Department of Chemical Engineering

Inactivation of Critically Ranked Carbapenem Resistant Bacteria and Genes in a Batch Atmospheric Plasma Reactor

by

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ABSTRACT

Wastewater treatment plants (WWTPs) have been observed to be direct key reservoir of both antibiotic resistant bacteria (ARBs) and antibiotic resistant genes (ARGs) associated with human infection as high concentrations of ARBs and ARGs have been detected in recycled hospital water. Among the ARBs, the carbapenem-resistant *Acinetobacter baumannii* and carbapenem-resistant *Pseudomonas aeruginosa* are ranked as priority 1 (critical) pathogens by the World Health Organisation (WHO) as they constitute a major threat to public health. Moreover, from the heuristic search of literature, it was observed that not only do conventional WWTPs fail to efficiently prevent the discharge of ARBs and ARGs into freshwater environments, but majority of extant advanced treatment technologies are also riddled with bottlenecks that oftentimes outweigh their proficiency. This has warranted the need for treatment technologies that have the capacity to completely obliterate pathogens (ARBs) as well as inactivate their resistance genes (ARGs). In this regard, this study investigated non-thermal plasma (NTP) technology as an alternative disinfection step to inactivate these bacteria and their ARGs. Culture based method and polymerase chain reaction (PCR) were employed in confirming the carbapenem resistance gene *bla*$_{\text{NDM-1}}$ in *Acinetobacter baumannii* (BAA 1605) and *Pseudomonas aeruginosa* (27853). Suspensions of carbapenem-resistant *Acinetobacter baumannii* (24 h culture) *and ATCC Pseudomonas aeruginosa* (16 hr culture) were prepared from the confirmed isolates and were subjected to plasma treatment at varying time intervals (3 min, 6 min, 9 min, 12 min and 15 min) in triplicates. The plasma treated samples were evaluated for re-growth and the presence of the resistance gene. The treatment resulted in a 1.13 log reduction after 3 min and the highest ≥8 log reduction (i.e. 99.999999 %) after 15 min for *Acinetobacter baumannii*. For *Pseudomonas aeruginosa*, the treatment resulted in a 0.68 log reduction after 3 min and the highest ≥8 log reduction after 12 min. The concentration of the *bla*$_{\text{NDM-1}}$ gene decreased with time, proving that NTP can inactivate ARGs. The log reduction and gel images suggest that plasma disinfection has a great potential to be an efficient tertiary treatment step for WWTPs. However, there are many factors that still need to be optimised, such as reaction time to completely inactivate the ARGs and removal of biofilms in the way of the treatment of ARBs such

as *Pseudomonas aeruginosa;* before implementation is possible as this technology is yet gradually gaining commercial and industrial espousal.

Keywords: waste water treatment plant, cold atmospheric plasma, carbapenems, A*cinetobacter baumannii*, *Pseudomonas aeruginosa*

DECLARATION

This thesis constitutes my master research work under the Chemical Engineering Department in the School of Engineering at the University of Pretoria, Pretoria from March 2021 to December 2023 under the supervision of Dr. S. A. Iwarere, Professor M. Daramola, Professor C. Tizaoui and Dr. John O. Unuofin.

I declare that:

(i) This thesis except where otherwise indicated is my original work.

(ii) The written expressions are in my own words, and where other written sources have been quoted, then:

a) their words have been re-written but the general information attributed to them has been referenced;

b) where their exact words have been used, their writing has been placed inside quotation marks, and referenced.

_____

T. B. M Mosaka

PUBLICATIONS AND CONFERENCES

Peer-reviewed Publication

Mosaka, T. B. M., Unuofin, J. O., Daramola, M. O., Tizaoui, C., and Iwarere, S. A. 2023. Inactivation of antibiotic-resistant bacteria and antibiotic-resistance genes in wastewater streams: Current challenges and future perspectives. *Front. Microbiol*, 13:1100102.

Conference Presentations

1. Mphahlele, M.P., Mosaka, T. B. M., Daramola, M. O., Tizaoui, C., and Iwarere, S. A. 2022. Cold Atmospheric Plasma as a tool to inactivate antibiotic resistant genes of high priority gram positive bacteria in wastewater treatment plants. *3rd IWA Disinfection and Disinfection By-Products Conference*, Milan Italy, 27 June-1 July 2022.

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NOMENCLATURE

Abbreviations

AOPs	Advanced oxidation processes
AMR	Antimicrobial resistance
AMP	Ampicillin
AnMBRs	Anaerobic membrane bioreactors
ARB	Antibiotic resistant bacteria
ARGs	Antibiotic resistant genes
CAP	Cold atmospheric plasma
CIP	Ciprofloxacin
DDD	Daily doses
DNA	Deoxyribonucleic acid
DOC	Dissolved organic carbon
(e-) ARGs	Extracellular antibiotic resistant genes
EC	Electrocoagulation
EPS	Exopolymeric substances
ERM	Erythromycin
ESBL	Extended-spectrum β-lactamase
HGT	Horizontal gene transfer
ICU	Intensive care units
(i-) ARGs	Intracellular antibiotic resistant genes
LOD	Level of detection
LPS	Lipopolysaccharide

MDR	Multi-drug resistant
MIC	Minimum inhibitory concentration
PBPs	Penicillin binding proteins
PAW	Plasma activated water
PEN	Penicillin
PCR	Polymerase chain reaction
PG	Peptidoglycan
NGWRP	New goreangab water reclamation plant
NGTWs	New Germany treatment works
NWWTP	Northern wastewater treatment plant
NTP	Non-thermal plasma
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SA	South Africa
STX	Sulfamethoxazole–trimethoprim
TET	Tetracycline
THB	Total heterotrophic bacteria
TRIM	Trimethoprim
UV	Ultraviolet radiation
VBNC	Viable but non-culturable state
VGT	Vertical gene transfer
WHO	World health organisation
WRP	Water reclamation plant
WRPF	Water reclamation plant facility

WWTPs	Wastewater treatment plants

IUPAC Nomenclature for Some Organic Compounds

H_2O_2	Hydrogen peroxide
OH	Hydroxyl radical
NO_3^-	Nitrates
NO_2^-	Nitrites
O_3	Ozone
$^1O_2$	Singlet oxygen
$O_2^{\cdot -}$	Superoxide radicals

Chapter 1

INTRODUCTION

1.1. Introduction and background

In recent years, there has been excessive use of antibiotics for control of bacterial infections in humans and animals (Yuan *et al*., 2015; Sarangapani *et al*., 2019), cancer treatment, and in some regions, as growth promotion agents (Sarangapani *et al*., 2019). This has consequently rendered the once antibiotic-susceptible bacteria, resistant to various categories of antibiotics administered so far, due to long-lived misuse and overuse (Yuan *et al*., 2015; Meletis, 2016; Sarangapani *et al*., 2019). Moreover, these antibiotic resistant bacteria (ARB) evade multiple antiseptic actions of diverse antimicrobial agents (multidrug-resistance (MDR) and are aetiological agents of nosocomial and community-acquired infections (Howard *et al*., 2012; Viehman *et al*., 2014). The World Health Organisation (WHO) even categorised these ARBs into three classes, namely, medium, high and critical based on the effect of antibiotic resistance on human health and the necessity to develop antibiotics that can heal resistant infections. The critical priority 1 class includes *Acinetobacter baumannii*, carbapenem-resistant and *Pseudomonas aeruginosa*, carbapenem-resistant (World Health Organisation, 2017; Soni *et al*., 2022).

Acinetobacter baumannii and *Pseudomonas aeruginosa* are gram-negative bacteria, that are notorious for surviving for prolonged periods in the most diverse environments (wet and dry) (Howard *et al*., 2012; Viehman *et al*., 2014; Logan *et al*., 2017; Valencia-Martín *et al*., 2019; Raut *et al*., 2020; Shi *et al*., 2020). This key feature facilitates their dissemination within the health care setting and often leads to outbreaks (Fishbain and Peleg, 2010; Viehman *et al*., 2014; Logan *et al*., 2017, Walters *et al*., 2019). Due to the opportunistic nature of *A. baumannii*, outbreaks are rampant among immunocompromised individuals, especially patients and convalescing persons who have been in the hospital for a long time (>90 days). (Fishbain and Peleg, 2010; Howard *et al*., 2012; Valencia-Martín *et al*., 2019; Raut *et al*., 2020, Shi *et al*., 2020). *P. aeruginosa* causes infections in patients in the intensive care units (ICU) or in patients with serious underlying disorders, such as a cystic fibrosis or suppressed immune system (Voor In 't Holt *et al*., 2014; Logan *et al*., 2017).

These bacteria then cause life-threatening infections of the urinary tract, ear, sinuses, connective tissues, pulmonary disease, bacteremia, meningitis, respiratory tract and wound infection which have limited options for treatment *(Fishbain and Peleg, 2010; Howard et al., 2012; Voor In 't Holt et al., 2014; Logan et al*., 2017; Valencia-Martín *et al*., 2019; Raut *et al*., 2020; Shi *et al*., 2020). This results in patients being

admitted to the ICU, having surgical procedures done on them and being hospitalized for longer periods (Fishbain and Peleg, 2010), hence consuming more health-care resources (World Health Organisation, 2020; Soni *et al*., 2022). In critical cases, these infections eventually lead to high morbidity and the demise of suffering patients (Voor In 't Holt *et al*., 2014; Logan *et al*., 2017; Walters *et al*., 2019) as these bacteria are resistant to commonly used antibiotics. Carbapenem antibiotics have thus become important for clinical management of these infections (Walters *et al*., 2019). However, these bacteria are becoming resistant to carbapenem group of antibiotics (Walters *et al*., 2019; Raut *et al*., 2020), which constitutes a global public health concern because carbapenems are considered to be the most reliable last-resort for treating bacterial infections when all other antibiotics do not work (Meletis, 2016; Codjoe and Donkor, 2017). Also the available second-line treatment options often accompany toxicity and are much less defined in their efficacy (Viehman *et al*., 2014).

Some outbreaks could however be controlled by eliminating an environmental reservoir (Valencia-Martín *et al*., 2019). However, recycled drinking water has been found to be direct key reservoir of ARBs and ARGs associated with human infection (Ekwanzala *et al*., 2018) as high concentrations of ARBs and antibiotic resistant genes (ARGs) have been detected in hospital water that has been recycled (Hassoun-Kheir *et al*., 2020). This is because Wastewater Treatment Plants (WWTPs) have principally been designed to reduce bacterial loads and remove nutrients to certain acceptable limits and even at their optimum operating conditions, they do not remove ARGs and ARB (Fadare and Okoh, 2021a). These ARBs and ARGs present in WWTPs are released into outgoing environmental systems such as rivers and reservoirs (Rodríguez-Molina *et al*., 2019), making wastewater both a resource and a problem (Unuofin, 2020). Hence the use of Cold Atmospheric Plasma (CAP), an Advanced Oxidation Processes (AOPs) which can serve as an alternative tool for both water treatment and wastewater reclamation and reuse; as it is able to break down organic matter while inactivating ARBs and ARGs (Umar, 2022).

1.2. Problem statement and research motivation

When the conventional disinfection processes such as chlorination, UV irradiation, and ozone oxidation are applied in WWTPs, a great fraction of ARBs dies, while others enter a state of dormancy due to stress and are resuscitated when the stressors are released. Disinfectants such as chlorine tend to have a selective effect on ARGs, decreasing abundance of genes (gene copies per mL of sample) while the prevalence of the gene (gene copies per total bacteria) increases (Manaia *et al*., 2018; Chen *et al*., 2020b). Sometimes disinfection processes may kill the bacteria by destroying its DNA or the cellular structure, but ARGs may persist for a long time in the cell debris and in the environment. Both intracellular (i-) and extracellular (e-) ARGs eventually transfer and adapt into new bacteria, leading to the inception and genetic transformation across bacteria and the development of antibiotic resistance, especially against carbapenems renowned as last-barrier antibiotics (Yuan *et al*., 2015; Sarangapani *et al*., 2019; Chen *et al*., 2020b, Jin *et al*., 2020). There are emerging studies on the application of non-thermal plasma in the treatment of water-borne pathogens. However, to our knowledge, there is a dearth of information regarding the response of critically ranked ARBs (*A. baumannii* and *P. aeruginosa*) and their corresponding genes ($bla_{\text{NDM-1}}$) to non-thermal plasma treatment.

1.3. Justification for study

The WHO identified antimicrobial resistance as one of the three most important problems threatening human health and the environment (Howard *et al*., 2012; Soni *et al.*, 2022). A forecast from the WHO reported that by 2050, 10 million people globally will be casualties to AR infections, if joint efforts are not put in place to prevent them (Anthony *et al*., 2020; Genthe *et al*., 2020; Fadare and Okoh, 2021b). Therefore, there is a need to control these ARB and ARGs in the environment. As far as could be ascertained, no strategy is in place to prevent the movement of the ARB and ARGs in the environment (Huang *et al*., 2013; Ekwanzala *et al*., 2018; Wang *et al*., 2020). Considering that conventional disinfectants like chlorine tend to be selective in what it oxidizes, AOPs produce reactive oxygen species (ROS) like the indiscriminate hydroxyl radical •OH (Foster, 2017; Chen *et al*., 2020c). The primary regimes of AOPs driven disinfection include the destruction of cell wall, cell membrane, enzymes, and intracellular genetic material (Chen *et al*., 2020c). The interest in the role of •OH is that it has an oxidation potential (2.8 V) that is higher than the conventional disinfectants, chlorine (1.36 V) and ozone (2.07 V), and it can damage DNA (Foster, 2017; Sharma *et al*., 2019; Rekhate and Srivastava, 2020; Azuma *et al*., 2022). The •OH radical has diverse impact on normal protein structure which is one of the primary targets in bacteria during disinfection, including

oxidation of amino acids, modification of sulphur groups, etc., causing irreversible damages to cells and inactivation of ARBs and ARGs (Chen *et al*., 2020c).

1.4. Research Questions and Objectives

The study seeks to answer the following questions:

1. What is the optimum cold atmospheric plasma contact time to inactive *Acinetobacter baumannii*, carbapenem-resistant and *Pseudomonas aeruginosa*, carbapenem-resistant and their genes using CAP?
2. Which factors result in the optimum inactivation of the ARB and ARGs?

Therefore, this study aimed to determine the efficacy of a cold atmospheric plasma (CAP) reactor in the inactivation of *Acinetobacter baumannii*, carbapenem-resistant *and Pseudomonas aeruginosa*, carbapenem-resistant and their genes.

In order to answer the proposed questions, the study set out with two specific objectives:

1. To set up, commission and operate a CAP batch reactor with discharge generated above the aqueous solution.

2. To understand the effect of pH, conductivity and concentrations of nitrate, nitrite and hydrogen peroxide on the efficacy of the CAP.

Chapter 2

LITERATURE REVIEW

This chapter is an adapted version of the review article published in Frontiers on Microbiology as:

Inactivation of antibiotic resistant bacteria and antibiotic-resistance genes in wastewater streams: Current challenges and future perspectives. Thabang B.M. Mosaka, John O. Unuofin, Michael O. Daramola, Chedly Tizaoui, Samuel A. Iwarere. *Frontiers in Microbiology* 13, p. 1100102.

Abstract

The discovery of antibiotics, which was once regarded as a timely medical intervention now leaves a bitter aftertaste: antimicrobial resistance (AMR), due to the unregulated use of these compounds and the poor management receiving wastewaters before discharge into pristine environments or the recycling of such treated waters. Wastewater treatment plants (WWTPs) have been regarded a central sink for the mostly unmetabolized or partially metabolised antibiotics and is also pivotal to the incidence of antibiotic resistance bacteria (ARBs) and their resistance genes (ARGs), which consistently contribute to the global disease burden and deteriorating prophylaxis. In this regard, we highlighted WWTP-antibiotics consumption-ARBs-ARGs nexus, which might be critical to understanding the epidemiology of AMR and also guide the precise prevention and remediation of such occurrences. We also discovered the unsophistication of conventional WWTPs and treatment techniques for adequate treatment of antibiotics, ARBs and ARGs, due to their lack of compliance with environmental sustainability, then ultimately assessed the prospects of cold atmospheric plasma (CAP). Herein, we observed that CAP technologies not only have the capability to disinfect wastewater polluted with copious amounts of chemicals and biologicals, but also have a potential to augment bioelectricity generation, when integrated into bio electrochemical modules, which future WWTPs should be retrofitted to accommodate. Therefore, further research should be conducted to unveil more of the unknowns, which only a snippet has been highlighted in this study.

KEYWORDS: Antibiotic-resistant bacteria; Antibiotic-resistant genes; Wastewater; Disinfection method.

2.1. Introduction

Antibiotics are used for the inhibition or complete destruction of bacteria that cause infections in humans and animals (Yuan *et al*., 2015; Duijkeren *et al*., 2018; Li and Gu, 2019; Sarangapani *et al*., 2019) and are also widely used in cancer treatment and in some regions, as growth promotion agents (Sarangapani *et al*., 2019). There has been a global increase in the consumption of antibiotics because these drugs are becoming more affordable and accessible (Genthe *et al*., 2020). Since most antibiotics are not completely metabolised by humans and animals, they are often ejected as common components of wastewater (Fadare and Okoh, 2021b) where they induce increased antibiotic resistance of common bacteria, and the gradual development of broad-spectrum antibiotic-resistant genes (Huang *et al*., 2013; Yuan *et al*., 2015). This further explains the high concentration of ARB and ARGs that are often reticulated into wastewater treatment plants (WWTPs) from the sewage systems of households, healthcare services, antibiotic manufacturing facilities, agricultural activities and animal feedlots *(Ekwanzala et al., 2018; Ben et al., 2019; Rodríguez-Molina et al*., 2019). WWTPs have been principally designed to remove nutrients and reduce bacterial load to certain

acceptable limits; regrettably, their optimum performances do not remove ARB and ARGs (Fadare and Okoh, 2021a). When the conventional disinfection processes such as chlorination, UV irradiation, and ozone oxidation are applied, a great fraction of ARB dies, while others enter a state of dormancy due to stress and are resuscitated when the stressors are released. Disinfectants such as chlorine tend to have a selective effect on ARGs, decreasing abundance of genes (gene copies per mL of sample) while the prevalence of the gene (gene copies per total bacteria) increases (Manaia *et al*., 2018; Chen *et al*., 2020b). Sometimes disinfection processes may kill the bacteria by destroying its DNA or the cellular structure, but ARGs may still persist for a long time in the cell debris and in the environment. Both intracellular (i-) and extracellular (e-) ARGs eventually transfer and adapt into new bacteria, leading to the inception and genetic transformation across bacteria and the development of antibiotic resistance (Yuan *et al*., 2015; Sarangapani *et al*., 2019; Chen *et al*., 2020b; Jin *et al*., 2020). These ARB and ARGs present in WWTPs are released into outgoing environmental systems such as rivers and reservoirs (Rodríguez-Molina *et al*., 2019). Wastewater has been previously discussed as both a resource and a problem (Unuofin, 2020); the compelled reuse of treated wastewater due to overstretched natural water resources in water-stressed countries further increases the risks of ARB and ARGs exposure.

WWTPs have been observed to be direct key reservoir of ARB and ARGs associated with human infection as high concentrations of ARB and ARGs have been detected in therein, worldwide Moreover, investigations have shown that patients with infections caused by bacteria regarded as critical by the World Health Organisation (WHO) consume more health-care resources, because they are more at risk of worse clinical outcomes and death than patients infected with non-resistant strains of the same bacteria (World Health Organisation, 2020). Therefore, there is a need to monitor and control these ARB and ARGs in the WWTPs, which might be instrumental in preventing their contact with pristine water bodies as well humans and animals (Huang *et al*., 2013; Ekwanzala *et al*., 2018; Wang *et al*., 2020). To our knowledge, no well-documented strategy is in place to prevent the movement of the ARB and ARGs in the environment. An alternative tool for both water treatment and wastewater reclamation and reuse is Advanced Oxygenation Processes (AOPs) which breaks down organic matter while inactivating ARB and ARGs (Umar, 2022). Considering that conventional disinfectants like chlorine tend to be selective in what it actually oxidizes, AOPs produce reactive oxygen species (ROS) like the indiscriminate hydroxyl radical •OH (Foster, 2017; Chen *et al*., 2020c). The primary regimes of AOPs-driven disinfection include the destruction of cell wall, cell membrane, enzymes, and intracellular genetic material (Chen *et al*., 2020c). The interest in the role of •OH is that it has an oxidation potential (2.8 V) that is higher than the conventional disinfectants, chlorine (1.36 V) and ozone (2.07 V), and it can damage DNA (Foster, 2017; Sharma *et al*., 2019; Rekhate and Srivastava, 2020; Azuma *et al*., 2022). The •OH radical has diverse impact on normal protein structure which is one of the primary targets in bacteria during disinfection, including oxidation of amino acids,

modification of sulphur groups, etc., causing irreversible damages to cells and inactivation of ARB and ARGs (Chen *et al*., 2020c).

The inadequate wastewater treatment (that can be assisted by AOPs) coupled with poor data collation contribute to greater challenge of tackling antibiotic resistance (Genthe *et al*., 2020). This review thus elucidates the WWTP-antibiotics consumption-ARBs-ARGs nexus, which is an invaluable blueprint for managing ARBs and ARGs occurrences as well as assessing the efficiency of WWTPs. The review also provides a commentary and analysis on the extant and the emerging treatment technologies, particularly cold atmospheric plasma (CAP), which is renowned for its environmental friendliness and swift response during operation.

2.1.1 Statement of Significance

This paper provides a critical review on the efficiency of commonly used disinfection methods (e.g. chlorination, ozone and Ultraviolet (UV) irradiance) for the inactivation of antibiotic resistant bacteria (ARB) and antibiotic resistant genes (ARGs) wastewater treatment plants (WWTPs). It was observed that these disinfection methods alone were not able to completely inactivate the ARB and ARGs. Enhanced removal efficiencies were only noticed when they were used alongside ultrafiltration, anaerobic membrane bioreactors, electrocoagulation, tertiary filtration and peracetic acid. Cold atmospheric plasma is suggested as an alternate all in one disinfection method, that generates intense UV radiation, shockwaves, reactive oxygen species and reactive nitrogen species that prevent procreation of cells and the spread of ARB and ARGs in the environment.

2.1.2 Literature Synthesis

The PRISMA guidelines were used for this systematic review and compilation of removal efficiencies of ARBs and ARGs by actual wastewater treatment plants WWTPs (Moher *et al*., 2010). The literature search was performed using seven online databases: PubMed Database, EBSCOhost Online Research Databases, MEDLINE, ISI Web of Knowledge, African Journals Online, and Scopus in August 2022. Predefined terms such as (Antibiotic OR Resistance OR Bacteria OR ARB OR Gene OR ARG OR WWTP OR Influent OR Effluent OR Inflow OR Outflow) AND (Treatment OR Disinfection Methods) were used to retrieve relevant articles published from March 1, 2021 to August 31, 2022. Figure A1 summarizes the steps taken to conduct the literature search and selection. The first step entailed removing duplicate articles that were found in the seven databases. The remaining articles were screened based on their title and abstract. Full-text articles were read and screened. The remaining full-text articles were read and included in the review. Only articles that contain information on the detection of ARBs and ARGs in the influent and effluent of

WWTPs were included in this review, regardless of the types of biological processes that were applied for quantification. Articles referring to ARGs detected from viruses and other micro-organisms other than bacteria were also excluded.

2.2. ARBs and ARGs in WWTPs

2.2.1. Incidence of ARBs and ARGs in wastewater

While there might be disagreeing accounts regarding the particular origin of AMR because the biochemical and molecular basis of such phenomenon was yet to be established in early studies (Hawkey, 1998; Moher *et al*., 2010), advancement in research has evinced not only its incidence but also suggested the rapidity in its evolution. The discovery of antibiotics has been observed as one of the most critically important healthcare interventions of the 20th century, where they were observed to reduce disease burden using different mechanisms against bacteria, such as inhibiting synthesis of the cell wall, depolarizing the cell membrane, inhibiting synthesis of the protein, inhibiting synthesis of the nucleic acid and inhibiting metabolic pathways in bacteria (Reygaert, 2018). Antibiotics have thereafter been abused, misused, overused and continually released into natural bodies through WWTPs, which are considered as sinks of major antibiotic reticulation pathways, such as households, aquaculture, healthcare facilities, antibiotic manufacturing facilities, agricultural activities, animal feedlots and slaughterhouses (Ekwanzala *et al*., 2018; Ben *et al*., 2019; Rodríguez-Molina *et al*., 2019). Correspondingly, numerous reviews have been able to identify the most predominantly consumed or utilised antibiotic categories as: macrolides, sulfonamides, trimethoprim, quinolones, tetracyclines, due to their prevalence in WWTPs and groundwater *(Nnadozie et al*., 2017; Wang *et al*., 2020; Noor *et al*., 2021) further reports a list of 16 antibiotic families based on their corresponding ARGs extrapolated from analysis of five continents. WWTPs have to capacity to hold, daily, phenomenal volumes of wastewater containing cocktails of chemical contaminants and organic matter, which are consistently biotransformed by denizen microorganisms (both beneficial and harmful). However, despite considerable achievement by these plants in reducing major pollutants through a combination of physicochemical and biological techniques, several reports have highlighted the surreptitiousness and subsequent evasion of certain classes of microcontaminants, especially antibiotics, as well as some ARBs and ARGs during such treatment processes (Unuofin, 2020; Wang *et al*., 2020; Zainab *et al*., 2020; Noor *et al*., 2021). Once in WWTPs, the persistent interaction of microbial denizens with antibiotics under genial conditions, such as adequate nutrients levels, biofilms abundance and other physicochemical conditions might facilitate microbial tolerance and evolution in resistance to gradually increasing concentration of antibiotics. Innately, bacterial populations of WWTP matrices might derive tolerance and resistance through certain morpho-physiological, biochemical and molecular phenomena. Intrinsic resistance

mechanisms that have been documented, so far, include reduced membrane permeability, induced modification of intracellular antibiotic target (i.e. protein, ribosome, etc), structural modification of the antibiotic, thus inactivating it, secretion of exopolymeric substances (EPS) or biofilms to immobilize and annul the bioavailability of the foreign chemical, use of active drug efflux pumps which expel antibiotics from inside the bacteria before they reach the specific binding site and apply the antimicrobial activity and also the expression of constitutive and inducible genes, which have evolved over time, due to selection pressure and recombination (Reygaert, 2018; Magureanu *et al*., 2021).

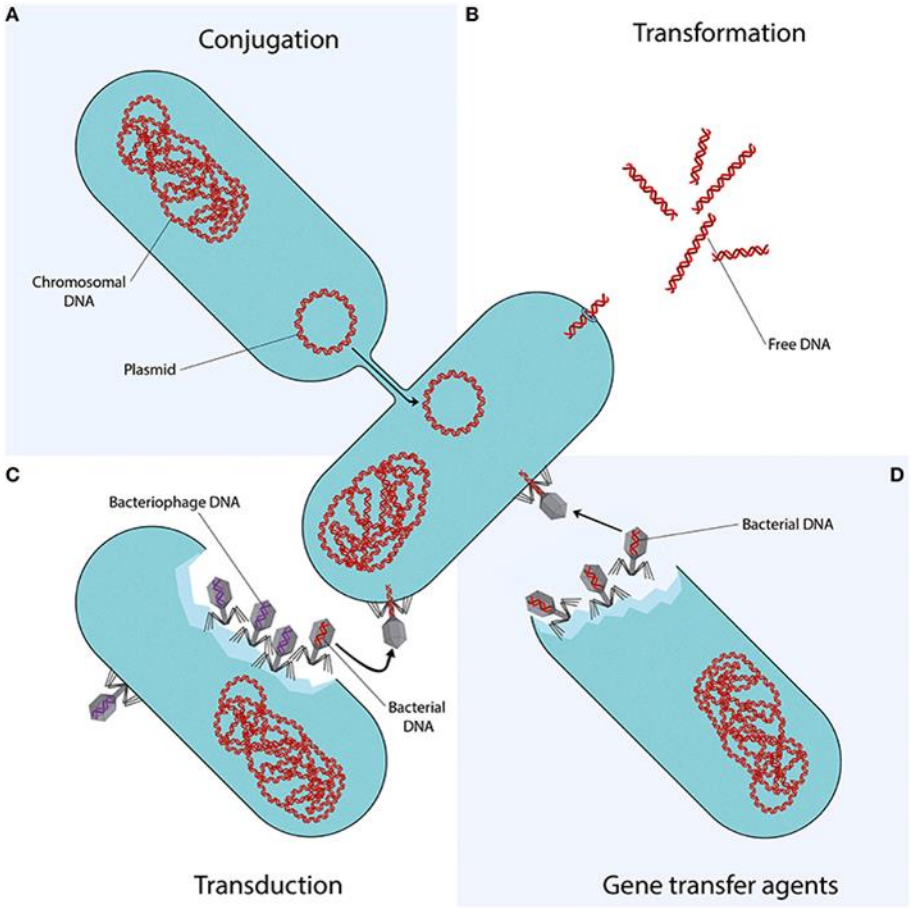

Figure 1: Horizontal gene transfer (HGT) of ARB and ARGs (von Wintersdorff *et al*., 2016). Conjugation is the direct transfer of DNA molecule known as a plasmid from a donor bacterium to a recipient bacterium, involving cell-to-cell contact between the two bacteria. Transduction is the transfer of DNA from a donor bacterium to a recipient bacterium, through viruses that infect bacteria, known as bacteriophages. Transformation is intra- and inter-species exchange of genetic information by uptake of naked DNA, released through cell lysis or actively excreted by some bacteria which can only be received by a competent bacterium. Following uptake and translocation to the cytoplasm, it is incorporated into the competent bacterium's chromosome or into a plasmid (Koutsoumanis *et al*., 2021; Uluseker *et al*., 2021; Courti *et al*., 2022).

Amongst the aforementioned mechanisms, the genetic factor is considered the most critical due to its capability to constantly evolve to match up to the constantly improving antibiotic efficacies. Moreover, ARBs of WWTPs might be able to confer resistance status on the innocuous communities through horizontal gene transfer (Figure 1), and thereafter vertical transfer of recombinant DNA during proliferation. While HGT is considered to be a non-reproductive gene transfer whereby genetic material are dispersed between bacteria that do not have an offspring-parent relationship, HGT can transfer ARGs faster and effectively, which is why HGT is the most concerning transfer mechanism when it comes to AR spread in WWTPs (Uluseker *et al*., 2021; Courti *et al*., 2022). This has warranted a knee-jerk response regarding the constant surveillance of AMR, worldwide, assessment of extant as well emerging water and wastewater treatment techniques and technologies that would obliterate the threat posed by AMR.

2.2.2. Current global status of ABRs and ARGs occurrences in WWTPs

The immense pressure on and critical reduction and pollution of globally available freshwater withdrawals coupled with the increasing incidences of deteriorating prophylaxis, especially regarding bacterial infections, has necessitated a more focused look at the once overlooked pollution sinks: WWTPs. Ever since the earliest surveillance, there has been overwhelmingly consistent studies, worldwide, that report the occurrence of antibiotics, ARBs and ARGs is different WWTP matrices, thereby suggesting their pivotal role in the infection cycle. Therefore, understanding the current global trend on occurrence of the WWTP-antibiotics consumption-ARBs-ARGs nexus is germane to developing a framework for preventive and remediation measures. To this end, we wish to provide a robust account on the current trend by assessing reviews by (Wang *et al*., 2020; Noor *et al*., 2021; Uluseker *et al*., 2021; Zhuang *et al*., 2021; Gao *et al*., 2022) and (Wang *et al*., 2022) *inter alia*, where especial attention was conferred on WWTPs on different geographical regions worldwide. From the reviews, it was apparent that there was no phenomenal reduction in concentrations of antibiotics, ARBs and ARGs, between influent and effluents. This might be due to lipophilicity and hydrophobicity of antibiotics that reduce their liability to certain physicochemical and biological treatment. Although cells of resistant bacteria might be damaged during treatment, their free lying genetic material which might be picked up by innocuous population in effluent and also downstream, thus rendering preceding treatment steps ineffective. The volatility and subsequent aerosolization of ARGs was observed as well, which could be a determinant in their evasion from treatment and transboundary movement to already treated effluent. It was also deduced that the type and abundance of ARGs in WWTP

influents could be used to fingerprint the categories of antibiotics with unregulated use. To corroborate this, our observation of high ARGs concentrations in WWTPs of high-income and upper-middle-income regions as compiled by (Wang *et al*., 2022), was in congruence with the outcomes of a global survey on consumption and usage of antibiotics, where Western Europe and East Asia consumed a daily doses (DDD) of 3364 million and 4413 million units, respectively. Some of the genes commonly detected in WWTPs in the studies examined include variants of *sul*, *tet*, *erm*, *mph*, *bla*, *qnr*, *msr*, *mex*, which confer resistance to sulfonamide, tetracycline, β-lactams, macrolides, quinolones, as well as multidrug resistance *(Zhuang et al*., 2021), thereby suggesting the accelerated use of this group of antibiotics, globally as discussed subsequently. Quinolones are excreted unchanged by urine and faeces into WWTPs (Pazda *et al*., 2019) but later eliminated via sorption to sludge as they are very hydrophilic compounds (Hendricks and Pool, 2012). The quinolones resistant genes, *qnr* (*qnrB, qnrD,* and *qnrS*) are however present in China (WWTP1 and WWTP2) (Table 1) as they are propagated by HGT (Mao *et al*., 2015; Pazda *et al*., 2019).

Table 1: Concentration of genes in the influent and effluent of different WWTPs

Genes	WWTP	Concentration ($copies/ml$) in the influent	Concentration ($copies/ml$) in the effluent	References
ermB	WWTP1	$9\ x\ 10^5$	$2\ x\ 10^3$	(Mao *et al*., 2015)
	WWTP2	$1\ x\ 10^6$	$2\ x\ 10^5$	(Mao *et al*., 2015)
	NGWRP	$1.2\ x\ 10^5$	0	(Pazda *et al*., 2019)
	WRPF	$6.31\ x\ 10^5$	$1\ x\ 10^2$	(Kappell *et al*., 2018)
	WWTP5 (UV)	$5.37\ x\ 10^4$ $cell\ eqivalents$ $/100ml$	$3.75\ x\ 10^4$ $cell\ eqivalents$ $/100ml$	(Jäger *et al*., 2018)
	WWTP5 (Ozone)	$5.37\ x\ 10^4$ $cell\ eqivalents$ $/100ml$	$1.01\ x\ 10^3$ $cell\ eqivalents$ $/100ml$	(Jäger *et al*., 2018)
	WWTP5 (UV and Ozone)	$5.37\ x\ 10^4$ $cell\ eqivalents$ $/100ml$	$1.07\ x\ 10^3$ $cell\ eqivalents$ $/100ml$	(Jäger *et al*., 2018)
qnr	WWTP1	$7\ x\ 10^4$	$1\ x\ 10^3$	(Mao *et al*., 2015)
	WWTP2	$2\ x\ 10^5$	$9\ x\ 10^3$	(Mao *et al*., 2015)
sul	WWTP1	$3\ x\ 10^7$	$5\ x\ 10^5$	(Mao *et al*., 2015)

	WWTP2	$9\ x\ 10^6$	$6\ x\ 10^5$	(Mao *et al*., 2015)
	WWTP3	$1.19\ x\ 10^8$	$4.52\ x\ 10^6$	(Zhang *et al*., 2017)
	WWTP4 (UV)	$1.86\ x\ 10^5$	$2.5\ x\ 10^3$	(Chen *et al*., 2020a)
	WWTP4 (UV and EC)	$1.86\ x\ 10^5$	$2x\ 10^2$	(Chen *et al*., 2020a)
	WWTP5 (UV)	$1.33\ x\ 10^6$ *cell eqivalents* $/100ml$	$9.33\ x\ 10^5$ *cell eqivalents* $/100ml$	(Jäger *et al*., 2018)
	WWTP5 (Ozone)	$1.33\ x\ 10^6$ *cell eqivalents* $/100ml$	$6.83\ x\ 10^4$ *cell eqivalents* $/100ml$	(Jäger *et al*., 2018)
	WWTP5(UV and Ozone)	$1.33\ x\ 10^6$ *cell eqivalents* $/100ml$	$5.53\ x\ 10^4$ *cell eqivalents* $/100ml$	(Jäger *et al*., 2018)
	NGWRP	$1.55\ x\ 10^5$	0	(Wallmann *et al*., 2021)
	WRP	$2\ x\ 10^6$	$3\ x\ 10^1$	(Quach-Cu *et al*., 2018)
	WRPF	$6.31\ x\ 10^6$	$3.98\ x\ 10^3$	(Kappell *et al*., 2018)
tet	WWTP1	$8\ x\ 10^5$	$3\ x\ 10^4$	(Mao *et al*., 2015)
	WWTP2	$2\ x\ 10^6$	$3\ x\ 10^5$	(Mao *et al*., 2015)
	WWTP3	$1.78\ x\ 10^8$	$2.49\ x\ 10^7$	(Zhang *et al*., 2017)
	WWTP4 (UV)	$3.18\ x\ 10^3$	$1.2\ x\ 10^3$	(Chen *et al*., 2020a)
	WWTP4 (UV and EC)	$3.18\ x\ 10^3$	$1\ x\ 10^2$	(Chen *et al*., 2020a)
	WRPF	$5.01\ x\ 10^5$	$6.31\ x\ 10^1$	(Kappell *et al*., 2018)
$bla_{SHV/TEM}$	WRP	$8\ x\ 10^3$	$6\ x\ 10^0$	(Quach-Cu *et al*., 2018)
	WWTP5 (UV)	$1.22\ x\ 10^5$ *cell eqivalents* $/100ml$	$1.83\ x\ 10^5$ *cell eqivalents* $/100ml$	(Jäger *et al*., 2018)
	WWTP5 (Ozone)	$1.22\ x\ 10^5$ *cell eqivalents* $/100ml$	$1.1\ x\ 10^4$ *cell eqivalents* $/100ml$	(Jäger *et al*., 2018)
	WWTP5(UV and Ozone)	$1.22\ x\ 10^5$	$1.12\ x\ 10^4$	(Jäger *et al*., 2018)

	cell eqivalents /100*ml*	*cell eqivalents* /100*ml*

The RNA methyltransferase, *ermB*, which is located on the transposon *(Pazda et al., 2019; Wallmann et al*., 2021), is prevalent in Gram-positive enterococci and it confers resistance to critically important macrolide-lincosamine-streptogramin (MLS) antibiotics like erythromycin, azithromycin and clarithromycin (Wallmann *et al*., 2021). The *ermB* genes are present in China (WWTP1, WWWP2), Namibia (NGWRP), USA (WRPF) and Germany (WWTP5) (Table 1).

Penicillin's (ampicillin, amoxicillin and clavulanic acid) and cephalosporins (cefotaxime) are the main β-lactam antibiotics used for veterinary and human medicine (DeFrancesco *et al*., 2017; Pazda *et al*., 2019). The instability of the β-lactam ring and its susceptibility to hydrolysis makes β-lactam antibiotics, especially penicillin, not easily detected in WWTPs (Pazda *et al*., 2019). Extended-spectrum β-lactamase (ESBL)-producing bacteria, including E. *coli*, are among the most commonly encountered multidrug-resistant (MDR) bacteria today and are frequently associated with a high mortality rate and prolonged hospitalisation. This may be because clinical E. *coli* isolates frequently co-carry multiple ESBL genes with varying hydrolysis spectra to different antibiotics, leading to treatment failure (Seyedjavadi *et al*., 2016; Gumede *et al*., 2021). The genes that confer resistances to ESBL is the $bla_{\text{CTX-M}}$ (Böckelmann et al., 2009; DeFrancesco *et al*., 2017), while the bla_{TEM} and bla_{SHV} genes confer resistance to the narrow-spectrum β-lactams (DeFrancesco *et al*., 2017). Specific variants of these, such as $\text{bla}_{\text{SHV-5}}$, have the ability to hydrolyze broad-spectrum cephalosporins and monobactams (Nzima *et al*., 2020). The bla_{TEM}, bla_{SHV} and $bla_{\text{CTX-M}}$ are located on the plasmid while the *ampC* (ampicillin resistant) can be found on the chromosome *(Pazda et al*., 2019). Total heterotrophic bacteria (THB) resistant to ampicillin was present in Italy (WWTP6, WWTP7, WWTP8) and $bla_{\text{SHV/TEM}}$ was present in USA (WRP) and Germany (WWTP5) (Table 1).

The most persistent antibiotics in the environment, with synergistic actions of the metabolite with other antibiotics which results in a longer degradation time of 60 days, are sulfamethoxazole and/or the sulfamethoxazole–trimethoprim (STX) combination (Hendricks and Pool, 2012; Genthe *et al*., 2020). Trimethoprim and sulphonamides (sulfamethoxazole, sulfadiazine, sulfachloropryridazine, sulfacetamide, sulfasalazine and acetylsulfamethoxazole) belong to the class of synthetic antibiotic. Sulfonamides are widely used in veterinary medicine as feed additives and the treatment of bacteria but humans they are usually used in combination with trimethoprim for chlamydia, respiratory and urinary tract infections (Hendricks and Pool, 2012; Pazda *et al*., 2019). *Sul1* is a resistant dihydropteroate synthase which mediates tolerance to a broad group of sulfonamide antibiotics (Wallmann *et al*., 2021). This gene is frequently found in both transposons and plasmids of Gram-negative enterobacteria but also in environmental pathogens like

Pseudomonas aeruginosa (Pazda *et al*., 2019; Wallmann *et al*., 2021). Resistance to trimethoprim is due to the plasmid although the genes are usually found on the chromosome (Pazda *et al*., 2019). The *sul* genes were present in China (WTP1, WWTP2, WWTP3, WWTP4), Germany (WWTP5), Namibia (NGWRP) and USA (WRP, WRPF) as can be seen in Table 1.

Another antibiotic that can persist for relatively long periods in the absence of sunlight and is less mobile, is Tetracycline (TET) (Koutsoumanis *et al*., 2021). TET is a naturally sourced antibiotic that is obtained from *Streptomyces* sp. (e.g. chlortetracycline, tetracycline and oxytetracycline) and they are also semi-synthetic antibiotics (e.g. demeclocycline and doxycycline) (Pazda *et al*., 2019; Panja *et al*., 2021). The naturally sourced TET are used in the treatment of aquaculture and livestock, and they are also as medicine for humans (Pazda *et al*., 2019). For humans, TET is used to treat diseases such as malaria, rosacea, chlamydia, etc. while for livestock, it is administered as a growth promoter in concentrated animal feeding operations (Panja *et al*., 2021). TET is mostly found in WWTPs because it is released in human and animal faeces and urine, in its active form (Böckelmann *et al*., 2009). The TET genes found on bacterial chromosome, integron, transposons and plasmids (*tetA, tetB, tetC, tetD, tetE, tetG, tetH, tetM, tetL, tetO, tetQ, tetX, tetT, tetW, and tetS*) are responsible for the resistance of bacteria to TET antibiotics (Böckelmann *et al*., 2009; DeFrancesco *et al*., 2017; Pazda *et al*., 2019). The TET genes were present in China (WWTP1, WWTP2, WWTP3, WWTP4) and USA (WRPF) (Table 1).

2.2.3. The current position on ARBs and ARGs occurrences in South African WWTPs

South Africa is critically overburdened by two socioeconomic nemeses: intense water stress and HIV/AIDS. This suggests the frugal use of scarcely available water sources and the desperate adoption of alternative sources, such as greywater and treated wastewater, for domestic purposes and irrigation, which have been advocated for and practiced in some municipalities (Ateba *et al*., 2020). The reuse of water in S.A may be harmful to consumers, especially the immunocompromised (HIV/AIDS) and vulnerable population, because of the threat presented by AMR; so far, not fewer than four major AMR outbreaks have been recorded at national level. A 2014 WHO report identified Africa and South East Asia as the regions without established AMR surveillance systems (Tadesse *et al*., 2017). In response, S.A has redoubled efforts to track the incidence and prevalence of ARBs and ARGs in different environmental matrices and also identify their respective pathways and thresholds through the establishment of the South African Antimicrobial Resistance Strategy Framework. Although surveillance of WWTPs has not gained the momentum anticipated, snippets from recent investigations and reviews suggest there might be a lot more to uncover regarding occurrences of ARBs and ARGs in WWTPs (Igwaran *et al*., 2018; Mbanga *et al*., 2021; Mpondo *et al*., 2021; Mtetwa *et al*., 2021; Conco *et al*., 2022; Ogunlaja *et al*., 2022; Ramsamy *et al*.,

2022) other worthy mentions are captured in Table 2. From the aforementioned studies, we observed a similar trend in occurrence and abundance of genes; moreover, genes coding for other virulence factors were also detected, which explains the persistence of their bearing organisms throughout the treatment processes of WWTPs. Strikingly, the investigation of (Mtetwa *et al*., 2021) suggests the emergence of new classes of antibiotics as well as their resistance genes in WWTPs, which stem from the treatment of HIV/AIDS associated infections. This already casts a cloud of bewilderment on management of the known, and triggers anxiety regarding the unknown yet impending dangers of AMR incidences in SA.

Table 2: Percentage (%) Resistance of bacteria to antibiotics in different WWTPs

Antibiotic	**WWTP**	**% Resistance of THB to antibiotic in the influent**	**% Resistance of THB to antibiotic in the effluent**	**% Resistance of E. *coli* to antibiotic in the influent**	**% Resistance of E. *coli* to antibiotic in the effluent**	**References**
Erythromycin (ERM)	WWTP14	2.5	4.8			(Ateba *et al*., 2020)
	WWTP15	5.4	3.1			(Ateba *et al*., 2020)
Ciprofloxacin (CIP)	WWTP14	2.5	1.6			(Ateba *et al*., 2020)
	WWTP15	2.6	1.6			(Ateba *et al*., 2020)
	NWWTP			17	23	(Pillay and Olaniran, 2016)
	NGTW			25	40	(Pillay and Olaniran, 2016)
Trimethoprim/sulfamethoxazole (STX)	WWTP9			70	90	(Gumede *et al*., 2021)
	WWTP10			60	100	(Gumede *et al*., 2021)
	WWTP11			40	90	(Gumede *et al*., 2021)

	WWTP12			90	90	(Gumede *et al.*, 2021)
Trimethoprim (TRIM)	WWTP14	42.5	73			(Ateba *et al.*, 2020)
	WWTP15	50	46.9			(Ateba *et al.*, 2020)
Tetracycline (TET)	WWTP9			30	80	(Gumede *et al.*, 2021)
	WWTP10			60	100	(Gumede *et al.*, 2021)
	WWTP11			20	100	(Gumede *et al.*, 2021)
	WWTP12			80	90	(Gumede *et al.*, 2021)
	NWWTP			63	86	(Gumede *et al.*, 2021)
	NGTW			41	68	(Gumede *et al.*, 2021)
	WWTP14	10	16.1			(Ateba *et al.*, 2020)
	WWTP15	40.5	17.2			(Ateba *et al.*, 2020)
Penicillin (PEN)	WWTP13			70	100	(Ateba *et al.*, 2020)
	WWTP14	42.9	58.8			(Ateba *et al.*, 2020)
	WWTP15	70.4	39.5			(Ateba *et al.*, 2020)
Ampicillin (AMP)	WWTP6	19	3			(Turolla *et al.*, 2018)
	WWTP7	25	14			(Turolla *et al.*, 2018)
	WWTP8	16	23			(Turolla *et al.*, 2018)
	WWTP9			90	90	(Gumede *et al.*, 2021)

	WWTP10			90	100	(Gumede *et al*., 2021)
	WWTP11			80	100	(Gumede *et al*., 2021)
	WWTP12			100	100	(Gumede *et al*., 2021)
	NWWTP			80	57	(Pillay and Olaniran, 2016)
	NGTW			53	60	(Pillay and Olaniran, 2016)
	WWTP13			0	40	(Nzima *et al*., 2020)
	WWTP14	53.6	61			(Nzima *et al*., 2020)
	WWTP15	92.6	55.8			(Nzima *et al*., 2020)

2.3. Removal of ARB and ARGs in WWTPs

2.3.1. Removal from the influent of WWTPs

ARB and ARGs are transported to WWTPs from ground or surface water, animal and human microbiota and mainly through sewage systems from health care facilities where antibiotics are consumed the most (Barancheshme and Munir, 2018). Health care services wastewater is the result of the residue collection from sewage of outpatients and those in the wards, kitchen and laundry, cooling and heating processes, and laboratorial discharge from the research centres and clinics (Giannakis *et al*., 2017). All these residues contain many substances, such as disinfectants, organic compounds, therapeutic metals, antibiotics, ARB and ARGs which are transported to WWTPs without being preliminarily disinfected (Giannakis *et al*., 2017; Barancheshme and Munir, 2018). These macro-pollutants and micropollutants arriving in WWTPs have different compositions and they are of a size range (µg or ng). Which affect the solubility, volatility, adsorbability, absorbability, biodegradability, polarity, and stability of WWTPs, hence the failure of treatment by conventional WWTPs (Giannakis *et al*., 2017). This is seen in many developing nations, including SA, which turn WWTPs into unintentional collection points for ARB and ARGs as they currently have no defined regulations regarding management of hospital wastes before they are disposed into the municipal WWTPs (Ben *et al*., 2019; Rodríguez-Molina *et al*., 2019; Fadare and Okoh, 2021b).

Practitioners have suggested pre-treatment while others suggest that hospital wastewater be treated onsite as a separate entity with conventional disinfectants such as chlorine, to effectively reduce bioaccumulation, and to importantly eliminate ARB and ARGs as they can directly threaten developing countries drinking water sources (Giannakis *et al*., 2017; Barancheshme and Munir, 2018). However, some techniques have been employed by WWTPs in selected countries, worldwide, to detect and reduce the occurrences of ARBs and ARGs in influents (Table 3).

Table 3: Treatment processes of ARB's and ARG's in different WWTPs worldwide

Country (Province/ City)	**Site of Influent**	**Capacity of treatment plant**	**Year Collected**	**Method of Disinfection**	**Method of quantification**	**References**
China (Northern China)	WWTP1	540 000 m^3/day	November – December 2011 and June-July 2012	Anaerobic and anoxic lagoons, conventional activated sludge and Chlorination	DNA extraction and Quantitative Polymerase Chain Reaction (qPCR)	(Mao *et al*., 2015)
	WWTP2	580 000 m^3/day	November – December 2011 and June-July 2012	Anaerobic and anoxic lagoons, conventional activated sludge and Chlorination	DNA extraction and qPCR	(Mao *et al*., 2015)
China (Xi'an)	WWTP3	Not mentioned	March – June 2013	Anoxic/anaerobic/ oxic process (Ultra violet (UV) Irradiation Experiment)	DNA extraction and PCR	(Zhang *et al*., 2017)

China	WWTP4	Not mentioned	Not mentioned	Oxidation ditch process Secondary clarifier (Electrochemical (EC) and UV Experiment)	DNA extraction and qPCR	(Chen *et al*., 2020a)
Germany	WWTP5	112 000 m^3/day	September 2016, March 2017, July 2017, and October 2017	Activated sludge treatment in combination with sedimentation (Ozonation and UV irradiation Experiments)	DNA extraction and qPCR	(Jäger *et al*., 2018)
Italy (Milan)	WWTP6	432 000 m^3/day	Not mentioned	Sand filtration and Peracetic acid (PAA)	Not mentioned	(Turolla *et al*., 2018)
	WWTP7	354 600 m^3/day	Not mentioned	Sand filtration and UV Radiation	Not mentioned	(Turolla *et al*., 2018)
	WWTP8	177 000 m^3/day	Not mentioned	Sand filtration and Sodium hypochloride (NaOCl)	Not mentioned	(Turolla *et al*., 2018)

Namibia (Windhoek)	New Goreangab Water Reclamation Plant (NGWRP)	21 000 m^3/day	September 2018	Pre Ozonation, Coagulation, Flotation, Dual Media Filtration, Main Ozonation, Activated Carbon, Ultrafiltration and Chlorination	DNA extraction and qPCR	(Wallmann *et al.*, 2021)
South Africa (Kwazulu-Natal Durban)	Northern Wastewater Treatment Plant (NWWTP)	70 000 m^3/day	March–August 2012	Chlorination	DNA extraction and PCR	(Pillay and Olaniran, 2016)
South Africa (Kwazulu-Natal Durban)	New Germany Treatment Works (NGTWs)	700 m^3/day	March–August 2012	Chlorination	DNA extraction and PCR	(Pillay and Olaniran, 2016)
South Africa (Kwazulu-Natal Durban)	WWTP9 (Msunduzi local municipality)	76 000 m^3/day	April 2020	Aeration basins, clarifiers and chlorination.	DNA extraction and PCR	(Gumede *et al.*, 2021)
	WWTP10 (uMngeni local municipality)	5 600 m^3/day	April 2020	Aeration basins, clarifiers and chlorination.	DNA extraction and PCR	(Gumede *et al.*, 2021)
	WWTP11 (Surrounding suburbs)	540 m^3/day	April 2020	Aeration basins, clarifiers and chlorination.	DNA extraction and PCR	(Gumede *et al.*, 2021)

	WWTP12 (Msunduzi local municipality)	500 m^3/day	April 2020	Aeration basins, clarifiers and chlorination.	DNA extraction and qCR	*(Gumede et al*., 2021)
South Africa (Kwazulu-Natal Durban)	WWTP13	Not mentioned	May -July 2017	Chlorination	DNA extraction and Multiplex PCR (M-PCR)	*(Nzima et al*., 2020)
South Africa (North West)	WWTP14	Not mentioned	2016 and 2017	Coagulation, flocculation, sedimentation, sand filtration, and chlorination	DNA extraction and PCR	(Ateba *et al*., 2020)
	WWTP15	Not mentioned	2016 and 2017	Sand filtration and chlorination	DNA extraction and PCR	(Ateba *et al*., 2020)
United States of America (Los Angeles)	Water Reclamation Plant (WRP)	235 000 m^3/day	Not mentioned	Sedimentation, nitrification, denitrification, flocculation, filtration and, chlorination	DNA extraction and qPCR	(Quach-Cu *et al*., 2018)
United States of America (Oak Creek, WI)	South Shore Water Reclamation Plant Facility (WRPF)	Not mentioned	Not mentioned	Primary clarifier (anaerobic membrane bioreactors (AnMBRs) experiment)	DNA extraction and qPCR	(Singh et al., 2022)

2.3.2. Removal from the effluent of WWTPs

2.3.2.1. Chlorination

Chlorination is the most common method of choice for the disinfection of water and wastewater worldwide due to its low cost (Pillay and Olaniran, 2016; Anthony *et al*., 2020; Barbosa *et al*., 2021), its largely known technology and proven effective disinfection of a great variety of pathogenic microorganisms (Beber de Souza *et al*., 2015; Feng *et al*., 2022). But chlorination alone is inadequate for the disinfection of wastewater because it does not permanently damage ARB/ARG and it results in high regrowth of bacteria (Pillay and Olaniran, 2016; Anthony *et al*., 2020) as is evident with WWTP9-14, NWWTP and NGTW plant in Table 5. WWTP15 was the exception that led to a reduction of the resistance of bacteria to antibiotics. This was due to the fact that WWTP15 was actually a dam that received surface water, not waste water isolates originating from areas of high antibiotic use (Ateba *et al*., 2020). In the NGWRP plant, chlorination was used as a stabilization measure, preventing the regrowth of ARB in storage tank and the drinking water distribution system (Wallmann *et al*., 2021). WWTP1 was also reported to have removal efficiencies of 41±5%, 42±3%, 69±7% and 55±6% for TET resistant, sulfonamide-resistant, CIP-resistant ethromycin-resistant bacteria, respectively. While in WWTP2, the removal efficiencies were 79±6%, 65±5%, 77±8% and 55±6% for TET resistant, sulfonamide-resistant, quinolone-resistant and macrolides-resistant bacteria, respectively. This is because chlorination initially lowers the total load of microbes, while significantly increasing the level of ARBs (Anthony *et al*., 2020; Chen *et al*., 2020b).

Chlorine increases cell membrane permeability by causing impairment to the cell membrane and cytoplasm (Anthony *et al*., 2020; Feng *et al*., 2022), and then directly inactivating ARGs (Gomes *et al*., 2019). However, when the disinfection dose is not enough, bacteria become injured and enter a viable but non-culturable state (VBNC). The injured bacteria have low metabolic activity which become active under certain conditions. They receive a large amount of DNA released from sensitive bacteria surrounding them, making horizontal transfer happen more frequently (Pillay and Olaniran, 2016; Jin *et al*., 2020; Feng *et al*., 2022). Also, when E. *coli* is exposed to chlorine it induces a specific set of proteins, making them less susceptible to disinfection (Luukkonen *et al*., 2014; Ateba *et al*., 2020). In WWTP1 the *ermB*, *sul*, *tet* and *qnr* genes were reduced by 99.8%, 98%, 96% and 99% respectively. In WWTP2 the *ermB*, *sul*, *tet* and *qnr* genes were reduced by 100%, 93%, 85% and 96% respectively, as can be seen in Table 4. WWTP1 with a lower capacity, reduced more genes than WWTP2 with a larger capacity and hence more antibiotic residues which facilitate the maintenance and propagation of ARGs in WWTPs (Mao *et al*., 2015; Magureanu *et al*., 2021). Dosage also might have played a role in more genes being inactivated because there was an instance whereby 30 mg/L of chlorine were required for the removal of 90% of ARB and ARG, while only 3 mg/L of ozone was required for the same reduction (Gomes *et al*., 2019), which is a shortcoming as chlorination forms harmful by-products, such as halo-organics (Luukkonen *et al*., 2014; Anthony *et al*., 2020) and de-

chlorination is required before release to the environment as chlorine is toxic to the water life (Beber de Souza *et al*., 2015). In the WRP the *sul* and $bla_{SHV/TEM}$ genes were both reduced by 100%. This is because of the combination of the tertiary filtration and chlorine disinfection, which produced a synergistic effect resulting in additional removal of extracellular ARGs compared to chlorine treated pre-filtered samples (Quach-Cu *et al*., 2018). Furthermore, the concentration of chlorine used was 25 mg/L, which is much higher than the concentration used in practice, which rarely exceeds 2 mg/L (Umar, 2022).

2.3.2.2. Ozonation

Ozone is a bluish gas with a pungent smell and is an extremely reactive and unstable allotrope of oxygen. Ozone has been widely used in water treatment since the 19th century (Rekhate and Srivastava, 2020). Ozone is a powerful oxidant that is able to inactivate a wide range of pathogens, such as bacteria, including its spores, viruses, protozoa, and prion protein but gram-positive bacteria are less susceptible to ozone as compared to gram negative bacteria (Gomes *et al*., 2019; Feng *et al*., 2022). This is because zone primarily diffuses to the membrane and then penetrates it, generating increased permeability (Gomes et al., 2019; Wallmann *et al*., 2021; Feng *et al*., 2022). Ozone forms reactive oxygen species (ROS) which impact the metabolism of bacteria by oxidising critical enzymes in bacterial cells, destroying their genetic material and eliminating bacterial cellular function, ultimately leading to bacterial death (Jäger *et al*., 2018; Feng *et al*., 2022). The disinfection efficiency of ozone depends on the water quality, the contact time and the ozone concentration. In WWTP5, a dose of 1g ozone per g dissolved organic carbon (DOC) led to the decrease of *ermB*, *sul* and bla_{TEM} genes of 98.1%, 95% and 91% respectively (Jäger *et al*., 2018), as can be seen in Table4. There is a clear removal effect of ARGs by ozone but less of an effect is seen on *tet* and *sul* resistant genes (Feng *et al*., 2022). For 100% *sul* resistant gene removal, a dose of 3-3.5 g ozone/g DOC was applied which is 3-7.5 times higher than what is usually applied at WWTPs (Wallmann *et al*., 2021). The doze of ozone that is required for an effective disinfection is generally higher than the one leading to organic compounds degradation and chemical micropollutants removal. The high dose demand especially when the water comprises high amounts of organic matter and solids, adds to ozone's operational cost (Gomes *et al*., 2019). The combination of UV and ozone at the same time was also tested in WWTP5 but the treatment did not result in a more effective reduction compared to ozone treatment. The *ermB*, *sul* and bla_{TEM} genes were reduced by 98%, 96% and 91% respectively (Table 4) (Jäger *et al*., 2018). Due to bromate and the dangerous by-products during the partial oxidation of dissolved organic compounds formed after ozone treatment, UV light was not able to interpenetrate the ozone treated wastewater (Jäger *et al*., 2018; Gomes *et al*., 2019). At the NGWRP plant, activated carbon and ultrafiltration were subsequently applied after ozonation. Activated carbon did not remove the ARG but instead the gene abundance returned to the value

upstream of the main ozonation. Ultrafiltration then reduced the *sul* genes again to below LOD by a membrane cut-off of 40 nm but ultrafiltration tends to be non-destructive in nature, resulting in the retentate water having higher concentrations of ARB and ARG than the influent (Wallmann *et al*., 2021).

Table 4: Removal efficiency of ARGs by different disinfection methods

	Disinfection	***ermB***	***Sul***	***Tet***	***qnr***	***bla*$_{SHV/TEM}$**
WWTP1	Chlorination	99.8%	98%	96%	99%	
WWTP2	Chlorination	100%	93%	85%	96%	
WWTP3	UV		96%	86%		
WWTP4	UV		99%	62%		
WWTP4	UV and EC		100%	97%		
WWTP5	UV	30.2%	30%			50% INC
WWTP5	Ozone	98.1%	95%			91%
WWTP5	UV and Ozone	98%	96%			91%
NGWRP	Main Ozonation, Activated Carbon and Ultrafiltration	100%	100%			
WRP	Filtration and Chlorination		100%			100%
WRPF	AnMBR	100%	100%	99%		

2.3.2.3. Ultraviolet Radiation (UV)

UV disinfection is a well-known process used for the inactivation of pathogens (Gomes *et al*., 2019). UV inactivates ARGs by impairing the synthesis of RNA and DNA replication and leading to cell death, when it is absorbed by pyrimidine and purine nucleobases which cause DNA mutations (Barbosa *et al*., 2021). Its efficiency however depends on the type of microorganism considered as viruses and bacteria spores are the most resistant to inactivation by it, followed by intestinal protozoa, and lastly, by bacteria *(Gomes et al*., 2019). In WWTP7 the percentage resistance of THB resistance to AMP was reduced by 11% under a UV disinfection of 150-300 mJ cm^{-2} as can be seen in Table 5, because of THBs high tolerance to UV (Turolla *et al*., 2018). The concentration of the *sul* and *ermB* were reduced by only 30% and 30.2% respectively, in WWTP5 and *bla*$_{TEM}$ even increased by 50% after UV disinfection (Jäger *et al*., 2018), as can be seen in Table 4. With only UV irradiance of 20 mJ cm^{-2} the *sul* and *tet* genes were reduced by 99% and 62% respectively in WWTP4 as can be seen in Table 4 (Chen *et al*., 2020a). While in WWTP3, the UV

resulted in a reduction of the *sul* and *tet* genes by 96% and 86% respectively (Zhang *et al*., 2017). More *sul* genes were reduced than *tet* genes because *tet* genes are more resistant to UV (Jäger *et al*., 2018).

Table 5: Reduction of the percentage resistance of bacteria to antibiotics

	Disinfection	**ERM**	**QN(CIP)**	**STX**	**TRIM**	**TET**	**PEN**	**AMP**
WWTP6	PAA							16%
WWTP7	UV							11%
WWTP8	NaOCl							7% INC
NWWTP	Chlorination		6% INC			23% INC		23%
NGTW	Chlorination		15% INC			27% INC		7% INC
WWTP9	Chlorination			20% INC		50% INC		0%
WWTP10	Chlorination			40% INC		40% INC		10%
WWTP11	Chlorination			50% INC		80% INC		20%
WWTP12	Chlorination			0%		10% INC		0%
WWTP13	Chlorination						30% INC	40% INC
WWTP14	Chlorination	2.3% INC	0.9%		7.5% INC	6.1% INC	15.6% INC	7.4% INC
WWTP15	Chlorination	2.3%	1%		27.9%	23.3%	30.9%	36.8%

(INC=Increase)

The dose of UV exposure was increased from 80 to 400 mJ cm^{-2} which completely inactivated ARB but a high concentration of ARGs remained and the relative abundance of ARGs increased as UV dose increased (Zhang *et al*., 2017). This is because wastewater has high turbidity that influences the reduction efficiencies of UV, preventing the interpenetration of the UV light through wastewater. Further processing steps such as filtration for the removal of particles will enable attack of the residual contaminants by UV treatment (Jäger *et al*., 2018). The high dose required to achieve complete removal of ARGs would be impractical in WWTPs with a high concentration of ARGs (Zhang *et al*., 2017). For the complete inactivation of ARB and ARGs to happen with UV treatment, a secondary residual disinfectant is usually required *(Gomes et*

al., 2019). This was evident with UV with a low fluence of 20 mJ cm^{-2}, was able to remove all of the extracellular ARGs and reduce the *sul* and *tet* genes by 100% and 97% respectively. When it was treated subsequently with Electrocoagulation (EC) with a current density of 20.0mA/cm^2 at pH 7 for 60 min.

EC is the most commonly used Electrochemical disinfection technology which is eco-friendlier and more cost-effective compared with conventional disinfection methods. When iron-based EC is applied at pH 7.0, insoluble $Fe(OH)_2$ or $Fe(OH)_3$ species are released, which are responsible for the removal of ARB and ARGs. However, for EC to achieve a higher removal efficiency of ARGs, a higher current density would have to be applied, so that more hydroxides flocs can be formed (Chen *et al*., 2020a). PAA and NaOCl were tested as alternatives to UV but ARGs were effectively reduced by PAA rather than by NaOCl and UV radiation. Because PAA acts selectively on AMP resistant bacteria but UV and NaOCl do not act selectively on AMP resistant bacteria, displaying their disinfecting action with the same intensity on the whole bacterial community present. ARGs can also be effectively reduced by PAA rather than by UV radiation and NaOCl disinfection because PAA acts selectively on resistant micro-organisms, behaving as an effective barrier against ARB spread into the environment (Turolla *et al*., 2018). UV disinfection continues to be applied wildly around the world, in spite of all its disadvantages, including its expensive equipment (Gomes *et al*., 2019). Table 6 Summarises the advantages and disadvantages of the treatment methods discussed.

Table 6: Pros and Cons of treatment methods

Treatment Method	**Advantages**	**Disadvantages**
Chlorination	It is low in cost (Pillay and Olaniran, 2016; Anthony *et al*., 2020; Barbosa *et al*., 2021)	When E. *coli* is exposed to chlorine it induces a specific set of proteins, making them less susceptible to disinfection (Ateba *et al*., 2020)
	Chlorine diffuses into the intracellular component of the cell causing impairment to the cell membrane and cytoplasm (Anthony *et al*., 2020)	It does not permanently damage ARB/ARG and it results in high regrowth of bacteria (Pillay and Olaniran, 2016; Anthony *et al*., 2020)
EC	Eco-friendly and affordable (Crini and Lichtfouse, 2019; Chen *et al*., 2020a)	A higher current density would have to be applied for EC to achieve a higher removal efficiency of ARGs (Chen *et al*., 2020a).

	pH control is not necessary (Crini and Lichtfouse, 2019)	Requires post-treatment to remove high concentrations of iron ions and the sludge treatment is costly (Crini and Lichtfouse, 2019)
Membrane	It traps undissolved ARGs (Anthony *et al*., 2020)	There is minimal ARG removal when dissolved ARGs pass through the pores (Anthony *et al*., 2020)
	Anaerobic membrane bioreactors (AnMBRs) do not require energy for aeration and they produce less solids than aerobic system (Kappell *et al*., 2018).	Ultrafiltration tends to be non-destructive in nature, resulting in the retentate water having higher concentrations of ARB and ARG than the influent, resulting in fouling (Ebomah and Okoh, 2020a; Wallmann *et al*., 2021)
	AnMBRs produce methane that could be recovered for energy *(*Kappell *et al*., 2018).	Removal efficiency of membrane systems depends on the shape of the ARB, i.e. round shaped ARB will be efficiently retained while rod shaped ARB will be unretained on the permeate (Anthony *et al*., 2020)
	No chemicals required (Crini and Lichtfouse, 2019)	Investment costs are often too high for small and medium industries. With high energy requirements, maintenance and operation costs (Crini and Lichtfouse, 2019)
		Limited flow rates (Crini and Lichtfouse, 2019)
Ozone	Gram negative bacteria are more susceptible to ozone (Anthony *et al*., 2020).	Gram-positive bacteria are less susceptible to ozone (Anthony *et al*., 2020).
	It induces oxidative stress responses in surviving wastewater populations (Jäger *et al*., 2018)	The reduction efficiency of oxidative treatments is different in different species because of the presence of anti-oxidative mechanisms in those species. The disinfection efficiency of ozone depends on the water quality, the contact time and

		the ozone concentration (Jäger *et al*., 2018).
	The oxygen radicals interact with the cell surface (Ebomah and Okoh, 2020a; Wallmann et al., 2021)	The oxygen radicals rarely oxidize the interior contents of the cells *(Wallmann et al*., 2021)
	Generation of ozone on-site (no storage-associated dangers) (Crini and Lichtfouse, 2019)	Short half-life (Crini and Lichtfouse, 2019)
UV	UV inactivates ARGs by impairing the synthesis of RNA and DNA replication and leading to cell death (Barbosa *et al*., 2021)	UV tends to reduce the absolute abundance and increase the relative abundance of some ARGs and it also induces bacteria into VBNC state *(*Jia *et al*., 2021)
	UV causes a selective change in the inhibition zone diameters of ARB (Hasan Abusaiba and Al-Harmoosh, 2020; Wallmann *et al*., 2021)	tet genes are more resistant to UV treatment (Jäger *et al*., 2018)
		UV light cannot interpenetrate wastewater that has high turbidity (Jäger *et al*., 2018)

2.4. Factors affecting the efficiency of WWTPs for removal of ARB and ARGs from wastewater

2.4.1. Biotic factors

Biological processes create an environment that is conducive for the development and spread of ARB and ARGs (Umar, 2022). The most influential biotic factors that play an integral role in shaping compositional variations of the resistomes, in the influent and effluent of WWTPs are the morpho-functional metabolic and genetic factors of the indwelling bacterial community (Ju *et al*., 2019). For instance, biological removal of organic material from WWTPs is linked to the fast growth of bacteria and other microorganisms. At steady state after flocculation and the subsequent settling of biomass that is aggregated, the biomass is continuously removed as biological sludge. The sludge contains ARB and ARGs that were present in the biomass (Uluseker *et al*., 2021). It has thus been suggested that part of the reduction ARGs in WWTPs is because of the removal of biomass. It was also shown that reduction of biomass positively correlated to the reduction of *tetA* and *tetB* (Du *et al*., 2015). Moreover, bacteria biofilm formation has been observed as a very critical mechanism to ensure the resistance to environmental pressures and stress posed by treatment

methods in the WWTP. Here, secretion of certain molecules, such as adhesins and exopolymeric substances, or the extracellular appendages (pilli and fimbrae) that enable their adhesion to biosolids and sludge. A large amount of ARB and ARGs are removed with the sludge because they stick to inorganic and organic particle in WWTPs which are released with the sludge, thus evading certain treatments or reducing contact time with certain disinfectants (Grehs *et al*., 2021). The activated sludge of two WWTPs in the Northern part of China have been reported to contain thirty isolates of ARGs that give resistance to macrolides, sulfonamides, tetracyclines and quinolones (Li *et al*., 2022; Soni *et al*., 2022). The dry weight of the waste sludge was also reported to contain ARGs at the rate of about 1.5×10^9 to 2.2×10^{11} copies/g (Soni *et al*., 2022).

The most critical genetic factors that influences WWTP efficiency is the mobile genetic elements; this is because they influence bacterial evolution and adaptability (Li *et al*., 2022). However, of particular importance regarding antimicrobial resistance in WWTPs are Class 1 integrons (*intI1*), conjugal transfer protein, and resolvase (Ju *et al*., 2019). The central players in resistance dissemination are *intI1* which are one of the ten ARGs markers (Ju *et al*., 2019; Ghernaout, 2020; Igere *et al*., 2020). Different ARGs as well as the efflux pump gene qacE11 are encoded by the resistance cassettes within integrons. When one antibiotic applies selection pressure, it can select for ARGs related with multiple antibiotics within the gene cassette of the integron. This is because of the ability of the multi-gene cassettes to help co-selection and to encode different ARGs (Barancheshme and Munir, 2018). Upon conjugative plasmid transfer *intI1* get activated, allowing host bacteria to quickly develop antibiotic resistance (Barancheshme and Munir, 2018; Ju *et al*., 2019; Igere *et al*., 2020). Moreover, *intI1* has been suggested as a general indicator of resistance since plasmid-mediated resistance in microbes and metal resistance has been reported in WWTPs. This is because the WWTP environment constantly changes and bacteria are exposed to chemical stress by antibiotics, heavy metals and or both, resulting in the co-selection of ARGs increasing (Ju *et al*., 2019; Igere *et al*., 2020; Umar, 2022). Hence the increase of *intI1* being reported in the effluents of WWTPs (Umar, 2022).

2.4.2. Abiotic factors

There are numerous abiotic factors that facilitate resistance in WWTPs such as salinity, temperature, oxidation reduction potential, pH and electric conductivity (Igere *et al*., 2020). In a WWTP that uses activated sludge system, an imbalance of antimicrobial agents in the waste, inorganic nitrogen, pH and dissolved oxygen play an additional role in the proliferation of resistome (Ju *et al*., 2019; Igere *et al*., 2020). The oxygen and nutrients are substantially consumed by activated sludge biomass and may thus act as driving forces for both community and resistome composition (Ju *et al*., 2019). Antibiotics become

susceptible to abiotic transformation when they are exposed to temperature, pH, and light. β-lactams are sensitized to degradation by the heat, light, and extreme pH, while methanol and water rapidly hydrolyse them. Therefore, despite their dominant presence in sewage influent, β-lactams are not detected in WWTP effluent (Bombaywala *et al*., 2021). Many ARB are strictly aerobic or facultative bacteria, neutrophilic, mesophilic, and chemoorgano heterotrophic, and their ability to thrive and multiply is determined by the adequate balance of all these variables (Manaia *et al*., 2018). Change in season has been shown to result in fluctuation of antibiotics and ARGs in WWTPs. During spring there are higher release loads of ARGs and ARB than those during winter months in effluent of WWTPs. The abundance of ARGs of tetracycline, sulfonamides, and vancomycin were abundantly higher in winter than in summer, while *mecA*, *tetA*, and *tetB* do not change with seasons (Du *et al*., 2015). High temperatures or high pH are said to be more effective for the removal of ARGs or *intI 1* than conventional treatments or low temperatures. Plausibly, the conditions that will contribute to the removal of ARB and ARGs are temperature or pH values veering from the neutral pH range and temperatures where most ARB thrive (Manaia *et al*., 2018). Most of the studies focus on the comparison of disinfection processes hence there is no evidence gathered of a specific measurement of a bio-physico-chemical condition or factor that can result in the inactivation of ARB and ARGs in WWTPs (Manaia *et al*., 2018; Pallares Vega *et al*., 2019).

2.5. Advanced Oxidation Processes (AOPs): a better alternative for removing ARB and ARGs in WWTPs

Advanced oxidation processes (AOPs) are a set of chemical processes that are able to decompose recalcitrant organics or persistent organic compounds, pharmaceuticals and heavy metals from wastewater which conventional technologies cannot degrade (Foster, 2017; Giannakis *et al*., 2017; Tichonovas *et al*., 2017; Rekhate and Srivastava, 2020; Courti *et al*., 2022) and they also enhance the biodegradability of wastewater (Giannakis *et al*., 2017; Rekhate and Srivastava, 2020; Courti *et al*., 2022). AOPs are able to do this by producing oxygen radicals and large quantities of the powerful, non-selective (•OH) which act as the oxidative agents (Foster, 2017; Giannakis *et al*., 2017; Tichonovas *et al*., 2017; Rekhate and Srivastava, 2020; Courti *et al*., 2022). The different types of AOPs which are discussed below are Fenton based reactions, UV-based reactions, Ozone based reactions (Giannakis *et al*., 2017; Rekhate and Srivastava, 2020; Courti *et al*., 2022) and plasma-based reactions (Courti *et al*., 2022).

2.5.1. Theory and mechanism of Fenton

Fenton oxidation is one of the AOPs widely used for wastewater and water treatment that was discovered in 1894 and named in honour of Fenton H.J.H (Giannakis *et al*., 2017; Chen *et al*., 2020c; Akbari *et al*.,

2021; Bracamontes-Ruelas *et al*., 2022). Fenton discovered that the reaction of ferrous (Fe^{2+}) salts with hydrogen peroxide (H_2O_2) could oxidize tartaric acid (Bracamontes-Ruelas *et al*., 2022). During Fenton process, H_2O_2 and Fe^{2+} salts (catalyst) are added into wastewater at the same time under acidic conditions to produce reactive oxygen species such as superoxide radicals ($O_2^{\cdot-}$) and singlet oxygen ($^1O_2$) and •OH (Chen *et al*., 2020b; Chen *et al*., 2020c; Akbari *et al*., 2021; Bracamontes-Ruelas *et al*., 2022). •OH, is the main species that plays the important part of degradation of organic pollutants and the elimination of ARB and ARGs (Chen *et al*., 2020b; Chen *et al*., 2020c; Cuerda-Correa *et al*., 2020). When Fenton treats ARB, the cell surface gets distorted lead to the loss of cell permeability, swelling and rupture of cells and ultimately the leakage of cell components (Chen *et al*., 2020c). The direct oxidation by •OH results in the removal of extracellular ARGs (Giannakis *et al*., 2017).

The advantage of Fenton process is that it is easy to operate, high degradation of toxic compounds, Fe is abundant in nature and has low inherent toxicity, H_2O_2 is environmentally safe and easy to handle and it decomposes spontaneously to H_2O and O_2 (Giannakis *et al*., 2017; Chen *et al*., 2020c; Akbari *et al*., 2021). However, different parameters like pH, temperature and H_2O_2 and Fe^{2+} concentrations influence the degradation process. Fenton oxidizing method is limited to acidic condition (Akbari *et al*., 2021). The process is limited by the strict acidic operational condition (pH =3-3.5) because less amount of the •OH is produced due to the formation of Fe^{2+} complexes with water at a lower pH (<2.5) or the precipitation of ferric oxyhydroxides at a higher pH (>4) (Chen *et al*., 2020b, Chen *et al*., 2020c). Maximum reduction of 2.58 to 3.79 logs are achieved for ARGs at pH 3 as compared to 2.26 to 3.35 logs reduction at pH 7 (Michael-Kordatou *et al*., 2018). Depending on the nature of the pollutant and the wastewater in which it is found, this pH range may not degrade some pollutant (Bracamontes-Ruelas *et al*., 2022). A basic pH is not an option for Fenton oxidation because the iron would catalytically decompose H_2O_2 into oxygen and water, without forming •OH (Cuerda-Correa *et al*., 2020). The concentration of H_2O_2 and Fe^{2+} are the main factors that determine the inactivation efficiency of the Fenton process (Chen *et al*., 2020b; Chen *et al*., 2020c). Fe^{2+} is regenerated, so that the Fenton process can be regarded as catalytic with respect to iron. As the iron concentration increases, the oxidation rate of organic compounds increases as the iron concentration increases to a point at which an additional increase in iron concentration is ineffective (Cuerda-Correa *et al*., 2020). A study showed that a molar ratio Fe^{2+}/ H_2O_2 from 0.033 to 0.1, results in an increase in the removal efficiency. The t*etG* genes are less susceptible to Fenton oxidation as compared to *int1*. *E. coli* resistant to ampicillin, ciprofloxacin and tetracycline is completely inactivated at a ratio concentration of Fe^{2+}/ H_2O_2 5:10 (Michael-Kordatou *et al*., 2018). Small increments of H_2O_2 dose result in evident decreases in the toxicity of the effluent once a minimum threshold has been reached (Cuerda-Correa *et al*., 2020). A lack of H_2O_2 may decrease the performance of the Fenton oxidation to treat wastewater while high concentrations of H_2O_2 leads to the scavenging of ·OH (Cuerda-Correa *et al*., 2020; Bracamontes-Ruelas

et al., 2022). The reaction rate in the Fenton process increases between 20–40◦C, the effect being more noticeable at temperatures below 20 ◦C, the effectiveness of the reagent decreases between 40–50 ◦C. This is due to the accelerated decomposition of H_2O_2 into oxygen and water (Cuerda-Correa *et al*., 2020).

The disadvantage of using Fe^{2+} salts is that it results in a large amount of iron-containing sludge, which is hard to recover or remove, causing high operational cost and secondary pollution (Chen *et al*., 2020c; Cuerda-Correa *et al*., 2020; Akbari *et al*., 2021). For this reason, the homogeneous Fenton-like method was developed to reduce iron species dissolved in the environment, without critically affecting the effciency of the process and to overcome the problems of Fenton oxidation (Cuerda-Correa *et al*., 2020; Akbari *et al*., 2021). The homogeneous Fenton-like method uses Fe^{3+} instead of the more expensive Fe^{2+} salts (Cuerda-Correa *et al*., 2020). The Fe^{3+} salt reacts with H_2O_2 which decomposes into •OH, and the Fe^{3+} is reduced to Fe^{2+}, and its catalytic process occurs throughout the liquid phase (Cuerda-Correa *et al*., 2020; Bracamontes-Ruelas *et al*., 2022). For most applications, the catalyst cycle starts quickly if organic material and H_2O_2 are in sufficient concentration regardless of whether Fe^{2+} or Fe^{3+} is used; (Cuerda-Correa *et al*., 2020). A homogeneous Fenton-like process is easy to operate and is effective in terms of pollutant removal as the circular use of the catalyst reduces Iron rich sludge. However, excessive sludge is still produced and the operational pH is limited to below 3 (Cuerda-Correa *et al*., 2020; Akbari *et al*., 2021). The other disadvantage of the homogeneous Fenton-like method is that Fe^{3+}/H_2O_2 results in slow decomposition rate of H_2O_2 and slower oxidation rate of organic solutes are markedly slower than when Fe^{2+}/ H_2O_2 is used (Cuerda-Correa *et al*., 2020).

The heterogeneous Fenton-like was developed to solve the problems presented by the homogeneous Fenton-like process (Cuerda-Correa *et al*., 2020; Akbari *et al*., 2021; Bracamontes-Ruelas *et al*., 2022). In heterogeneous Fenton oxidation, a reaction takes place between H_2O_2 and Fe (III) in different forms, e.g., Fe_2O_3 or α-FeOOH or zero-valent iron (ZVI), etc. (Cuerda-Correa *et al*., 2020; Akbari *et al*., 2021). If solid catalysts are used, sludge generation is reduced as physical adsorption occurs at the surface of the solid catalyst (Cuerda-Correa *et al*., 2020; Bracamontes-Ruelas *et al*., 2022). Heterogeneous Fenton-like processes is gaining importance, but it appears to be less effective than a homogeneous Fenton process due to mass-transfer limitation (Cuerda-Correa *et al*., 2020). For Fenton-like processes, a high concentration of catalyst will consume •OH, limiting practical application and prevent the degradation of pollutant and therefore raising treatment costs. Low concentration of H_2O_2 leads to low availability or lack of •OH and reduce degradation efficiency. Excessive concentration of H_2O_2 is also not appropriate for removal of contaminants in Fenton-like process (Akbari *et al*., 2021). It should be clarified that if the wastewater contains heterogenous cocktails apart from the target pollutant to be treated, the other contaminants may

decrease the removal performance of the target pollutant since Fenton AOP is a non-selective treatment process (Bracamontes-Ruelas *et al*., 2022).

2.5.2. Theory and mechanism of UV assisted Advanced Oxidation Process

2.5.2.1. UV/hydrogen peroxide

To effectively control HGT and ARGs, combination of UV technology with different radical promoters have been investigated. Some of the most researched AOPs include UV/chlorine, H_2O_2/ Fe^{2+}/ UV (photo-Fenton), UV/H_2O_2, UV/O_3, UV/peroxydisulfate (PDS), UV/peroxymonosulfate (PMS), and UV/TiO_2 (Sharma *et al*., 2019; Umar *et al*., 2019). Among all these combinations, the most widely researched in water and wastewater treatment at small scale is UV/H_2O_2 (Umar *et al*., 2019; Umar, 2022). A few full-scale investigations have been done in recent years, using UV/ H_2O_2 to damage ARGs (Umar, 2022). UV/ H_2O_2 inactivates ARB and ARGs by production of radicals by UV/ H_2O_2 when it receives radiation energy (Grehs *et al*., 2021, Li *et al*., 2022). During photolysis two HO• radicals are produced per photon absorbed by H_2O_2 (Giannakis *et al*., 2017, Umar, 2022). The efficiency of the process strongly depends on the oxidative ability and production velocity of the •OH (Giannakis *et al*., 2017; Umar *et al*., 2019). The •OH can attack electron-rich organic contaminants at high rate constant and ultimately leads to their transformation to CO_2 and H_2O (Sharma *et al*., 2019; Umar *et al*., 2019). The •OH inactivates the ARB by making the oxidation potential of the chemical system better, leading to modifications in the bacterial cell structure (Ghernaout, 2020).

The other main factor which will affect the inactivation of ARB and ARGs in the UV/ H_2O_2 processes are the UV absorption, the more ARB absorbs UV, the more internal components get damaged and hence gets inactivated (Giannakis *et al*., 2017; Michael-Kordatou *et al*., 2018). Hence, when the UV absorbance of the target pollutant is high or when strong photon absorbers are present, the efficiency of UV/ H_2O_2 process significantly decreases (Giannakis *et al*., 2017). The UV fluence required for a real-water matrix with organics could be very high, therefore wastewater would have to be pre-treated prior to UV AOPs for the removal of contaminants to improve the process performance (Umar, 2022). A UV fluence of 50 - 130 mJ/cm^2 for UV/H_2O_2 achieves 4 logs reduction of ARGs (Sharma *et al*., 2019, Ghernaout and Elboughdiri, 2020a). The UV fluence delivered to clear water is 1.4 folds higher than that delivered to wastewater (Umar, 2022). Other important factors include the concentration of H_2O_2 and of the target compound, the pH of the matrix, the presence/absence of scavenging compounds (e.g., bicarbonates) and the reaction time (Giannakis *et al*., 2017; Umar, 2022). With a concentration of 20-25 mg/L of H_2O_2, antibiotic resistant *E.coli* and *tetW* were deactivated after 240 min while the blaTEM gene was still present at 540 min. A pH of 3 which is practically not feasible and a high H_2O_2 concentration of 340 mg/L are considered best for

damaging ARGs. This was evident as 2.8-3.5 logs of sul1, tetG and tetX were reduced at these conditions within 30 min, with a higher reduction of the tetracycline than sulphonamide genes as compared to 1.55 to 2.32 logs reduction at wastewater pH of 7 (Michael-Kordatou *et al*., 2018; Umar, 2022). The reduction of *ampC* and *mecA* was approximately 2.3–2.9- and 1.4–2.7-logs, respectively, with different concentrations of H_2O_2 of (340, 1700, and 3400 mg/L) for a UV fluence of 120 mJ/cm^2 (Umar, 2022). The inactivation of ARGs is generally lower than that of *E.coli* (Michael-Kordatou *et al*., 2018; Sharma *et al*., 2019).

With UV/ H_2O_2 treatment inactivation of ARB is faster than the damages to ARGs. An increase in pH does not have any influence on damage to ARGs by UV/ H_2O_2 (Sharma *et al*., 2019). Lower amounts of i-ARGs are generally inactivated as compared to e-ARGs because of the preservative functions of cellular components versus UV and the scavenging of •OH and oxidising species by cellular components (Sharma *et al*., 2019; Ghernaout and Elboughdiri, 2020a). The high reaction between •OH and H_2O leads to scavenging of •OH and hence •OH cannot penetrate the cell (Sharma *et al*., 2019; Umar *et al*., 2019). Removal of residual H_2O_2 after treatment is could be beneficial to reduce scavenging of •OH since only approximately 5–10% of the H_2O_2 is used during the treatment process (Umar *et al*., 2019). Organic matter in complex water matrices also scavenge radicals leading to reduced oxidation efficiency of •OH and hence fairly similar rates of damage of e-ARG damage by UV-only and UV/H_2O_2 treatments (Umar, 2022).

2.5.3. Theory and mechanism of Ozone assisted

2.5.3.1. Ozonation and UV radiation

The different types of ozone based AOPs that are used for water and wastewater treatment are O_3/ H_2O_2, O_3/UV and O_3/UV/ H_2O_2 (Chen *et al*., 2020c; Cuerda-Correa *et al*., 2020; Rekhate and Srivastava, 2020). A recent study which is apparently the first to compare the effectiveness mentioned ozone AOPs for the inactivation of ARB from real sewage treatment plant (STP) wastewater. The study revealed that O_3/UV resulted in better inactivation rates of ARB than the other ozone AOPs (Chen *et al*., 2020c). There are however relatively few studies done in the literature devoted to the removal of pollutants by O_3/UV processes in comparison with other ozone AOPs (Igere *et al*., 2020). In O_3/UV the wavelength is less than 300 nm because ozone strongly absorbs UV light of wavelength λ = 254 nm (Cuerda-Correa *et al*., 2020; Bracamontes-Ruelas *et al*., 2022). The •OH are produced by the reaction of O• with H_2O after the photolysis of ozone (Rekhate and Srivastava, 2020). The •OH can also be generated indirectly by the O_3/UV combination. The ozone is dissolved and splits, a fast reaction of atomic oxygen (O) with H_2O takes place, producing thermally excited H_2O_2 which then results in the production of •OH (Cuerda-Correa *et al*., 2020; Bracamontes-Ruelas *et al*., 2022). The •OH react with the organic substances while UV radiation speeds up the kinetics of the process. UV radiation also degrades some compounds by direct photolysis and it

excites the organic molecules of the pollutant, increasing their vulnerability towards an attack by the •OH (Cuerda-Correa *et al*., 2020).

Within a minute, over 99% inactivation of carbapenem-resistant Enterobacteriaceae (CRE), extended spectrum β-lactamase producing Enterobacteriaceae (ESBL-E), multidrug-resistant Acinetobacter (MDRA), multidrug-resistant Pseudomonas aeruginosa (MDRP), methicillin-resistant Staphylococcus aureus (MRSA), and vancomycin-resistant Enterococcus (VRE) was inactivated by the O_3/UV combination. The inactivation rates after half a minute of treatment were 94% and for CRE, 87% for ESBL-E, 32% for MDRA, 94% for MDRP, 50% for MRSA, and 94% for VRE, respectively (Chen *et al*., 2020c). The efficiency of production of •OH is however low by the O_3/UV combination due to low photolysis efficiency (Umar *et al*., 2019; Bracamontes-Ruelas *et al*., 2022). The combination is also expensive since ozone generators and UV lamps consume large amounts of electric energy (Cuerda-Correa *et al*., 2020; Bracamontes-Ruelas *et al*., 2022). Ozone also low solubility in water which results in mass transfer limitations (Umar *et al*., 2019). Mostly a toxicity monitoring is required since the ozone AOPs result in problematic oxidation by-products (Ghernaout and Elboughdiri, 2020b). However, the good thing about O_3/UV is the fact that the generation of bromate is inhibited (Cuerda-Correa *et al*., 2020). Depending on the pollutants to be treated in the wastewater by O_3/UV combination, the optimum ozone dosage and radiation exposure need to be optimised, simultaneousness with pH (Bracamontes-Ruelas *et al*., 2022).

2.5.4. Theory and mechanism of Cold Atmospheric Plasma

Plasma is the fourth basic state of matter after solid, liquid and gas (Ghernaout, 2020; Sanito *et al*., 2022). Plasma is a partially or completely ionized gas and an electroneutral mixture carrying free reactive radicals (including reactive nitrogen and oxygen species), charged particles, electrons, ions, UV photons and quanta of electromagnetic radiation (Ghernaout, 2020; Giacometti *et al*., 2021; Courti *et al*., 2022). The ionized gas is generated by applying thermal energy, or electromagnetic radiation energy, or mostly an electric field to a carrier gas at atmospheric pressure and room temperature (von Wintersdorff *et al*., 2016; Giacometti *et al*., 2021). The reactive species may be generated in bubbles in the liquid or directly in liquid, water surfaces, aerosols and clusters (Jamróz *et al*., 2014; Sanito *et al*., 2022). Plasmas can be divided into thermal and non-thermal depending on the thermal equilibrium between the electrons (Te) and gas (Tg). Non-thermal plasma is out of thermodynamic equilibrium (Te >> Tg) while thermal plasmas are in thermodynamic equilibrium (Te=Tg) (Courti *et al*., 2022). Non-thermal plasma is also known as cold atmospheric plasmas (CAPs), atmospheric pressure plasmas (APPs) or Non-equilibrium atmospheric pressure discharge (NEPD (Ghernaout and Elboughdiri, 2020b).

CAP is an emerging technology, that has been frequently applied in sterilization, waste water treatment., cleaning and bio-decontamination, to inactivate bacteria in water and food produce, as a fertilizer and in chemical reduction (Jamróz *et al*., 2014; Li *et al*., 2015; Royintarat *et al*., 2019). CAP is one of the advanced oxidation processes (AOPs), possessing both chemical and physical processing traits (Li *et al*., 2015). It is however environmentally friendly as it does not inject poisonous chemicals, does not form waste or toxic by-products and it neutralizes pathogens. It also has simpler equipment which is secure and easy to operate and has higher energy efficiency (Jamróz *et al*., 2014; Ghernaout, 2020). CAPS can reduce the number of bacteria in both gram-negative and gram-positive bacteria as it makes use of permeabilization of the cell membrane or cell wall as one of the mechanisms of inactivation of ARBs and ARGs (Li *et al*., 2015; Niedźwiedź *et al*., 2019; Royintarat *et al*., 2019). Permeabilization by overcoming the tensile strength of the membrane causing it to rupture at a point of small local curvature and ultimately leading to leakage of intracellular components. Then irreversible oxidative damage to intracellular proteins and DNA occurs, which leads to cell death, preventing the growth of ARBs and ARGs (Ercan *et al*., 2016; Niedźwiedź *et al*., 2019; Rezaei *et al*., 2019). This is all due to the reactive species of CAP which are classified as primary species, secondary species and tertiary species (Sanito *et al*., 2022).

The primary species, ROSs and RNSs oxidize nucleic acids, proteins, and lipids (Ghernaout, 2020), but ROS is the main component which reacts with lipid cell membranes and then lipid peroxides will damage DNA and protein permanently. More ROS are generated under water, which results in bacteria being killed more efficiently than those above water (Royintarat *et al*., 2019). The shock waves help to mix the liquid, enhancing the efficiency of CAP in the sterilization process (Niedźwiedź *et al*., 2019; Rezaei *et al*., 2019). UV which is also a primary species that deteriorates nucleic acids (Ghernaout, 2020) and is involved in the generation of secondary species such as •OH (Niedźwiedź *et al*., 2019; Rezaei *et al*., 2019; Royintarat *et al*., 2019; Anthony *et al*., 2020). •OH, are highly reactive oxidant agents that play the most important role in the removal of organic pollutants, oxidization of organic and inorganic compounds and as disinfectants (Beber de Souza *et al*., 2015; Magureanu *et al*., 2021).

The primary species have short lifetimes, but they contribute to degradation more than long lived ones which have a less dominant effect (Magureanu et al., 2021). When ROS and RNS dissolve in liquid, they form ozone (O_3), hydrogen peroxide (H_2O_2), nitrate (NO_3^-) and nitric oxide (NO^-), which are tertiary, long-lived species (Sanito *et al*., 2022). These species result in a decrease in the pH of the target liquid of up to 2 pH values (von Wintersdorff *et al*., 2016). At low pH, species reduce the resistance of bacteria to an acidic environment, which helps in better penetration of species into the bacteria cell wall (Royintarat *et al*., 2019). The long-term, post-plasma effect is mainly caused by the reaction between H_20_2 and ozone during the peroxone process, that forms •OH (Magureanu *et al*., 2021).

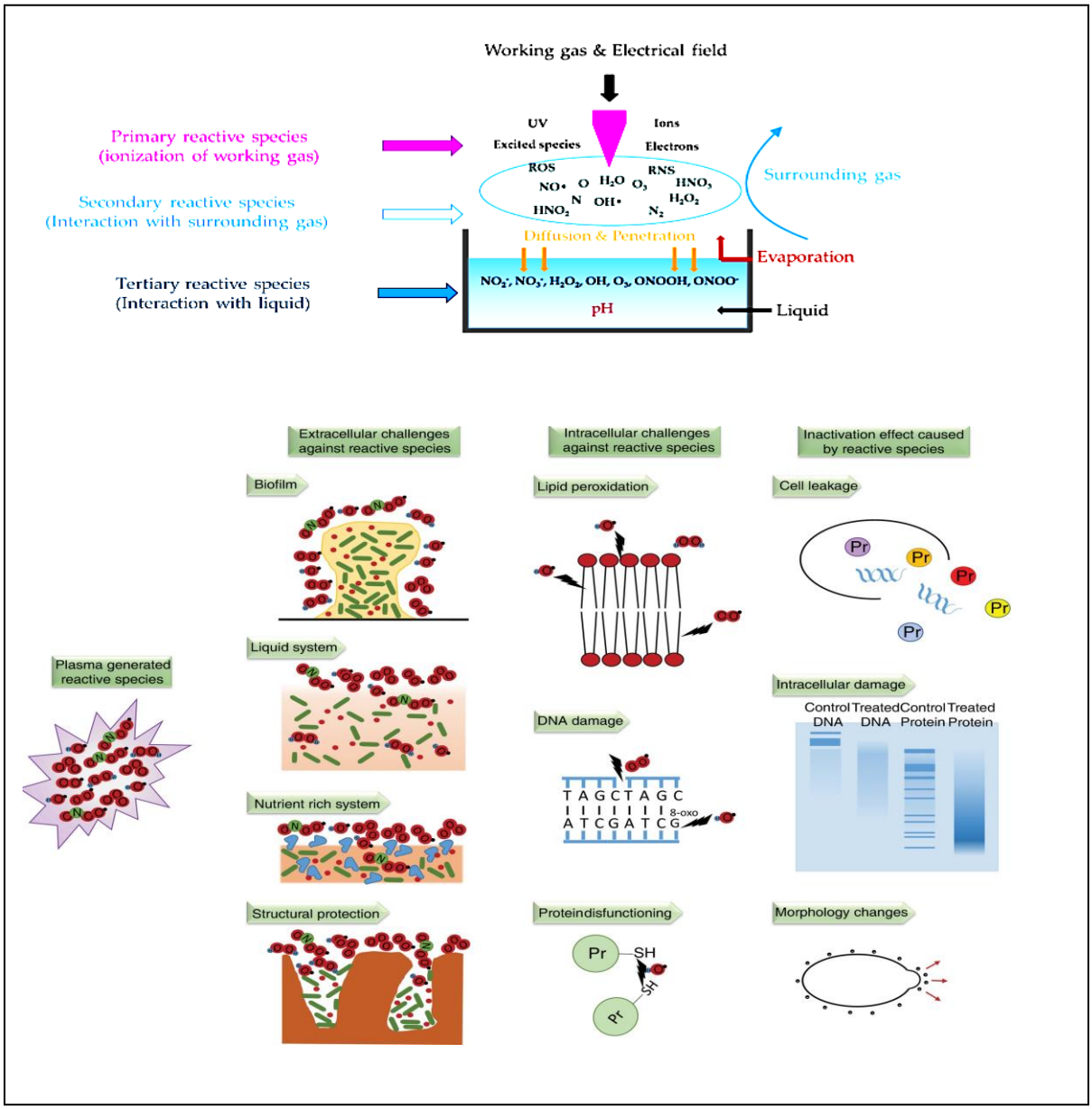

Figure 2: Mechanisms of plasma inactivation of ARBs and ARGs in liquid showing the reactive species released into pollutant containing aquatic matrix and their subsequent interaction with microbial communities and morphological and genetic components (Bourke *et al.*, 2017; Courti *et al.*, 2022)

When plasma is applied above liquids, a 5log reduction of bacteria and 0.19-log degradation for i-mecA genes is achieved with a low plasma influence of 0.12 kJ/cm^2. To achieve 1 log reduction of i-mecA, a plasma treatment of more than 0.53 kJ/cm^2 is required, while only 0.12 kJ/cm2 only could result in more than 1-log degradation of e-mecA. A plasma intensity of 0.35 kJ/cm2, reduces e- and i-mecA genes by 2.6 and 0.8 logs, respectively (Liao *et al*., 2018a; Courti *et al*., 2022). ARB are less difficult to inactivate than ARGs. Vancomycin-resistant enterococci (VRE) count reduced by more than 5 log, below the detection limit, while vanA resistance genes remained in the order of 10^5 copies/mL even though it showed a reduction of more than 4 log (Furukawa *et al*., 2022). Higher inactivation requires a longer treatment time which is proportional to the applied energy of CAP (Liao *et al*., 2017b). Longer time of 30 min increases gene reduction in tetA, tetR, aphA, and tnpA was increased to 5.8, 5.4, 5.3, and 5.5 log, respectively (Courti *et al*., 2022). To shorten the time, a higher frequency could be used. The initial voltage is the most important for inactivation of ARB and ARGs (Furukawa *et al*., 2022). A higher plasmas voltage of 18 kV reduces resistant *E. coli* by approximately 6.3 log, while a lower voltage of 10 kV reduced the bacteria by 4.4 log. A higher flow rate of 2.5 L min^{-1} reduced resistant *E. coli* by 7 log while a flow rate of 1.5 L min-1 reduced the bacteria by only 5.5 log in 10 min (Courti *et al*., 2022). The fixed gap distance and the input power also affect inactivation. For example, it takes only takes 30s to inactivate S. aureus under a 2 mm gap distance at 60 W. A larger gap results in less inactivation, for instance inactivation of *E. coli* increased from 80% to 99,93% after 3 min treatment when the gap was reduced from 5 to 3 cm (Liao *et al*., 2017b).

2.5.4.1. State-of-the-art of CAP

The trend of the studies with the use of CAP for the inactivation of ARB and ARGs can be seen in Figure2, and the publications are shown in Table 7. There isn't much information about CAP for the inactivation of ARB and ARGs in wastewater. Most publications out there focused on the removal of non-resistant bacteria and wound treatments in medicine.

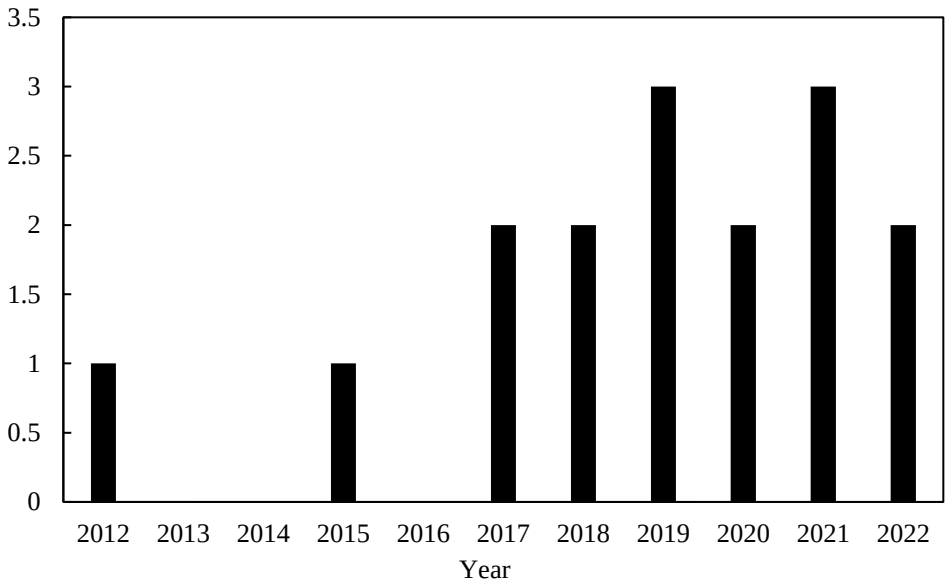

Figure 3: Trends articles of CAP for inactivation of ARB and ARGs in water treatment

Table 7: Sample of studies done using CAP for treatment of Wastewater

Journal articles and patents	Year	Reference
Nonthermal Atmospheric Plasma Rapidly Disinfects Multidrug-Resistant Microbes by Inducing Cell Surface Damage	2012	(Kvam *et al.*, 2012)
A comparative study for the inactivation of multidrug resistance bacteria using dielectric barrier discharge and nano-second pulsed plasma	2015	(Hoon Park *et al.*, 2015)
Effect of preliminary stresses on the resistance of Escherichia coli and Staphylococcus aureus toward non-thermal plasma (NTP) challenge	2017	(Liao *et al.*, 2017a)
Lethal and Sublethal Effect of a Dielectric Barrier Discharge Atmospheric Cold Plasma on Staphylococcus aureus	2017	(Liao *et al.*, 2017b)
Application of a Dielectric Barrier Discharge Atmospheric Cold Plasma (Dbd-Acp) for Eshcerichia Coli Inactivation in Apple Juice: Inactivation of E. coli by cold plasma…	2018	(Liao *et al.*, 2018b)
Combating Staphylococcus aureus and its methicillin resistance gene (mecA) with cold plasma	2018	(Liao *et al.*, 2018a)
Degradation kinetics of cold plasma-treated antibiotics and their antimicrobial activity	2019	(Sarangapani *et al.*, 2019)

Study on the killing effect of cold atmospheric pressure plasma on MRSA Staphylococcus aureus in vitro and in vivo infection model	2019	(Namini *et al*., 2019)
Degradation of antibiotic resistance contaminants in wastewater by atmospheric cold plasma (ACP): Kinetics and mechanisms	2019	(Liao *et al*., 2019)
Antibiotic-Resistant and Non-Resistant Bacteria Display Similar Susceptibility to Dielectric Barrier Discharge Plasma	2020	(Sakudo and Misawa, 2020)
Inactivation of antibiotic resistant Escherichia coli and degradation of its resistance genes by glow discharge plasma in an aqueous solution	2020	(Yang *et al*., 2020)
Cumulative damage by nonthermal plasma (NTP) exceeds the defense barrier of multiple antibiotic-resistant Staphylococcus aureus: a key to achieve complete inactivation	2021	(Liao *et al*., 2021)
Cold Atmospheric-Pressure Plasma Caused Protein Damage in Methicillin-Resistant *Staphylococcus aureus* Cells in Biofilms	2021	(Guo *et al*., 2021)
Plasma induced efficient removal of antibiotic-resistant Escherichia coli and antibiotic resistance genes, and inhibition of gene transfer by conjugation	2021	(Li *et al*., 2021)
Inactivation of antibiotic resistant bacteria and their resistance genes in sewage by applying pulsed electric fields	2022	(Furukawa *et al*., 2022)
Degradation of Bacterial Antibiotic Resistance Genes during Exposure to Non-Thermal Atmospheric Pressure Plasma	2022	(Courti *et al*., 2022)

2.5.4.2. Challenges

Although the aforementioned wastewater treatment methods in our study have demonstrated several potentials of repressing the concentrations of antibiotics, ARBs and ARGs in wastewater streams, researchers are likewise concerned about their environmental sustainability and unregulated ecotoxicology. For instance, during chlorination, disinfection intermediates or transformation products may be formed from the earliest interactions with organic and inorganic matter before subsequent contacts with the pathogenic microflora, Consequently, this phenomenon elevates the ecotoxicity of the final receiving natural water bodies (Yang *et al*., 2019; Li *et al*., 2023); worse still, it enhances the selection of chlorine tolerant bacteria as well as confer multiple resistance through the exchange of other virulence genes. As earlier mentioned, the lesser contact time with original chlorine concentrations and more contact time with by-products afford the indwelling bacteria resistance mechanisms, whereby their cell walls are at worst made porous to receive the floating DNA of other dead chlorine-susceptible ARB species (Luo *et al*., 2021). Similar to chlorination, the efficiency of other treatment methods is heavily reliant on dosage, exposure

time and even the physicochemical properties of the wastewater matrix. In this regard, the interactions of radicals generated from treatment techniques like AOPs, ozonation and UV radiation with the organic and inorganic matter interfere with their optimal contact with ARBs and ARGs, thereby preventing their complete inactivation. In order to achiever near complete inactivation using these methods, a high dosage and exposure would be warranted, which is not only impracticable in ARB and ARG-laden wastewater matrices but also might distort the biogeochemical cycle of natural water bodies downstream of the WWTP, thereby creating ecological imbalance, and ultimately having adverse impacts on humans through the food-water nexus. In order to sustainably mitigate these occurrences, more environmentally and cost-friendly alternatives should be adopted, such as enzymatic biodegradation of pollutants as well as the bio electrochemical treatment of the ARB-ARGs cocktails, which could also be coupled with inexpensive acute DNA-binding filtration techniques. Moreover, optimizing the synergistic effect of extant advanced treatment technologies and green technologies would ensure well-balanced inactivation or elimination of ARBs and ARGs.

There are few publications on the study of CAP as a disinfection method for ARB and ARGs in wastewater. The studies that have been conducted on CAP are laboratory scale (Crini and Lichtfouse, 2019), There is lack of research devoted to the upscaling to industrial level (Tichonovas *et al*., 2017), the efficiency may not be the same when tested in full scale WWTPs. CAP affects the properties of the exposed surfaces on the upper layer of liquids, it is impossible to store and unlike UV radiation, it has a non-remote action (Scholtz *et al*., 2021). Intracellular (i-)antibiotic resistance genes (ARGs) require higher plasma intensity was for degradation as compared to extracellular (e-)ARGs because of the shielding effects of the outer envelopes or intracellular components against plasma (Liao *et al*., 2018a; Liao *et al*., 2019).

2.6. Conclusions and future perspectives

It is evident that the urgency required for tackling antibiotic resistance cannot be overemphasized, especially due the consistent change in dynamics involved; however, the constancy of occurrence of antibiotics, ARBs and ARGs in WWTPs clearly imply the poor regulation or abuse of certain classes of antibiotics. It is therefore necessitous that drastic regulatory measures are imposed on manufacturers and healthcare settings (regarding wastewater treatment and discharge) as well as proper education of potential consumers to practice safe and ethical consumption of antibiotics.

Using the WWTP-antibiotics consumption-ARBs-ARGs nexus, it is apparent that WWTPs not only serve as sinks but also as intelligent epidemiological and community diagnostic tools; therefore, regional and global databases should be setup based on consistent research-based information, in order to assist policy

makers, engineers and citizens in the campaign against the prevalence of AMR and associated medical inconveniences they may cause. Ultimately, prevention of such incidences is insufficient as there already exist copious amounts of constantly evolving ARGs and ARBs; hence the prompt intervention of wastewater treatment technologies is necessitated.

From the extant treatment techniques appraised, we observed the potential contribution of chlorination to the abundance of ARBs and ARGs, whilst reducing the total load of microbes, as well as further inducing selective pressure through the formation of harmful intermediates, such as halo-organics that correspondingly adversely impact aquatic fauna. Also, ozone, though needing a lower dose to achieve the same efficiency as chlorination is rendered ineffective by Gram-positive bacteria and the environmentally critical *tet* and *sul* genes, and hence need a higher dose to guarantee 100% removal. However, this comes at the expense of bromate production and other toxic intermediates during the partial oxidation of dissolved organic compounds. All other techniques, such as Fenton- and UV-mediated treatments have major drawbacks as their aforementioned confrere, such as secondary pollution, formation of toxic intermediates or impracticability of total removal of ARBs and ARGs.

Cold activated plasma is gradually gaining espousal in water and wastewater management due to its desirable antimicrobial properties and also its ability to degrade certain microcontaminants, which antibiotics is not an exception. However, research is currently being refined at laboratory scale only; there has not been a scale up or large-scale deployment of such technologies to assist with the overwhelmed conventional WWTPs. This therefore warrants further research work to build on already existing data, thereby optimizing laboratory studies and advancing them into scale up studies.

The foundation of the future with regard to ARBs and ARGs diagnostics and remediation has already been laid for improvements thereupon. The world has gone abuzz with advancements in artificial intelligence-themed technologies, which facilitate the dissemination of molecular information of this phenomenon, through the omics (genomics, transcriptomics, metabolomics, proteomics, plasmidomics etc.) and bioinformatics, which uncover the complete information of unculturable organisms. Also, bio-nanosensors (nanotechnology) have already been developed for the early, rapid detection of minute quantities of micropollutants, which will trigger drastic actions to prevent their accumulation. Moreover, with consciousness toward a sustainable environment, nano-based biotechnology and artificial intelligence have also been attempted in the generation of electrical energy from pharmaceutical residues through the performances of bioelectrochemical systems, such as microbial fuel cells (MFCs), microbial electrolytic cells (MECs) *inter alia*. Interestingly, the manipulability of the aforementioned cells could permit the seamless integration of plasma technology, which is assumed to not only ensure the improved management of pollutants (both chemical and microbial), but also enhance its electricity generating capacity. The future

anticipations of behaviour of ARBs, ARGs cannot be accurately decipherable at the moment, as these organisms and molecules constantly evolve, much to the befuddlement of research scientists, engineers and policy makers. Therefore, a robust surveillance and management scheme, involving all stakeholders should be implemented and reviewed frequently in order to increase our readiness for unsuspected episodes of AMR outbreaks.

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Chapter 3

Acinetobacter baumannii

An adapted version of this chapter is in preparation for submission to Journal of Environmental Chemical Engineering as:

Non-thermal Obliteration of Critically Ranked Carbapenem-Resistant *Acinetobacter baumannii* and its Resistance Genes in a Batch Atmospheric Plasma Reactor. Thabang B.M. Mosaka, John O. Unuofin, Michael O. Daramola, Chedly Tizaoui, Samuel A. Iwarere.

Abstract

Wastewater treatment plants (WWTPs) have been implicated as direct key reservoir of both antibiotic resistant bacteria (ARB) and antibiotic resistant genes (ARGs) associated with human infection, as high concentrations of ARBs and ARGs have been detected in recycled hospital wastewater. Among the ARBs, the carbapenem-resistant *Acinetobacter baumannii* has been ranked as priority 1 (critical) pathogen by the World Health Organisation (WHO), due to its overwhelming burden on public health. Therefore, this study aimed to investigate non-thermal Plasma (NTP) technology as an alternative disinfection step to inactivate these bacteria and its ARGs. Culture-based method and PCR were employed in confirming the carbapenem resistance gene $bla_{\text{NDM-1}}$ in *A. baumannii* (BAA 1605). Suspension of carbapenem-resistant *A. baumannii* (24 h culture) was prepared from the confirmed isolate and subjected to plasma treatment at varying time intervals (3 min, 6 min, 9 min, 12 min and 15 min) in triplicates. The plasma treated samples were evaluated for re-growth and the presence of the resistance gene. The treatment resulted in a 1.13 log reduction after 3 min and the highest log reduction of ≥8 after 15 min, the results also showed that NTP was able to inactivate the $bla_{\text{NDM-1}}$ gene. The log reduction and gel image results suggest that plasma disinfection has a great potential to be an efficient tertiary treatment step for WWTPs.

Keywords: cold atmospheric plasma, disinfection, carbapenem resistant, *Acinetobacter baumannii* carbapenem resistant genes.

3.1. Introduction

Today, clinical isolates of *Acinetobacter baumannii*, resistant to carbapenems (last resort antibiotics) are being reported globally and have earned the term "red alert" human pathogen, among the medical fraternity (Howard *et al*., 2012; Dekic *et al*., 2019). The World Health Organisation (WHO) even categorised *A. baumannii*, carbapenem-resistant as critical priority 1 class (World Health Organisation, 2017; Soni *et al*., 2022). *A. baumannii* is a gram-negative bacterium, that is non-motile, pleomorphic and strictly aerobic (Howard *et al*., 2012; Viehman *et al*., 2014; Valencia-Martín *et al*., 2019; Raut *et al*., 2020; Shi *et al*., 2020). It is commonly associated with aquatic environments (Howard *et al*., 2012) but it is also notorious for surviving desiccation and surviving for prolonged periods on all kinds of surfaces (dry and wet) (Fishbain and Peleg, 2010; Viehman *et al*., 2014; Valencia-Martín *et al*., 2019; Raut *et al*., 2020). This key feature facilitates its dissemination within the health care setting and it often leads to outbreaks (Fishbain and Peleg, 2010; Viehman *et al*., 2014) which are rampant among immunocompromised individuals, especially patients and convalescing persons who have been in the hospital for a long time (>90 days). Causing life-threatening infections such as respiratory tract infection, bacteremia, meningitis, urinary tract infections, and wound infection which have limited options for treatment (Fishbain and Peleg, 2010; Howard *et al*., 2012; Valencia-Martín *et al*., 2019; Raut *et al*., 2020; Shi *et al*., 2020). These clinical manifestations often result in patients being admitted to the intensive care unit, having surgical procedures done on them, being hospitalized for longer periods (Fishbain and Peleg, 2010). In critical cases, it eventually leads to the demise of suffering patients (Raut *et al*., 2020). *A. baumannii* has developed resistance to commonly used antibiotics during the last 30 years (Dekic *et al*., 2019), accentuating its status amongst the most common and serious multi-drug resistant (MDR) bacteria, ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *A. baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* spp). Its innate resistance mechanisms, together with the acquisition of foreign determinants enable it to switch its genomic structure, quickly capturing resistance markers under antibacterial pressure (Howard *et al*., 2012).

Over the last decade, viable *A. baumannii* of clinical significance has been reported in natural environments outside of hospital settings, being recovered in rivers, wastewater treatment plants, untreated hospital wastewaters and urban wastewaters. There is however, very little information about the environmental factors that influence the survival of *A. baumannii* in waters from which it was found (Dekic *et al*., 2019). Most studies have investigated and observed the presence of carbapenem resistant *A. baumannii* (CRAB) after secondary treatment in WWTPs (Pulami *et al*., 2023). One of the few studies that have investigated the effectiveness of disinfection, have reported CRAB and the NDM-1 gene in the effluent of the WWTP after chlorine disinfection (Hrenović *et al*., 2016). This is because chlorination tends to have a selective effect on antibiotic resistant genes (ARGs), decreasing abundance of genes (gene copies per mL of sample)

while increasing the prevalence of the gene (gene copies per total bacteria) *(Manaia et al., 2018; Chen et al*., 2020b).The ARGs are eventually transferred and adapted into new bacteria, leading to the inception and genetic transformation across bacteria and the development of antibiotic resistance (Yuan *et al*., 2015; Sarangapani *et al*., 2019; Chen *et al*., 2020b, Jin *et al*., 2020).

The inability of WWTPs to inactivate antibiotic resistant bacteria (ARBs) and ARGs is not limited to chlorination; studies have proven that when either chlorination or UV irradiation, or ozone oxidation are applied in WWTPs, they might destroy the bacteria by disintegrating its DNA or cellular structure. However, ARGs may still persist for a long time in the cell debris and in the environment, eventually resulting in bacteria developing antibiotic resistance (Yuan *et al*., 2015; Sarangapani *et al*., 2019; Chen *et al*., 2020b, Jin *et al*., 2020). This recycled drinking water becomes a direct key reservoir of ARBs and ARGs associated with human infection (Ekwanzala *et al*., 2018), making wastewater both a resource and a problem (Unuofin, 2020). This study aims to the use Non-thermal Plasma (NTP), a type of advanced oxygenation process (AOP), as an alternative tool for both water treatment and wastewater reclamation and reuse, as it is able to produce reactive oxygen species (ROS) like the indiscriminate hydroxyl radical •OH, breaking down organic matter while inactivating ARBs and ARGs (Foster, 2017; Chen *et al*., 2020c; Umar, 2022). Correspondingly, this study appraises the effectiveness of NTP, based on the concentration of its by-products, the pH and conductivity.

3.2. Materials and Methods

3.2.1. Non-Thermal Plasma Reactor

A 250 mL capacity non-thermal plasma reactor was designed and set up for the treatment of samples (Figure 4). Major components of the module comprised Polytetrafluoroethylene (PTFE), copper electrodes and high voltage cable. The PTFE, which sealed the rector was machined to create an orifice, which was fitted with a hollow copper electrode with an outer diameter of 12.7 mm. One end of the hollow copper electrode had four copper prongs (30 mm × 3 mm) welded on to it. The hollow electrode was connected to a high voltage end of the Jeenel Technology Services (Pty) Ltd high voltage direct current power supply with a maximum capacity of 40 kV and 15 mA. The ground electrode flat copper disk and there was a gap of 50 mm between it and the copper electrode (prongs). To discharge the plasma, the voltage was set at 23 kV but it went down to 10 kV after the initialization of the discharge, and current of 0.7 mA, sustaining a 7 W discharge power throughout the treatment. The insert at the bottom shows the discharge generated which is a streamer in nature. The white masking tape was wrapped around the hollow copper electrode to prevent arc formation at the neck of the reactor.

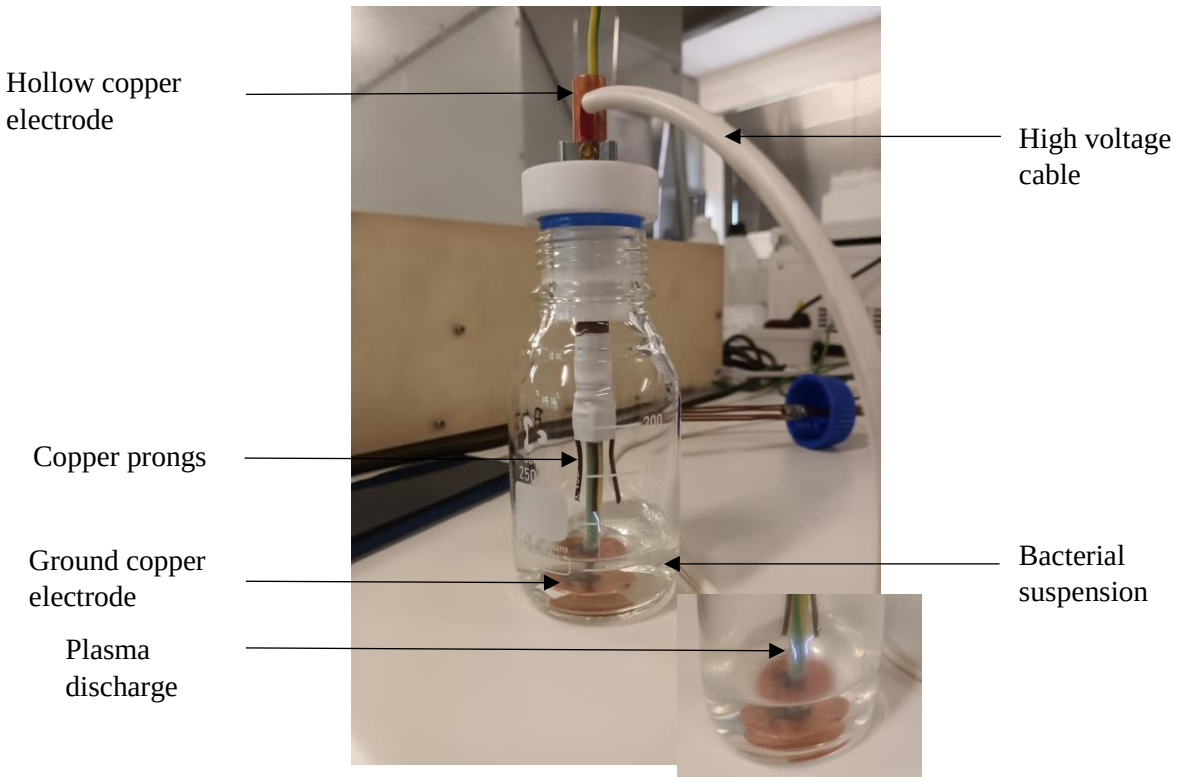

Figure 4: The NTP set up used for treatment

3.2.2. Organism and reagents

The ATCC BAA 1605 strain of *A. baumannii* adopted for this study was collected from Laboratory Specialties PTY LTD Trading as Thermo Fisher Scientific in Randburg, South Africa. Imipenem antibiotic, Luria Bertani (LB) broth as well as LB agar were purchased from Sigma Merck. Nucleomag DNA/RNA water kit was procured from Separations in Randburg, South Africa. Primers were delivered by Inqaba in Pretoria, South Africa. All reagents were of analytical grade.

3.2.3. Antibiotic screening and Non-Thermal Plasma

3.2.3.1. Antibiotic screening

The study confirmed the resistance of *A. baumannii* using culturing methods (Rashmei et al., 2016). The bacteria were cultured in Luria Bertani (LB) broth at 37°C for 24 hours with agitation. Serial passaging on LB agar plates supplemented with increasing concentrations of imipenem (2 µg/mL, 4 µg/mL, 8 µg/mL, and 16 µg/mL) confirmed resistance (Ebomah and Okoh, 2020; Reinke et al., 2020), particularly when growth occurred at 16 µg/mL imipenem. Isolates thriving under these conditions were identified as the standard carbapenem-resistant bacterial strain (CRBS) for subsequent inactivation experiments in the study.

3.2.3.2. Non-Thermal Plasma treatment

The standard *A. baumannii* was inoculated into nutrient broth prepared according to manufacturer's instructions. The standard was incubated at 37 ^{o}C for 24 h (aerobically at160 rpm). After incubation the solution was centrifuged at 4500 rpm for 5 min to retrieve the bacteria pellets. The pellets were then washed twice with physiological saline solution and centrifuged at 4500 rpm for 5 min after each wash. Thereafter, the pellets were resuspended in physiological saline solution. The solution was plated on LB agar plates containing 16 µg/mL imipenem antibiotic, and incubated at 37 ^{o}C for 48 h. The colonies were counted which gave an average plate count of 5.5×10^{9} cfu/mL before plasma treatment. The plasma treatment was done for different durations in a range of 3, 6, 9, 12 and 15 min (Rashmei *et al*., 2016) in triplicates. After plasma treatment, the samples were plated on LB agar plates containing 16 µg/mL imipenem antibiotic, and incubated at 37 ^{o}C for 48 h. After incubation the colony forming units (per mL) were determined and used for log reduction calculations (Reinke *et al*., 2020). Copper is said to also have antimicrobial properties (Benhalima *et al*., 2019; Ortega-Nieto *et al*., 2023), to check if it assisted plasma, the bacterial suspensions were exposed to a copper electrode, without electric discharge for 15 min.

3.2.4. Physiochemical and structural characterization

The Spectrometer (StellarNet) was used to investigate the discharge characteristics and the formation of the chemical species. The H_2O_2 and nitrite/nitrate ions give absorbances at < 350 nm, but they are usually not obvious on the optical emission spectra. In order to obtain more accurate results (Zhang *et al*., 2021), the Lovibond Spectro direct water testing instrument (Tintometer Group, Germany) was used to determine the concentration of (H_2O_2), (NO_2^-) and (NO_3^-) after treating the bacterial suspensions with CAP for 3, 9 and 15 min. Different reagents were reacted with constant volume (10 mL) of the bacterial suspensions, for measurements of H_2O_2 and NO_2^-, while only 0.5 mL of the bacterial suspensions was used for measurements of NO_3^-. For H_2O_2 measurements, Titanium Tetrachloride reagent was used, whereas for NO_2^- and NO_3^- measurements, N-(1-Naphthyl)-ethylenediamine and 2,6-Dimethylphenole reagents were used respectively. Moreover, the reaction time and spectrophotometric reading related to the observed species was carried out according to the manufacturer's manual (GmbH, 2021). The PL-700AL pH meter was used to determine the pH and the WTW Cond 3310 was used to determine the conductivity of the bacterial suspension before the plasma treatment and after 3, 6, 9, 12 and 15 min of the plasma treatment.

3.2.4.1. Scanning Electron Microscopic analysis

The bacterial suspensions (before NTP and 15 min after respective treatments) were centrifuged for 5min at 4500 rpm, the supernatant was discarded and the pelleted cells were retrieved. Phosphate washing buffer was used to wash serum/media away for 15 min. the buffer was then removed by centrifuging and the cells were retrieved. 2.5% of Glutaraldehyde / Formaldehyde solution was added and fixed for 1 – 24 h. The fixative solution was removed and the pellets were washed with phosphate washing buffer 3 times (15 min each wash) and the buffer was removed. 1% of Osmium Tetroxide (OsO_4) solution and post-fixing was done for 1 h. OsO_4 fixative solution was removed in the Fume cupboard and the first buffer wash was added in the fume cupboard. Washing was done with phosphate washing buffer 3 times (15 min each wash) and the buffer was removed. A graded series of ethanol (30%, 50%, 70%, 90% and **3x** 100%) for 15 min each was used to dehydrated the pellets. The pellets were left in the last 100% ethanol for 30 min. A 50:50 mixture of HMDS and 100% ethanol was added and left for 1 h (sample was covered). The ethanol: HMDS mixture was removed and HMDS was added and left for 1 h (sample was covered). HMDS was removed and fresh one was added; the container was left open for samples to dry. The samples were mounted onto aluminum stubs and coated with carbon and then examined in the SEM.

3.2.5. Molecular analysis

DNA was extracted from the bacterial suspension before and after NTP treatment using the kit according to Nucleomag's instructions. The extracted DNA was used as template DNA for the PCR assay to confirm the presence of *bla*$_{NDM-1}$ gene in *A. baumannii*. The primer used in this study can be found in Table 8.

Table 8: Primer used in this study

Name	Forward primer	Reverse primer	Size (bp)	Reference
bla$_{NDM-1}$	GGTGCATGCCCGGTGAAATC	ATGCTGGCCTTGGGGAACGS	660	(Anand *et al*., 2015)

The PCR mixture contained 5 µL of PCR master mix with chosen 0.5 µL of forward primer, 0.5 µL of reverse primer, 2.5 µL of template DNA and 1.5 µL of milli-q water to make up a reaction volume of 10 µL. The PCR conditions for *bla*$_{NDM-1}$ was initial denaturation at 95ºC for 3 min, followed by 30 cycles for 1 min at 95 ºC, annealing at 55ºC for 1 min and extension at 72ºC for 1 min 30 sec, with a final extension at 72ºC for 10 min. The BIO RAD T100 Thermal cycler was used. The PCR products were stained with ethidium bromide (Anand *et al*., 2015; Odjadjare and Olaniran, 2015) and observed using electrophoresis in 1% agarose gel (Somma and Querci, 2006). According to Lee *et al*. (2012), the most efficient method of separating DNA fragments is agarose gel electrophoresis and the addition of ethidium bromide allows fluorescence of DNA under UV light. The BIO RAD PowerPac basic with Mini Sub Cell GT was used for electrophoresis. The presence or absence of the genes on the gel images gave an indication of the ability of plasma treatment to inactivate resistance genes (Anand *et al*., 2015).

3.3 Results and Discussion

3.3.1. Physicochemical properties of plasma discharge

NTP produces reactive nitrogen species (RNS) such as nitrites (NO_2^-) and nitrate (NO_3^-) (Sanito *et al*., 2022, Sreedevi and Suresh, 2023, Zhang *et al*., 2023); and reactive oxygen species (ROS) such hydroxyl (OH) and hydrogen peroxide (H_2O_2) (Sreedevi and Suresh, 2023; Sanito *et al*., 2022) in both the adjoining gaseous and liquid mediums (Sreedevi and Suresh, 2023). The ROS and RNS play an important role in the inactivation of bacteria (Domonkos *et al*., 2021, Das *et al*., 2022). Figure 5 indicates that the NTP reactor generated a streamer discharge that mainly consisted of ROS and RNS, such as NO line (239.5 nm), O^+

lines (435.5 nm, 464.5 nm), OH lines (307,5 nm, 309 nm), N_2 lines (316 nm, 337 nm, 404 nm), N^+ lines (344.5 nm, 394.5 nm) and O line (777.5 nm). The oxygen (O_2) and nitrogen (N_2) present in the atmospheric air, individually undergo electron impact ionization reactions, resulting in electrons and positive ions (O_2^+, N_2^+), that separate and eventually enable streamer propagation which resulted in the formation of NO, O^+, N^+ and O in the gas phase (Nijdam *et al*., 2020). In wet air or in liquids, the •OH, H_2O_2 and ozone (O_3) are formed. The nitrogen oxides (NO) dissolve in water forming nitrite ions and nitrate ions (Zhang *et al*., 2023). Among ROS, •OH have the highest oxidation potential, the most reactive, and are considered to play the important role in NTP bacterial treatment (Beber de Souza *et al*., 2015; Magureanu *et al*., 2021; Zhang *et al*., 2023). The strong oxidation potential (2.8 V) of the •OH is higher than the conventional disinfectants, chlorine (1.36 V) and ozone (2.07 V), and it can damage DNA (Foster, 2017; Sharma *et al*., 2019; Rekhate and Srivastava, 2020; Magureanu *et al*., 2021; Azuma *et al*., 2022). The •OH radical has diverse impact on normal protein structure which is one of the primary targets in bacteria during disinfection, including oxidation of amino acids, modification of sulphur groups, etc., causing irreversible damages to cells and inactivation of ARB and ARGs (Chen *et al*., 2020c; Sreedevi and Suresh, 2023).

Treatment time resulted in an increase of both concentrations of nitrates (NO_3^-) and nitrites (NO_2^-). The highest concentration for both species were observed at 15 min, the concentrations were 6 ± 0.3 mg/L and 1.55 ± 0.078 mg/L for NO_3^- (Figure 6) and NO_2^- (Figure 7), respectively. This correlated with a study which showed an increase in the concentration of nitrates and nitrites with time, although they attained a concentration of 41.41 mg/L and 5.27 mg/L of NO_3^- and NO_2^-, respectively, after the same treatment time. Although their reactor configuration was a dielectric barrier discharge with deionised water (Pandey *et al*., 2023). Nitrites tend to be oxidised to nitrates (Picetti *et al*, 2022), which explains why nitrates were higher than nitrites in this study.

A concentration of 0.065 mg/L hydrogen peroxide (H_2O_2) was only observed after 15 min of plasma treatment (Figure 8). A study resulted in nil production of H_2O_2 after plasma treatment (Pandey *et al*., 2023), while in another study H_2O_2 was observed immediately after treatment but the concentration decreased with time of incubation (Sreedevi and Suresh, 2023). H_2O_2 usually increases quadratically or linearly with plasma treatment time but the cells in the medium uptake it with incubation time (Pandey *et al*., 2023; Sreedevi and Suresh, 2023).

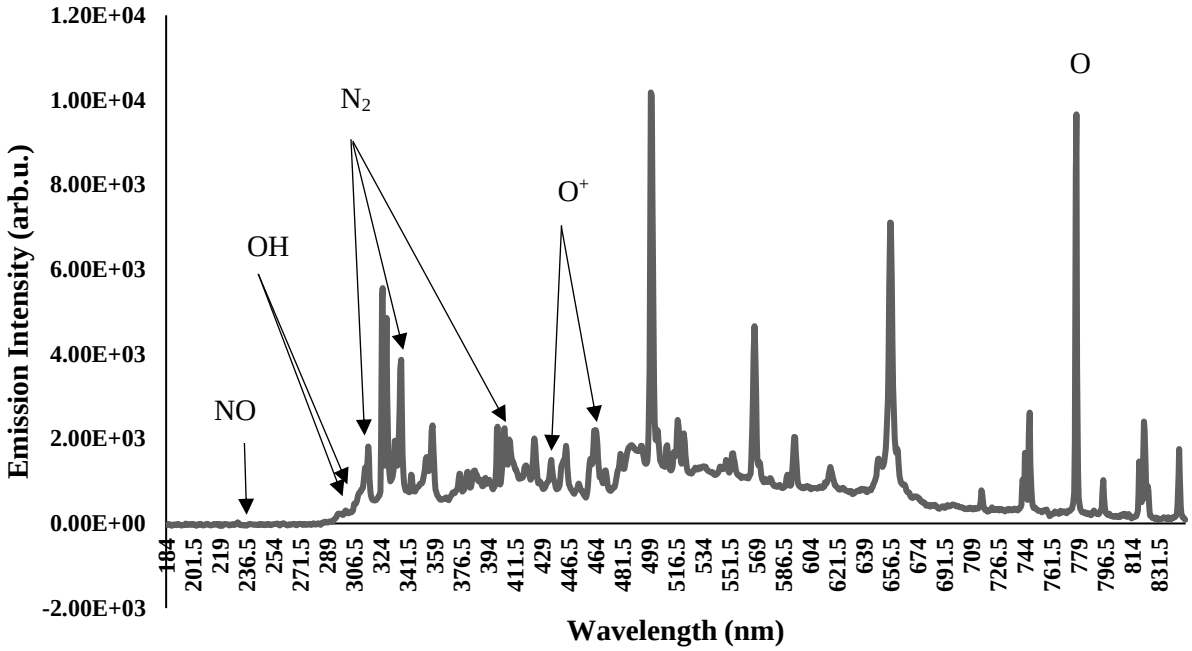

Figure 5: Optical emission spectra from hydroxyl radical OH species in non-thermal plasma treatment during treatment of *A. baumannii*

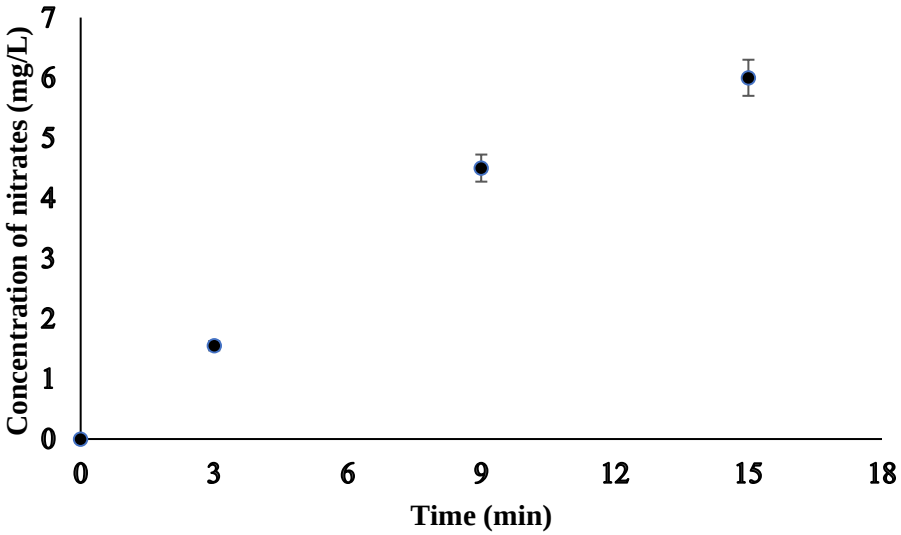

Figure 6: Concentration of nitrates during non-thermal plasma treatment of *A. baumannii*

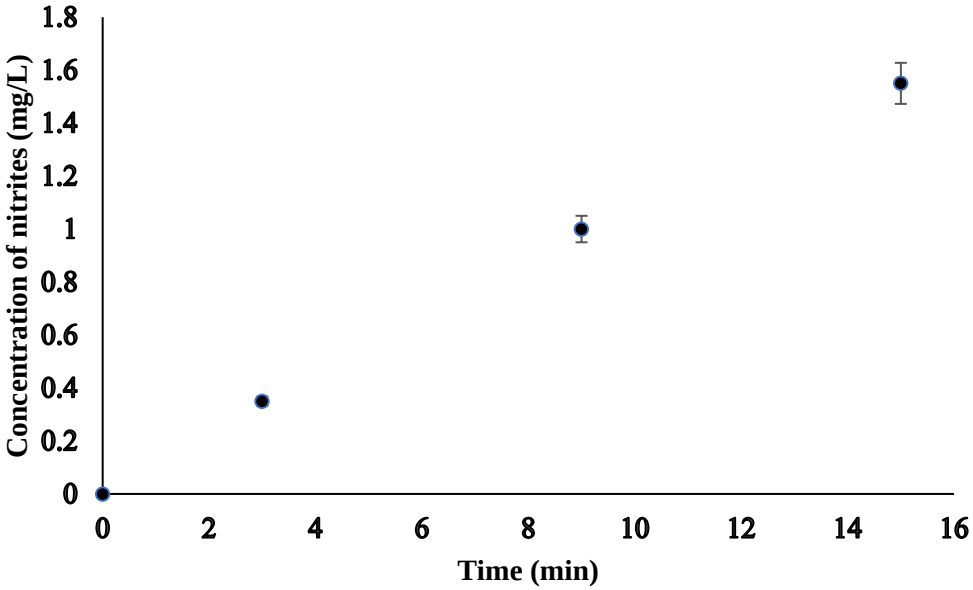

Figure 7: Concentration of nitrites during non-thermal plasma treatment of *A. baumannii*

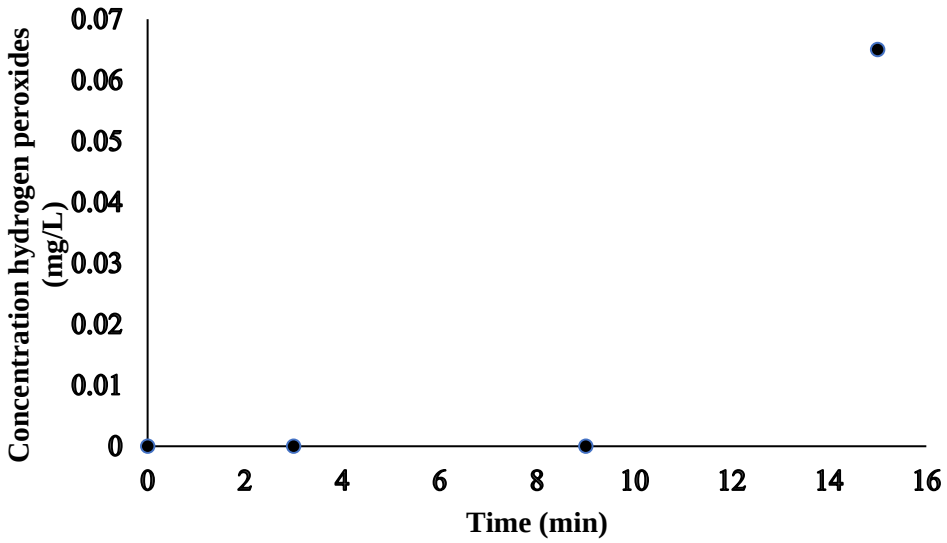

Figure 8: Concentration of hydrogen peroxides during non-thermal plasma treatment of *A. baumannii*

The (H_2O_2), (NO_2^-) and (NO_3^-) have a relatively long lifetime and can react to secondary products post-discharge. The post-discharge reactions between the by-products occurring in plasma activated water (PAW) can result in the generation of peroxynitrous (HNO_3)/peroxynitrite ($ONOO^-$) acid, which significantly participates in the antibacterial activity of PAW. The long-lived reactive species result (Rezaei *et al*., 2019; Tsoukou *et al*., 2020) in continued inactivation of cells in water and microbial cells being killed by contact with water that had first been activated by discharges without being subjected to the plasma plume (Naïtali *et al*., 2010). The long-term, post-plasma effect is mainly caused by the reaction between H_2O_2 and ozone during the peroxone process, that forms •OH (Magureanu *et al*., 2021).

The pH was 7.07 before treatment and it went down to 3.76 after 15 min of plasma treatment (Figure 9). The drop in pH is similar to other studies which also achieved pH of 3.78 and 3.85, respectively, both at 15 min plasma treatment time (Pandey *et al*., 2023). The nitrates and nitrites led to the formation of HNO_3, which resulted in the reduction of pH in this study. The low pH keeps the oxidizing potential of ozone at 2.08 V which can decrease to 1.4 V under alkaline conditions (Zeghioud *et al*., 2020). The production of hydrogen radicals also increases under acidic conditions, which then react with H_2O_2 and H_2O to produce more •OH. An acid pH range of 3-4 is said to be conducive for production of •OH (Magureanu *et al*., 2021)

and results in the increase in cell membrane permeability, enabling easy penetration of reactive molecules through the cell walls (Zhang *et al*., 2023).

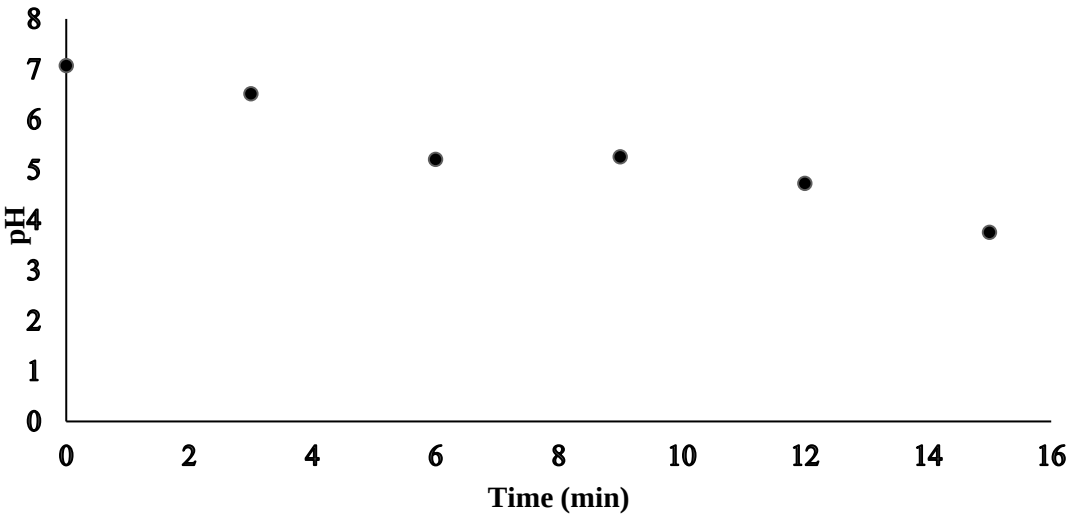

Figure 9: pH readings of *A. baumannii* bacterial suspension during non-thermal plasma treatment

The conductivity was 11.5 mS/cm before treatment, and it increased to 12.24 mS/cm after 15 min of plasma treatment (Figure 10). The ROS and RNS produced during NTP treatment result in varying conductivity of the water (Pandey *et al*., 2023). In one study the conductivity fluctuated between 2.57 mS/cm to 3.31 mS/cm over 30 min of NTP treatment (Liew *et al*., 2023) and in another study the conductivity increased from 1 µS/cm to 123 µS/cm over 15min treatment time (Pandey *et al*., 2023). Although the conductivity in this study increased with reaction time, it still remained fairly low as compared to the initial conductivity. This may be because a low conductivity favours the production of O_3 and H_2O_2 which contribute to the destruction of the pollutants (Jiang *et al*., 2014; Zeghioud *et al*., 2020). The increment is said to be an indication of a loss in cell membrane integrity of bacteria (Wang *et al*., 2022).

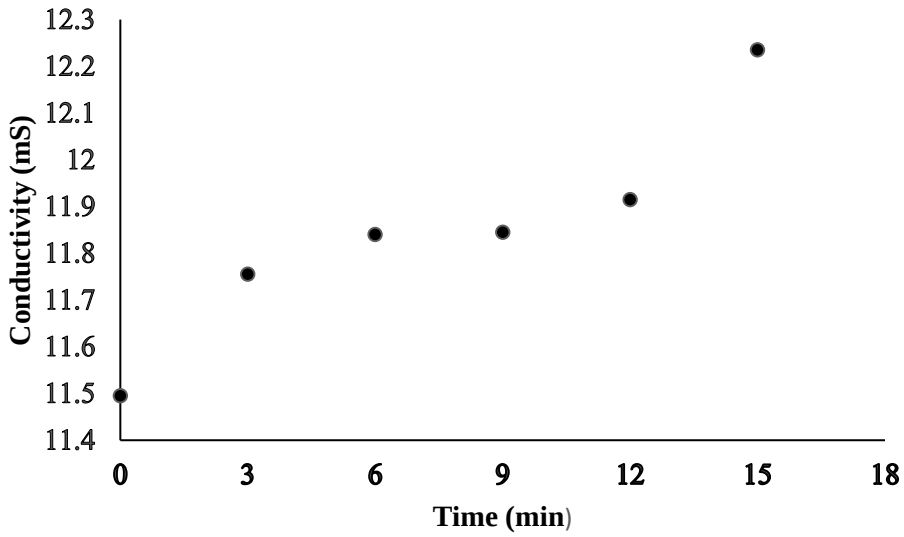

Figure 10: Conductivity readings of *A. baumannii* bacterial suspension during non-thermal plasma treatment

3.3.2. Inactivation of ARBs and ARGs

3.3.2.1. Evaluation of re-growth

The log reductions increased with treatment time and the highest log reduction of 9.74 ± 0.49 (close to 100% reduction) was observed after 15 min of treatment (Figure 11). This indicated that NTP resulted in reduction of CRAB with time, proving that NTP be a better alternative disinfection step. Copper on its own resulted in the lowest log reduction (0.40) of *A. baumannii*, perhaps due to the solid elemental state employed. A study has shown that copper in salt form results in the greatest antimicrobial effectiveness for all the bacteria that was tested (Benhalima *et al*., 2019), which might be due to its ability to ionise to Cu^{2+} which makes it very toxic and reactive; hence soluble copper catalyses the formation of ROS, like H_2O_2, responsible for lipid peroxidation and DNA/RNA damage. It disrupts the binding of sulphur or iron to their respective enzymes, resulting in poor protein metallisation and inactivation of the bacteria (Salah *et al*., 2021; Virieux-Petit *et al*., 2022). Moreover, it is observed that Cu^{2+} could facilitate the destruction of bacterial membrane, double-stranded DNA and single-stranded RNA through copper-catalysed Fenton-like reactions produced by the reactions of Cu^{2+}/H_2O_2 (Li *et al*., 2021). The copper electrode used in this study did not have a significant effect on the reduction of *A. baumannii*, it was solely the plasma discharge that resulted in the log reductions.

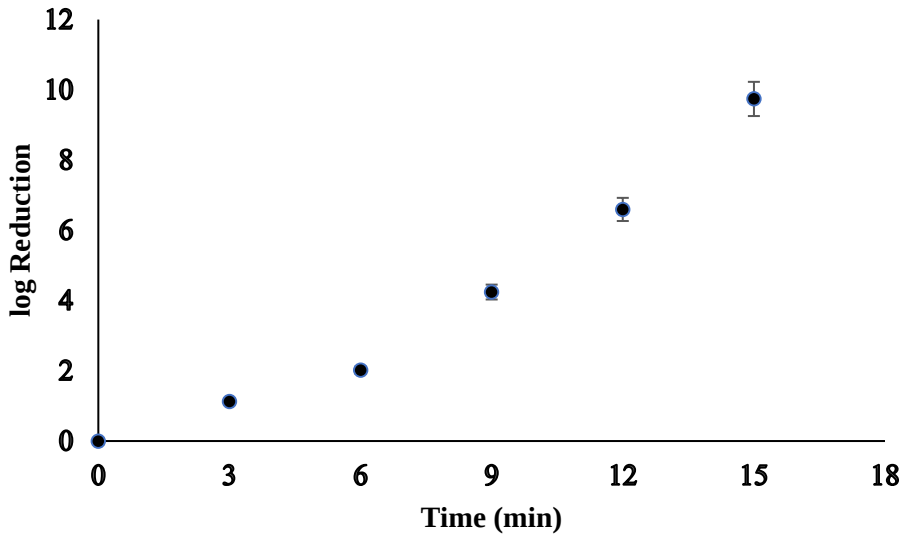

Figure 11: Log reduction of *A. baumannii* (BAA 1605) after non-thermal plasma treatment

3.3.2.2. Evaluation of cellular disintegration

The cells of *A. baumannii* cells appeared smooth and coccobacilli before NTP treatment, further confirming retention of its cellular structure (Figure 12) (Jamiu and Okesola, 2023).

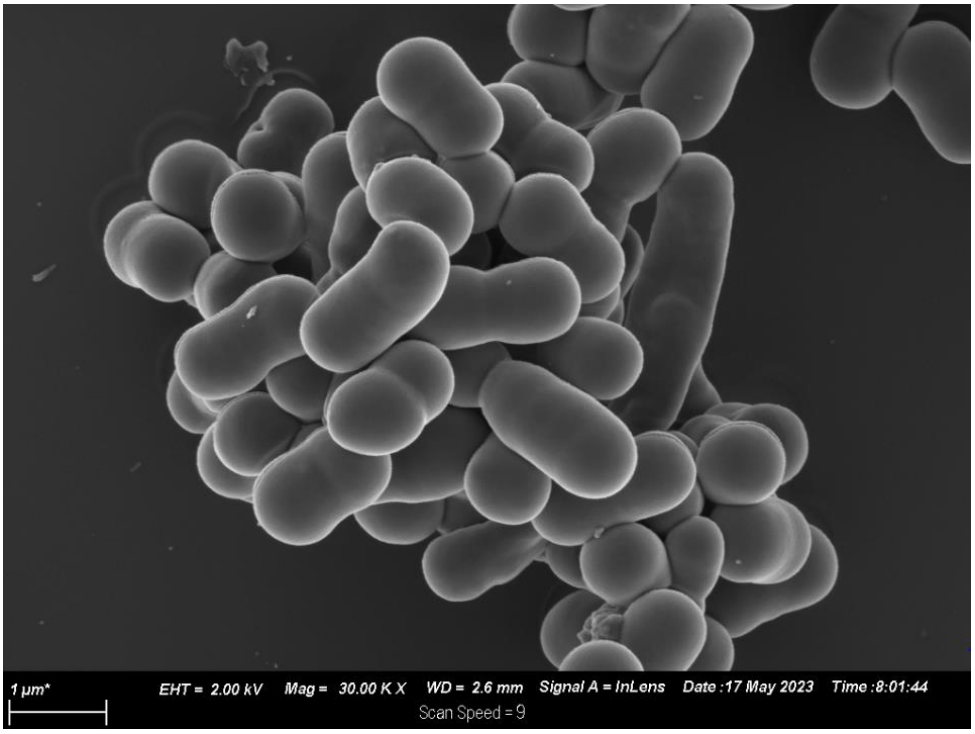

Figure 12: SEM image of *A. baumannii* before non-thermal plasma treatment

However, after 15 min NTP treatment, the surface of *A. baumannii* cells had undergone significant changes (Figure 13). The *A. baumannii* cells were destroyed and lost their characteristic coccobacilli morphology or rod shape as compared to their normal cell structure before NTP treatment. This further demonstrates that NTP interaction with the cell membrane, causes it to rupture, expelling its intracellular components. Ultimately, this leads to cell death, preventing the growth of *A. baumannii* and exposing its ARGs to direct NTP attenuation (Zhang *et al*., 2023). This phenomenon could be attributed to the accumulation of the ROS and RNS free radicals on the cell membrane (Mazandarani *et al*., 2022; Zhang *et al*., 2023), exceeding the tensile strength of the cell membrane, rupturing the cell membrane, and eventually inactivating the bacteria (Zhang *et al*., 2023). Ultimately, the authors presume that the disintegration of the cellular membrane might be achieved through either or both of two phenomena: lipid peroxidation and electroporation. During lipid peroxidation, plasma generated reactive radicals (especially OH groups) detach the polar head moieties and fatty acid tails of phosphatidyl choline residues that make up the lipid bilayer of the cell plasma membranes. This results in crosslinks between adjacent fatty acid tails, which allows unrestrained influx of radicals and water molecules, thereby causing membrane lesions and pore formation. Conversely, electric fields generated by plasma could increase transmembrane potential, which initiates lipid bilayer breakdown and membrane pore creation. The pores might be recoverable or might become irrecoverable in cases of

increased electric field and time, leading to necrosis and cell rupture (Sreedevi and Suresh, 2023). The fact that *A. baumannii* is a gram-negative bacterium also made it easy for NTP to destroy its membrane as it is easier for NTP to inhibit gram-negative bacteria than gram-positive bacteria (Yan *et al*., 2021; Zhang *et al*., 2023). This is because gram negative bacteria have a thinner peptidoglycan layer and an outer membrane with components such as lipopolysaccharide (LPS) and proteins which are sensitive to ROS (Zhang *et al*., 2023).

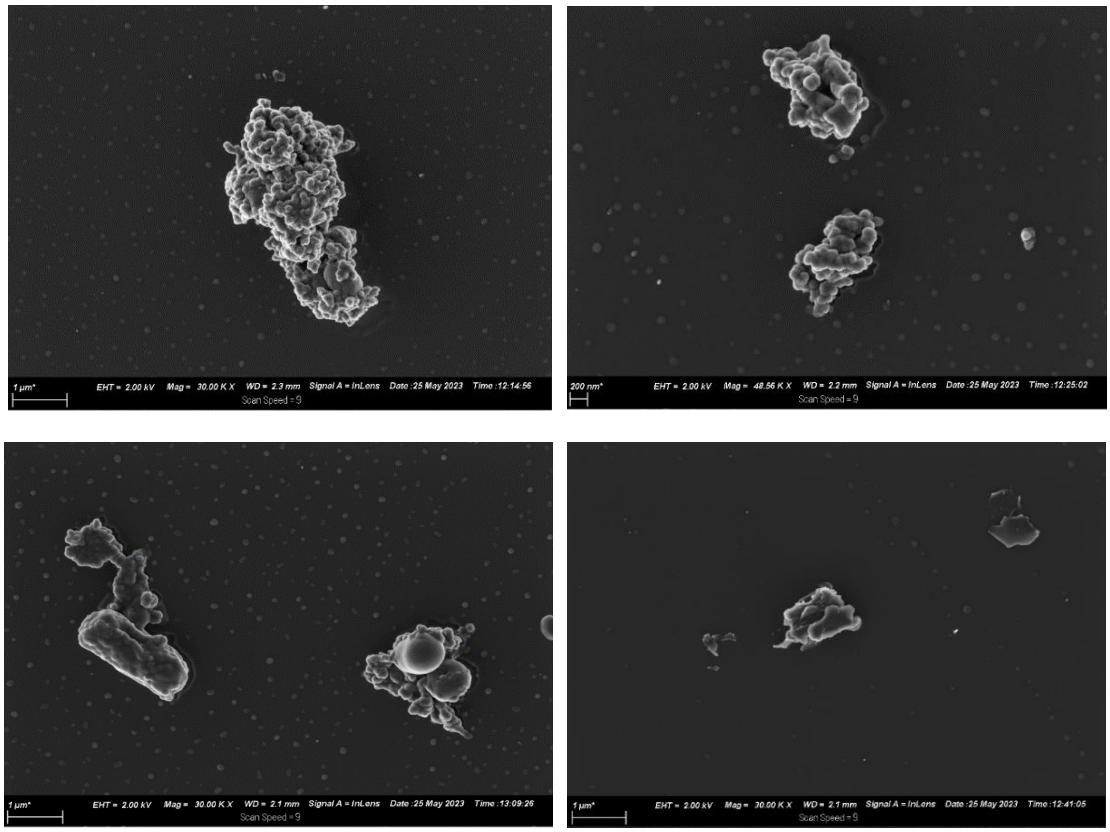

Figure 13: SEM images of *A. baumannii* after 15 min of non-thermal plasma treatment

Copper is capable of forming ions (Cu^{+} and Cu^{2+}), which damage the membrane and infiltrate the cell and induce oxidative stress response involving endogenous ROS. However, the *A. baumannii* cells in this study were not distorted by copper treatment, maintaining their coccobacilli or rod shape throughout the treatment (Figure 14). This might be because the copper employed in the study was not in ionic form, or because the outer membrane of Gram-negative bacteria makes them less susceptible to antibacterial agents (Salah et al., 2021). A study compared SEM images of (E. *coli*) gram negative and (*Staphylococcus aureus*) gram-positive cell after plasma treatment, the destruction was more visible on the E. *coli* cells as there was cell

breakage effects on the E. *coli* cells and only shrinkage and irregular shape on the *S. aureus* cells (Han *et al*., 2016).

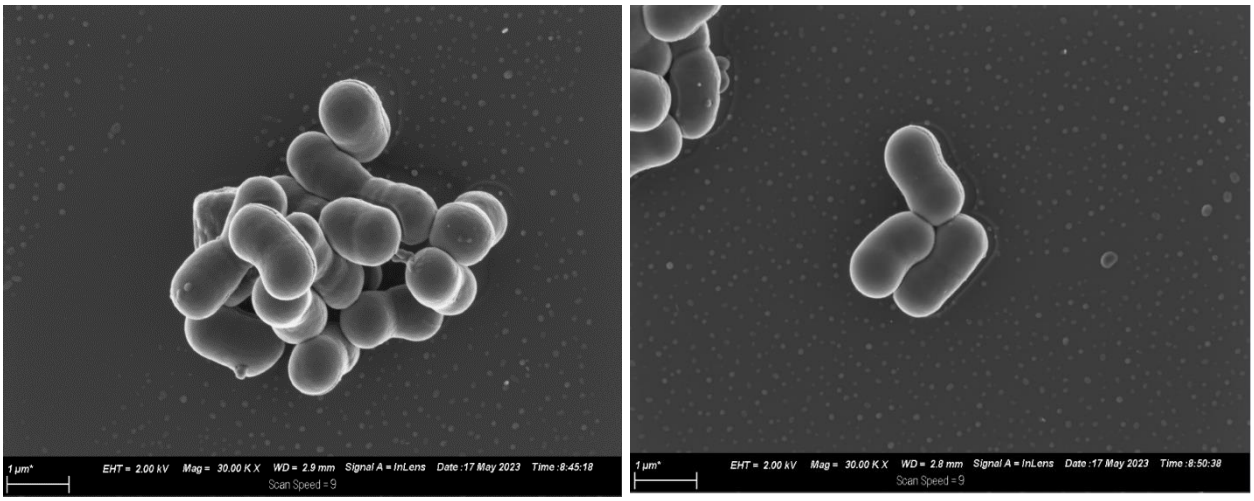

Figure 14: SEM images of *A. baumannii* after copper treatment

3.3.2.3. Detection of resistance gene (bla_{NDM-1}) attenuation

According to (Resources, 2023), the concentration of the present DNA is indicated by the fluorescence of the bands. Therefore, dull bands have a lower concentration of DNA as compared to the bands that are brighter. The band size of our bla_{NDM-1} was evaluated to be 230 bp and its brightness decreased with time, illustrating that the genes were inactivated with time (Figure 15). Although the genes were not completely inactivated after 15mins, the progression in time showed a considerable reduction in bla_{NDM-1}, which we presume might be totally eliminated with increased time and/or electric field. Interestingly, our results are indeed remarkable, when compared with a study that employed plasma generated Fenton-oriented reactions (Cu^{2+}/H_2O_2 and Fe^{2+}/H_2O_2) and recorded measurable gene copies of bla_{TEM-1} after 10 minutes, despite achieving enhanced ARG inactivation by Cu^{2+} and Fe^{2+} (Li *et al*., 2021). Chlorination achieved a maximum reduction of 100 % (Mao et al., 2015), UV achieved a maximum reduction of 99 % (Chen *et al*., 2020a) and ozone achieved a maximum of 98.1 % (Jäger e*t al*., 2018) of ARGs. But these came at a cost to the environment as chlorine forms harmful by-products, such as halo-organics (Luukkonen *et al*., 2014; Anthony *et al*., 2020), ozone forms bromate (Luukkonen et al., 2014; Anthony *et al*., 2020) as concentrations of the disinfectants used were impractical and much higher than those currently used in WWTPs (Zhang *et al*., 2017; Wallmann *et al*., 2021; Umar, 2022). The good thing about NTP is even with an increase in reaction time, no harmful chemicals are used. The grounded electrode surface or diameter

can also be increased at fixed discharge gap in order to optimise the area of plasma discharge. Oxygen-containing feeding gas can also be used, instead of air, argon and the least removal occurs in nitrogen-containing feed gas. as it has been widely demonstrated to result in the fastest degradation of contaminants degradation. This is because oxygen-containing gas has been widely demonstrated to result in the fastest degradation of contaminants as it induces the production of O-based active species and O_3 that boosts the production of •OH (Jiang *et al*., 2014; Magureanu *et al*., 2021).

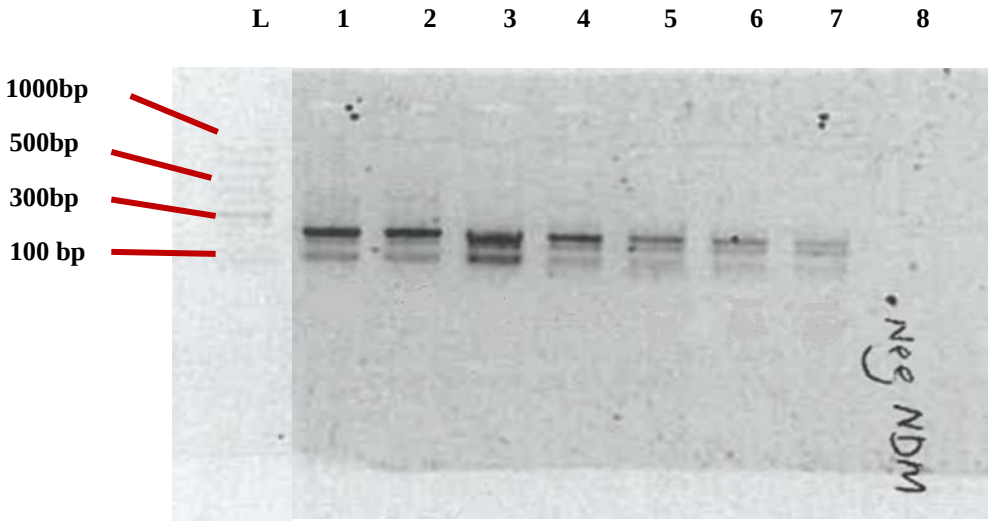

Figure 15: Effect of periodic non-thermal plasma treatment on the carbapenem resistant gene of *A. baumannii*. L: DNA ladder, 1: +ve control, 2: Copper control, 3: 3 min, 4: 6 min, 5: 9 min, 6: 12 min, 7: 15 min, 8: -ve control.

3.4. Conclusions

The 15-minute treatment demonstrated the most pronounced impact on *A. baumannii* reduction, coinciding with a diminishing intensity of the resistance gene in *A. baumannii* over time. This observation, coupled with the log reductions, substantiates the temporal efficacy of NTP in completely eradicating both ARBs and ARGs. The mechanism underlying this effectiveness is attributed to the increasing presence of •OH and long-lived species (H_2O_2, NO_2^- and NO_3^-), which exhibit a time-dependent augmentation. These species facilitate the generation of more •OH and participate in post-discharge reactions, significantly contributing to the antibacterial activity of Plasma-Activated Water (PAW). Concurrently, they contribute to pH reduction to levels conducive for bacterial destruction, while the expected low conductivity is maintained. Interestingly, SEM results validate the theory that CAP disrupts the tensile strength of the cell membrane, inducing rupture and consequent cell death, thereby preventing bacterial growth, including the presumed preservation of ARGs. The study excludes the antimicrobial contribution of copper, emphasizing the sole attribution of log reductions to NTP, as corroborated by SEM findings. These collective results underscore the promising potential of plasma treatment as an efficient disinfection step for wastewater. However, it is advisable to extend the treatment duration as well as increased electric field supply for ARGs, given that the observed inactivation of ARBs may not correspond to the inactivation of ARGs. This recommendation aligns with the pursuit of conclusive gene elimination outcomes.

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Chapter 4

Pseudomonas aeruginosa

An adapted version of this chapter is in preparation for submission to Environmental Technology & Innovation as:

Rapid Susceptibility of Carbapenem Resistant *Pseudomonas aeruginosa* and its Resistance Genes to Non-thermal Plasma Treatment in a Batch Reactor. Thabang B.M. Mosaka, John O. Unuofin, Michael O. Daramola, Chedly Tizaoui, Samuel A. Iwarere.

Abstract

The inability of Wastewater treatment plants (WWTPs) to completely inactivate both antibiotic resistant bacteria (ARB) and antibiotic resistant genes (ARGs) has resulted in their recycling and persistence in reticulation systems of public health spaces, especially health care facilities. Among these ARBs, the critically ranked carbapenem-resistant *Pseudomonas aeruginosa* has been observed to prey on patients with compromised immune system, leading to critical infections and more hospital costs. To save lives and unburden the government of preventable costs, non-thermal plasma (NTP) technology was investigated as an alternative disinfection step that could be applied in WWTPs to inactivate this bacterium and its ARGs. Culture and molecular-based techniques were employed to confirm carbapenem resistance in *P. aeruginosa* (27853). Culture suspensions of carbapenem-resistant *ATCC P. aeruginosa* (16 h culture) were prepared from confirmed isolates and subjected to plasma treatment at varying time intervals (3 min, 6 min, 9 min, 12 min and 15 min) in triplicates. The plasma treated samples were evaluated for re-growth and the presence of the resistance gene. The treatment resulted in a 0.68 log reduction after 3 min and the highest log reduction of ≥8 after 12 min, suggesting that plasma disinfection has a great potential to be an efficient tertiary treatment step for WWTPs. Moreover, the gel image showed that concentration of the ARGs decreased with treatment time. Notwithstanding, longer treatment time, a grounded electrode with a larger surface and or oxygen-containing feeding gas is warranted to completely inactivate ARGs, which might be bound by biofilms as they seem to protect *P. aeruginosa* from the action of NTP disinfection.

Keywords: wastewater treatment plants, disinfection, cold atmospheric plasma, carbapenems, *Pseudomonas aeruginosa*.

4.1. Introduction

Wastewater treatment plants (WWTPs) are considered an important hotspot for spreading of antibiotic resistances in different bacteria, as compared to other water environments (Golle *et al*., 2017). This is because conventional WWTPs were not designed to remove the antibiotic resistant bacteria (ARBs) and genes (ARGs) (Fadare and Okoh, 2021) that are often reticulated from the sewage systems of households, healthcare services, antibiotic manufacturing facilities, agricultural activities and animal feedlots (Ekwanzala *et al*., 2018; Ben *et al*., 2019; Rodríguez-Molina *et al*., 2019). Moreover, conventional disinfection processes, such as chlorination, UV irradiation and ozone oxidation, which are applied in WWTPs, only kill a great fraction of ARBs, whereas others enter a state of dormancy due to stress and are resuscitated when the stressors are released. Sometimes, they decrease the abundance of the genes (gene copies per mL of sample) while the prevalence of the gene (gene copies per total bacteria) increases (Manaia *et al*., 2018; Chen *et al*., 2020b). Whilst some of the disinfection processes kill the bacteria, ARGs may still persist for a long time in the cell debris and in the environment, transferring and adapting into new bacteria, eventually leading to the development of antibiotic resistance (Yuan *et al*., 2015; Sarangapani *et al*., 2019; Chen *et al*., 2020b, Jin *et al*., 2020). As a result, WWTPs keep recycling drinking water with high concentrations of ARBs and ARGs, back to municipal water supplies and health care facilities (Hassoun-Kheir *et al*., 2020). *Pseudomonas aeruginosa*, a carbapenem-resistant that has been categorised as critical priority 1 class by the World Health Organisation (WHO) (World Health Organisation, 2017; Soni *et al*., 2022), is an example of an ARB been found in the effluent of WWTPs, in lakes, rivers and swimming pools (Golle *et al*., 2017).

P. aeruginosa, is a Gram-negative, aerobic bacteria that is also considered as a facultative anaerobe (Logan *et al*., 2017; McAuley, 2018; Walters *et al*., 2019). It survives the most diverse environments, spreading quickly in a new habitat, as it has minimal nutritional needs and is able to get energy from a variety of carbon sources (Logan *et al*., 2017; Walters *et al*., 2019). In municipal drinking water distribution systems, it proliferates forming biofilm (Golle *et al*., 2017). *P. aeruginosa* carries a variety of virulence factors, such as the type III secretion system, which enhances disease severity by injecting effector proteins into host cells (Logan *et al*., 2017; Walters *et al*., 2019). In hospital settings, it causes infections in patients in the intensive care units (ICU) or in patients with serious underlying disorders, such as a cystic fibrosis (CF) or suppressed immune system, infection of the urinary tract, ear, sinuses, wounds, skin, connective tissues and pulmonary disease (Voor In 't Holt *et al*., 2014; Logan *et al*., 2017). Even with the adoption of control measures (Voor In 't Holt *et al*., 2014), infections continue to become more complex and difficult to treat and are associated with significant hospital and societal burdens. Moreover, infections eventually lead to high morbidity and mortality rate (Voor In 't Holt *et al*., 2014; Logan *et al*., 2017; Walters *et al*., 2019) as

compared to infections caused by members of Enterobacteriaceae or other non–lactose fermenting gram-negative bacilli (Logan *et al*., 2017; Walters *et al*., 2019).

Considering that (i) *P. aeruginosa* is intrinsically resistant to many commonly used antibiotics and additionally acquires resistance by horizontal gene transfers or mutation (Golle *et al*., 2017) (ii) *P. aeruginosa* is resistant to the last resort antibiotics (carbapenems) (Meletis, 2016; Codjoe and Donkor, 2017) (iii) second-line treatment options for *P. aeruginosa* often accompanying toxicity and are less defined in their efficacy (Viehman *et al*., 2014) and (iv) *P. aeruginosa* in healthcare settings, environment and water sources prevents control of its acquisition among the most vulnerable patients (Logan *et al*., 2017). Elimination of an environmental reservoir could be the answer to controlling some of the outbreaks (Valencia-Martín *et al*., 2019). The use of conventional treatment methods and even other advanced oxidation processes have been evaluated in a previous study, where the merits were observed to outweigh any drawbacks (Mosaka *et al*., 2022). Hence, the use of an Advanced Oxygenation Process (AOP), this study adopted a Non-Thermal Plasma (NTP) treatment technique, which we hypothesized that it would facilitate rapid elimination of ARBs and ARGs. We also presumed it could serve as an alternative disinfection step in WWTPs, as it is able break down organic matter while causing irreversible damage to cells and inactivating ARBs (in this case *P. aeruginosa,* carbapenem-resistant) and its ARGs (Chen *et al*., 2020c; Umar, 2022).

4.2. Materials and method

4.2.1. Non-Thermal Plasma Reactor

The reactor adopted in this study involved a configuration comprising a designed air-tight machined Polytetrafluoroethylene (PTFE), copper electrodes and high voltage cable, assembled and fitted to a 250 mL capacity Duran Schott glass bottle. The reactor was powered with a high voltage direct current (HVDC) developed by Jeenel Technology Services Pty Ltd in South Africa, having a maximum capacity of 40 kV and 15 mA (Fig 16). A flat copper disk functioned as the ground electrode, with a 50 mm gap between it and the hollow copper electrode. The gap between the hollow copper electrode and the surface of the solution was 15 mm. The voltage on the HVDC was set at 23 kV to ignite the plasma but was regulated to 10 kV with a constant current of 0.7 mA, sustaining a 7 W discharge power throughout the treatment.

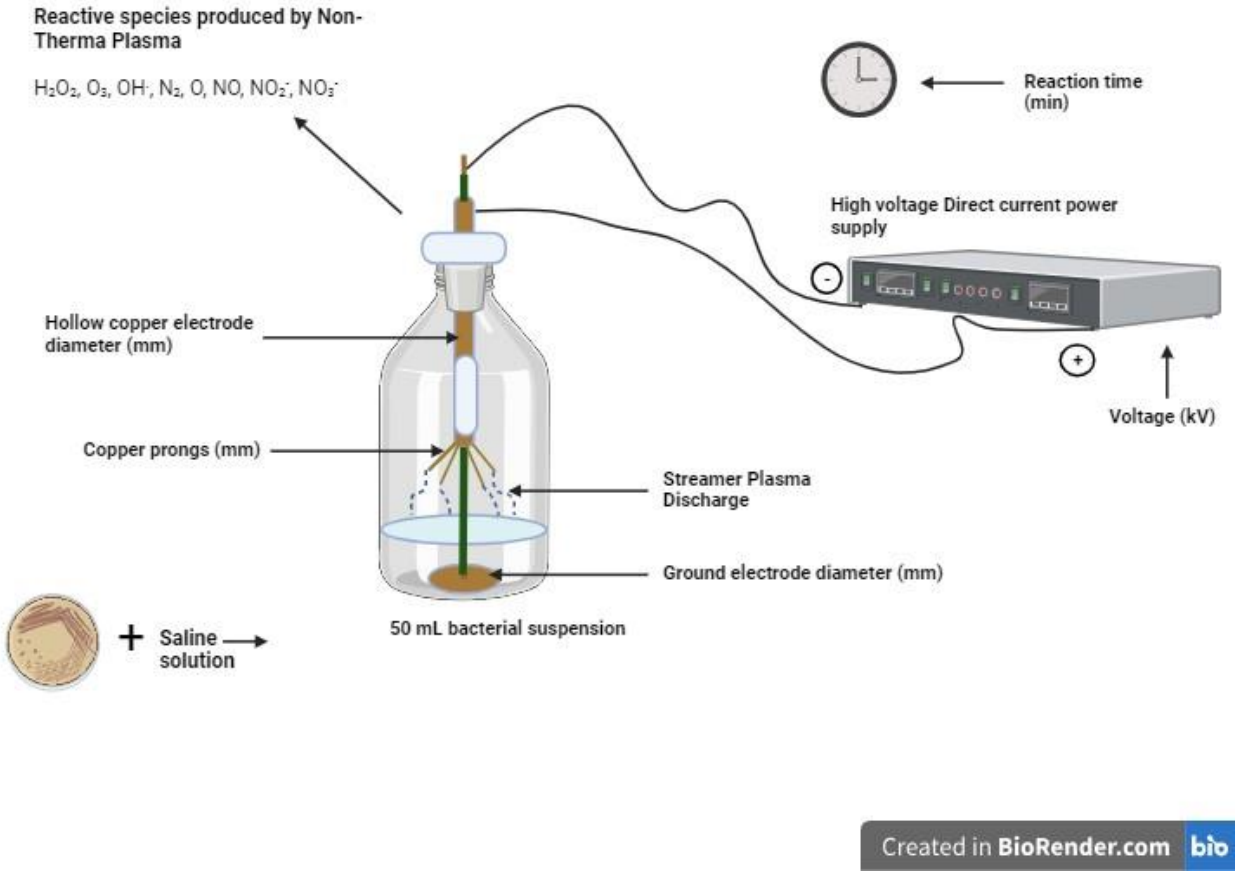

Figure 16: Schematic diagram of the non-thermal plasma experimental setup, showing all variables

4.2.2. Organism and chemicals

The bacterial strain used in this study (ATCC *P. aeruginosa* (27853) was obtained from Laboratory Specialties PTY LTD Trading as Thermo Fisher Scientific in Randburg, South Africa. Imipenem antibiotic, Luria Bertani (LB) broth and LB agar plates were procured from Sigma Merck. Nucleomag DNA/RNA water kit was purchased from Separations in Randburg, South Africa and primers were delivered by Inqaba in Pretoria, South Africa.

4.2.3. Antibiotic screening and Non-Thermal Plasma treatment

4.2.3.1. Antibiotic screening

The preliminary screening of *P. aeruginosa* for antibiotic resistance was done using culture-based methods (Rashmei *et al*., 2016). The bacteria were inoculated into Luria Bertani (LB) broth and orbitally incubated at 37 ^{o}C, 160 rpm for 24 h. Thereafter, the cultures were successively screened increasing gradients of imipenem (2 µg/mL, 4 µg/mL, 6 µg/mL, 8 µg/mL, 12 µg/mL and 24 µg/mL), which were supplemented in LB agar and incubated at 37 ^{o}C for 24 h (Ebomah and Okoh, 2020; Reinke *et al*., 2020). The growth of *P. aeruginosa* on the plates supplemented with imipenem confirmed their resistance to carbapenems. The isolate that grew on the plates supplemented with 24 µg/mL imipenem antibiotic was used as the standard carbapenem resistance bacteria strain (CRBS) for the inactivation experiment in this study.

4.2.3.2. Non-Thermal Plasma treatment

The standard *P. aeruginosa* strain underwent a series of procedures. It was initially introduced into nutrient broth and orbited at 37 °C, 160 rpm for 16 h to minimize biofilms. After incubation, bacterial pellets were collected, washed, and resuspended in saline. The resulting solution was plated on LB agar with 24 µg/mL imipenem, yielding an average plate count of 3.0×10^{9} cfu/mL before plasma treatment. Plasma exposure involved treating a 50 mL bacterial suspension at different time intervals (3, 6, 9, 12, and 15 min) in triplicates. Post-plasma treatment, samples were plated on LB agar with 24 µg/mL imipenem and incubated at 37 °C for 48 h. Colony-forming units per mL were then estimated for log reduction calculations. Additionally, to explore copper's potential antimicrobial properties, bacterial suspensions were treated with the copper electrode alone for 15 min, referencing studies by (Benhalima *et al*., 2019, Ortega-Nieto *et al*., 2023).

4.2.4. Physical chemical and structural characterization

The discharge characteristics and chemical species formed during treatment were assessed using the Black Comet C-25 Spectrometer (StellarNet, Inc). Although the H_2O_2 and nitrite/nitrate ions gave absorbances at < 350 nm, due to not been conspicuous on the optical emission spectra, the Lovibond® SpectroDirect water testing instrument (Tintometer Group, Germany) was used to accurately determine the concentration of (H_2O_2), (NO_2^-) and (NO_3^-) after treating the bacterial suspensions with CAP for 3, 9 and 15 min (Zhang *et al*., 2021). The resultant bacterial suspensions (10 mL) were assayed for NO_2^- and H_2O_2 concentrations measurement using Titanium Tetrachloride reagent and N-(1-Naphthyl)-ethylenediamine, respectively. For NO_3^- concentrations measurement, 0.5 mL of the resultant bacterial suspensions was assayed using 2,6-Dimethylphenole reagent. The reactions were monitored spectrophotometrically after respective time intervals specified by the user manual (GmbH, 2021). The pH of the bacterial suspension before the plasma treatment and after 3, 6, 9, 12 and 15 min of treatment were measured using PL-700AL pH meter. The conductivity of the bacterial suspension before the plasma treatment and after 3, 6, 9, 12 and 15min of treatment were determined using a WTW – Portable conductivity meter ProfiLine Cond 3310 (Einzelgerät, Zubehör).

4.2.4.1. Scanning Electron Microscopic analysis

Bacterial suspensions were treated with non-thermal plasma (NTP), and both pre- and post-treatment, underwent centrifugation to discard the supernatant and recover pelleted cells. The cells were washed with a phosphate buffer to remove serum and media, followed by fixation using a 2.5% Glutaraldehyde/Formaldehyde solution for 1 to 24 h. Subsequently, the fixed pellets were subjected to multiple washes with phosphate buffer and post-fixed with a 1% Osmium Tetroxide (OsO4) solution for 1 h. Dehydration was achieved through a graded ethanol series (30%, 50%, 70%, 90% and **3x** 100%), and a mixture of Hexamethyldisilazane (HMDS) and ethanol was applied before drying. The samples were then mounted on aluminium stubs, coated with carbon, and examined using a scanning electron microscopy (SEM) (Zeiss Gemini Ultra Plus FEG-SEM (Field Emission Gun – Scanning Electron Microscope) with BS, energy dispersive spectroscopy (EDS) and Electron Backscatter Diffraction (EBSD) detectors).

4.2.5. Molecular analysis

The extraction of DNA was conducted both before and after the plasma treatment of the bacterial suspension, following the guidelines provided by Nucleomag. The extracted DNA served as the template for the PCR assay, aiming to validate the presence of the blaNDM-1 gene in *P. aeruginosa* (Table 9).

Table 9: Primer used in this study

Name	Forward primer	Reverse primer	Size (bp)	Reference
bla_{NDM-1}	GGTGCATGCCCGGTGAAATC	ATGCTGGCCTTGGGGAACGS	660	(Anand *et al*., 2015)

The amplification of the bla_{NDM-1} gene was carried out using a 10 µL PCR mixture, consisting of PCR master mix (5 µL), forward and reverse primers (0.5 µL each), template DNA (2.5 µL), and milli-q water (1.5 µL) in a BIO RAD T100 Thermal cycler. The reaction conditions included initial denaturation (95 ^{o}C for 3 min) and 30 cycles for denaturation (95 ^{o}C for 1 min), annealing (55 ^{o}C for 1 min), and extension (72 ^{o}C for 1 min 30 sec), and a terminal extension (72 ^{o}C for 10 min). The ethidium bromide-stained PCR products were visualized through electrophoresis in a 1% agarose gel (Somma and Querci, 2006; Odjadjare and Olaniran, 2015), employing the BIO RAD PowerPac basic with Mini Sub Cell GT. Gel electrophoresis facilitates the efficient separation of DNA fragment and ethidium bromide enables DNA fluorescence and detection under UV light (Lee *et al*., 2012). This method allowed for the observation of gene presence or absence on gel images, providing insights into the effectiveness of plasma treatment in inactivating resistance genes (Anand *et al*., 2015).

4.3. Results and discussion

4.3.1. Physical Chemical and structural characterization

Reactive nitrogen species (RNS) and reactive oxygen species (ROS) play an important role in the inactivation of bacteria (Domonkos *et al*., 2021; Das *et al*., 2022). During NTP treatment, the electric field discharges ROS, such hydroxyl (OH) and hydrogen peroxide (H_2O_2) (Sanito *et al*., 2022; Sreedevi and Suresh, 2023) and RNS, such as nitrites (NO_2^-) and nitrate (NO_3^-) (Sanito e*t al*., 2022; Sreedevi and Suresh, 2023; Zhang *et al*., 2023) are produced in both the adjoining liquid and gaseous media (Sreedevi and Suresh, 2023). The oxygen (O_2) and nitrogen (N_2) lines (316 nm, 337 nm, 404 nm), individually undergo electron impact ionization reactions, resulting in electrons and positive ions (O_2^+, N_2^+), that separate and eventually increase and spread the streamer (Figure 17). Then the NO lines (227 nm, 239.5 nm), O^+ lines (327 nm, 435.5 nm, 464.5 nm), N^+ lines (344 nm, 395.5 nm) and O lines (777 nm) are generated in the gas phase

(Nijdam *et al*., 2020). In wet air or in liquids, the •OH lines (307,5 nm, 309 nm), H_2O_2 and ozone (O_3) are produced (Nijdam *et al*., 2020; Zhang *et al*., 2023). The nitrogen oxides (NO) dissolved in water forming NO_2^- and NO_3^- (Zhang *et al*., 2023). The •OH causes impairment to DNA, as it has strong oxidation potential (2.8 V) that is greater than the conventional disinfectants, chlorine (1.36 V) and ozone (2.07 V) (Foster, 2017; Sharma *et al*., 2019; Rekhate and Srivastava, 2020; Mai-Prochnow *et al*., 2021; Azuma *et al*., 2022). Among ROS, •OH have the greatest oxidation potential, are the most reactive, and are considered to play a crucial role in NTP bacterial treatment (Beber de Souza *et al*., 2015; Magureanu *et al*., 2021; Zhang *et al*., 2023). The •OH radical causes irreversible damage to cells and inactivates ARBs and ARGs as it has diverse impact on normal protein structure which is one of the primary targets in bacteria during disinfection, including oxidation of amino acids, modification of sulphur groups (Chen *et al*., 2020c; Sreedevi and Suresh, 2023). The (H_2O_2), (NO_2^-) and (NO_3^-) have a relatively long lifetime and can react to secondary products post-discharge. The post-discharge reactions between the by-products occurring in plasma activated water (PAW) can result in the generation of peroxynitrous (HNO_3)/peroxynitrite ($ONOO^-$) acid, which significantly participates in the antibacterial activity of PAW. These long-lived species (Rezaei *et al*., 2019; Tsoukou *et al*., 2020) continue to inactivate cells even after the discharge had been switched off (Naïtali *et al*., 2010). The long-term, post-plasma effect of the PAW is mainly caused by the reaction between ozone and H_2O_2 during the peroxone process, that forms •OH (Magureanu *et al*., 2021). The concentration of these species increased with time during NTP treatment of *P. aeruginosa*, as can be seen in Figure 18-21. The highest concentrations of the species are at the time when no more re-growth of *P. aeruginosa*, was observed.

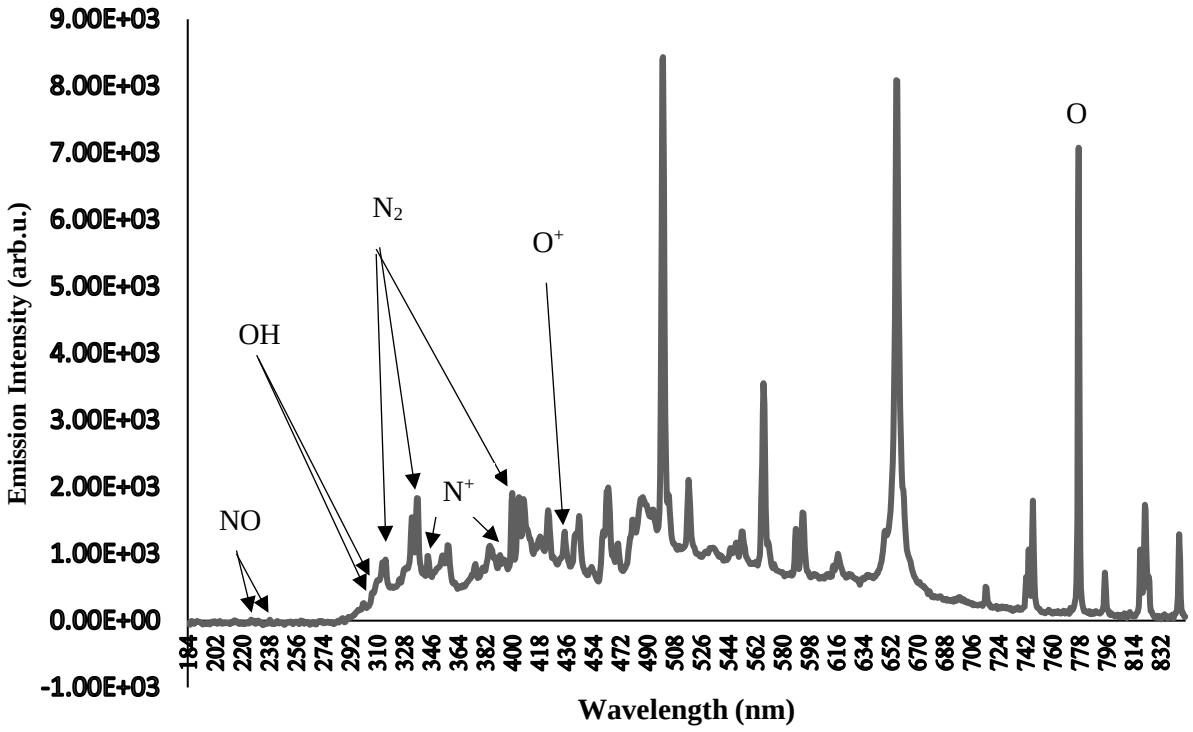

Figure 17: Optical emission spectra from hydroxyl radical OH species in non-thermal plasma during treatment of *P. aeruginosa*

Nitrates (NO_3^-) and nitrites (NO_2^-) were incrementally added into the solution with time and the highest concentration of 6.5 ± 0.325 mg/L and 1.9 ± 0.095 mg/L of NO_3^- (Figure 18) and NO_2^- (Figure 19), respectively, were observed at 15 min. Another study confirmed an increase in the concentration of NO_3^- with treatment time, although a much higher concentration of 41.41 mg/L of NO_3^- and 5.27 mg/L of NO_2^- was attained after the same treatment time. The result were however from treating deionised water only with a dielectric barrier discharge configuration (Pandey *et al*., 2023). The oxidation of nitrite to nitrate (Picetti *et al*., 2022) may contributed to there being more NO_3^- than NO_2^- in this study.

There was no measurable concentration of hydrogen peroxide (H_2O_2) observed at 3 min; however, 0.15 mg/L and 0.36 mg/L concentrations of were observed at 9 and 15 min (Figure 20). A study resulted in the formation of H_2O_2 which was immediately observed after plasma treatment but the concentration decreased with time of incubation (Sreedevi and Suresh, 2023), another study resulted in nil production of H_2O_2 after plasma treatment (Pandey *et al*., 2023). Plasma treatment usually results in quadratic or linear increase of H_2O_2 with treatment time but the cells in the medium uptake H_2O_2 with incubation time (Pandey *et al*., 2023, Sreedevi and Suresh, 2023).

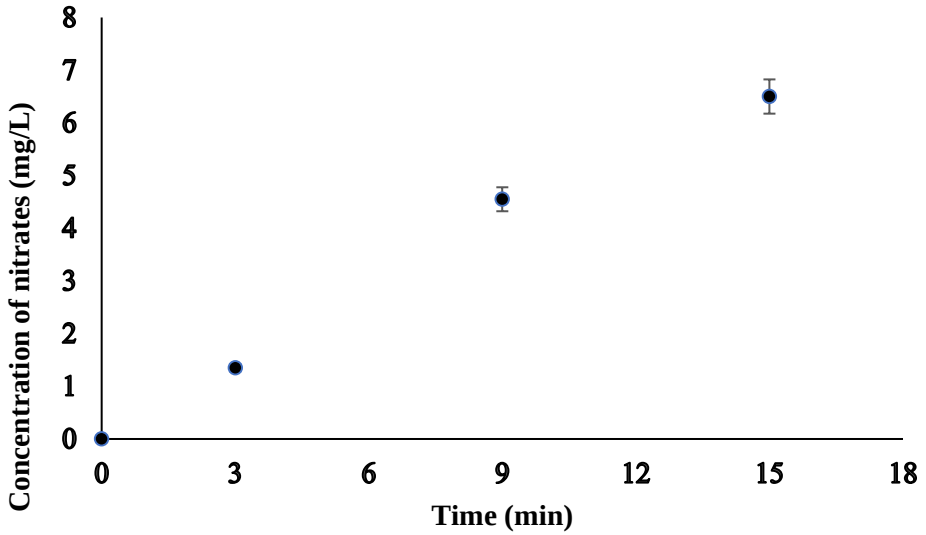

Figure 18: Concentration of nitrates during non-thermal plasma treatment of *P. aeruginosa*

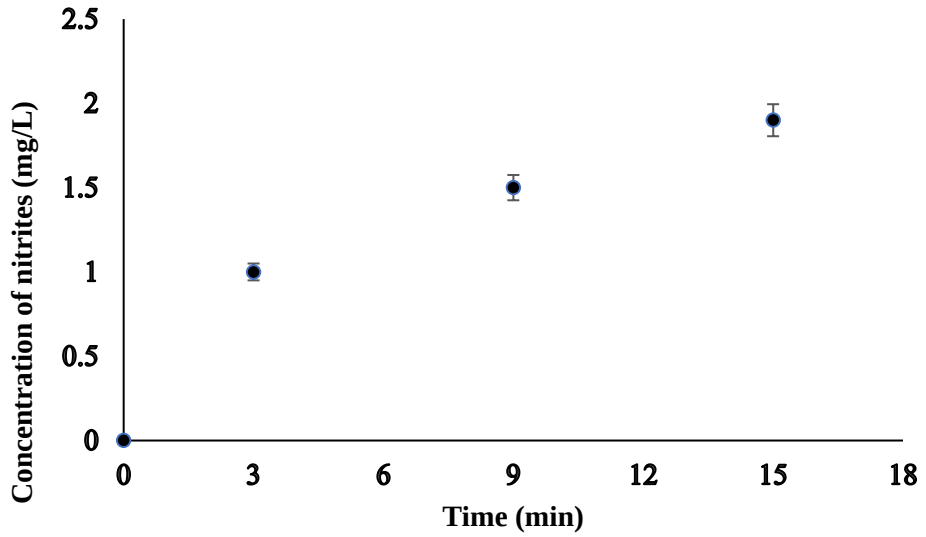

Figure 19: Concentration of nitrites during non-thermal plasma treatment of *P. aeruginosa*

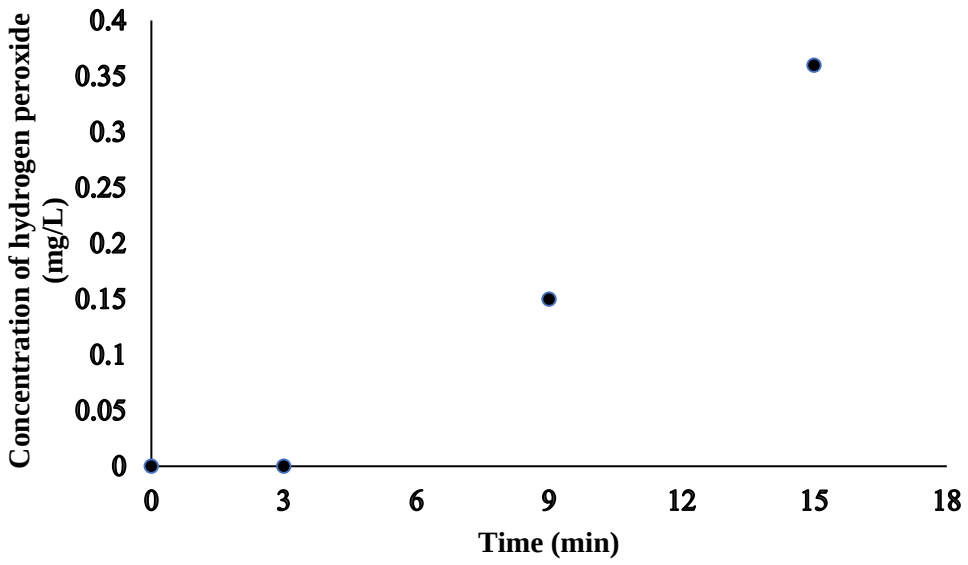

Figure 20: Concentration of hydrogen peroxide during non-thermal plasma treatment of *P. aeruginosa*

The pH was reduced to 3.85 from 7.12 after 15 min of plasma treatment (Figure 21). This outcome is similar to other studies which also treated water for 15 min with plasma, both achieving pH of 3.85 and 3.78, respectively (Pandey *et al*., 2023). The reduction in pH was caused by the presence of nitrates and nitrites in the suspension, which led to the generation of HNO_3. The low pH keeps the oxidizing potential of ozone at 2.08 V which can decrease to 1.4 V under alkaline conditions (Zeghioud *et al*., 2020). The production of hydrogen radicals also increases under acidic conditions, which then react with H_2O_2 and H_2O to produce more •OH. An acid pH range of 3-4 is said to be conducive for production of •OH (Magureanu *et al*., 2021) and the reactive molecules readily penetrate cell walls under the low pH conditions because the cell membrane permeability increases (Zhang *et al*., 2023).

The conductivity was 12.06 mS/cm before treatment, and it increased to 12.24 mS/cm after 15 min of plasma treatment (Figure 22). The conductivity of water tends to vary as a result of the ROS and RNS produced during NTP treatment. This is evident as one study resulted in an increase of conductivity from 1 µS/cm to 123 µS/cm over 15min treatment time (Pandey *et al*., 2023) while in another study the conductivity fluctuated between 2.57 mS/cm to 3.31 mS/cm over 30 min NTP treatment (Liew *et al*., 2023). The conductivity in this study increased with treatment time, but the increment was low. Perhaps because a low conductivity is the one that favours the production of H_2O_2 and O_3 which contribute to the destruction of the pollutants (Jiang *et al*., 2014; Zeghioud *et al*., 2020). The increase in conductivity is an indication of a loss in cell membrane integrity of bacteria (Wang *et al*., 2022).

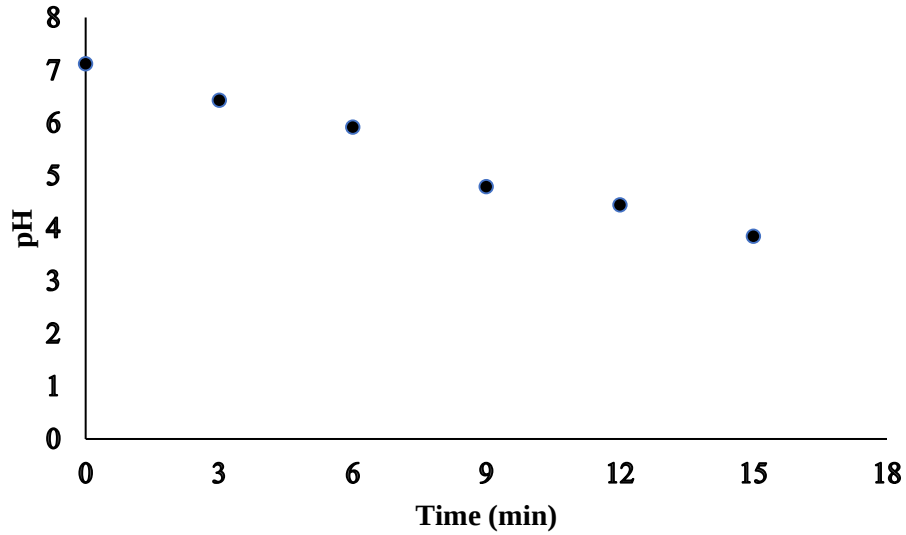

Figure 21: pH readings of *P. aeruginosa* bacterial suspension during non-thermal plasma treatment.

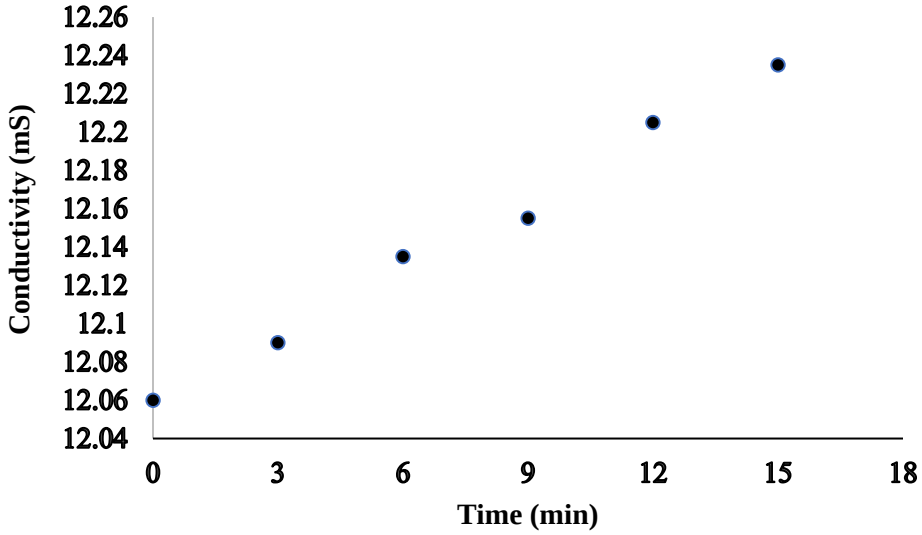

Figure 22: Conductivity readings of *P. aeruginosa* bacterial suspension during non-thermal plasma treatment

4.3.2. Inactivation of ARBs and ARGs

4.3.2.1. Re-growth Assessment

The observed trend indicated an escalating log reduction of *Pseudomonas aeruginosa*, implying a logarithmic inactivation of Antibiotic Resistant Bacteria (ARBs) with extended treatment time (9.48 ± 0.474 after 12 and 15 min) (Figure 23). The close to 100% reduction attained in this investigation indicates that NTP can be used as an alternative disinfection step.

Isolation of copper on its own yielded the smallest log reduction (0.15) of *P. aeruginosa*, potentially due to the solid state of the copper employed in this study. Soluble copper, acknowledged for its high toxicity and reactivity owing to oxidative power, catalyzed Reactive Oxygen Species (ROS) formation (i.e., H_2O_2), leading to lipid peroxidation and DNA/RNA damage, ultimately resulting in bacterial inactivation (Salah et al., 2021, Virieux-Petit *et al*., 2022). A comparative study of copper compounds (copper oxide, copper acetate, copper nitrate, and copper sulfate), all possessing bactericidal properties, revealed that copper in salt form exhibited the highest antimicrobial effectiveness (Benhalima *et al*., 2019). Moreover, considering the low capacity of Cu^{2+} to stimulate bacterial membrane and nucleic acid destruction through copper-catalyzed Fenton-like reactions (driven by the reactions of Cu^{2+}/H_2O_2) (Li *et al*., 2021), it is therefore reasonable to deduce that the copper electrode had negligible impact on the reduction of *P. aeruginosa*. Consequently, the observed log reductions were attributed solely to the plasma discharge. The challenging characteristics of *P. aeruginosa* as a gram-negative bacterium that thrives in biofilms likely contributed to the limited efficacy of solid copper in its inactivation (Salah *et al*., 2021).

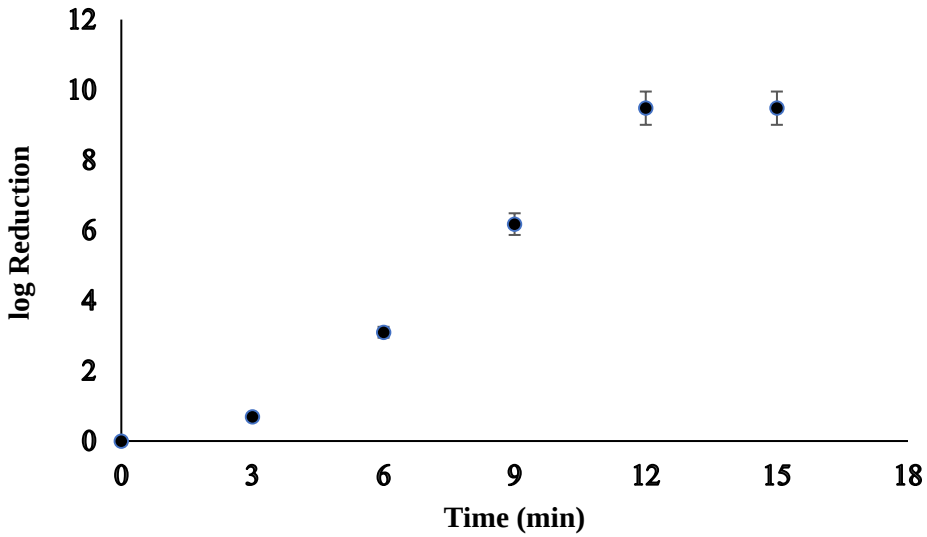

Figure 23: Log reduction of *P. aeruginosa* (27853) after non-thermal plasma treatment

4.3.2.2. Evidence of cellular disintegration

The rod-like shape disseminated by SEM imaging confirms the structural integrity of *P. aeruginosa* cells before NTP treatment (Figure 24) (Wood *et al*., 2023).

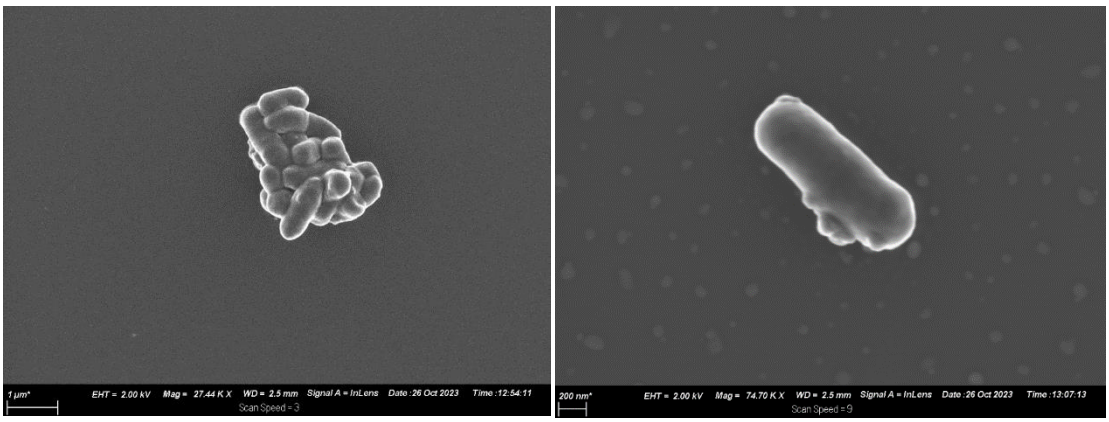

Figure 24: SEM image of *P. aeruginosa* before non-thermal plasma treatment

Following NTP treatment, SEM images indicated a loss of structural integrity on the surface of *P. aeruginosa* cells (Figure 25). The cellular remnants of *P. aeruginosa* assumed an amorphous shape, deviating from their usual rod-like structure observed before NTP treatment. This observation highlights NTP's capability to overcome the tensile strength of the cell membrane, leading to rupture, leakage of

intracellular components, and cell death. These effects effectively impede the growth of *P. aeruginosa* and negate its protection of Antibiotic Resistance Genes (ARGs) (Zhang *et al*., 2023). The rupturing phenomenon is attributed to the accumulation of Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS) free radicals on the cell membrane, surpassing the membrane's tensile strength and causing it to rupture (Mazandarani *et al*., 2022; Zhang *et al*., 2023). Notably, NTP appears to have a greater inhibitory effect on gram-negative bacteria than gram-positive bacteria, as demonstrated by its ability to disrupt the membrane of *P. aeruginosa*, a gram-negative bacterium (Yan *et al*., 2021; Zhang *et al*., 2023). A study compared SEM images of gram negative and gram-positive cell after plasma treatment, the destruction was more evident on gram negative as there was cell breakage effects on the gram negative and only irregular shape and cell shrinkage of the gram-positive cells (Han *et al*., 2016). This susceptibility is attributed to the thinner peptidoglycan layer and the presence of an outer membrane with components such as proteins and lipopolysaccharide (LPS), which are sensitive to ROS in gram-negative bacteria (Zhang *et al*., 2023). A hypothesized dual mechanism for the destruction of cellular membranes involves lipid peroxidation and electroporation. In lipid peroxidation, reactive radicals generated by plasma, particularly OH groups, detach polar head moieties and fatty acid tails from phosphatidylcholine residues in the lipid bilayer of cell plasma membranes. This leads to crosslinks between adjacent fatty acid tails, allowing the unrestricted influx of radicals and water molecules, resulting in membrane lesions and pore formation. Alternatively, electric fields generated by plasma could enhance transmembrane potential, initiating the breakdown of the lipid bilayer and the formation of membrane pores. The recoverability of these pores may vary, and with increased electric field strength and duration, they may become irrecoverable, ultimately causing necrosis and cell rupture (Sreedevi and Suresh, 2023).

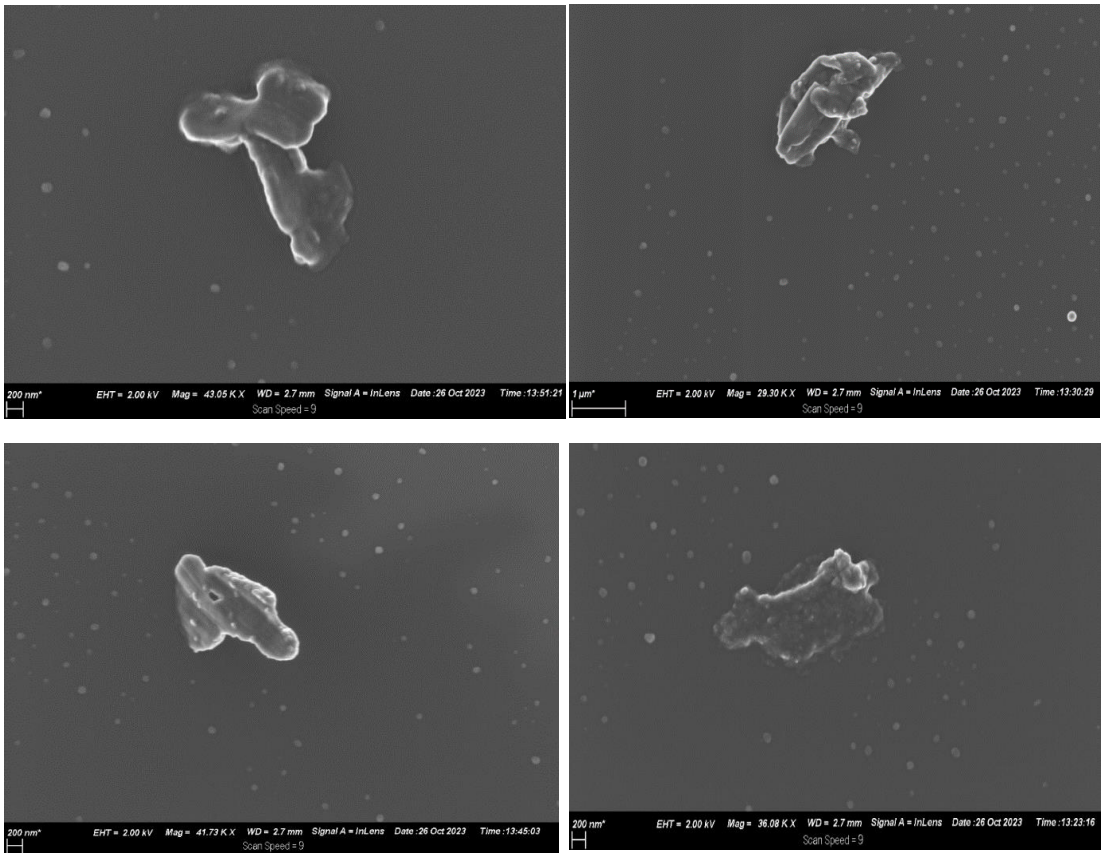

Figure 25: SEM image of *P. aeruginosa* after 15 min of non-thermal plasma treatment

4.3.2.3. Detection of resistance gene (bla_{NDM-1}) attenuation

In our investigation, the diminishing intensity of *P. aeruginosa* bla_{NDM-1} (240 bp) bands over time, as depicted in Figure 26, suggests the capacity of Non-Thermal Plasma (NTP) to inactivate ARGs. Fluorescence of the bands is presumed to indicate the DNA concentration (Resources, 2023). However, at the final treatment duration (15 min), the ARGs were not entirely inactivated, possibly due to the protective biofilm surrounding *P. aeruginosa*. Biofilm-inhabiting bacteria are known to exchange more genes than those in a planktonic/free-living state (Song *et al*., 2021). The NDM-1 gene exhibits increased expression in optimal biofilm formation conditions (Al-Bayati and Samarasinghe, 2022). Biofilms, with their high cell densities, facilitate intercellular gene transfer due to the short physical distance between microorganisms (Song *et al*., 2021). The protective nature of the biofilm also hinders NTP reactive species from penetrating it (Grehs *et al*., 2021). Despite this, a study utilizing plasma-generated Fenton-oriented reactions (Cu^{2+}/H_2O_2 and Fe^{2+}/H_2O_2) recorded measurable gene copies of bla_{TEM-1} after 10 minutes, despite enhanced

ARG inactivation by Cu^{2+} and Fe^{2+} (Li *et al*., 2021). This underscores the efficiency of our treatment process, which does not rely on exogenous Fenton-oriented inorganic ions (Cu^{2+} and Fe^{2+}). Comparative studies show UV achieving a maximum reduction of 99% (Chen *et al*., 2020a), chlorination achieving 100% reduction (Mao *et al*., 2015), and ozone achieving a maximum of 98.1% (Jäger *et al*., 2018) of ARGs. However, the concentrations of these disinfectants were considerably higher and impractical compared to those used in Wastewater Treatment Plants (WWTPs), raising environmental concerns due to the formation of harmful by-products like halo-organics (Luukkonen *et al*., 2014; Anthony *et al*., 2020) and bromate (Jäger *et al*., 2018; Gomes *et al*., 2019) from chlorine and ozone, respectively. Furthermore, we hypothesize that extending NTP treatment time may lead to complete inactivation of ARGs, offering the advantage of eliminating the need for additional use of potentially harmful chemicals. Additional parameters, such as increasing the grounded electrode surface or diameter at a fixed discharge gap, can be optimized to enhance the area of plasma discharge. The choice of feeding gas also plays a role, with oxygen-containing gas being demonstrated to lead to the fastest contaminant degradation, followed by air and argon, while nitrogen results in the least removal. Oxygen-containing gas induces the production of O^-based active species and O_3, enhancing the degradation rate of pollutants (Jiang *et al*., 2014, Magureanu *et al*., 2021).

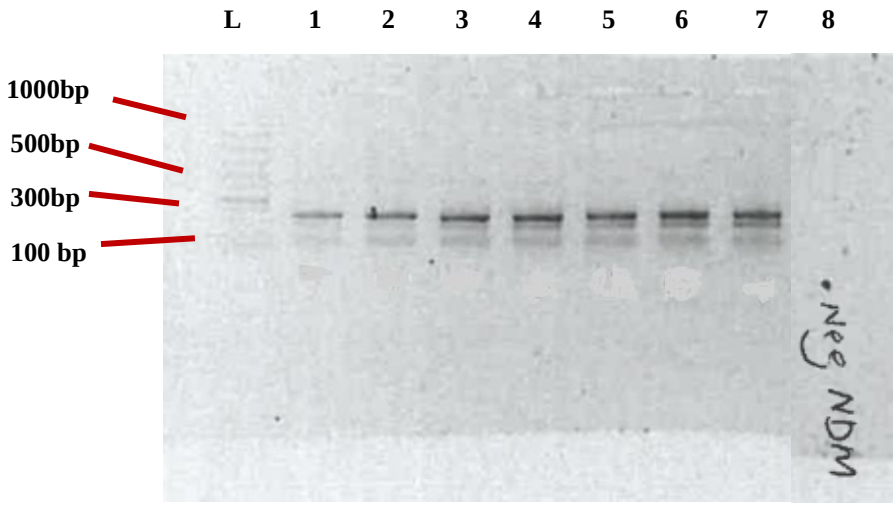

Figure 26: Effect of periodic non-thermal plasma treatment on the carbapenem resistant gene of *P. aeruginosa*. L: DNA ladder, 1: 15 min, 2: 12 min, 3: 9 min, 4: 6 min, 5: 3 min, 6: copper control, 7: +ve control, 8: -ve control.

4.4. Conclusions

A dramatic extermination of *P. aeruginosa* was achieved over 12 min treatment, which correspondingly evinced the diminishing presence of the resistance gene terminal treatment time (15 min). The observed log reductions further validated the efficacy of Non-Thermal Plasma (NTP) in completely eliminating Antibiotic-Resistant Bacteria (ARBs) over time. This effectiveness was attributed to the increasing presence of ˙OH and the long-lived species (H_2O_2, NO_2^- and NO_3^-) which can generate more ˙OH and reacting to secondary products post-discharge, significantly contributing to the antibacterial activity of Plasma-Activated Water (PAW). These species also played a role in lowering the pH to levels conducive to bacterial destruction, while the conductivity remained consistently low.

The SEM results provided additional confirmation that NTP disrupts the tensile strength of the cell membrane, leading to rupture and ultimately cell death. This mechanism, in turn, prevents bacterial growth, including the preservation of ARGs. Notably, the study excluded the antimicrobial contribution of copper, emphasizing that the observed log reductions were solely attributed to NTP. Overall, these findings underscore the potential of plasma treatment as an effective disinfection step for wastewater. However, it is suggested that addressing biofilms near *P. aeruginosa*, extending the reaction time, using oxygen-containing feed gas and enlarging the diameter of the grounded electrode may further enhance the complete inactivation of ARGs.

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Chapter 5

5.1. CONCLUSIONS

The antibiotics that were once an aid to ailing persons have now contributed to the worsening of conditions. With the overuse and misuse of antibiotics, ARBs such as *A. baumannii* and *P. aeruginosa* carbapenem resistant, their genes, are now emerging victorious against the most vulnerable patients, leading to more costly hospital stays and the demise of most patients. These ARBs and ARGs continue to thrive in all types of environments and facilities, propagating through vertical and mostly gene transfer. The water we drink is now also a hotspot of these ARBs and ARGs, which prevents their acquisition among the frail. As one of the ways of destroying them completely, they would have to be eliminated from our water supplies, i.e. WWTPs which keep supplying us with this important resource but not equipped to set us free from these dangerous pathogens. Hence this study was conducted on *A. baumannii* and *P. aeruginosa* using NTP in an attempt to not only eradicate them but to also eradicate their genes. The 15-minute treatment conducted had the highest impact on the reduction of *A. baumannii*, while the 12-minute treatment had the highest impact on the reduction of *P. aeruginosa*. The presence of the resistance gene in both *P. aeruginosa* and *A. baumannii*, seemed to fade with time. This was attributed to the presence of •OH and the long-lived species (H_2O_2, NO_2^- and NO_3^-) which increased with time. The H_2O_2 formed more •OH and the NO_2^- and NO_3^- species also reduced the pH to levels which are conducive for the destruction bacteria. The conductivity also remained low as expected. The SEM results also confirmed the theory that that NTP overcomes the tensile strength of the cell membrane, causing it to rupture and ultimately leading to cell death, preventing the growth of bacteria and its ARGs. The effect of copper antimicrobial ability was excluded in this study, attributing the log reductions solely to NTP.

5.2. RECOMMENDATIONS

These results suggest that plasma treatment has a great potential to be an efficient disinfection step for wastewater. However, in order for the genes to be completely inactivated, research has to be ongoing. Many factors need to be optimised before implementation is possible, which includes increased treatment time of the genes, increasing the diameter of a grounded electrode, using an oxygen-containing feed gas and test run in large scale WWTP, to see how it would fair, as there are more challenges for technologies in real life situations. The complete removal of biofilms in *P. aeruginosa* would also assist in the better penetration of NTP species to completely inactivate the genes. When the water is used for human consumption, the nitrites must be removed and the pH must be raised in order to meet WHO's drinking water standards.

Appendix A

Study selection criteria

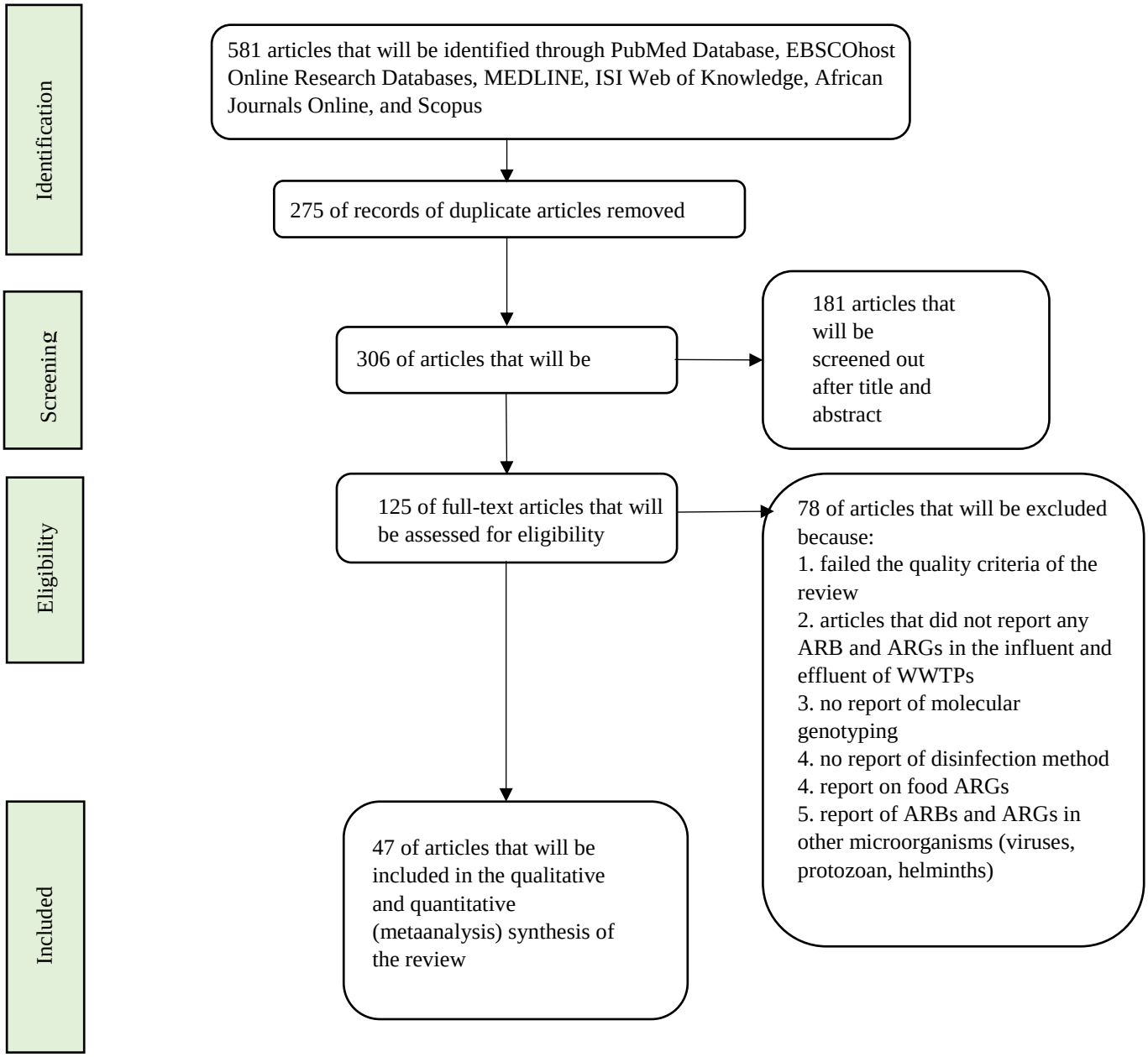

Figure A 1: Flow diagram summarizing the process of literature search and selection.

Appendix B

Acinetobacter baumannii

B1. Standard

Figure B1 shows the growth of *A. baumannii* on media containing imipenem.

Figure B 1: Growth of *A. baumannii* (BAA 1605) on LB agar containing imipenem

The growth of the colonies on the plate can be described as shiny, creamy and whitish in colour. This a positive indication of *A. baumannii*, as it is reported to give smooth, and sometimes mucoid colonies that are pale yellow in colour (Aryal, 2022).

B2. Molecular analysis

B2.1. Purity of DNA

Prior to the CAP treatment of the bacterial suspensions, the standard *A. baumannii* was grown in LB broth for 24 h (aerobically @3250 rpm). DNA was extracted from the culture using the kit according to manufacturers' instructions. Absorbance ratios were measured to assess DNA purity; for protein contamination (A260/280 nm) and for salt and phenol contamination (A260/ 230 nm) with a nano-

spectrophotometer (Vesty *et al*., 2017). Table B1 shows the concentrations and purity of the DNA extracted twice from the same sample of *A. baumannii*, using Nucleomag DNA/RNA water kit.

Table B1: Concentration and purity results of DNA of *A. baumannii* standard

***A. baumannii* DNA**	**Sample 1**	**Sample 2**
Concentration (ng/µL)	1828.76	2031.03
A260/280	1.88	1.88
A260/230	2.0	1.9

The results highlight the high DNA concentrations present in *A. baumannii*.The ratios for A260/280 nm were between 1.8– 2.0 and the ratio for A260/230 nm was around 1.8 (Vesty *et al*., 2017), this indicated that the samples were not contaminated during the culturing and extraction with the DNA kit used. From these results PCR was run.

B2.2. Confirmation of the presence of Carbapenem Resistance gene

The extracted DNA was used as template DNA for the PCR assay in order to confirm the presence of $bla_{\text{NDM-1}}$ gene in *A. baumannii*. The primer used in this study can be found in Table 8. The PCR mixture contained 5 µL of PCR master mix with chosen 0.5 µL of forward primer, 0.5 µL of reverse primer, 2.5 µL of template DNA and 1.5µL of milli-q water to make up a reaction volume of 10 µL. The PCR conditions for $bla_{\text{NDM-1}}$ was initial denaturation at 95ºC for 3 min, followed by 30 cycles for 1 minute at 95ºC, annealing at 59ºC for 1 minute and extension at 72ºC for 1 minute 30 seconds, with a final extension at 72ºC for 10 min. Figure B2 shows the BIO RAD T100 Thermal cycler was used for PCR.

Figure B 2: BIO RAD T100 Thermal cycler

The PCR products were stained with ethidium bromide (Anand *et al*., 2015; Odjadjare and Olaniran, 2015) and observed using electrophoresis in 1% agarose gel (Somma and Querci, 2006). The BIO RAD PowerPac basic with Mini Sub Cell GT (Figure B3) was used for electrophoresis.

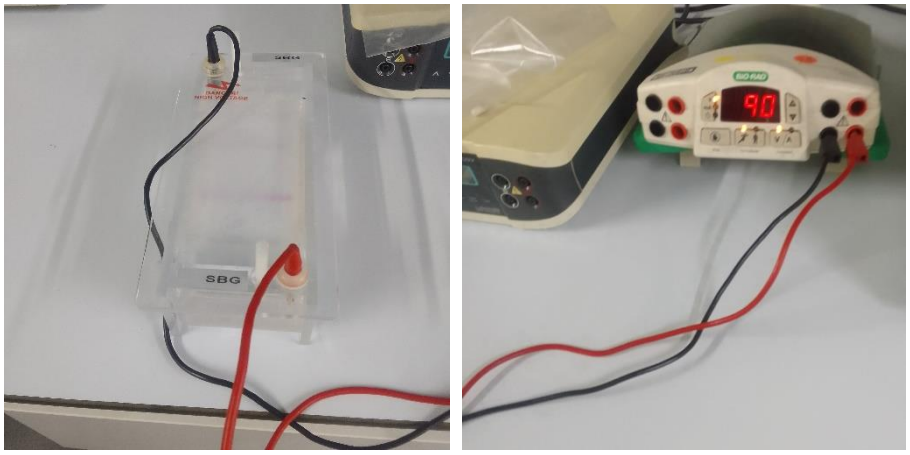

Figure B 3: BIO RAD PowerPac used for electrophoresis

B2.3. Optimisation of Annealing temperature

Once the *bla*$_{NDM-1}$ gene was confirmed in *A. baumannii*, the extracted DNA was thereafter used for optimising the annealing temperature for the gene. The gradient temperature used for the optimisation ranged from 55 ºC – 61 ºC. Figure B4 shows the results from optimisation of the annealing temperature for the bla$_{NDM-1}$ gene.

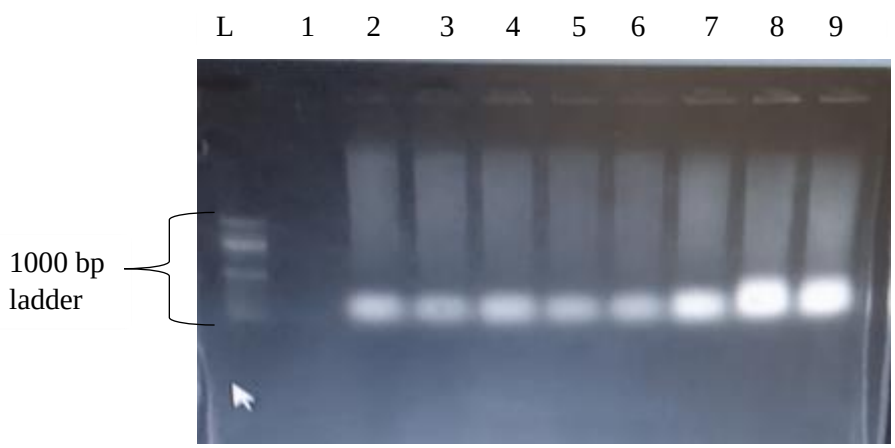

Figure B4: Optimization of annealing temperature of NDM gene in A. baumannii. L: DNA ladder, 1: -ve control, 2: 61 ºC, 3: 60.6 ºC, 4: 59.8 ºC, 5: 58.7 ºC, 6: 57.3 ºC, 7: 56.2 ºC, 8: 55.4 ºC, 9: 55 ºC.

The presence of the NDM gene in *A. baumannii* was confirmed, with the brightest band being observed at 55.4ºC and 55ºC. From this result 55ºC was chosen as the optimum annealing temperature.

B.3. Plasma treatment and disinfection effectiveness

Figure B5 shows the A. baumannii bacterial suspension that was used in this study.

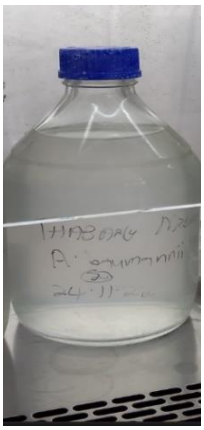

Figure B 5: *A. baumannii* bacterial suspension

Figure B6 shows the NTP power supply used in this study.

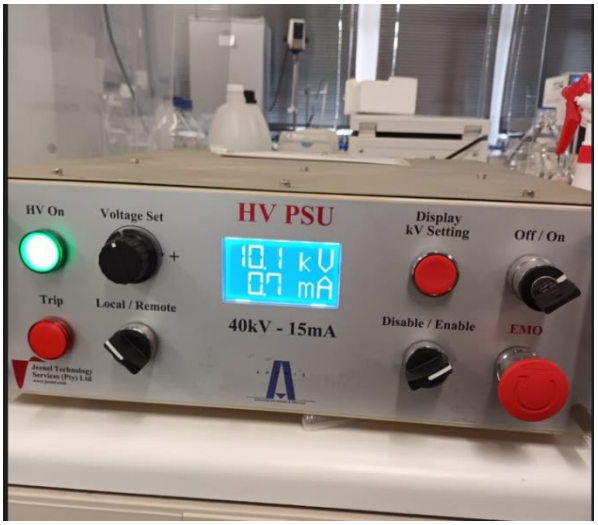

Figure B 6: Power supply for the NTP reactor

Appendix C

Pseudomonas aeruginosa

C.1. Standard

Figure C1 shows the growth of *Pseudomonas aeruginosa* on media containing imipenem.

Figure C 1: Growth of *P. aeruginosa* (27853) on LB agar containing imipenem

The growth of the colonies on the plate can be described as shiny, creamy and greenish in colour. This a positive indication of *P. aeruginosa*, as it is reported to give rise to smooth, mucoid colonies that are greenish blue in colour (Batra, 2018).

C.2. Molecular analysis

C.2.1. Purity of DNA

Prior to the NTP treatment of the bacterial suspensions, the standard *P. aeruginosa* was grown in LB broth for 16 h (aerobically @3250 rpm). DNA was extracted from the culture using the kit according to manufacturers' instructions. Absorbance ratios were measured to assess DNA purity; for protein contamination (A260/280 nm) and for salt and phenol contamination (A260/ 230 nm) with a nano-

spectrophotometer (Vesty *et al*., 2017). Table C1 shows the concentrations and purity DNA extracted twice from the same sample of *P. aeruginosa*, using Nucleomag DNA/RNA water kit.

Table C1: Concentration and purity results of DNA of *P. aeruginosa* standard

***P. aeruginosa* DNA**	**Sample 1**	**Sample 2**
Concentration (ng/µL)	1896.05	2272.23
A260/280	1.8	1.78
A260/230	1.55	1.52

The results highlight the high DNA concentrations present in *P. aeruginosa*. The ratios for A260/280 nm were between 1.8– 2.0 and greater than the ratio for A260/230 nm which were around 1.8 (Vesty *et al*., 2017), this indicated that the samples were not contaminated during the culturing and extraction with the DNA kit used. From these results PCR was run.

C.2.2. Confirmation of the presence of Carbapenem Resistance gene

The extracted DNA was used as template DNA for the PCR assay in order to confirm the presence of *bla*$_{NDM-1}$ gene in *P. aeruginosa*. The primer used in this study can be found in Table 8. The PCR mixture contained 5 µL of PCR master mix with chosen 0.5 µL of forward primer, 0.5 µL of reverse primer, 2.5 µL of template DNA and 1.5µL of milli-q water to make up a reaction volume of 10 µL. The PCR conditions for *bla*$_{NDM-1}$ was initial denaturation at 95ºC for 3 min, followed by 30 cycles for 1 minute at 95ºC, annealing at 59ºC for 1 minute and extension at 72ºC for 1 minute 30 seconds, with a final extension at 72ºC for 10 min. Figure 28 shows the BIO RAD T100 Thermal cycler was used for PCR. The PCR products were stained with ethidium bromide (Anand *et al*., 2015; Odjadjare and Olaniran, 2015) and observed using electrophoresis in 1% agarose gel (Somma and Querci, 2006). The BIO RAD PowerPac basic with Mini Sub Cell GT (Figure B3) was used for electrophoresis.

C.2.3. Optimisation of Annealing temperature

Once the *bla*$_{NDM-1}$ gene was confirmed in *P. aeruginosa*, the extracted DNA was thereafter used for optimising the annealing temperature for the gene. The gradient temperature used for the optimisation ranged from 55 ºC – 61 ºC. Figure C2 shows the results from optimisation of the annealing temperature for the bla$_{NDM-1}$ gene.

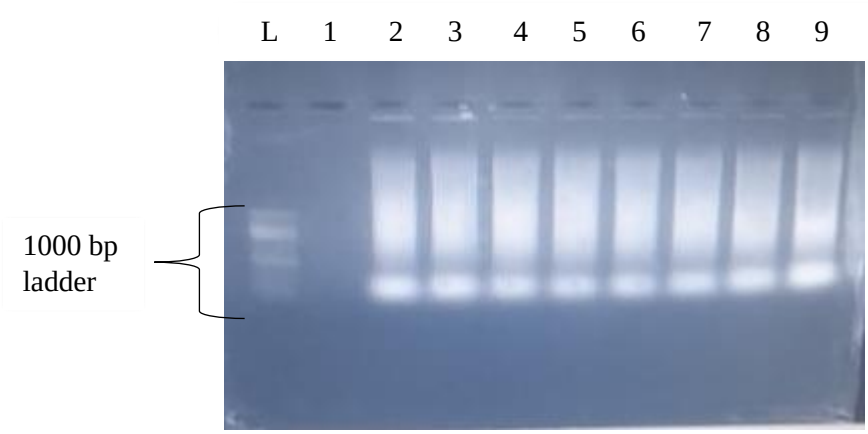

Figure C 2: Optimization of annealing temperature of NDM gene in *P. aeruginosa* L: DNA ladder, 1: -ve control, 2: 61 ºC, 3: 60.6 ºC, 4: 59.8 ºC, 5: 58.7 ºC, 6: 57.3 ºC, 7: 56.2 ºC, 8: 55.4 ºC, 9: 55 ºC.

The presence of the NDM gene in *P. aeruginosa* was confirmed, with the brightest band being observed at 55ºC. From this result 55ºC was chosen as the optimum annealing temperature.

C.2.4. Plasma treatment and disinfection effectiveness

Figure C3 shows the bacterial suspension of *P. aeruginosa* that was used in this study

.

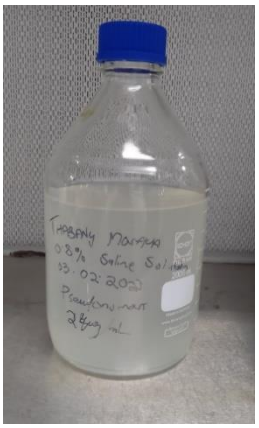

Figure C 3: P. aeruginosa bacterial suspensions

C.3. Physical chemical and structural characterization

C.3.1. Analysis of by-products

Spectrometer (StellarNet) was used to investigate the discharge characteristics and the formation of the chemical species (Figure C4) (Zhang *et al*., 2021).

Figure C 4: StellerNet Spectrometer

The Lovibond Spectro direct water testing instrument (Tintometer Group, Germany) (Figure C5) was used to determine the concentration of (H_2O_2), (NO_2^-) and (NO_3^-). There are two, four and two procedures to determine the concentrations of NO_3^-, NO_2^- and H_2O_2, respectively. The Nitrite with Tablet, Nitrate LR with Tube Test and Hydrogen peroxide with Tablet, with reaction times of 10 min, 15 min and 2 min, respectively, were chosen as they read the lowest expected concentrations. More details can be found in the manual (GmbH, 2021).

Figure C 5: Lovibond Spectro direct water testing instrument (Tintometer Group, Germany) (Limited, 2023)

C.3.1.1. Method of Analysis for Hydrogen Peroxide using the Spectro Direct

1.1 Methods

2 1 0 Hydrogen peroxide with Tablet

0.03 – 1.5 mg/l H_2O_2

1. Fill a clean vial (24 mm Ø) with **10 ml of the water sample,** close tightly with the cap.

2. Place the vial in the sample chamber making sure that the ⋈ marks are aligned.

prepare Zero
press ZERO

3. Press **ZERO** key.

4. Remove the vial from the sample chamber and **empty it, leaving a few drops remaining in the vial**.

5. Add **one HYDROGENPEROXIDE LR tablet** straight from the foil and crush the tablet using a clean stirring rod.

6. Add water sample to the 10 ml mark.

7. Close the vial tightly with the cap and swirl several times until the tablet is dissolved.

8. Place the vial in the sample chamber making sure that the ⋈ marks are aligned.

Zero accepted
prepare Test
press TEST

9. Press **TEST** key.

   Wait for a **reaction period of 2 minutes**.

Countdown
2:00

After the reaction period is finished the measurement starts automatically.

The result is shown in the display in mg/l Hydrogen peroxide.

1.1 Methods

Notes:

1. Vial cleaning:
   As many household cleaners (e.g. dishwasher detergent) contain reducing substances, the subsequent determination of Hydrogen peroxide may show lower results. To avoid any measurement errors, only use glassware free of Chlorine demand.
   Preparation: Put all applicable glassware into Sodium hypochlorite solution (0.1 g/l) for one hour, then rinse all glassware thoroughly with deionised water.
2. Preparing the sample:
   When preparing the sample, the lost of Hydrogen peroxide, e.g. by pipetting or shaking, must be avoided. The analysis must take place immediately after taking the sample.
3. The DPD colour development is carried out at a pH value of 6.2 to 6.5. The reagent tablet therefore contains a buffer for the pH adjustment.
   Strong alkaline or acid water samples must be adjusted between pH 6 and pH 7 before the tablet is added (use 0.5 mol/l Sulfuric acid resp. 1 mol/l Sodium hydroxide).
4. Exceeding the measuring range:
   Concentrations above 5 mg/l Hydrogen peroxide can lead to results showing 0 mg/l.
   In this case, the water sample must be diluted with water free of Hydrogen peroxide.
   10 ml of the diluted sample should be mixed with the reagent and the measurement repeated.
5. Oxidising agents such as Chlorine, Ozone etc. interfere as they react in the same way as Hydrogen peroxide.

Reagent / Accessories	Form of reagent/Quantity	Order-No.
Hydrogenperoxide LR	Tablet / 100	512380BT

C3.1.2. Method of Analysis for Nitrates using the Spectro Direct

1.1 Methods

2 6 7 **Nitrate LR with Tube Test**

0.5 – 14 mg/l N

prepare Zero
press ZERO

1. Place the supplied blank (red label) in the sample chamber making sure that the marks are aligned.
2. Press **ZERO** key.
3. Remove the vial from the sample chamber.
4. Add **0.5 ml of water sample** into one reaction tube.
5. Close the vial tightly with the cap and invert several times to mix the contents.

   (Caution: tube becomes warm!)
6. Add **0.2 ml Nitrate-111**.
7. Close the vial tightly with the cap and invert several times to mix the contents.
8. Place the vial in the sample chamber making sure that the marks are aligned.

Zero accepted
prepare Test
press TEST

9. Press **TEST** key.

   Wait for a **reaction period of 15 minutes**.

Countdown
15:00

After the reaction period is finished the measurement starts automatically.

The result is shown in the display in mg/l Nitrate.

1.1 Methods

Notes:

1. Nitrite concentrations greater than 2 mg/L NO_2^- lead to higher test results.
2. Great quantities of COD lead to higher test results.
3. ▲ N
   ▼ NO_3

Reagent / Accessories	Form of reagent/Quantity	Order-No.
Set: Reaktion tube NITRATE-111	Tube Test (Liquid reagent) / 24 Tests	2420702

C3.1.3. Method of Analysis for Nitrites using the Spectro Direct

1.1 Methods

2 7 0 Nitrite with Tablet

0.01 – 0.5 mg/l N

1. Fill a clean vial (24 mm Ø) with **10 ml of the water sample**, close tightly with the cap.
2. Place the vial in the sample chamber making sure that the marks ⧖ are aligned.

prepare Zero
press ZERO

3. Press **ZERO** key.
4. Remove the vial from the sample chamber.
5. Add **one NITRITE LR tablet** straight from the foil to the water sample and crush the tablet using a clean stirring rod.
6. Close the vial tightly with the cap and swirl several times until the tablet is dissolved.
7. Place the vial in the sample chamber making sure that the marks ⧖ are aligned.

Zero accepted
prepare Test
press TEST

8. Press **TEST** key.
   Wait for a **reaction period of 10 minutes**.

Countdown
10:00

After the reaction period is finished the measurement starts automatically.

The result is shown in the display in mg/l Nitrite.

1.1 Methods

Notes:

1. The following ions can produce interferences since under the reaction conditions they cause precipitation:
   Antimony (III), Iron (III), Lead, Mercury (I), Silver, Chloroplatinate, Metavanadate and Bismuth.
   Copper (II)-ions may cause lower test results as they accelerate the decomposition of the Diazonium salt.
   It is unlikely in practice that these interfering ions will occur in such high concentrations that they cause significant reading errors.
2. Conversion:
   mg/l NO_2 = mg/l N x 3.29
3. ▲ N
   ▼ NO_2

Reagent / Accessories	Form of reagent/Quantity	Order-No.
NITRITE LR	Tablet / 100	512310BT

C3.2. pH and conductivity measurements

Figure C6 shows the PL-700AL pH meter that was used to determine the pH of the bacterial suspensions.

Figure C 6: PL-700AL pH meter

Figure C7 shows the WTW Cond 3310 that was used to determine the conductivity of the bacterial suspensions.

Figure C 7: WTW Cond 3310 (hbzhan, 2023)

Appendix D

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Inactivation of antibiotic-resistant bacteria and antibiotic-resistance genes in wastewater streams: Current challenges and future perspectives

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The discovery of antibiotics, which was once regarded as a timely medical intervention now leaves a bitter aftertaste: antimicrobial resistance (AMR), due to the unregulated use of these compounds and the poor management receiving wastewaters before discharge into pristine environments or the recycling of such treated waters. Wastewater treatment plants (WWTPs) have been regarded a central sink for the mostly unmetabolized or partially metabolised antibiotics and is also pivotal to the incidence of antibiotic resistance bacteria (ARBs) and their resistance genes (ARGs), which consistently contribute to the global disease burden and deteriorating prophylaxis. In this regard, we highlighted WWTP-antibiotics consumption-ARBs-ARGs nexus, which might be critical to understanding the epidemiology of AMR and also guide the precise prevention and remediation of such occurrences. We also discovered the unsophistication of conventional WWTPs and treatment techniques for adequate treatment of antibiotics, ARBs and ARGs, due to their lack of compliance with environmental sustainability, then ultimately assessed the prospects of cold atmospheric plasma (CAP). Herein, we observed that CAP technologies not only has the capability to disinfect wastewater polluted with copious amounts of chemicals and biologicals, but also have a potential to augment bioelectricity generation, when integrated into bio electrochemical modules, which future WWTPs should be retrofitted to accommodate. Therefore, further research should be conducted to unveil more of the unknowns, which only a snippet has been highlighted in this study.

KEYWORDS

antibiotic-resistant bacteria, antibiotic-resistance genes, wastewater, disinfection method, cold atmospheric plasma

1. Introduction

Antibiotics are used for the inhibition or complete destruction of bacteria that cause infections in humans and animals (Yuan et al., 2015; Duijkeren et al., 2018; Li and Gu, 2019; Sarangapani et al., 2019) and are also widely used in cancer treatment and in some regions, as growth promotion agents (Sarangapani et al., 2019). There has been a global increase in the consumption of antibiotics because these drugs are becoming more affordable and accessible (Genthe et al., 2020). Since most antibiotics are not completely metabolised by humans and animals, they are often ejected as common components of