the chlorine-resistant bacteria. Overall, with the chlorine-resistant bacteria, argon had the highest effect for the direct treatment and the 24 h storage post-treatment.

In Figures 4.6 (a-c) the inactivation coefficients such as the decimal reduction time are summarised in Table 4.3 and are not denoted on the graphs. The combined D-values (min) is the inverse of the pooled slope.



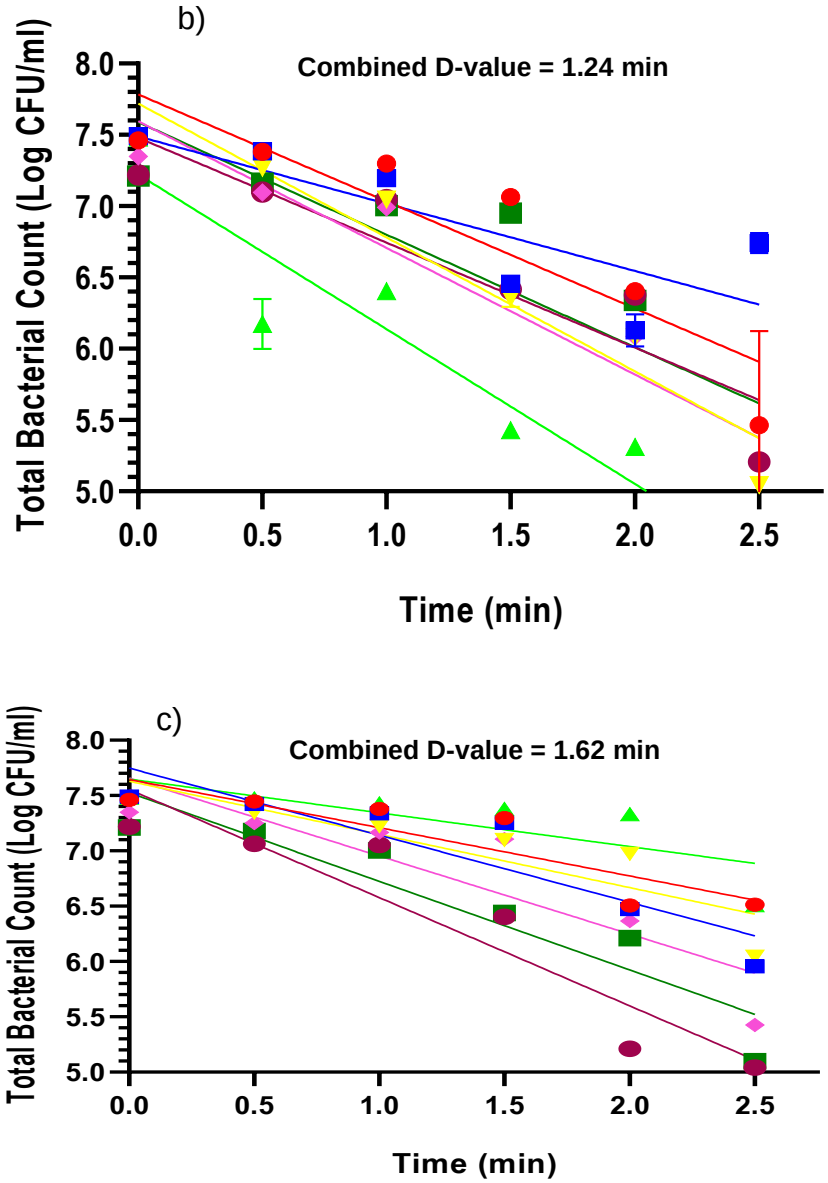


Figure 4.6: Total bacterial counts of chlorine-resistant bacteria at 13.53 kV after 24 h storage post-treatment using a) oxygen, b) argon and c) air after 2.5 min. The best fit straight line is represented with plotted values as mean ± standard error of the mean and n=2. The combined D-value (min) is the inverse of the pooled slope.

4.3.3 Enumeration of non-chlorine-resistant bacteria after direct treatment

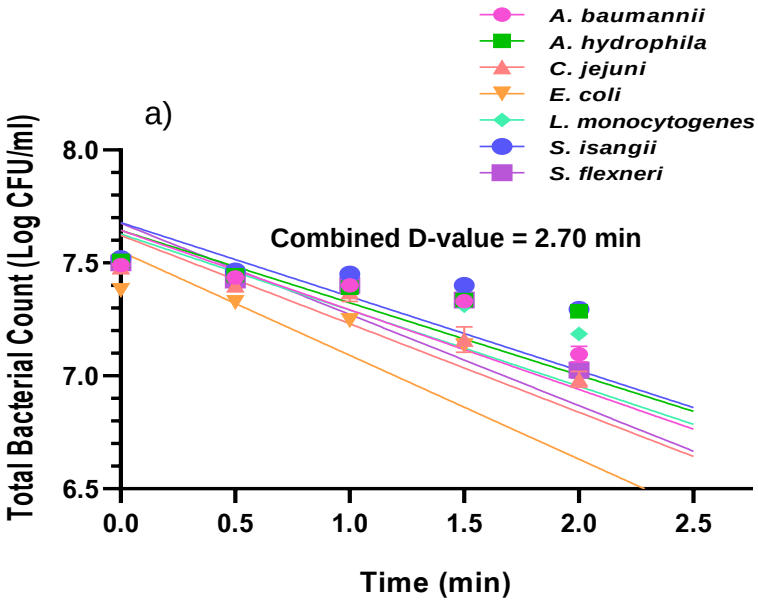
The total bacterial counts (Log CFU/mL) obtained for all non-chlorine-resistant bacteria after direct plasma treatment at 13.53 kV for oxygen, argon and air plasma between 0-2.5 min is depicted in Figure 4.7 (a-c). The surviving bacterial counts for the non-chlorine-resistant bacteria for direct treatment

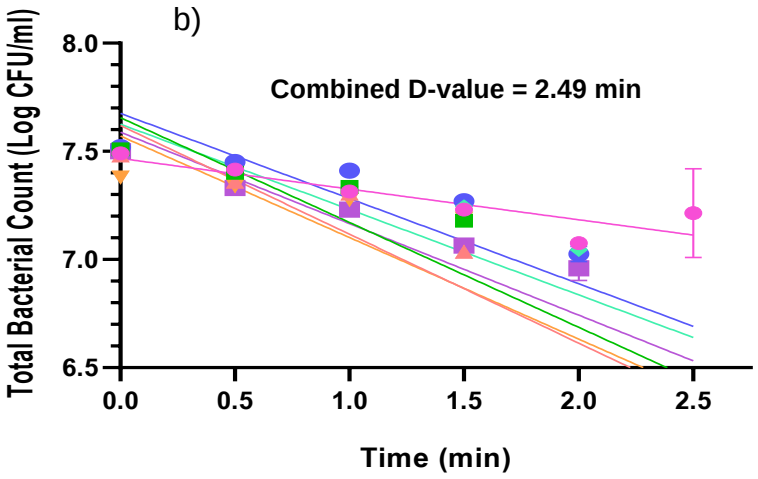
ranged from 6.26 Log CFU/mL to 7.36 Log CFU/mL using oxygen, argon and air plasma after 2.5 min. The simple linear regression in Figure 4.7(a-c) revealed that the difference between the slopes was not significantly different for the non-chlorine-resistant bacteria (oxygen: F = 0.2054; DFn = 6; DFd = 28; P=0.9722, argon: F = 1.732; DFn = 6; DFd = 28; P=0.1503 and air: F = 0.7094; DFn = 6; DFd = 28; P=0.6448) and therefore was possible to calculate one slope for all the data (oxygen pooled slope = -0.3704; argon pooled slope = -0.4014 and air pooled slope = -0.4122.). The differences between the elevations/intercepts were not significant (oxygen: F = 1.218; DFn = 6; DFd = 34; P=0.3211, argon: F = 1.721; DFn = 6; DFd = 34; P=0.1461 and air: F = 1.417; DFn = 6; DFd = 34; P=0.2367). Since the Y-intercepts were not significantly different, it was possible to calculate one Y-intercept for all the data. The pooled intercept results were oxygen: 7.635, argon: 7.600 and air: 7.657.

In Figure 4.7 (a), using oxygen plasma, *E. coli* had the lowest surviving bacterial counts (6.31 Log CFU/mL) whilst *A. hydrophila* had the highest surviving bacterial counts (6.51 Log CFU/mL) after direct treatment in 2.5 minutes. *E. coli* was highly inactivated while *A. hydrophila* was least inactivated to oxygen plasma after direct treatment. In Figure 4.7 (b), using argon plasma, *S. flexneri* had the lowest surviving bacterial counts (6.26 Log CFU/mL) whilst *A. baumannii* had the highest surviving bacterial counts (7.36 Log CFU/mL) after direct treatment in 2.5 minutes. *S. flexneri* was highly inactivated while *A. baumannii* was least inactivated to argon plasma after direct treatment. In Figure 4.7 (c), using air plasma, *E. coli* had the lowest surviving bacterial counts (6.39 Log CFU/mL) whilst *A. hydrophila* had the highest surviving bacterial counts (6.52 Log CFU/mL) after direct treatment in 2.5 minutes. *E. coli* had the highest inactivation while *A. hydrophila* was least inactivated to air plasma after direct treatment. *E. coli* was highly inactivated to oxygen and air plasma and *A. hydrophila* least inactivated to oxygen and air plasma. For the direct treatment at 13.53 kV, air had the highest effect with the lowest combined D-value of 2.43 min and oxygen the least effective with the highest combined D-value of 2.70

min for non-chlorine-resistant bacteria after 2.5 minutes as seen in Figure 4.7 (a, b and c).

In Figures 4.7 (a-c) the inactivation coefficients such as the decimal reduction time are summarised in Table 4.4 and are not denoted on the graphs. The combined D-value (min) is the inverse of the pooled slope.





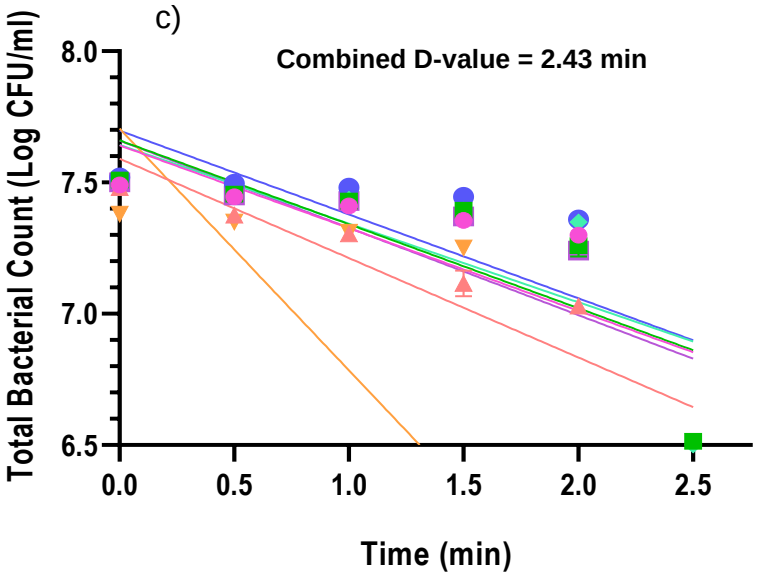


Figure 4.7: Total bacterial counts of non-chlorine-resistant bacteria at 13.53 kV after direct treatment using a) oxygen, b) argon and c) air after 2.5 min. The best fit straight line is represented with plotted values as mean ± standard error of the mean and n=2. The combined D-value (min) is the inverse of the pooled slope.

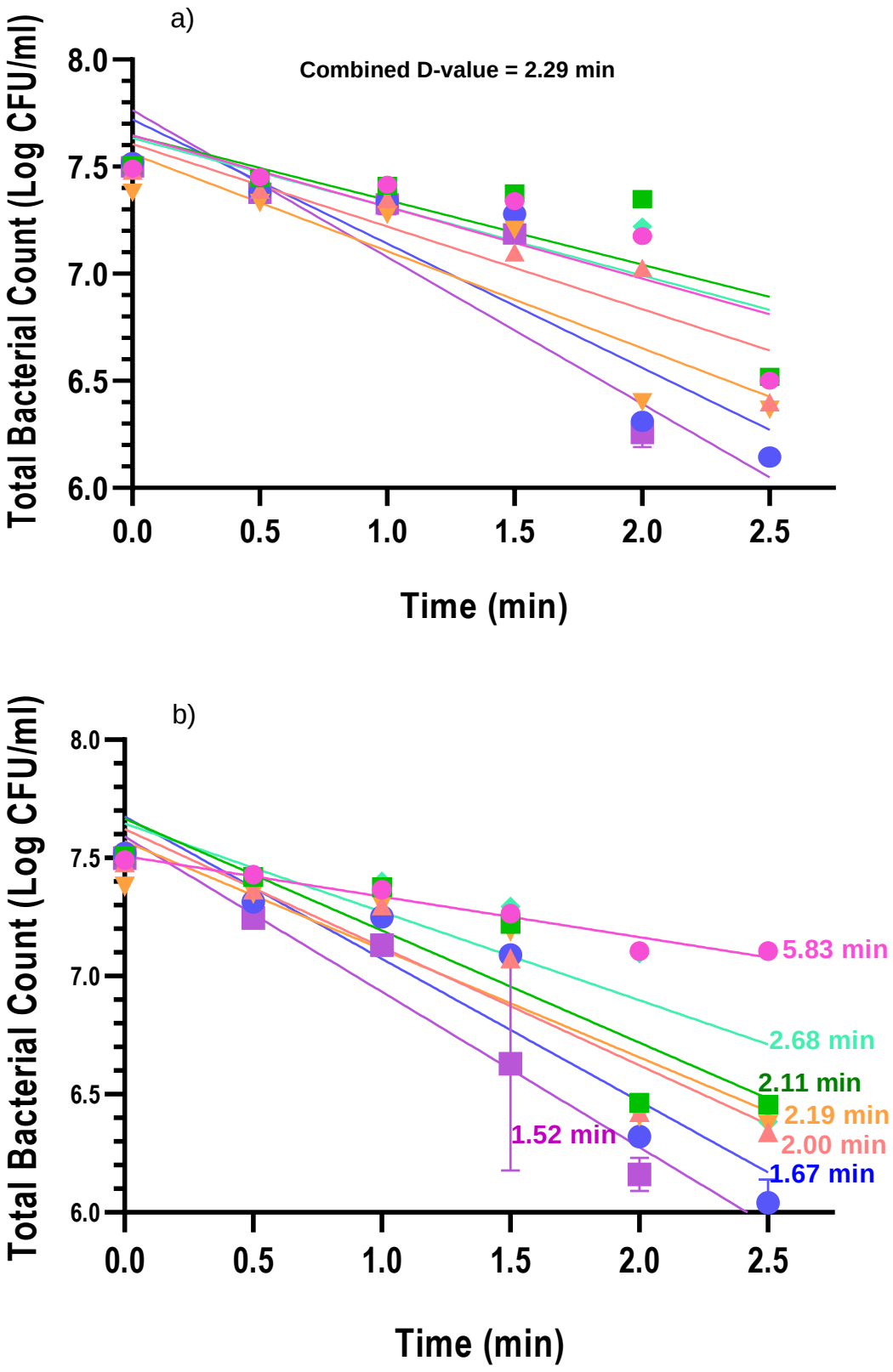
4.3.4 Enumeration of non-chlorine-resistant bacteria after 24 h storage post-treatment

The total bacterial counts (Log CFU/mL) obtained for all non-chlorine-resistant bacteria after 24 h storage post-treatment at 13.53 kV for oxygen, argon and air plasma after 0-2.5 min is depicted in Figure 4.8 (a-c). The surviving bacterial counts for the non-chlorine-resistant bacteria after 24 h storage post-treatment ranged from 5.76 Log CFU/mL to 7.11 Log CFU/mL for oxygen, argon and air plasma in 2.5 min. The simple linear regression for oxygen and air plasma respectively in Figure 4.8 (a and c) revealed that the difference between the slopes was not significantly different for the non-chlorine-resistant bacteria (oxygen: F = 1.398; DFn = 6; DFd = 28; P=0.2503 and air: F = 0.2020; DFn = 6; DFd = 28; P=0.9733) and therefore was possible to calculate one slope for all the data (oxygen pooled slope = -0.4373 and air pooled slope = -0.3439). The differences between the elevations/intercepts were not significant for oxygen and air plasma (oxygen: F = 1.718; DFn = 6; DFd = 34; P=0.1468 and air: F = 0.4271; DFn = 6; DFd = 34; P=0.8557) and since the Y-intercepts were not

significantly different, it was possible to calculate one Y-intercept for all the data (oxygen: pooled intercept = 7.653 and air: pooled intercept = 7.637). The linear regression for argon in Figure 4.8 (b) revealed that the differences between the slopes were significant (F = 2.928; DFn = 6; DFd = 28; P=0.0241) and because the slopes differed so much, it was not possible to test whether the intercepts differed significantly.

In Figure 4.8 (a), using oxygen plasma, *S. flexneri* had the lowest surviving bacterial count (5.76 Log CFU/mL) whilst *A. baumannii and A. hydrophila* had the highest surviving bacterial counts (6.52 Log CFU/mL) after 24 h storage post-treatment in 2.5 minutes. *S. flexneri* was highly inactivated while *A. baumannii* and *A. hydrophila* were least inactivated to oxygen plasma after 24 h storage post-treatment. In Figure 4.8 (b), using argon plasma, *S. flexneri* had the lowest surviving bacterial counts (5.92 Log CFU/mL) whilst *A. baumannii* had the highest surviving bacterial counts (7.11 Log CFU/mL) after 24 h storage post-treatment in 2.5 minutes. *S. flexneri* was highly inactivated while *A. baumannii* was least inactivated to argon plasma after 24 h storage post-treatment. In Figure 4.8 (c), using air plasma, *S. isangii* had the lowest surviving bacterial counts (6.18 Log CFU/mL) after 24 h storage post-treatment whilst *A. hydrophila and L. monocytogenes* had the highest surviving bacterial counts (6.52 Log CFU/mL). *S. isangii* was highly inactivated while *A. hydrophila* and *L. monocytogenes* were least inactivated to air plasma after 24 h storage post-treatment. For the 24 h storage post-treatment, oxygen had the highest effect with the lowest surviving bacterial counts and lowest combined D-value of 2.29 min while air had the least effect with the highest combined D-value of 2.91 min for non-chlorine-resistant bacteria. Overall, with the non-chlorine-resistant bacteria, air was most effective for the direct treatment and oxygen highly effective for the 24 h storage post-treatment.

In Figures 4.8 (a-c) the inactivation coefficients such as the decimal reduction time are summarised in Table 4.4 and are not denoted on the graphs.


a)
Combined D-value = 2.29 min
Total Bacterial Count (Log CFU/ml)
8.0
7.5
7.0
6.5
6.0
0.0
0.5
1.0
1.5
2.0
2.5
Time (min)
b)
Total Bacterial Count (Log CFU/ml)
8.0
7.5
7.0
6.5
6.0
0.0
0.5
1.0
1.5
2.0
2.5
Time (min)
5.83 min
2.68 min
2.11 min
2.19 min
2.00 min
1.67 min
1.52 min

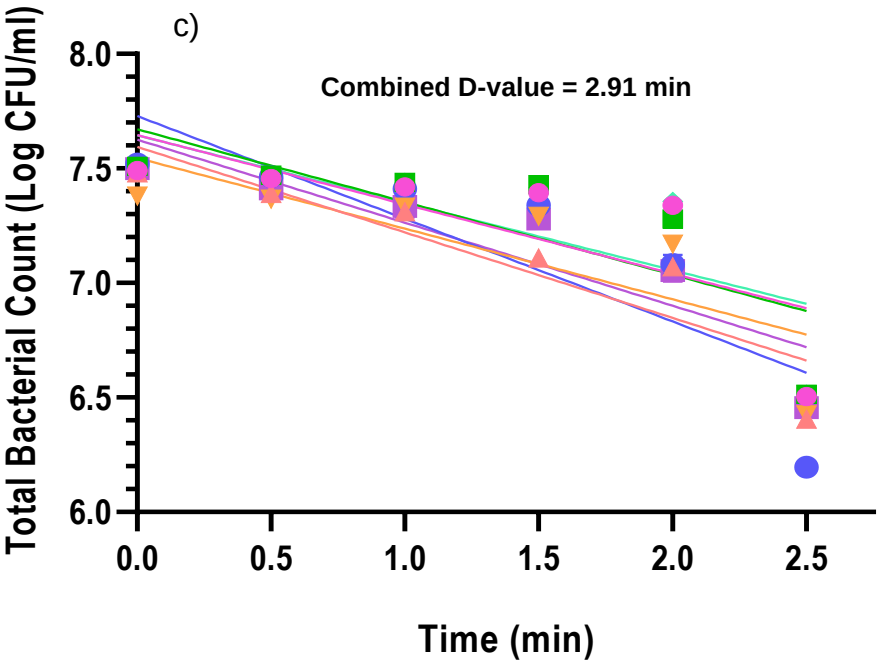


Figure 4.8: Total bacterial counts of non-chlorine-resistant bacteria at 13.53 kV after 24 h storage post-treatment using a) oxygen, b) argon and c) air after 2.5 min. The best fit straight line is represented with plotted values as mean ± standard error of the mean and n=2. The combined D-value (min) is the inverse of the pooled slope for oxygen and air. For argon, the differences between the slopes were significant and different therefore a pooled slope was not calculated instead individual D-values were calculated for each chlorine-resistant bacteria.

4.3.5 Enumeration of chlorine-resistant bacteria at voltages 9.56 kV and 10.82 kV

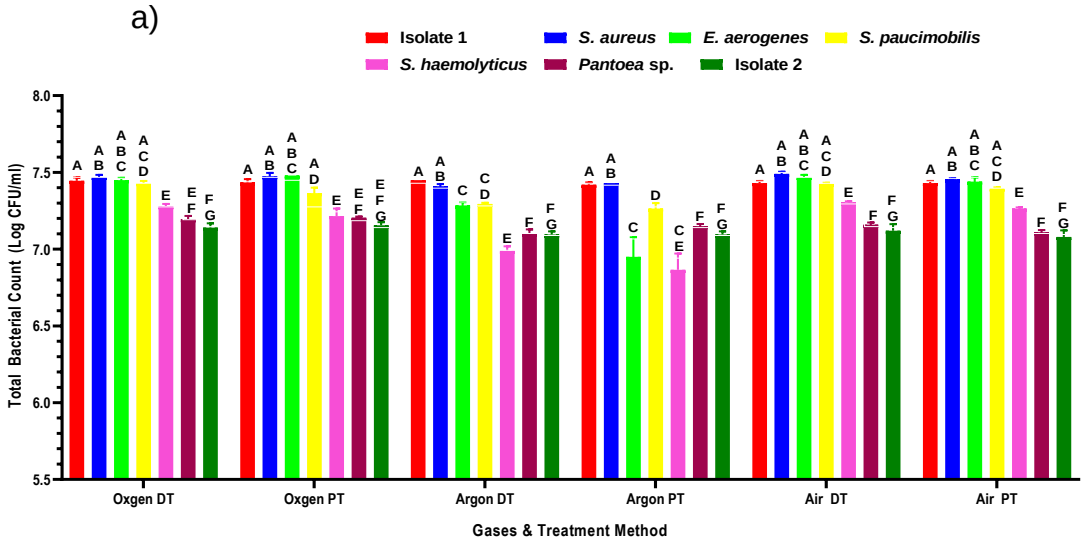
The total bacterial counts (Log CFU/mL) for the chlorine-resistant bacteria at voltage 9.56 kV and 10.82 kV for oxygen, argon and air plasma after direct treatment and 24 h storage post-treatment after 2.5 min is depicted in Figures 4.9 (a and b). In Figure 4.9 (a), the surviving bacterial counts for the direct treatment and 24 h storage post-treatment for the chlorine-resistant bacteria using oxygen, argon and air plasma at 9.56 kV ranged from 6.79 Log CFU/mL to 7.49 Log CFU/mL. At 9.56 kV argon plasma was most inactivated after 24 h storage post-treatment for the chlorine-resistant bacteria *S. haemolyticus* (6.79

Log CFU/mL) and oxygen plasma least inactivated after 24 h storage post-treatment for *S. aureus* (7.49 Log CFU/mL). In Figure 4.9 (b), the surviving bacterial counts for the direct treatment and 24 h storage post-treatment for the chlorine-resistant bacteria using oxygen, argon and air plasma at 10.82 kV ranged from 6.00 Log CFU/mL to 7.45 Log CFU/mL. At 10.82 kV argon plasma was highly inactivated after 24 h storage post-treatment for the chlorine-resistant bacteria *S. haemolyticus* (6.00 Log CFU/mL) and oxygen plasma was least inactivated after direct treatment for Isolate 1. At 9.56 kV and 10.82 kV after direct treatment and 24 h storage treatment, there was no correlation in results except that argon plasma had the highest effect to 24 h storage post-treatment on the chlorine-resistant bacteria.

In Figure 4.9 (a and b), two-way ANOVA and Tukey's posthoc comparison test revealed extremely significant differences ($P < 0{,}0001$) among the chlorine-resistant bacteria, the gases and treatment methods for the voltages 9.56 kV and 10.82 kV except for the labelled chlorine-resistant bacteria which shared the same capital letter/s implying that there were no significant differences ($P \geq 0.05$) for those gases and treatment method.

In Figure 4.9 (a and b) the inactivation coefficients such as the decimal reduction time are summarised in Table 4.3 and are not denoted on the graphs.

a)



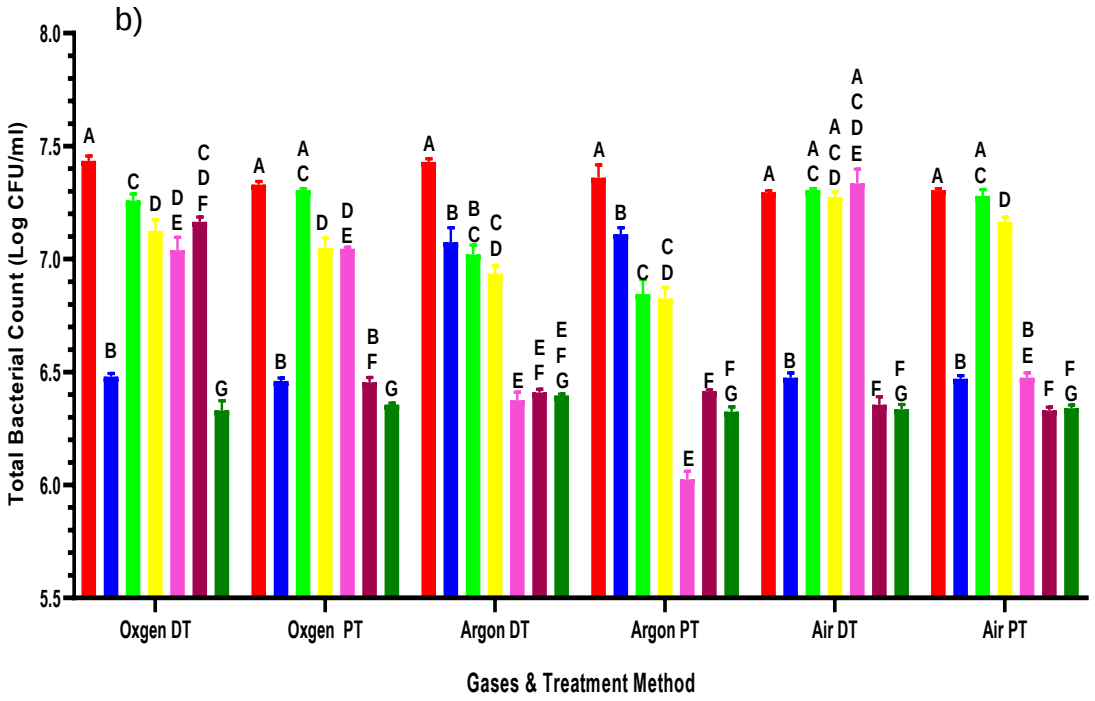


Figure 4.9: Total bacterial count of chlorine-resistant bacteria after direct treatment and 24 h storage post-treatment at voltages a) 9.56 kV and b) 10.82 kV for oxygen, argon and air after 2.5 min. Bars are represented as mean ± standard error of the mean; n=2 and bars sharing similar letter/s indicate no significant differences (P ≥ 0.05).

4.3.6 Enumeration of non-chlorine-resistant bacteria at voltages 9.56 kV and 10.82 kV

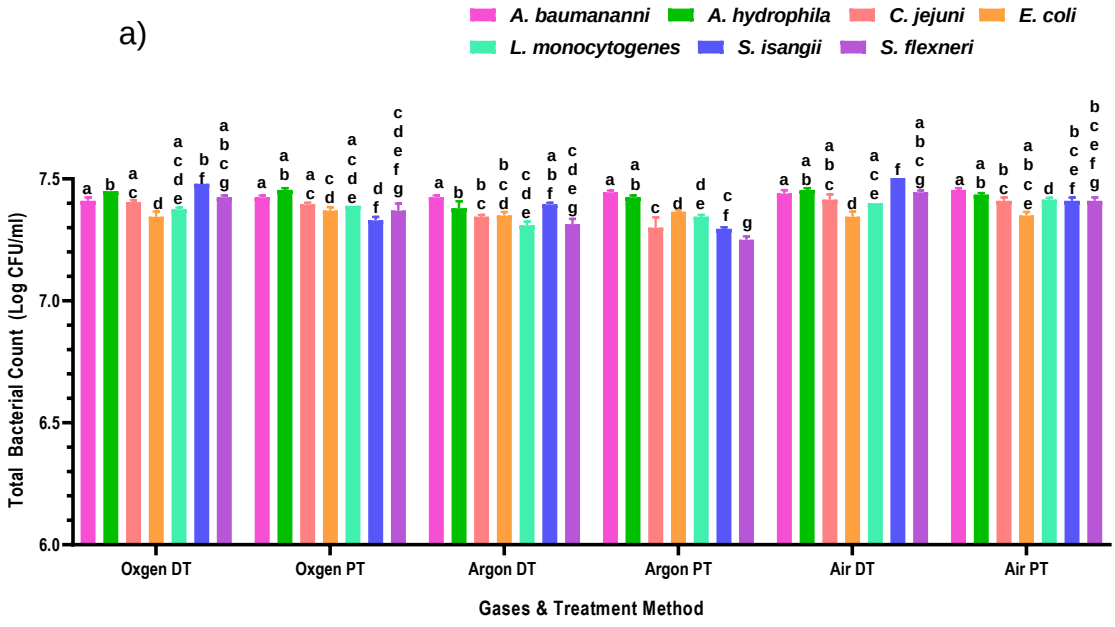
The total bacterial counts (Log CFU/mL) for the non-chlorine-resistant bacteria at voltage 9.56 kV and 10.82 kV for oxygen, argon and air plasma after direct treatment and 24 h storage post-treatment after 2.5 min is depicted in Figure 4.10 (a and b). In Figure 4.10 (a), the surviving bacterial counts for the direct treatment and 24 h storage post-treatment for the non-chlorine-resistant bacteria using oxygen, argon and air plasma at 9.56 kV ranged from 7.24 Log CFU/mL to 7.48 Log CFU/mL. At 9.56 kV argon plasma after 24 h storage post-treatment had the highest inactivation to the non-chlorine-resistant bacteria *S. flexneri* (7.24 Log CFU/mL) and oxygen plasma after direct treatment had the least inactivation on *S. isangii* (7.48 Log CFU/mL). In Figure 4.10 (b), the surviving bacterial counts for the direct treatment and 24 h storage post-treatment for the

non-chlorine-resistant bacteria using oxygen, argon and air plasma at 10.82 kV ranged from 6.38 Log CFU/mL to 7.44 Log CFU/mL. At 10.82 kV, argon plasma after 24 h storage post-treatment had the highest inactivation for the non-chlorine-resistant bacteria *S. isangii* (6.38 Log CFU/mL) and air plasma after direct treatment and 24 h storage post-treatment had the least inactivation on *A. hydrophila* (7.44 Log CFU/mL) and *S. isangii* (7.44 Log CFU/mL). At 9.56 kV and 10.82 kV after direct treatment and 24 h storage treatment, there was no correlation in results except that argon plasma produced the most effective disinfection to 24 h storage post-treatment on the non-chlorine-resistant bacteria.

Two-way ANOVA and Tukey's post-hoc comparison test revealed extremely significant differences ($P < 0{,}0001$) among the non-chlorine-resistant bacteria, the gases and treatment methods for the voltages 9.56 kV and 10.82 kV except for the labelled non-chlorine-resistant bacteria which shared the same capital letter/s implying that there were no significant differences ($P \geq 0.05$) for those gases and treatment method.

In Figure 4.10 (a and b) the inactivation coefficients such as the decimal reduction time are summarised in Table 4.4 and are not denoted on the graphs.

a)



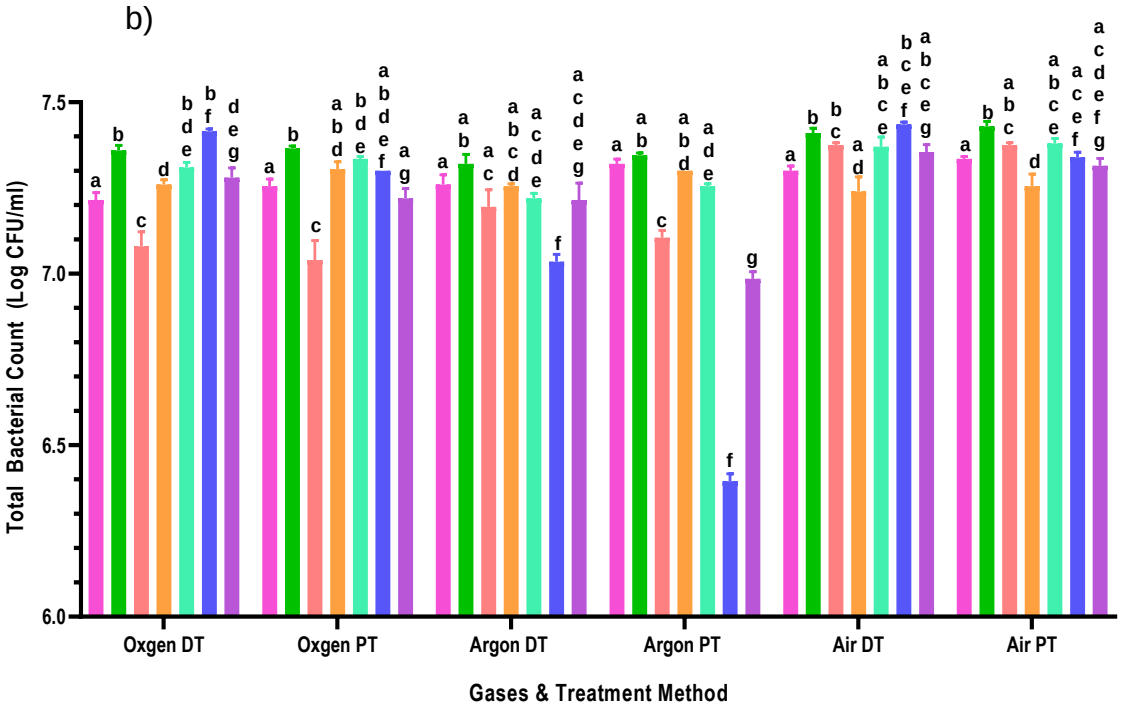


Figure 4.10: Total bacterial count of non-chlorine-resistant bacteria after direct treatment and 24 h storage post-treatment at voltages a) 9.56 kV and b) 10.82 kV for oxygen, argon and air after 2.5 min. Bars are represented as mean ± standard error of the mean; n=2 and bars sharing similar lowercase letter/s indicate no significant differences (P ≥ 0.05).

4.4 Kinetics of microbial inactivation of chlorine-resistant and non-chlorine-resistant bacteria

4.4.1 Microbial inactivation kinetics of chlorine-resistant and non-chlorine-resistant bacteria

The calculation of the log reduction, % log reduction (bacterial inactivation) and the D-value after direct treatment and 24 h storage post-treatment using oxygen, argon and air plasma at 13.53 kV after 2.5 min for chlorine-resistant bacteria and non-chlorine-resistant bacteria are depicted in Table 4.3 and Table 4.4.

Table 4.3: Microbial inactivation results of chlorine-resistant bacteria

Chlorine-resistant Bacteria							
		Voltage (13.53 kV)					
		Oxygen		Argon		Air	
		DT	PT	DT	PT	DT	PT
Isolate1 (G2)	Log Reduction	0.98	2.50	0.95	2.47	0.96	0.95
		0.96	2.46	0.96	1.53	0.95	0.95
	% Log Reduction	89.51	99.68	88.73	99.66	88.94	88.87
		89.07	99.65	89.01	97.08	88.66	88.69
	D-Value	2.55	1.00	2.66	1.02	2.63	2.63
		2.60	1.02	2.60	1.63	2.66	2.66
S. aureus (G1)	Log Reduction	1.47	1.49	0.76	0.71	1.48	1.53
		1.46	0.55	0.85	0.80	1.50	1.54
	% Log Reduction	96.60	96.76	82.69	80.45	96.70	97.08
		96.54	72.12	85.90	84.30	96.83	97.15
	D-Value	1.72	1.67	3.29	3.57	1.69	1.63
		1.72	4.55	2.94	3.13	1.68	1.62
E. aerogenes (E2)	Log Reduction	1.01	2.01	1.40	3.06	0.99	0.97
		1.00	1.99	1.30	3.11	1.00	0.98
	% Log Reduction	90.20	99.02	96.02	99.91	89.74	89.21
		90.07	98.98	94.97	99.92	89.90	89.51
	D-Value	2.48	1.25	1.79	0.82	2.52	2.60
		2.50	1.26	1.92	0.80	2.52	2.55
S. paucimobilis (C1)	Log Reduction	1.39	1.46	1.36	2.40	1.37	1.44
		1.39	1.45	1.37	2.47	1.41	1.43
	% Log Reduction	95.92	96.52	95.59	99.60	95.72	96.36
		95.02	96.49	95.69	99.66	96.09	96.25
	D-Value	1.80	1.71	1.84	1.02	1.82	1.74
		1.90	1.71	1.83	1.01	1.77	1.75
S. haemolyticus (J1)	Log Reduction	1.00	1.98	1.36	2.43	0.88	1.94
		0.99	2.01	1.30	2.35	0.85	1.91
	% Log Reduction	90.05	98.95	95.63	99.63	86.88	98.84
		89.69	99.02	95.00	99.55	85.98	98.76
	D-Value	2.50	1.26	1.84	1.03	2.84	1.29
		2.53	1.24	1.92	1.06	2.94	1.31
Pantoea sp. (F1)	Log Reduction	0.91	0.90	2.07	2.02	1.93	2.19
		0.83	0.88	2.00	2.02	2.17	2.17
	% Log Reduction	87.78	87.31	99.14	99.05	98.81	99.35
		85.15	86.77	99.01	99.04	99.32	99.32
	D-Value	2.75	2.81	1.21	1.24	1.30	1.14
		3.01	2.84	1.25	1.24	1.15	1.15
Isolate 2 (J3)	Log Reduction	1.92	1.89	2.24	2.31	2.05	2.10
		2.16	1.87	2.17	2.21	2.00	2.13
	% Log Reduction	98.79	98.72	99.43	99.52	99.12	99.20
		99.31	98.63	99.33	99.38	99.00	99.26
	D-Value	1.31	1.31	1.12	1.09	1.22	1.20
		1.16	1.34	1.15	1.13	1.25	1.17

DT = Direct treatment & PT = 24 h storage post-treatment.

Table 4.4: Microbial inactivation results of non-chlorine-resistant bacteria

Non-Chlorine-resistant Bacteria							
		Voltage (13.53 kV)					
		Oxygen		Argon		Air	
		DT	PT	DT	PT	DT	PT
A. baumannii	Log Reduction	1.01	0.97	0.13	0.39	1.01	0.99
		1.02	1.01	0.42	0.38	1.00	0.98
	% Log Reduction	90.23	89.29	25.89	59.55	90.23	89.84
		90.52	90.23	62.14	58.25	90.03	89.64
	D-Value	2.48	2.58	19.23	6.41	2.48	2.53
		2.50	2.48	5.95	6.58	2.50	2.55
A. hydrophila	Log Reduction	1.03	0.99	1.09	1.04	1.00	1.00
		1.00	0.99	1.12	1.05	0.99	0.99
	% Log Reduction	90.62	89.69	91.93	90.94	90.03	90.00
		90.00	89.78	92.43	91.15	89.72	89.75
	D-Value	2.43	2.53	2.27	2.38	2.50	2.50
		2.50	2.53	2.23	2.36	2.53	2.53
C. jejuni	Log Reduction	1.09	1.09	1.17	1.13	1.08	1.08
		1.06	1.06	1.13	1.14	1.08	1.06
	% Log Reduction	91.81	91.94	93.28	92.61	91.67	91.61
		91.30	91.34	92.54	92.68	91.61	91.34
	D-Value	2.29	2.27	2.12	2.19	2.32	2.32
		2.34	2.34	2.21	2.19	2.32	2.34
E. coli	Log Reduction	1.07	1.02	1.06	1.02	0.99	0.96
		1.06	1.01	1.08	1.01	0.98	0.95
	% Log Reduction	91.58	90.54	91.20	90.42	89.71	89.09
		91.29	90.33	91.66	90.12	89.50	88.76
	D-Value	2.34	2.45	2.38	2.45	2.53	2.60
		2.36	2.48	2.31	2.50	2.55	2.63
L. monocytogenes	Log Reduction	1.01	0.98	1.15	1.08	0.98	0.97
		1.02	0.97	1.17	1.10	0.97	0.96
	% Log Reduction	90.30	89.60	92.90	91.73	89.53	89.27
		90.43	89.30	93.30	92.03	89.37	88.97
	D-Value	2.45	2.53	2.17	2.29	2.55	2.58
		2.45	2.58	2.12	2.27	2.55	2.60
S. isangii	Log Reduction	1.03	1.35	1.13	1.41	1.02	1.34
		1.02	1.40	1.05	1.55	1.03	1.31
	% Log Reduction	90.76	95.50	92.61	96.08	90.52	95.38
		90.52	95.99	91.16	97.17	90.61	95.11
	D-Value	2.40	1.85	2.21	1.77	2.43	1.87
		2.43	1.79	2.36	1.61	2.43	1.91
S. flexneri	Log Reduction	1.14	1.75	1.22	1.53	1.03	1.04
		1.18	1.67	1.24	1.58	1.04	1.06
	% Log Reduction	92.76	98.21	93.95	97.02	90.56	90.88
		93.45	97.84	94.23	97.37	90.78	91.22
	D-Value	2.19	1.44	2.07	1.64	2.45	2.40
		2.12	1.51	2.02	1.58	2.43	2.38

DT = Direct treatment & PT = 24 h storage post-treatment.

4.4.2 Log reduction of chlorine-resistant & non-chlorine-resistant bacteria after direct treatment & 24 h storage post-treatment

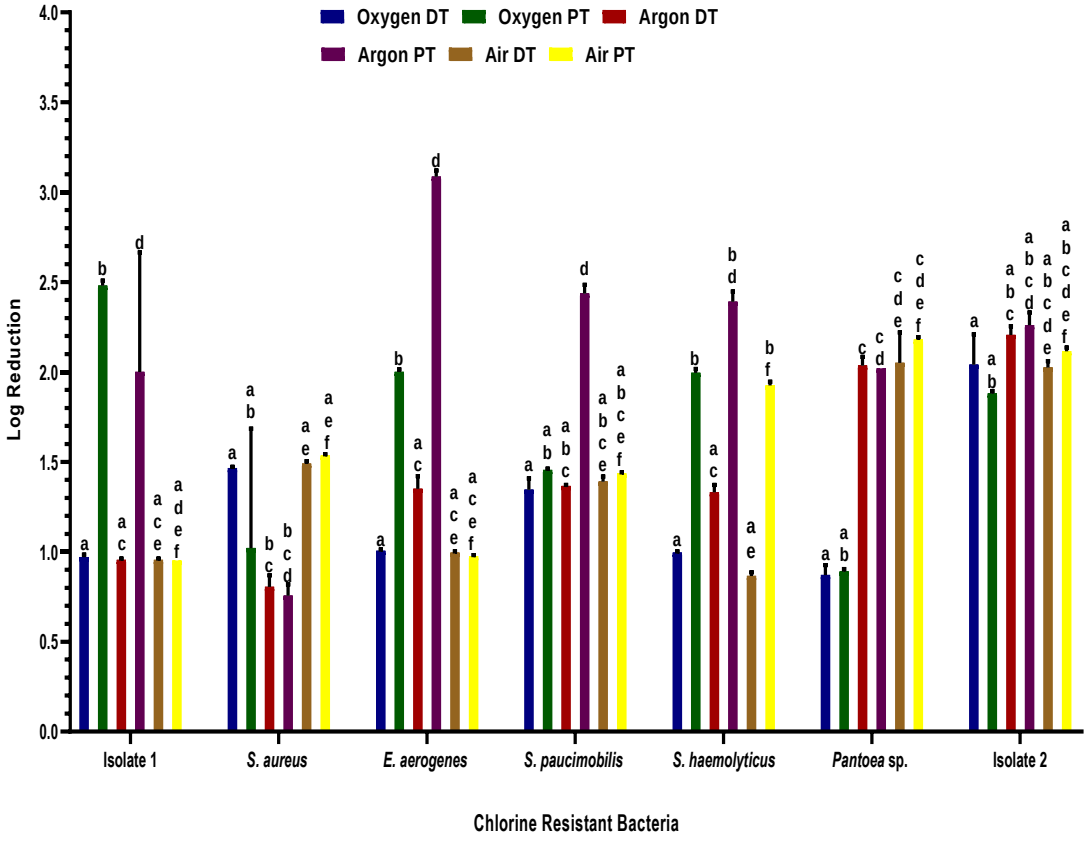
The log reduction for the chlorine-resistant bacteria and non-chlorine-resistant bacteria at voltage 13.53 kV for oxygen, argon and air plasma after direct treatment and 24 h storage post-treatment is depicted in Figure 4.11 (a and b). In Figure 4.11 (a), the log reduction for chlorine-resistant bacteria for direct treatment ranged from 0.76 log reduction to 2.24 log reduction and for the 24 h storage post-treatment the log reduction ranged from 0.55 log reduction to 3.06 log reduction. In Figure 4.11 (a), after direct treatment on the chlorine-resistant bacteria argon plasma had the least inactivation on *S. aureus* (0.76 log reduction) with the lowest log reduction and argon plasma was highly inactivated on Isolate 1 (2.24 log reduction) with the highest log reduction. After the 24 h storage post-treatment, oxygen plasma had the least inactivation on *S. aureus* (0.55 log reduction) with the lowest log reduction *w*hilst argon plasma was highly inactivated on *E. aerogenes*. (3.06 log reduction) having the highest log reduction. Overall, with the chlorine-resistant bacteria, argon had the highest effect for the direct treatment and the 24 h storage post-treatment.

In Figure 4.11 (b), for the non-chlorine-resistant bacteria, the log reduction ranged from 0.13 log reduction to 1.24 log reduction for direct treatment and 0.38 log reduction to 1.75 log reduction for the 24 h storage post-treatment. In Figure 4.11 (a) after direct treatment on the non-chlorine-resistant bacteria, argon plasma was most inactivated on *S. flexneri* (1.24 Log reduction) with the highest log reduction and argon was least inactivated on *A. baumananni* (0.13 log reduction) with the lowest log reduction. After the 24 h storage post-treatment, oxygen plasma was the most inactivated on *S. flexneri* (1.75 log reduction) with the highest log reduction whilst argon plasma was least inactivated with the lowest log reduction on *A. baumananni* (0.38 log reduction). Overall, with the non-chlorine-resistant bacteria, argon had the highest effect for the direct treatment and oxygen had the highest effect during the 24 h storage post-treatment.

Two-way ANOVA and Tukey's post-hoc comparison test revealed extremely significant differences ($P < 0{,}0001$) for the interaction among the chlorine-resistant bacteria, non-chlorine-resistant bacteria, the gases and treatment methods except for the labelled chlorine-resistant and non-chlorine-resistant bacteria which shared the same letter/s implying that there were no significant differences ($P \geq 0.05$) for those gases and treatment method as depicted in Figure 4.11 (a and b).

In Figure 4.11 (a and b) the inactivation coefficients such as the decimal reduction time are summarised in Table 4.3 and are not denoted on the graphs.

a)



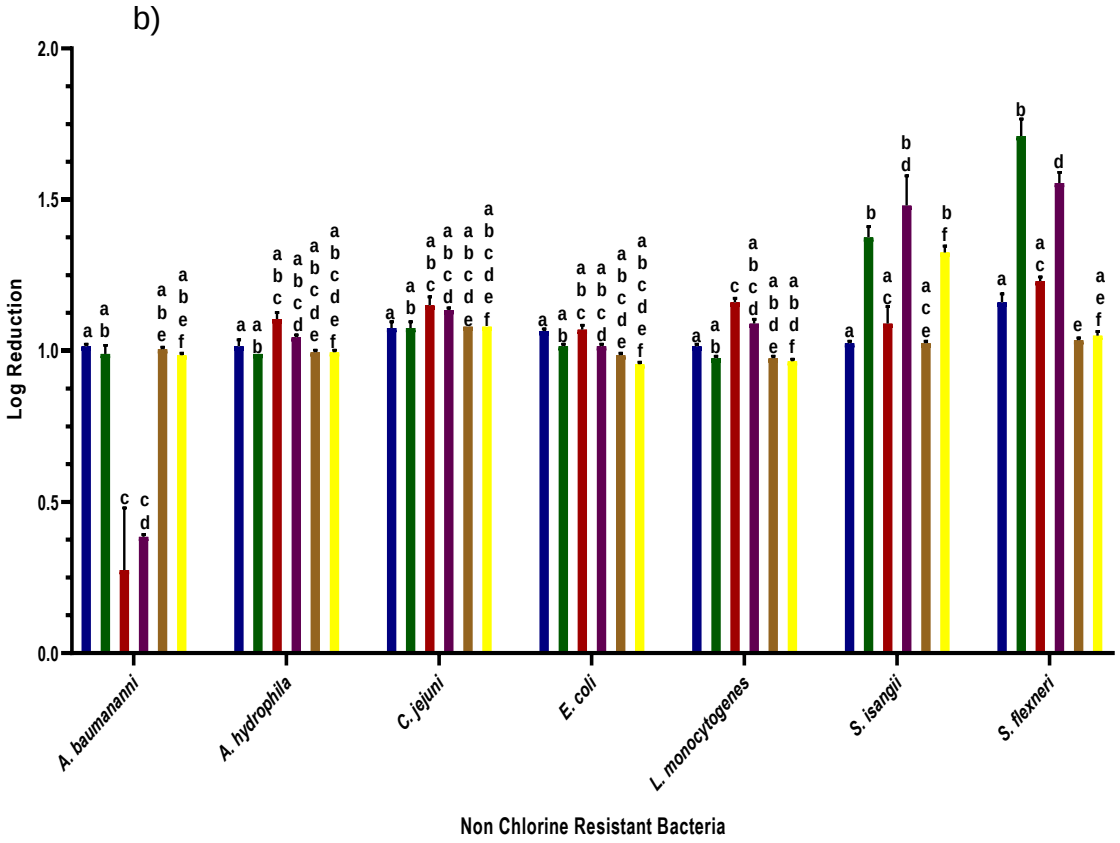


Figure 4.11: Log reduction of a) chlorine-resistant bacteria and b) non-chlorine-resistant bacteria after direct treatment and 24 h storage post-treatment for oxygen, argon and air at 13.53 kV after 2.5 min. Stacked bars are represented as mean ± standard error of the mean; n=2 and bars sharing similar letter/s indicate no significant differences (P ≥ 0.05).

4.4.3 Percentage (%) log reduction of chlorine-resistant & non-chlorine-resistant bacteria after direct treatment & 24 h storage post-treatment

The percentage log reduction for the chlorine-resistant bacteria and non-chlorine-resistant bacteria at a voltage of 13.53 kV for oxygen, argon and air plasma after direct treatment and 24 h storage post-treatment is depicted in Figure 4.12 (a and b). In Figure 4.12 (a), the percentage log reduction for chlorine-resistant bacteria for direct treatment ranged from 82.69% to 99.60% and for the 24 h storage post-treatment the percentage log reduction ranged from 80.45% to 99.66%. The chlorine-resistant bacteria with the highest log reduction after the direct treatment was *S. aureus* using oxygen plasma

(99.60%) and the bacteria with the lowest log reduction was *S. aureus* using argon plasma (82.69%). After the 24 h storage post-treatment, *S. paucimobilis* had the highest log reduction using argon plasma (99.66%) and *S. aureus* had the lowest log reduction using argon plasma (80.45%).

In Figure 4.12 (b), for the non-chlorine-resistant bacteria, the percentage log reduction ranged from 25.89% to 94.23% for direct treatment and 58.25% to 98.21 for the 24 h storage post-treatment. The non-chlorine-resistant bacteria with the highest log reduction after the direct treatment was *S. flexneri* using argon plasma (94.23%) and the bacteria with the lowest log reduction was *A. baumananni* using argon plasma (25.89%). After the 24 h storage post-treatment, *S. flexneri* had the highest log reduction using oxygen plasma (98.21%) and *A. baumananni* had the lowest log reduction using argon plasma (58.25%). Two-way ANOVA and Tukey's post-hoc comparison test revealed extremely significant differences ($P < 0{,}0001$) for the interaction among the chlorine-resistant bacteria, non-chlorine-resistant bacteria, the gases and treatment methods except for the labelled chlorine-resistant and non-chlorine-resistant bacteria which shared the same letter/s implying that there were no significant differences ($P \geq 0.05$) for those gases and treatment method as depicted in Figure 4.12 (a & b).

In Figure 4.12 (a and b) the inactivation coefficients such as the decimal reduction time are summarised in Table 4.3 and are not denoted on the graphs.

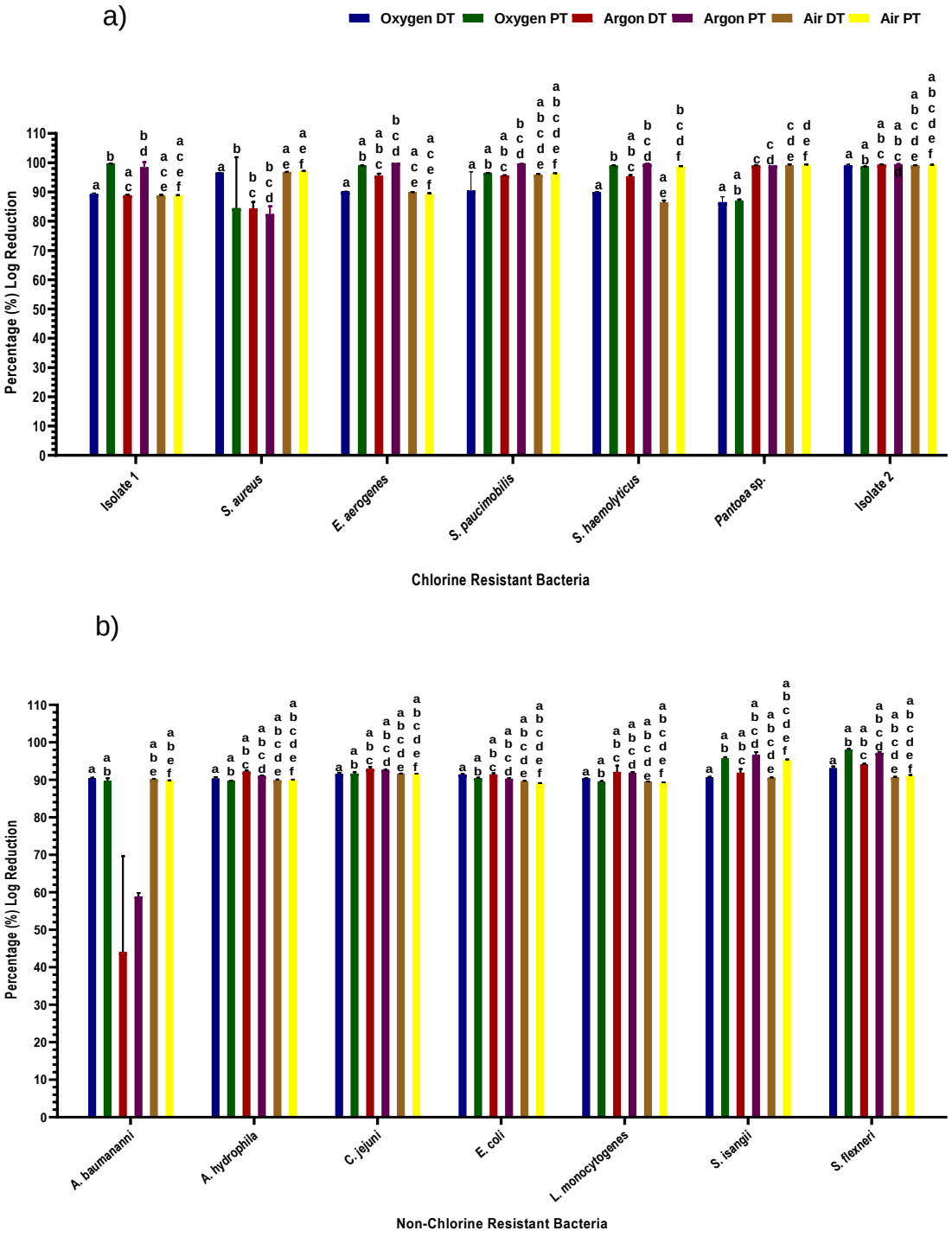


Figure 4.12: Percentage (%) log reduction of a) chlorine-resistant and b) non-chlorine-resistant bacteria after direct treatment & 24 h storage post-treatment for oxygen, argon and air at 13.53 kV after 2.5 min. Bars are represented as mean ± standard error of the mean; n=2 and bars sharing similar letter/s indicate no significant differences ($P \geq 0.05$).

4.4.4 Decimal reduction time of chlorine-resistant & non-chlorine-resistant bacteria after direct treatment & 24 h storage post-treatment

The decimal reduction time (D-value), i.e. the time taken to achieve 90% bacterial inactivation of chlorine-resistant bacteria and non-chlorine-resistant bacteria at voltage 13.53 kV for oxygen, argon and air plasma after direct treatment and 24 h storage post-treatment is depicted in Figure 4.13 (a and b). In Figure 4.13 (a), the D-value for chlorine-resistant bacteria for direct treatment ranged from 1.12 min to 3.29 min and for the 24 h storage post-treatment the D-value ranged from 0.80 min to 4.55 min. For the direct treatment, the chlorine-resistant bacteria with the highest D-value was *S. aureus* (3.29 min) using argon plasma and the bacteria with the lowest D-value was Isolate 2 (1.12 min) using argon plasma. For the 24 h storage post-treatment, the bacteria with the highest D-value was *S. aureus* (4.55 min) using oxygen plasma and the bacteria with the lowest D-value was *E. aerogenes* (0.80 min) using argon plasma.

In Figure 4.13 (b), for the non-chlorine-resistant bacteria, the D-value ranged from 2.02 min to 19.23 min for direct treatment and 1.44 min to 6.58 min for the 24 h storage post-treatment. For the direct treatment, the chlorine-resistant bacteria with the highest D-value was *A. baumananni* (19.23 min) using argon plasma and the bacteria with the lowest D-value was *S. flexneri* (2.02 min) using argon plasma. For the 24 h storage post-treatment, the bacteria with the highest D-value was *A. baumananni* (6.58 min) using argon plasma and the bacteria with the lowest D-value was *S. flexneri* (1.44 min) using oxygen plasma.

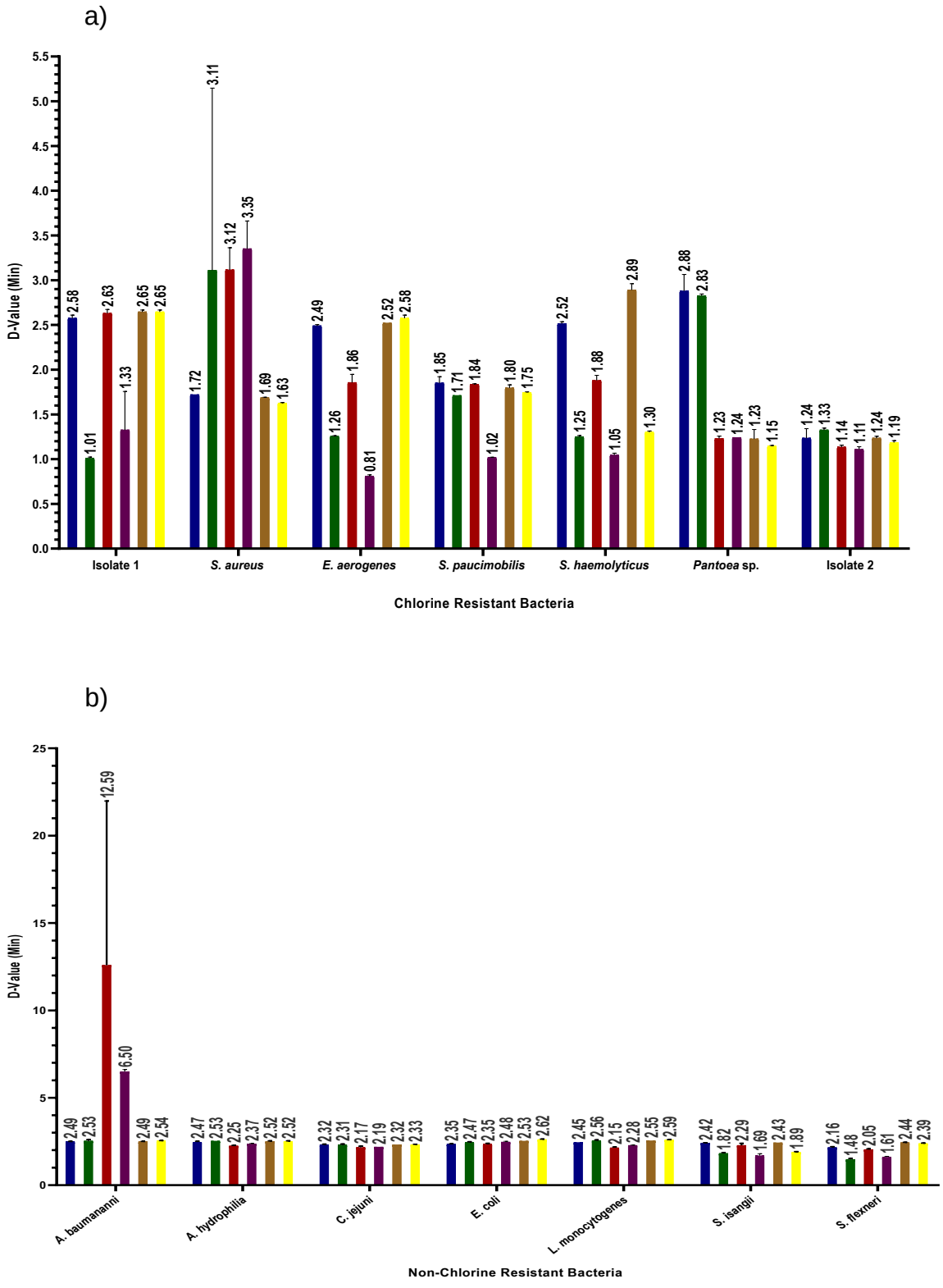


Figure 4.13: D-Value for a) chlorine-resistant and b) non-chlorine-resistant bacteria after direct treatment & 24 h storage post-treatment using oxygen, argon & air at 13.53 kV after 2.5 min. Bars are represented as mean ± standard error of the mean; n=2.

4.5 Enumeration of bacteria from water samples

The water samples used in this study are depicted in Table 3.2.

4.5.1 Enumeration of bacteria from water samples after direct treatment and 24 h storage post-treatment

The total bacterial counts (Log CFU/mL) obtained for all 10 water samples using direct treatment at 13.53 kV for oxygen, argon and air plasma after 3 min is depicted in Figure 4.14. The surviving bacterial counts for the direct treatment and 24 h storage post-treatment for the water samples using oxygen, argon and air plasma ranged from 3.00 Log CFU/mL to 8.26 Log CFU/mL and 3.96 Log CFU/mL to 7.90 Log CFU/mL respectively.

In Figure 4.14, after the direct treatment, the sample with the highest surviving bacterial count was Shongweni Dam (8.26 Log CFU/mL) using argon plasma and the sample with the lowest surviving bacterial count was uMhlanga Lagoon (3.00 Log CFU/mL) using argon plasma. For the 24 h storage post-treatment, the sample with the highest surviving bacterial count was Shongweni Dam (7.90 Log CFU/mL) using air plasma and the sample with the lowest surviving bacterial count was uMhlanga Lagoon (3.96 Log CFU/mL) using argon plasma. Two-way ANOVA and Dunnett's post-hoc comparison test revealed extremely significant differences ($P < 0,0001$) among the control, the gases and treatment methods for each of the water samples analysed.

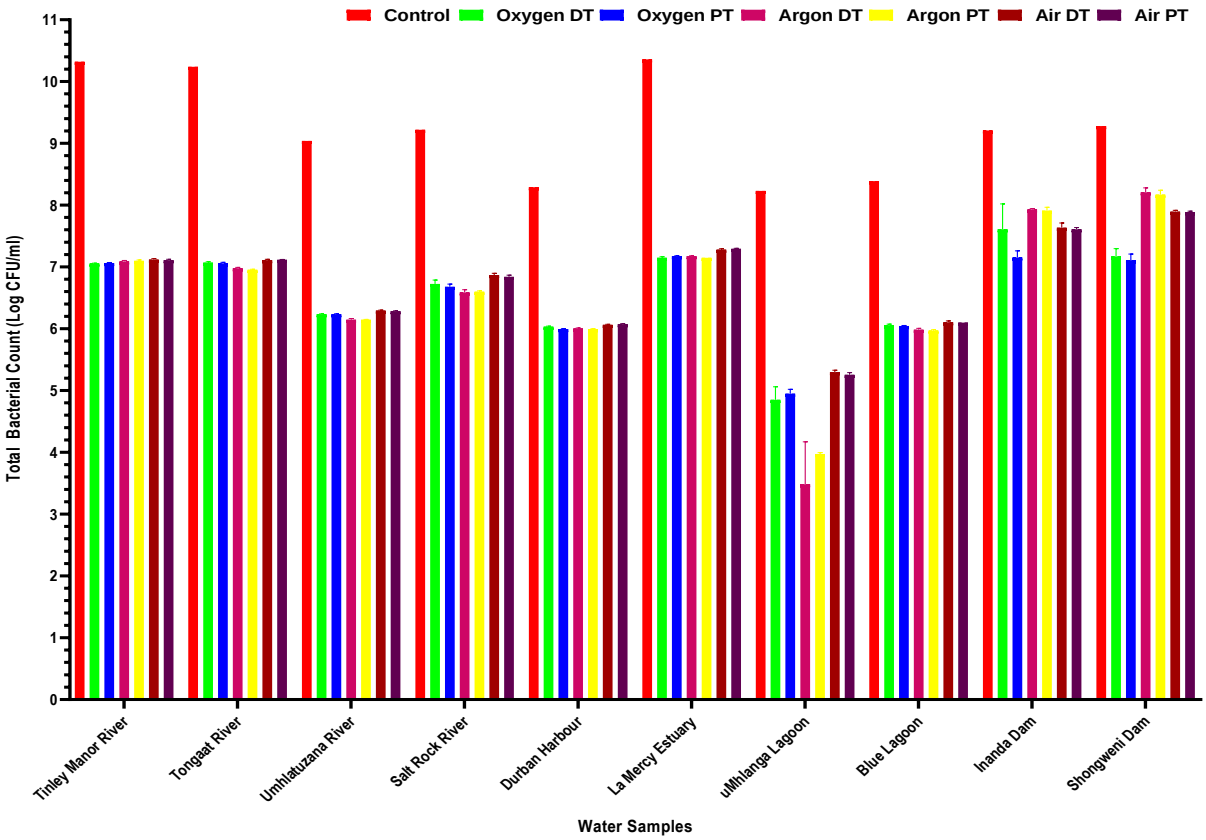


Figure 4.14: Total bacterial count of water samples after direct treatment and 24 h storage post-treatment at voltage 10.53 kV. for oxygen, argon and air after 3 min. Bars are represented as mean ± standard error of the mean; n=2.

4.5.2 Log reduction of water samples after direct treatment & 24 h storage post-treatment

The log reduction for the water samples at voltage 13.53 kV for oxygen, argon and air plasma after direct treatment and 24 h storage post-treatment is depicted in Figure 4.15. The log reduction for direct treatment ranged from 1.01 log reduction to 5.23 log reduction and for the 24 h storage post-treatment the log reduction ranged from 1.06 log reduction to 4.27 log reduction. In Figure 4.15, after the direct treatment, the sample with the highest log reduction was uMhlanga Lagoon (5.23 Log reduction) using argon plasma and the sample with the lowest log reduction was Shongweni Dam (1.01 log reduction) using argon plasma. For the 24 h storage post-treatment, the sample with the highest log

reduction was uMhlanga Lagoon (4.27 log reduction) using argon plasma and the sample with the lowest log reduction was Shongweni Dam (1.06 log reduction) using argon plasma. Two-way ANOVA and Tukey's posthoc comparison test revealed extremely significant differences ($P < 0,0001$) for the interaction among the water samples, the gases and the treatment methods except for the labelled water samples which shared the same letter/s implying that there were no significant differences ($P \geq 0.05$) for those gases and treatment method as depicted in Figure 4.15.

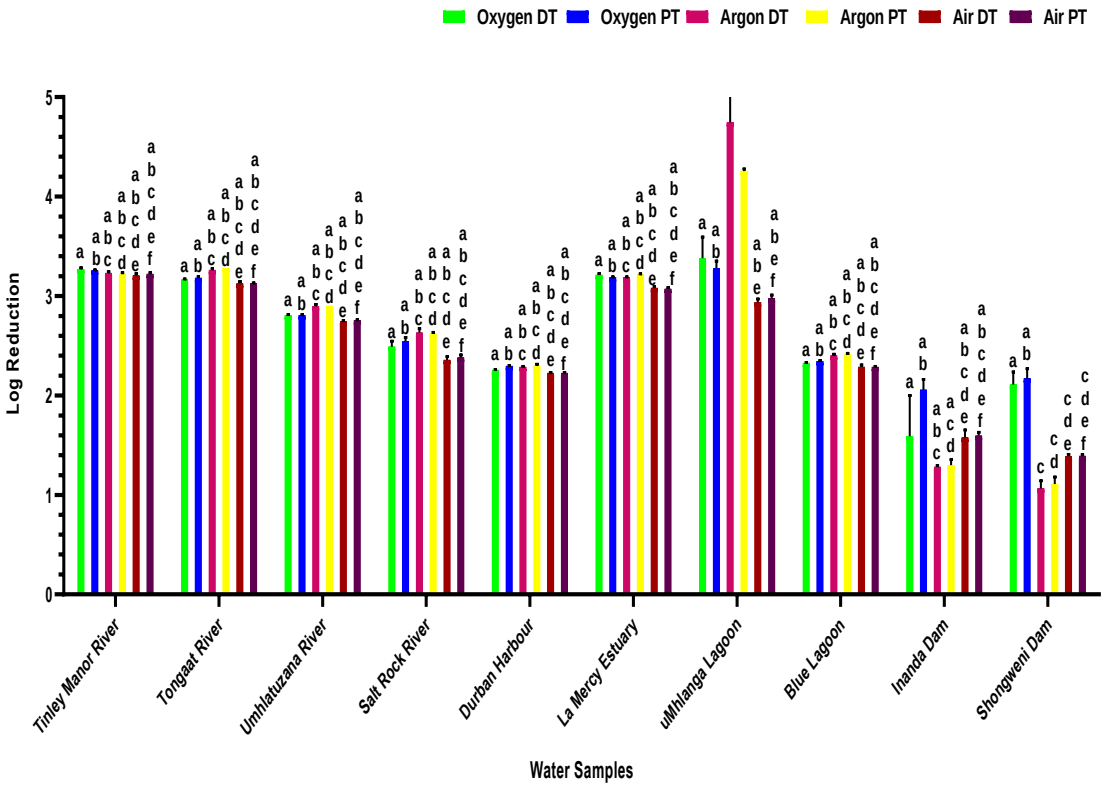


Figure 4.15: Log reduction of water samples after direct treatment and 24 h storage post-treatment for oxygen, argon and air at 13.53 kV after 3 min. Bars are represented as mean ± standard error of the mean; n=2 and bars sharing similar letter/s indicate no significant differences ($P \geq 0.05$).

4.5.3 Percentage log reduction of water samples after direct treatment & 24 h storage post-treatment

The percentage log reduction for the water samples at voltage 13.53 kV for oxygen, argon and air plasma after direct treatment and 24 h storage post-

treatment is depicted in Figure 4.16. The percentage log reduction for direct treatment ranged from 90.27% log reduction to 100% log reduction and for the 24 h storage post-treatment the log reduction ranged from 91.22% log reduction to 100% log reduction. In Figure 4.16, after the direct treatment, the sample with the highest percentage log reduction was uMhlanga Lagoon (100%) using argon plasma and the sample with the lowest percentage log reduction was Shongweni Dam (90.27%) using argon plasma. For the 24 h storage post-treatment, the sample with the highest percentage log reduction was uMhlanga Lagoon (100%) using argon plasma and the sample with the lowest percentage log reduction was Shongweni Dam (91.22%) using argon plasma. Two-way ANOVA and Tukey's posthoc comparison test revealed extremely significant differences ($P < 0,0001$) for the interaction among the water samples, the gases and the treatment methods except for the labelled water samples which shared the same letter/s implying that there were no significant differences ($P \geq 0.05$) for those gases and treatment method as depicted in Figure 4.16.

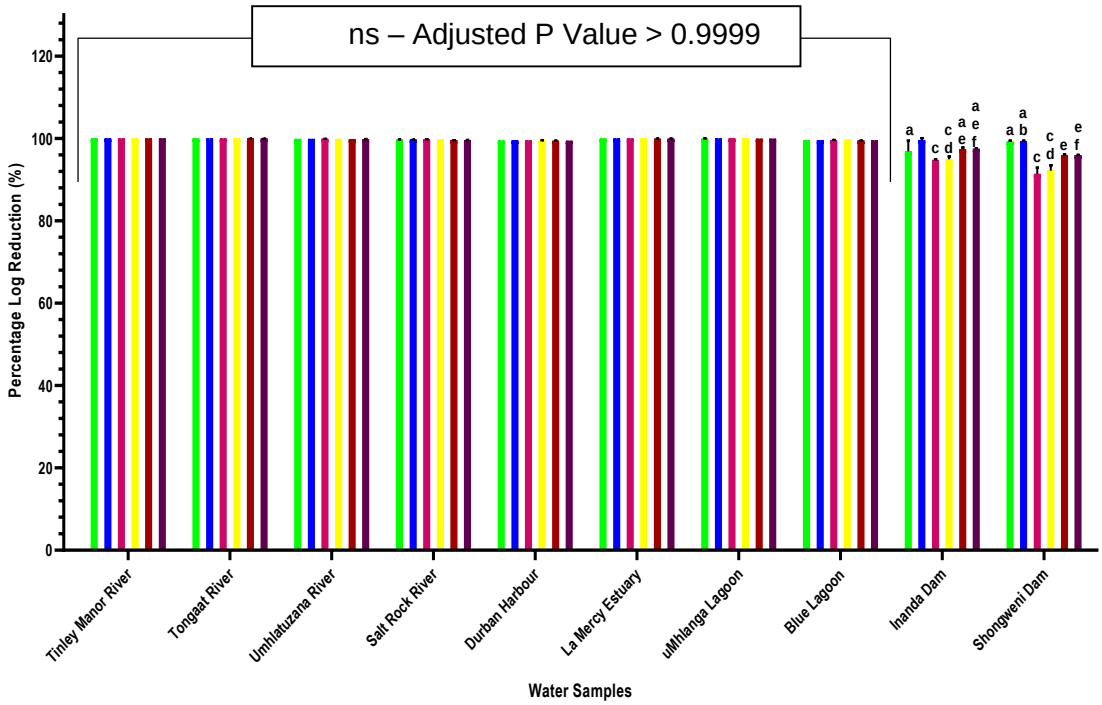


Figure 4.16: Percentage (%) log reduction of water samples after direct treatment & 24 h storage post-treatment for oxygen, argon and air at 13.53 kV after 3 min. Bars are represented as mean ± standard error of the mean; n=2 and bars sharing similar letter/s indicate no significant differences ($P \geq 0.05$).

CHAPTER 5: DISCUSSION

5.1 Optimisation of cold plasma production by verification of operating parameters

The optimisation of plasma generation from the constructed device involved adjusting the distance between the electrodes based on the specific gases used, as outlined in Table 4.1. The applied potential difference was systematically varied until a robust plasma discharge was achieved under atmospheric pressure and room temperature conditions. This process is visually depicted in Figure 4.1 (a-c), Figure 4.2 (a-c) and Figure 4.3 (a-c). Dielectric breakdown voltage and plasma arcs were thus successfully generated using oxygen, argon and air plasma ensuring the effectiveness of these gases in plasma production.

To verify the production of atmospheric cold plasma, dielectric constants associated with oxygen, argon and air were considered. The distances between the electrodes were precisely measured using a vernier calliper for different constant voltages. The electric field (E) strength was calculated along with gas flow, voltage and gas type for the various potential differences (ΔV) required to create plasma using these gases., confirming the operating parameters necessary for both direct and 24 h storage post-treatment. The electric field strength relationship with the potential difference and its inverse proportionality to the separation distance (d) were crucial factors during optimisation. Controlling the plate separation distance was essential for enhancing the electric field intensity, even when a maximum in the DC potential difference was reached (Singh, 2012).

For instance, using oxygen the ideal plate separation distance was 3 mm at 9.56 kV (ΔV) with an electric field intensity of 3.19×10^6 V/m, 3.50 mm at 10.82 kV (ΔV) with an electric field intensity of 3.09×10^6 V/m and 4.10 mm at 13.53 kV

(ΔV) with an electric field intensity of 3.30 x 10^6 V/m. With argon, the ideal distances were 6.50 mm at 9.56 kV (ΔV) with an electric field intensity of 1.47 x 10^6 V/m, 8.24 mm at 10.82 kV (ΔV) with an electric field intensity of 1.31 x 10^6 V/m and 9.50 mm at 13.53 kV (ΔV) with an electric field intensity of 1.42 x 10^6 V/m. When using air, the ideal distances were 1.75 mm at 9.56 kV (ΔV) with an electric field intensity of 5.46 x 10^6 V/m, 2.75 mm at 10.82 kV (ΔV) with an electric field intensity of 3.93 x 10^6 V/m and 3.50 mm at 13.53 kV (ΔV) with an electric field intensity of 3.87 x 10^6 V/m.

The choice of operating gas is a critical input parameter influencing the generation of ACP discharges (Das *et al.,* 2022). Oxygen, argon and air, the chosen gases were commonly used for plasma disinfection treatments due to the unique properties that make them effective in different treatment applications. Oxygen, known for producing ROS, is highly effective in damaging microbial cellular components, including proteins, lipids and DNA, making it a broad-spectrum disinfectant against bacteria, fungi, viruses and spores (Sachdev *et al.,* 2021; Zhang *et al.*, 2023). Argon, an inert gas, provides a stable and controlled plasma environment, ideal for applications requiring precise plasma control. Its ability to transfer energy efficiently and operate at lower temperatures makes it suitable for treating heat-sensitive materials (Lim *et al.,* 2023). Air is cost-effective and readily available for large-scale disinfection producing a combination of reactive oxygen and nitrogen species that have synergistic antimicrobial effects on microbial disinfection (Schnabel *et al.,* 2019). By leveraging the unique properties of these gases, cold plasma disinfection treatments can be tailored to optimise microbial control across different applications, ensuring flexibility and effectiveness.

5.2 Surviving bacterial counts of chlorine-resistant bacteria

The surviving bacterial counts of chlorine-resistant bacteria were examined using total bacterial counts (Log CFU/mL) for two groups based on Gram classification: Gram-positive and Gram-negative. The bacterial counts

exhibited reductions during plasma treatment with oxygen, argon and air plasma at 13.53 kV for both direct treatment (2.5 min) and after 24 h storage post-treatment, as shown in Figure 4.5 (a-c) and Figure 4.6 (a-c). Different inactivation rates were observed among the bacteria within each group depending on the gas used and the treatment method. For oxygen plasma treatment, as illustrated in Figures 4.5 (a) and Figure 4.6 (a), chlorine-resistant Isolate 1 showed the highest surviving bacterial counts during direct treatment but the lowest after the 24 h storage post-treatment. In contrast, Isolate 2 had the lowest surviving bacterial count during direct treatment and a minimal increase in bacterial counts after the 24 h storage post-treatment. This indicates that Isolate 2 had lower surviving bacterial counts under direct oxygen plasma treatment, while Isolate 1 exhibited higher surviving bacterial counts after the 24 h storage post-treatment.

When argon plasma was used as shown in Figure 4.5 (b) and Figure 4.6 (b), Isolate 2 had the lowest surviving bacterial counts following direct treatment, with a further decrease after the 24 h storage post-treatment. *S. aureus*, however, had the highest surviving bacterial counts in both direct treatment and the 24 h storage post-treatment. *E. aerogenes* showed initially high surviving bacterial counts after direct treatment, but these significantly decreased after the 24 h storage post-treatment. This suggests that reactive species generated during argon plasma ACP treatment continued to inactivate *E. aerogenes* during the storage and rest period, preventing further colony formation (Kalakonda *et al.,* 2022). Argon plasma caused high inactivation of Isolate 2 during direct treatment and higher inactivation of *E. aerogenes* after the 24 h storage post-treatment.

With air plasma treatment, as depicted in Figure 4.5 (c) and Figure 4.6 (c), *Pantoea* sp. showed the lowest surviving bacterial counts, indicating high inactivation rates among non-chlorine-resistant bacteria. Conversely, Isolate 1 had the highest surviving bacterial counts during both direct treatment and 24 h storage post-treatment, indicating low inactivation rates to air plasma. Overall,

Gram-negative chlorine-resistant bacteria showed lower surviving bacterial counts after 2.5 min of treatment with oxygen, argon and air plasma compared to Gram-positive chlorine-resistant bacteria which had higher surviving bacterial counts.

The impact of cold plasma on Gram-positive *Staphylococcus* spp*.* including *S. aureus* and *S. haemolyticus*, was notably influenced by microbial morphology, particularly their thicker cell walls. Electron microscopy showed that *S. aureus* had a relatively thick cell wall (20-40 nm) (Giesbrecht *et al.,* 1998) while *S. haemolyticus* had even thicker cell walls (60-80 nm) (Jigisha & Tadi, 2022). These thicker cell walls contributed to higher resistance to ACP treatment with *S. haemolyticus* showing the lowest log reduction after 2.5 min of cold plasma exposure (Mai-Prochnow *et al.,* 2016).

The main difference between Gram-negative and Gram-positive bacteria lies in cell wall thickness and the presence of an outer membrane, exclusive to Gram-negative bacteria. Gram-positive bacteria generally have thicker peptidoglycan layers (20–80 nm), while Gram-negative bacteria have thinner peptidoglycan layers (1.5–10 nm). The destruction of peptidoglycan, essential for maintaining cell integrity, can lead to cell lysis (Vollmer *et al.,* 2008; Rogers *et al.,* 1980; Mai-Prochnow *et al.,* 2016). Membrane lipid peroxidation is a common feature of ACP inactivation (Vatansever *et al.,* 2013; Mai-Prochnow *et al.,* 2016), and the presence of porins in the outer membrane of Gram-negative bacteria makes them more permeable to ACP, contributing to their higher sensitivity to plasma treatment (Mai-Prochnow *et al.,* 2016). Chlorine-resistant bacteria show increased lethal effects with higher voltages and longer treatment times (Kazemzadeh *et al.,* 2022).

The effects of lower voltages (9.56 kV and 10.82 kV) using oxygen, argon and air plasma for 2.5 min of direct treatment and 24 h storage post-treatment were also assessed. As shown in Figure 4.9 (a-b), argon plasma produced the highest effect with the strongest plasma stream after 24 h storage post-

treatment on chlorine-resistant *S. haemolyticus* while oxygen generated the least effect with the weakest plasma stream for *S. aureus* at 9.56 kV after 24 h storage post-treatment and Isolate 1 at 10.82 kV after direct treatment.

Although there have been many *in-vitro* studies on the antibacterial activity of ACP, published clinical trials remain relatively few. The antimicrobial efficacy of ACP appears to be influenced by the specific properties of the plasma generation device, adding complexity to understanding its mode of action (Isbary *et al.,* 2013; Von Woedtke *et al.,* 2014; Mai-Prochnow *et al.,* 2016). The results emphasise the need to consider voltage levels and gas composition when optimising ACP treatment for specific bacterial strains (Mai-Prochnow *et al.,* 2016). At the highest voltage of 13.53 kV, argon plasma proved to be the most effective, resulting in the lowest surviving bacterial counts for chlorine-resistant bacteria in both direct treatment and 24 h storage post-treatment. Conversely, oxygen plasma showed the least effectiveness with the highest surviving bacterial counts observed. Thus, argon was the optimal gas choice for treating chlorine-resistant bacteria at 13.53 kV.

5.3 Surviving bacterial counts of non-chlorine-resistant bacteria

The surviving bacterial counts of non-chlorine-resistant, Gram-negative bacteria to different gases and plasma treatment methods at 13.53 kV are presented in Figure 4.7 (a-c) and Figure 4.8 (a-c). Among the tested bacteria, *A. hydrophila* showed the highest surviving bacterial counts when treated with oxygen and air plasma, both immediately after direct treatment and following 24 h storage post-treatment. These results differed from other studies (Abbas *et al.,* 2017) that have shown cold oxygen plasma (COP) to be more effective against planktonic *A. hydrophila* than biofilm, typically achieving significant log reductions. In contrast, this study did not observe a reduction in *A. hydrophila* counts exceeding 1 log.

Figure 4.7 (a) and Figure 4.8 (a) demonstrate that *E. coli* had the lowest surviving bacterial count when treated with oxygen plasma at 13.53 kV, both after direct treatment and 24 h storage post-treatment after 2.5 min. *S. flexneri* showed the lowest surviving bacterial counts only after the 24 h storage post-treatment. Consistent with these findings, studies have shown that pure argon ACP exposure can reduce *E. coli* CFU counts by 70% within 300 seconds while combining argon with air does not significantly enhance this reduction (Kaupe *et al.,* 2019; Das *et al.,* 2022). Moreover, while pure helium ACP exposure had minimal effects on *E. coli* CFU counts, helium mixed with 1% air resulted in a 98% reduction (Das *et al.,* 2022), illustrating the variability in antibacterial efficacy depending on the gas composition.

Using argon gas, Figure 4.7 (b) and Figure 4.8 (b), show that *S. flexneri* had the lowest surviving bacterial counts, whereas *A. baumannii* demonstrated the highest surviving bacterial counts after both direct treatment and 24 h storage post-treatment. Despite *A. baumannii'*s resistance to argon plasma, effective disinfection was achieved with oxygen and air plasma, possibly due to the production of ROS and RNS which chemically interact with cellular macromolecules like lipids, nucleic acids, and proteins, leading to cell membrane rupture and bacterial death (Abbas *et al.,* 2017; Khalaf *et al.,* 2015; Atta *et al.,* 2019). Charged particles from plasma accumulate on bacterial surfaces, generating electrostatic tension that ruptures outer membranes, catalysing oxidation and inhibiting bacterial metabolism (Abbas *et al.,* 2017; Pandit *et al.,* 2017; Atta *et al.,* 2019).

In Figure 4.7 (c) and Figure 4.8 (c), *C. jejuni* and *E. coli* showed the lowest surviving bacterial counts after direct treatment with air plasma, while *S. isangii* had the lowest surviving bacterial counts after the 24 h storage post-treatment. *L. monocytogenes,* the only Gram-positive bacteria, showed the highest surviving bacterial counts when treated with oxygen, argon and air plasma after 24 h storage post-treatment. This increased survival rate in *L. monocytogenes* can be attributed to higher intracellular ROS levels induced by ACP treatment,

which suggests a heightened oxidative stress response that might contribute to its survival (Patange *et al.,* 2019b). These results highlight the complexity of microbial inactivation mechanisms under ACP treatment.

The effects of lower voltages (9.56 kV and 10.82 kV) using oxygen, argon and air plasma were also studied after 2.5 min of direct treatment and 24 h storage. In Figure 4.10 (a) and Figure 4.10 (b), argon plasma had the lowest surviving bacterial counts for *S. flexneri* after 24 h storage post-treatment, while oxygen plasma had the highest surviving bacterial counts for *S. isangii* at 9.56 kV during direct treatment. At 10.82 kV argon plasma had the lowest surviving bacterial counts for *S. isangii* after 24 h storage post-treatment, while air plasma had the highest surviving bacterial counts for both *A. hydrophila* and *S. isangii* during direct treatment and 24 h storage post-treatment.

These findings emphasise the importance of optimising ACP treatments by considering voltage and gas composition in addition to type and treatment duration to target specific bacterial strains effectively. For non-chlorine-resistant bacteria, argon plasma was the most effective gas with the highest effect at 13.53 kV for both direct treatment and 24 h storage post-treatment, showing the lowest surviving bacterial counts immediately after 2.5 min of treatment. However, after the 24 h storage post-treatment, oxygen plasma showed the highest efficacy with the lowest surviving bacterial counts, while argon plasma demonstrated the least effectiveness with the highest surviving bacterial count. Thus, argon plasma is optimal for immediate inactivation (direct treatment), whereas oxygen plasma is preferable for sustained bacterial suppression following storage (24 h storage post-treatment).

The observed difference in efficacy between argon plasma and oxygen plasma after the 24 h storage post-treatment can be attributed to the residual effects of reactive species generated during the plasma treatment. Oxygen plasma has a higher tendency to produce ROS, such as hydrogen peroxide and ozone, both of which are strong oxidants with prolonged antimicrobial properties (Huang *et al.,* 2022). These species can continue to exert their bactericidal effects even

after the initial plasma treatment has ended, contributing to the ongoing inactivation of bacterial cells during the 24 h storage post-treatment. In contrast, argon plasma is chemically inert and does not form significant quantities of ROS after the treatment (Bende *et al.,* 2024). As a result, once the direct exposure to plasma ends, the bacteria are no longer subjected to the same sustained oxidative stress, leading to higher survival rates over the 24 h period. This could explain why oxygen plasma exhibited greater long-term efficacy while argon plasma demonstrated a reduced ability to further inhibit bacterial growth after the initial exposure, leading to higher bacterial counts after the 24 h storage period (Fallon *et al.,* 2022).

5.4 Effect of plasma treatment on Gram-positive and Gram-negative bacteria

The effectiveness of plasma treatment varies depending on the bacterial classification, with distinct targets in Gram-positive and Gram-negative bacteria. For Gram-negative bacteria, the primary target for ROS is the cell envelope leading to cell membrane disruption. In contrast, for Gram-positive bacteria, intracellular components like DNA are more directly affected, even though severe intracellular damage may not result in immediate cell leakage due to their thicker cell walls (Han *et al.,* 2016).

The study by Han *et al.,* (2016) indicated that longer plasma treatment times generally enhance bacterial inactivation efficacy, although a detection limit may be reached under certain conditions. Incorporating a post-treatment storage period, especially over 24 hours, can significantly increase inactivation efficacy. This enhancement could be attributed to the continued generation of reactive species during post-treatment storage, which maintains antimicrobial efficacy. Such a strategy is particularly beneficial for sensitive samples, as it allows for minimal initial exposure while still ensuring effective bacterial inactivation (Han *et al.,* 2016).

Cold plasma affects multiple bacterial cell components, including the cell membrane, cell wall, DNA and intracellular proteins (Vatansever *et al.,* 2013; Mai-Prochnow *et al.,* 2014). Plasma species can break essential bonds in the peptidoglycan structure of Gram-positive bacteria (Yusupov *et al.,* 2013; Rao *et al.,* 2020) and induce lipid peroxidation in the membranes of Gram-negative bacteria (Joshi *et al.,* 2011; Rao *et al.,* 2020). The disruption of cell walls allows reactive species to penetrate the cell, causing further damage to DNA and intracellular proteins (Mai-Prochnow *et al.,* 2014). This multifaceted approach contributes to the overall antimicrobial efficacy of cold plasma (Rao *et al.,* 2020).

The effectiveness of bacterial inactivation through ACP or non-thermal plasma treatment largely depends on the bacterial type. Gram-positive bacteria generally exhibit less susceptibility to plasma treatment compared to Gram-negative bacteria, primarily due to their thicker peptidoglycan layers that protect reactive plasma species (Liao *et al.,* 2017; Huang *et al.,* 2020). However, it's important to note that sensitivity can vary among bacterial strains, and this generalization may not apply to all situations (Paldrychová *et al.,* 2019; Scholtz *et al.,* 2021).

Earlier studies, such as those by Van Gils *et al.,* (2013), used an atmospheric plasma pressure jet for bacterial inactivation at remote distances, where the plasma did not directly contact the liquid. The distance caused substantial air entrainment in the plasma effluent, resulting in the production of RNS and a decrease in the pH of the plasma-treated solution. Under these conditions, peroxynitrite chemistry was associated with bacterial inactivation (Van Gils *et al.,* 2013). The effectiveness of ACP can be influenced by various factors, including the characteristics of the plasma source and the specific bacterial strains targeted (Kondeti *et al.,* 2018).

There have been cases of contradictory results in the susceptibility of Gram-positive and Gram-negative bacteria to ACP or non-thermal plasma. For instance, studies using DBD-ACP have reported contradictory findings

regarding the susceptibility of these two bacterial types (Daeschlein *et al.,* 2012; Daeschlein *et al.,* 2014). Such variations may result from differences in experimental conditions, the types of ACP devices used and the characteristics of the bacterial strains involved (Das *et al.,* 2022).

5.5 Microbial inactivation kinetics of chlorine-resistant bacteria

This study utilised a first-order reaction model to demonstrate the kinetics of bacterial inactivation. This model effectively described the reduction of bacterial populations over time, measured in terms of log reduction, which expresses the percentage of bacteria inactivated. The results showed that chlorine-resistant bacteria exhibited higher bacterial inactivation after 2.5 min of ACP treatment compared to non-chlorine-resistant bacteria. Additionally, chlorine-resistant bacteria showed higher inactivation after 24 h storage post-treatment compared to direct treatment. Gram-negative chlorine-resistant bacteria had a higher level of bacterial inactivation after 24 h storage post-treatment compared to Gram-positive chlorine-resistant bacteria. Among the gases tested, argon proved most effective, followed by air and oxygen. However, the effectiveness varied for Gram-positive bacteria depending on the strain and the gas utilised. Overall, cold plasma was the most effective against Gram-negative bacteria than Gram-positive bacteria.

The study evaluated the microbial inactivation kinetics, including log-reduction, percentage log-reduction and the D-value for both chlorine-resistant and non-chlorine-resistant bacteria. Figure 4.11 (a) and Figure 4.12 (a) illustrated that microbial inactivation varied depending on the gas used, treatment method and the plasma exposure duration. For *S. aureus,* the highest inactivation was observed with air plasma after 24 h storage post-treatment, showing a 2-log reduction (97.12% inactivation). In contrast, *S. aureus* had the lowest inactivation with oxygen and argon plasma after 24 h storage post-treatment and with argon plasma after direct treatment, exhibiting only a 1-log reduction (< 90% inactivation). Similar findings have been reported in other studies,

showing complete inactivation of *S. aureus* with ACP exposure times of 7 min (Huang *et al.,* 2020). Reduced exposure times of 5 min resulted in a 3.4-log reduction. Other research demonstrated partial or complete bacterial inactivation with ACP treatment times between 2 and 4 minutes (Alkawareek *et al.,* 2014; Flynn *et al.,* 2015; Parkey *et al.,* 2015; Yoo *et al.,* 2015; Lunov *et al.,* 2016).

Except for *S. aureus,* all chlorine-resistant bacteria showed satisfactory inactivation to different gases, voltages and treatment methods, displaying 2-log reductions (99% inactivation), indicating the importance of tailoring plasma parameters to the target bacteria. *S. haemolyticus* showed high inactivation rates to argon, oxygen and air plasma after the 24 h storage post-treatment (99.56%, 98.99% and 98.80% respectively), but showed lower inactivation rates (< 90% inactivation) with the same gases after direct treatment. *S. aureus* exhibited the highest inactivation rate of 99.60% when treated directly with oxygen plasma, whereas it showed the lowest inactivation rate of 82.69% with argon plasma. After 24 h storage post-treatment, *S. paucimobilis* showed the highest inactivation with argon plasma (99.66%), while *S. aureus* showed the lowest inactivation with argon plasma (80.45%).

Gram-negative bacteria had higher inactivation efficacy after 24 h storage post-treatment, while the inactivation of Gram-positive bacteria depended on the bacterial strain and gas type. For Gram-negative bacteria, the highest log reduction was observed after the 24 h storage post-treatment using argon plasma followed by oxygen plasma. A study using a non-thermal atmospheric pressure plasma jet for bacterial inactivation in an aqueous medium conducted the quantification of reactive species (H_2O_2, OH and HNO_3) with various gas mixtures (He, He + Air (50% - 50%), Ar, Ar + air (50% - 50%), air). The results indicated that argon produced the highest number of reactive species like H_2O_2 (47 ppm), enhancing bacterial inactivation (Chandana *et al.,* 2018). Argon plasma was the most effective for Gram-negative bacteria, while the effectiveness of oxygen and air plasmas varied among the different chlorine-

resistant bacterial strains but was generally less than argon plasma. These patterns indicate that for optimal microbial inactivation using ACP, argon should be the gas of choice, especially for targeting chlorine-resistant Gram-negative bacteria. In summary, argon plasma was identified as the most effective gas for bacterial inactivation particularly for Gram-negative bacteria. Chlorine-resistant bacteria were more susceptible to ACP treatment compared to non-chlorine-resistant bacteria. Tailoring the treatment duration and gas type based on the specific chlorine-resistant bacterial strain and resistance characteristics is crucial for optimal bacterial inactivation.

Figure 4.13 (a) highlights the D-value (time required for 1-log reduction or 90% inactivation) for chlorine-resistant bacteria. *E. aerogenes* had the lowest D-value (0.81 min) using argon plasma after 24 h storage post-treatment, whereas *S. aureus* had the highest D-value (3.35 min) with argon plasma after 24 h storage post-treatment. Only Isolate 2 and *S. paucimobilis* showed a 1-log reduction (lowest treatment time ranging from 1.11 min to 1.33 min and 1.02 min to 1.85 min respectively) within the study's maximum treatment time of 2.5 min.

Summary of bacterial inactivation:

1. Isolate 1: Highest inactivation with oxygen and argon plasma after 24 h storage post-treatment (99.67% and 98.37% respectively); lowest with direct treatment using oxygen, argon, and air plasma and air plasma after 24 h storage post-treatment (< 90% inactivation).

2. *S. aureus*: Highest inactivation with air plasma after 24 h storage post-treatment and air, oxygen plasma for direct treatment (97.12%, 96.77% and 96.57% respectively); lowest with oxygen and argon plasma after 24 h storage post-treatment and argon for direct treatment (< 90% inactivation).

3. *E. aerogenes*: Highest inactivation with argon and oxygen plasma after 24 h storage post-treatment (97.92% and 99.00% respectively); lowest with air plasma for direct treatment and 24 h storage post-treatment (< 90% inactivation).

4. *S. paucimobilis*: Highest inactivation with argon plasma after 24 h storage post-treatment (99.63%); lowest with oxygen plasma for direct treatment (90.47%).

5. *S. haemolyticus*: Highest inactivation with argon, oxygen and air plasma after 24 h storage post-treatment (99.56%, 98.99% and 98.80% respectively); lowest with the same gases for direct treatment (< 90.00%).

6. *Pantoea* sp.: Highest inactivation with argon and air plasma for direct and 24 h storage post-treatment (99.08%, 99.07%, 99.05% and 99.34% respectively); lowest with oxygen plasma for direct and 24 h storage post-treatment (< 90.00%).

7. Isolate 2: Highest inactivation with argon plasma after 24 h storage post-treatment (99.45%); lowest with oxygen, argon and air plasma for direct treatment and oxygen, air plasma for 24 h storage post-treatment (> 95.00%).

This study highlights the varied susceptibility of different bacterial isolates to ACP treatment, emphasizing the need to tailor plasma parameters based on specific bacterial strains and resistance characteristics for optimal bacterial inactivation. Chlorine-resistant bacteria exhibited higher inactivation rates compared to non-chlorine-resistant bacteria. Among the gases tested, argon plasma was the most effective, especially against Gram-negative bacteria. Oxygen and air plasmas, while generally less effective than argon, showed efficacy depending on bacterial strain and treatment method. Longer exposure times consistently led to higher inactivation rates, highlighting the importance of optimising both gas type and treatment duration for effective microbial control.

Plasma exposure times consistently led to higher bacterial inactivation rates for all chlorine-resistant bacteria. This emphasises the importance of considering both the type of gas and the duration of treatment to achieve optimal microbial inactivation. The inactivation efficacy varied based on gas type, treatment method and exposure time.

5.6 Microbial inactivation kinetics of non-chlorine-resistant bacteria

This study assessed the efficacy of non-thermal DBD-ACP treatment on non-chlorine-resistant bacteria, focusing on oxygen, argon and air plasmas. Figure 4.11 (b) and Figure 4.12 (b) show that certain non-chlorine-resistant bacteria did not display a 2-log reduction (99% inactivation) or greater indicating variable effectiveness depending on the gas used and the exposure method. *A. baumannii* exhibited the highest inactivation with air and oxygen plasma during direct treatments (1-log reduction, 90.38% and 90.13% inactivation respectively). In contrast, *A. baumannii* showed the lowest inactivation with argon plasma, achieving only a 0-log reduction (44.02% inactivation). A study investigating the impact of ACP on *A. baumannii* ATCC 17904 found that exposure to double barrier discharge (DBD-ACP) at a fixed voltage of 20 kV, fixed distance of 1 and 4 mm resulted in an increase in the inactivation diameter with increasing exposure time. The inactivation gap increased from 2.5 cm to 4 cm after 30 seconds, highlighting the effectiveness of double barrier discharge-ACP as a sterilisation method. This finding suggests that *A. baumannii* might possess specific resistance mechanisms against certain plasma treatments, which is consistent with observations that some bacteria may have thicker or more robust cell walls that offer protection against ROS-induced damage (Atta *et al.,* 2019).

S. flexneri showed the highest inactivation rates with argon plasma after direct treatment (1.24-log reduction, 94.23% inactivation), supporting studies by Abbas *et al.,* (2017), which attribute high ROS concentrations in argon plasma to effective bacterial inactivation. However, oxygen plasma was more effective

during the 24 h storage post-treatment phase, achieving a 1.75-log reduction (98.21% inactivation). This is consistent with the findings of Khalaf *et al.,* (2015) and Pandit *et al.,* (2017), who reported sustained antibacterial activity due to prolonged ROS presence.

Figures 4.7(c), 4.8(c), 4.11(b) and 4.12(b) showed different surviving bacterial rates based on the type of gas, voltage and exposure duration used. For instance, *E. coli* had the lowest surviving bacterial count with air plasma (6.39 Log CFU/mL), whereas *A. hydrophila* had the highest (6.52 Log CFU/mL). These results are consistent with the literature suggesting that air plasma, rich in ROS, is effective in disrupting bacterial cell membranes (Abbas *et al.,* 2017). The findings also concur with reports of Khalaf *et al.,* (2015); Pandit *et al.*, (2017) and Attar *et al.,* (2019), which confirm the role of ROS and RNS in disrupting bacterial cell membranes. Non-thermal ACP effectively reduces bacterial counts by generating reactive species that continue to exert oxidative stress post-treatment, as seen with oxygen plasma's enhanced efficacy during the 24 h storage post-treatment phase.

Interestingly, while argon plasma showed strong direct inactivation capability (as seen with *S. flexneri*), its efficacy diminished over the 24 h storage post-treatment phase, especially with *A. baumannii* showing a low log reduction (0.38-log reduction, 58.25% inactivation). This variability indicates that while argon plasma is effective under certain conditions, its universal applicability across all non-chlorine-resistant species is limited (Khalaf *et al.,* 2015; Pandit *et al.,* 2017). *L. monocytogenes*, *A. hydrophila*, *C. jejuni*, *E. coli* and *S. isangii* generally showed lower inactivation rates across all plasma treatments, both directly and during 24 h storage post-treatment. These results emphasise the need to optimise plasma protocols to target specific bacterial characteristics effectively (Patange *et al.,* 2019a; Patange *et al.,* 2019b).

The reduced efficacy of argon plasma during the 24 h storage post-treatment period, particularly for *A. baumannii,* can be attributed to several factors. Argon

plasma primarily generates RNS and high-energy electrons that cause immediate bacterial damage, but because argon is chemically inert, it does not produce long-lasting ROS like hydrogen peroxide or ozone, which could provide bactericidal effects (Bende et al., 2024; Huang et al., 2022). Additionally, *A. baumannii* is a highly resilient pathogen, capable of surviving oxidative stress and adapting to harsh conditions, which may allow limited regrowth after the plasma treatment (Monem *et al.,* 2021). Furthermore, favourable storage conditions (e.g. temperature and nutrients) could promote bacterial recovery, reducing the overall efficacy of the plasma treatment (Das *et al.,* 2022). Thus, the lack of residual reactive species, combined with *A. baumannii's* adaptability and storage conditions, likely contributed to the reduced efficacy during the 24 h storage post-treatment phase.

A study by Kondeti *et al.,* (2018) on *C. jejuni* demonstrated that argon plasma effectively inactivates bacteria through the production of short-lived species is similar to the results of this study where significant bacterial inactivation was exhibited using oxygen, argon and air plasma during direct and 24 h storage post-treatment phases. Overall, the results of this study reinforce the established understanding that plasma-generated ROS and RNS are essential for bacterial inactivation. While DBD-ACP treatments using argon and oxygen gases are effective, their variability across different bacterial species highlights the need for further research to optimise treatment parameters and understand resistance mechanisms. The findings of this study highlight the critical role of ROS and RNS in bacterial cell disruption, consistent with existing literature (Khalaf *et al.,* 2015; Pandit *et al.,* 2017; Attar *et al.,* 2019).

Figure 4.13 (b) illustrates the D-value (time required) to achieve a 1-log reduction (90% inactivation) for non-chlorine-resistant bacteria. Among the bacteria studied, *S. flexneri* had the lowest D-value (1.48 min) for oxygen after 24 h storage post-treatment, indicating a higher susceptibility to oxygen plasma. In contrast, *A. baumannii* demonstrated the highest D-value (12.59 min) for argon plasma under the same post-treatment conditions, suggesting significant

resistance. Considering the maximum treatment duration of 2.5 min, only a few bacterial strains could achieve a 1-log reduction within this time frame. Specifically, *C. jejuni*, *S. isangii* and *S. flexneri* showed the potential for effective reduction, with their D-values ranging from 2.17 to 2.33 min for oxygen, argon and air plasma after direct treatments and from 1.48 to 2.44 min for oxygen, argon and air plasma after 24 h storage post-treatment. These findings highlight the varying degrees of resistance and susceptibility among chlorine-resistant bacterial species when exposed to different plasma treatments, emphasizing the importance of optimising treatment parameters based on specific bacterial characteristics.

The effectiveness of the treatments varied among the bacteria, with some showing higher inactivation to specific gases and treatment methods, while others showed minimal inactivation. Overall, with the non-chlorine-resistant bacteria *S. flexneri* had the highest inactivation after direct treatment using argon plasma (94.23%) and *A. baumananni* had the lowest inactivation using argon plasma (25.89%). After the 24 h storage post-treatment, *S. flexneri* had the highest inactivation using oxygen plasma (98.21%) and *A. baumananni* had the lowest inactivation using argon plasma (58.25%). For non-chorine-resistant bacteria, it was found that Gram-negative bacteria had both the highest and lowest inactivation rates after direct treatment and 24 h storage post-treatment. This variability in inactivation rates suggests that while argon plasma is potent under certain conditions, its efficacy is not universal across all non-chlorine-resistant bacteria.

5.7 Enumeration and microbial inactivation kinetics of water samples

This study tested cold plasma treatment on ten different water samples from various water sources including dams, lagoons, rivers and harbours. The broad applicability of the plasma treatment across these diverse water samples and achieving microbial inactivation up to 100% demonstrates the versatility and robustness of ACP plasma treatment as a disinfection method.

The results demonstrate that cold plasma treatment, using gases such as oxygen, argon and air, effectively disinfects various contaminated water samples. Cold plasma treatment led to substantial reductions in bacterial counts across the different water samples. Figure 4.14 illustrates the total bacterial counts (Log CFU/mL) for all 10 water samples subjected to direct treatment and 24 h storage post-treatment at 13.53 kV using oxygen, argon and air plasma after 3 min. Following both direct treatment and 24 h storage post-treatment for oxygen, argon and air plasma, a noticeable reduction in bacterial counts was observed. The uMhlanga Lagoon results had the lowest surviving bacterial counts, ranging from 3.49-Log CFU/mL to 5.30-Log CFU/mL with a 100% bacterial inactivation with argon plasma after direct treatment and 24 h storage post-treatment (log reduction of 4.75 and 4.26 respectively), while air and oxygen plasma also produced high levels of inactivation. Air plasma was the least effective during direct treatment and 24 h storage post-treatment with log reductions of 2.94 and 2.98 (99.89% and 99.90% inactivation). Despite being the least reactive for uMhlanga Lagoon, air plasma still demonstrated significant effectiveness in microbial inactivation, making it suitable for disinfection purposes. These results exhibit the broad-spectrum antimicrobial efficacy of cold plasma, corroborating the findings from previous studies (Magureanu *et al.,* 2018; Guo *et al.,* 2018). The consistent results between direct plasma treatment and 24 h storage post-treatment further substantiate the effectiveness of cold plasma technology. Minimal regrowth or resurgence in bacterial counts indicates that the plasma treatment caused irreversible damage to the bacterial cells, preventing their recovery.

This study revealed the effectiveness of different gases in the inactivation of bacterial contaminants in water samples using ACP. Among the gases tested, oxygen emerged as the most effective for direct treatment and the 24 h storage post-treatment, while argon was less effective overall, and air exhibited moderate effectiveness. Oxygen was the most effective gas, achieving the highest log reductions of 2.11 and 2.17 (99.21% and 99.32% inactivation). The effectiveness of oxygen is attributed to its ability to generate a high

concentration of ROS during plasma treatment. These ROS are highly reactive and cause oxidative stress as well as capable of damaging bacterial cellular components such as proteins, lipids and DNA, leading to cell death (Sachdev *et al.,* 2021). In Figure 4.14 and Figure 4.15, oxygen plasma treatment showed significant reductions in bacterial counts with the best log reductions observed in the uMhlanga Lagoon sample (4.75 log reduction after direct treatment and 4.26 log reduction after 24 h storage post-treatment). These findings are consistent with the reports of Sachdev *et al.,* (2021) and Zhang *et al.,* (2023), which support the efficacy of oxygen-based plasma for broad-spectrum disinfection against bacteria, fungi, viruses and spores.

There were substantial reductions in bacterial counts across the various water samples after cold plasma treatment, both in direct treatment and 24 h storage post-treatment. The uMhlanga Lagoon sample exhibited a 5.23 log reduction after direct treatment and a 4.27 log reduction after 24 h storage post-treatment with argon plasma yielding a 100% bacterial inactivation. These results demonstrate the ability of cold plasma to effectively reduce bacterial loads. While argon plasma demonstrated effectiveness, especially in yielding high log reductions in the uMhlanga Lagoon sample, it was less effective compared to oxygen plasma across the other samples. Figure 4.14, revealed that the Shongweni Dam results had the highest surviving bacterial counts among all the water samples, ranging from 7.11-Log CFU/mL to 9.28-Log CFU/mL.

The Shongweni Dam sample showed a log reduction of 1.01 after direct treatment and 1.06 after 24 h storage post-treatment using argon plasma. This inconsistency may be due to argon's inert nature, producing less reactive species than oxygen. Its role in stabilizing the plasma discharge and enhancing energy transfer to other reactive species makes it a useful component of cold plasma disinfection. The literature suggests that argon plasma can enhance the efficiency of disinfection processes by transferring energy efficiently to surfaces and other gases, however, its standalone antimicrobial activity may not be as effective (Lim *et al.,* 2023). In Figure 4.15, it is evident that argon plasma,

both after direct treatment and 24 h storage post-treatment was the least effective gas, resulting in a log reduction of 1.07 and 1.11 with 91.35% and 92.15% inactivation, respectively.

Figure 4.15 illustrates a notable log reduction in bacterial counts for all water samples subjected to plasma treatment. Studies suggest that achieving higher microbial inactivation with ACP requires optimal mixing of gases. The addition of N_2, O_2, or air to noble gases has been observed to increase reactive species in ACP, thereby enhancing antimicrobial efficacy (Das *et al.,* 2022). The findings of this study are consistent with other research by Khalaf *et al.,* (2015), Pandit *et al.,* (2017) and Attar *et al.,* (2019) highlighting the critical role of plasma-generated reactive species in bacterial inactivation. According to Das *et al.,* (2022), mixing noble gases like argon with reactive gases such as oxygen or air enhances the generation of reactive species thereby improving antibacterial efficacy. The results of this study support this claim, as argon was less effective on its own. Studies conducted by Magureanu *et al., (*2018), Guo *et al.,* (2018) and Patange *et al.,* (2018) also confirmed the potential of non-thermal plasma technology in wastewater treatment and pathogen inactivation findings that are corroborated by the high efficacy of oxygen and air plasma in this study.

Air, a combination of oxygen and nitrogen, demonstrated moderate effectiveness with significant reductions in bacterial counts (99.89% to 99.90% inactivation in uMhlanga Lagoon). While not as potent as oxygen, air still achieved substantial reductions in bacterial counts, making it a practical option for large-scale or routine disinfection. Air's efficacy is due to the production of both ROS and RNS which can synergistically enhance antimicrobial activity. This dual mechanism broadens the range of pathogens targeted enhancing overall disinfection efficacy, which emphasises the versatility of air plasma for targeting a broad range of pathogens due to its balanced generation of reactive species (Schnabel *et al.,* 2019).

The observation of minimal differences in bacterial counts between direct treatment and 24 h storage post-treatment, as illustrated in Figure 4.14, suggests that the bacterial cells were effectively killed and unable to regrow after plasma exposure. This supports the notion that the ROS and RNS generated during plasma treatment inflict irreversible damage to the microbial cells, thereby preventing them from recovering or proliferating even after a 24 h period. The persistence of this antimicrobial effect highlights the potential of ACP as a reliable method for long-term water disinfection. The low bacterial counts remained low even after 24 hours implying that the cold plasma treatment disrupts critical cellular functions and structures, leading to cell death that prevents recovery or proliferation substantiating the claim of effective disinfection.

While oxygen plasma emerged as the most effective plasma overall, some inconsistencies were noted with argon and air plasma. Although argon plasma had lower effectiveness (Shongweni Dam), it still achieved 100% inactivation in the uMhlanga Lagoon. These contradictions may be due to variations in water composition, microbial load or resistance profile of the bacterial communities present in the different water samples. Differences in bacterial resistance mechanisms, such as biofilm formation or the presence of thick cell walls might also play a role in reducing the efficacy of plasma treatments under certain conditions. The moderate effectiveness of air plasma to oxygen plasma suggests that while air-generated plasma produces both ROS and RNS, the concentration or reactivity of these species might not be as high or as targeted as with pure oxygen plasma. This explains why air plasma showed lower log reduction compared to oxygen plasma despite yielding significant effectiveness.

In Figure 4.15 and Figure 4.16, regarding the dam samples, the Inanda Dam results had the lowest surviving bacterial counts to oxygen plasma after 24 h storage post-treatment (99.60% inactivation), while the Shongweni Dam results had the lowest surviving bacterial counts to oxygen plasma after direct treatment and 24 h storage post-treatment (99.21% and 99.32% inactivation,

respectively). However, for the other gases and treatment methods, the dam sample results had lower surviving bacterial counts, resulting in less than 99.00% inactivation.

As shown in Figure 4.15 and Figure 4.16, oxygen, argon and air plasma, both in direct treatment and 24 h storage post-treatment, were effective in disinfecting water samples. In the river, estuary and lagoon samples; Tinley Manor, Tongaat, Umhlatuzana and Salt Rock, La Mercy Estuary and uMhlanga Lagoon, the microorganisms were reduced by 99.50% inactivation after plasma treatment with oxygen, argon and air. In the Durban Harbour and Blue Lagoon samples, the microorganisms were reduced by 99.00% inactivation for all the gases. In terms of effectiveness, argon plasma was the most effective followed by oxygen and air plasma. The results from this study, using optimal parameters determined from the chlorine-resistant and non-chlorine-resistant bacteria tests, indicate that cold plasma was a successful treatment method for water disinfection. This corroborates with earlier developments in non-thermal plasma technology, applying it to wastewater treatment (Magureanu *et al.,* 2018, Guo *et al.,* 2018, Patange *et al.,* 2018), and the inactivation of harmful pathogenic microorganisms in water to make it suitable for human consumption (Gururani *et al.,* 2021).

The cold plasma treatment using oxygen, argon and air plasma demonstrated increased effectiveness for water disinfection in this study evidenced by significant reductions in bacterial counts and sustained antibacterial after both direct and 24 h storage post-treatment. Oxygen emerged as the most potent disinfectant due to its ability to generate ROS & RNS causing extensive damage to bacterial cells. Argon, while less effective in some cases still contributed to substantial bacterial reductions. Air, due to its practicality and combined generation of ROS and RNS, offered a balanced approach suitable for large-scale applications. These findings correspond with and are supported by existing literature emphasizing the potential of cold plasma technology for effective and reliable water disinfection across diverse environmental and public

health contexts. The results of the water samples demonstrated that direct plasma treatment was effective in disinfecting contaminated water sources, and the 24 h storage post-treatment results remained consistent with the direct treatment results.

The result of this study correlates with existing literature on the effectiveness of ACP for water disinfection. A review by Ekanayake *et al.,* (2021) highlighted the potential of non-thermal plasma technology for water purification and desalination, emphasizing its ability to improve energy efficiency and reduce harmful contaminants. Gururani *et al.,* (2021) also pointed out the environmental benefits of plasma technology in reducing pollutants discharged into ecosystems. The minimal differences in bacterial counts observed between direct treatment and 24 h storage post-treatment suggest sustained antibacterial efficacy. This indicates that the cold plasma treatment not only inactivated bacteria immediately but also prevented regrowth, which is crucial for ensuring long-term water safety.

While the study demonstrated bacterial inactivation, it is important to consider whether the treated water met safe drinking water standards. Safe drinking water levels typically adhere to guidelines set by the World Health Organization (WHO) or local regulatory bodies, which define acceptable microbial counts (bacteria, coliform levels, *E. coli*, yeasts, etc.) and chemical contaminant concentrations. The significant reductions in bacterial counts, yielding up to 100% inactivation in some water samples, indicate that cold plasma treatment could bring contaminated water within safe microbial levels. However, without specific reference to the initial microbial loads and a comparison to established safe drinking water thresholds, it is difficult to conclude that the treated water was safe for human consumption.

This study focused primarily on bacterial disinfection and did not provide a detailed analysis regarding the reduction of chemical contaminants such as heavy metals, organic pollutants or other harmful substances. Cold plasma has been shown to degrade certain organic compounds and inactivate

microorganisms, but comprehensive testing of chemical contaminants is necessary to fully assess the safety of treated water for drinking purposes. To confirm the usage of potable water, further testing should be conducted to evaluate the presence of residual microorganisms and chemical contaminants post-treatment. Adhering to the WHO or other local water quality guidelines will be essential in these evaluations.

5.8 Limitations of the study

The study faced several challenges and limitations. One significant challenge was the lack of expertise to build a closed plasma device that could incorporate both direct and indirect treatment within a closed unit with provisions for multiple gas feeds. Achieving optimal mixing of gases is essential for higher microbial inactivation of ACP, and the inability to build the device according to specifications limited the study. The handheld device used had various limitations including the inability to increase treatment time beyond 3 minutes due to the risk of resistor explosion. The single input for gas feed prevented the use of gas mixtures, and the device was delicate, requiring rest periods between treatments to avoid damage.

The cost was another major challenge. Initially, the study planned to use five gases (argon, oxygen, nitrogen, helium and air) for all tests performed in triplicate. However, the cost of completing all microbiological tests with the specified parameters, including multiple gases, treatment methods, voltages, bacterial strains, and water samples, was prohibitively high. As a result, the study performed an initial test run using all five gases on non-chlorine-resistant bacteria, and the three gases that performed the best (oxygen, argon and air) were selected for further study. Identification limitations were also present as two chlorine-resistant bacteria could not be identified to the strain level and were solely based on Gram-reaction. This lack of detailed identification made it challenging to interpret the bacteria's reaction to cold plasma treatment.

CHAPTER 6: CONCLUSION AND RECOMMENDATIONS

This study demonstrates that ACP treatment, using oxygen, argon and air plasma is effective for bacterial inactivation in both chlorine-resistant and non-chlorine-resistant bacteria. The findings highlight that key factors such as voltage, treatment method, gas type and treatment duration play a crucial role in optimising the effectiveness of ACP disinfection. Higher voltages and extended treatment durations consistently resulted in greater bacterial inactivation, with oxygen plasma proving the most effective, particularly for chlorine-resistant bacteria.

However, this study also revealed important limitations. Although ACP treatment showed potential, non-chlorine-resistant bacteria were less susceptible, and while bacterial inactivation was significant, further research is needed to establish the safety of treated water for human consumption. Specifically, comparisons to established drinking water safety standards and assessments of potential chemical contaminants in treated water are essential next steps. The lack of these evaluations presents a gap in confirming the complete suitability of the treated water for potable use.

One of the major challenges in upscaling ACP technology for large-scale water treatment lies in its energy consumption and the need for precise control over the treatment parameters (voltage, gas type and exposure time). The current laboratory-scale setup may not be easily transferable to larger systems, and scalability concerns could involve difficulties in maintaining uniform plasma treatment across large volumes of water. Additionally, the cost of maintaining equipment for continuous ACP treatment could hinder large-scale implementation. These barriers must be addressed to make ACP a feasible option for widespread water disinfection.

Future research should focus on optimising ACP systems for large-scale applications, exploring energy-efficient methods, and investigating the potential for integration with existing water treatment infrastructure. Moreover, there is a need to address the long-term impact of ACP treatment on water quality, including possible effects on chemical contaminants. A comprehensive approach combining microbial and chemical safety assessments will be crucial for establishing ACP as a sustainable water disinfection solution. These steps will also help determine the viability of ACP as an alternative to traditional water treatment methods on a global scale.

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APPENDIX

Appendix 1: Log CFU/mL counts of the water samples after plasma exposure treatment of 3 minutes and 24 h storage post-treatment at voltage 13.53 kV using oxygen, argon and air.

Sample	Time (min)	Water Samples Voltage (13.53 kV)						
		Control	Oxygen		Argon		Air	
			DT	PT	DT	PT	DT	PT
R1	0	10.32						
	3.0		7.05 7.06	7.07 7.06	7.10 7.08	7.09 7.11	7.11 7.13	7.10 7.12
R2	0	10.24						
	3.0		7.06 7.08	7.05 7.07	6.97 6.99	6.95 6.96	7.10 7.12	7.11 7.12
R3	0	9.04						
	3.0		6.23 6.24	6.24 6.23	6.16 6.13	6.14 6.15	6.29 6.30	6.28 6.29
R4	0	9.22						
	3.0		6.68 6.77	6.71 6.65	6.56 6.62	6.59 6.61	6.85 6.89	6.82 6.86
E1	0	8.29						
	3.0		6.03 6.04	5.99 6.00	6.01 6.00	6.00 5.99	6.07 6.06	6.08 6.07
E2	0	10.36						
	3.0		7.16 7.14	7.17 7.18	7.17 7.18	7.15 7.15	7.27 7.29	7.29 7.30
L1	0	8.23						
	3.0		5.00 4.70	5.00 4.90	3.97 3.00	3.99 3.96	5.28 5.32	5.23 5.28
L2	0	8.39						
	3.0		6.05 6.07	6.05 6.04	6.00 5.97	5.98 5.96	6.09 6.12	6.10 6.10
D1	0	9.21						
	3.0		7.90 7.32	7.08 7.23	7.94 7.92	7.95 7.87	7.58 7.69	7.59 7.63
D2	0	9.28						
	3.0		7.08 7.26	7.04 7.18	8.26 8.16	8.22 8.12	7.88 7.91	7.88 7.90

Appendix 2: Microbial inactivation results of water samples

Water Samples							
Sample		Voltage (13.53 kV)					
		Oxygen		Argon		Air	
		DT	PT	DT	PT	DT	PT
R1	Log Reduction	3.28	3.25	3.22	3.23	3.22	3.23
		3.26	3.26	3.24	3.21	3.19	3.21
	% Reduction	99.95	99.94	99.94	99.94	99.94	99.94
		99.95	99.95	99.94	99.94	99.94	99.94
R2	Log Reduction	3.17	3.19	3.27	3.28	3.14	3.13
		3.16	3.17	3.25	3.28	3.11	3.12
	% Reduction	99.93	99.94	99.95	99.95	99.93	99.93
		99.93	99.93	99.94	99.95	99.92	99.92
R3	Log Reduction	2.81	2.80	2.88	2.90	2.75	2.76
		2.80	2.81	2.91	2.90	2.74	2.75
	% Reduction	99.85	99.84	99.87	99.87	99.82	99.83
		99.84	99.85	99.88	99.87	99.82	99.82
R4	Log Reduction	2.53	2.51	2.66	2.63	2.38	2.40
		2.45	2.57	2.60	2.61	2.33	2.36
	% Reduction	99.71	99.69	99.78	99.76	99.58	99.60
		99.65	99.73	99.75	99.75	99.54	99.57
E1	Log Reduction	2.26	2.30	2.28	2.29	2.22	2.22
		2.25	2.29	2.29	2.31	2.23	2.23
	% Reduction	99.45	99.50	99.48	99.49	99.40	99.40
		99.44	99.49	99.49	99.51	99.42	99.41
E2	Log Reduction	3.20	3.19	3.19	3.22	3.09	3.08
		3.22	3.18	3.18	3.21	3.07	3.06
	% Reduction	99.94	99.94	99.94	99.94	99.92	99.92
		99.94	99.93	99.93	99.94	99.91	99.91
L1	Log Reduction	3.23	3.23	4.26	4.24	2.96	3.00
		3.53	3.33	5.23	4.27	2.91	2.95
	% Reduction	99.94	99.94	100.0	99.99	99.89	99.90
		99.97	99.96	100.00	100.00	99.88	99.89
L2	Log Reduction	2.33	2.34	2.39	2.40	2.30	2.28
		2.32	2.35	2.41	2.42	2.27	2.29
	% Reduction	99.54	99.54	99.59	99.61	99.50	99.48
		99.52	99.55	99.61	99.62	99.46	99.49
D1	Log Reduction	1.30	2.13	1.27	1.26	1.63	1.62
		1.88	1.98	1.29	1.34	1.52	1.57
	% Reduction	95.03	99.26	94.60	94.47	97.64	97.58
		98.70	98.94	94.85	95.40	96.96	97.33

D2	Log Reduction	2.20	2.24	1.01	1.06	1.40	1.40
		2.02	2.10	1.12	1.16	1.37	1.38
	% Reduction	99.37	99.42	90.27	91.22	96.03	95.98
		99.05	99.21	92.43	93.07	95.71	95.82