RICE UNIVERSITY

Inactivation of Antibiotic Resistant Bacteria and Genes by Conventional and Advanced Disinfection Methods.

by

Manisha Patel

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE Master of Science

APPROVED, THESIS COMMITTEE

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Assistant Professor, Civil and Environmental Engineering

HOUSTON, TEXAS
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Abstract

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This research investigates the impact of chlorination, ultraviolet-C (UVC) irradiation, and titanium dioxide photocatalysis (UV-A/TiO₂) on the destruction of the multi-drug resistant New Delhi metallo-beta-lactamase (blaNDM-1) gene and the inactivation of Escherichia coli (E. coli) carrying the gene. The blaNDM-1 E. coli was obtained by transferring plasmids carrying the blaNDM-1 gene to the control strain, E. coli K12, and experiments were performed with both the blaNDM-1 E. coli and the untreated E. coli K12. Our results show that the blaNDM-1 E. coli was significantly more resistant to all three disinfection methods especially TiO₂ photocatalysis, compared to the non-resistant E. coli K12. blaNDM-1 E. coli achieved a maximum log (C/C₀) removal of -2.1, -2.6, and -3.6 whereas E. coli K12 achieved -2.7, -3.4, and -3.9 for chlorine, UVA/TiO₂, and UVC. ARG abundances following treatment were not significantly different, whereas microbial inactivation of viable bacteria was. ARG results showcasing higher resistance implies a need to better understand the proliferation of resistant extracellular genes and how to properly control them in wastewater treatment plants. Results from this study can be insightful in optimizing chlorine, UV, and photocatalysis oriented disinfection systems to achieve ARB and ARG reduction.
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1. Introduction

Antibiotic resistance is an immense threat to public health. Municipal wastewater treatment plants are considered potential hot spots of antibiotic resistance because they serve as a collecting location for all resistant organisms from sources such as hospitals and households (Luo, 2014). Due to antibiotic resistant genes (ARGs) ability to be taken up by other microorganisms via horizontal gene transfer, it is important to not only inactivate the antibiotic resistant bacteria (ARB) but also the ARGs (Molton, 2013; Wang, 1999; Worthington, 2013). The New Delhi metallo-beta-lactamase-1 (NDM-1) enzyme, known as blaNDM-1 gene, was chosen for this study because it is poorly understood and confers resistance to last line antibiotics.

This study investigated three disinfection processes consisting of chlorination, ultraviolet light-C (UVC), and ultraviolet-A light with titanium dioxide photocatalysis (UVA/ TiO₂) for the removal of ARB and ARGs by bacteria plate counts and genes absolute abundance. The ARB sample consisted of the blaNDM-1 gene encoded in E. coli and the ARG sample consisted solely of the isolated blaNDM-1 gene that was purified via a plasmid extraction kit. The experiments were repeated for non-resistant bacteria, E. coli K12 (ATCC 10798), to compare reduction patterns between E. coli K12 and blaNDM-1 E. coli.

The main objective of this study is to (1) determine the level of inactivation of resistant and non-resistant bacteria for the same disinfection methodologies and experimental characteristics and (2) inactivate isolated ARGs in order to determine disinfection efficiency of extracellular DNA. Successful removal of ARB and ARGs can provide an improved understanding of resistance mechanisms and an efficient process for inactivation.
2. Literature Review

2.1 Antibiotic Resistance Phenomenon

Antibiotic resistance bacteria (ARB) have the ability to completely withstand, and occasionally thrive, in the presence of antimicrobial agents. While majority of bacteria are benign, there is a population which are pathogenic- leading to ARB being a threat to human health. Such bacteria acquire resistance due to encoded antibiotic resistant genes (ARGs). ARGs allow for any microorganism to proliferate and broaden its own ecological niche. ARB become resistant to antibiotics via three processes: spontaneous mutation, vertical gene transfer (VGT), and horizontal gene transfer (HGT).

Spontaneous mutation frequencies occur naturally and dramatically depending on the physiology of the species and if it portrays a mutator phenotype (Normark, 2002). Once acquiring the ability of resistance, there can be a biological cost associated with it for the microorganism. The rate and level of resistance by spontaneous mutations in genes have been found to be dependent on the dosage and type of antibiotic present in the system (Wang, 2001). On the other hand, VGT evolution represents the transfer of such genetic material from a paternal microorganism to progeny, via the natural reproduction mechanism. Therefore, all ARGs obtained by a bacteria cell are those passed down from cells holding the same genes that are resistant to the antibacterial agent.

HGT on the other hand can occur by three main mechanisms, all which require (1) a way for the donor’s DNA segment to be expressed to the recipient cell, (2) for the delivered sequences to be in integrated into the recipient’s genome, and (3) the genes to be properly incorporated by the recipient cell in a manner that is significant (Lerminiaux, 2019). Through the transduction process of HGT, DNA is transferred via bacteriophages. Bacteriophages play a vital role due to
their ability to package DNA fragments (DNA length is dependent on capsid size, but generally around 100 kilobases) and replicate themselves inside of the donor microorganism. Furthermore, due to the presence of a bacteriophage, the transduction process does not require for the donor and recipient cells to be near one another at the same time or place. Such phase-encoded proteins can also express the DNA into the chromosome (Ochman, 2000).

Another route for the genetic exchange between bacterial species is by the transformation process. Here, fragments of DNA are taken up naturally by a transformable bacterium. Essentially, it is the process of inserting free DNA that has been released by a donor cell into a recipient cell. Transformation allows for the transmit of DNA between microorganisms that are highly distantly related. However, transformation genes are regulated by numerous and diverse environmental and growth factors. Studies have shown that some bacterial species such as *Neisseria gonorrhoeae* are capable of accepting DNA from a donor cell at any stage of their life while *Bacillus subtilis* has to reach a specific physiological stage in order to accept DNA for an effective transformation (Dubnau, 1999).

The conjugation process differs due to its involvement of physical contact amongst the two cells in order for the transfer of DNA to be conducted. Conjugation can also transfer chromosome sequences by chromosome integrated plasmids and protein encoded conjugative transposons. Conjugation occurs through a wide array of divergent domains such as between plants and bacteria (Buchanan- Wollaston, 1987). However, in order to have successful gene transfer, the recipient must stabilize and properly maintain the sequences which have been transferred. Through these three mechanisms, it is possible for genes to be transferred between bacteria, leading to the transfer of ARGs and proliferation of ARB.
2.2 Antibiotic Resistant Genes and Mechanisms

ARB have become resistant to antibiotics through a diverse range of resistant mechanisms. Known mechanisms are the following (Spratt, 1994; McDermott, 2003; Magnet, 2005; Wright, 2005):

(1) permeability changes in the cell wall
(2) efflux of the antibiotic from the cell
(3) modification of the antibiotic via an enzyme
(4) degradation of the antimicrobial agent
(5) obtaining alternative pathways to those inhibited by the drug
(6) modifying the target of the antibiotic
(7) overproducing the target enzyme.

The first discovered antimicrobial compound was in 1929 called penicillin, whose first resistance gene was found in 1943. Penicillin belongs to the beta-lactam group of antibiotics, the most common group of antibiotics utilized to fight infections. Other well-utilized beta-lactam antibiotics include monobactams, cephalosporins, and carbapenems. All beta-lactam antibiotics have the beta-lactam nucleus as part of their molecular structure. Majority of all beta-lactams, with the exception of monobactams, contain a fused ring in the nucleus. Beta-lactam antibiotics main mechanism is through cell-wall synthesis during which it binds to the penicillin-binding proteins (PBPs) of the bacteria cell and then interferes with the cross linking of the peptidoglycans which prevents terminals transpeptidation inside the cell. This leads to the cell wall of the bacteria to weaken and therefore die due to osmotic pressure (Mobashery, 1998).

Of the numerous deadly and persistent genes found in the environment, the New Delhi metallo-beta-lactamase-1 (NDM-1) enzyme, known as blaNDM-1 gene, is the focal point of this study
because it remains incredibly challenging to treat even with current antibiotics including carbapenems- a beta-lactam antibiotic utilized as the last resort for infections. NDM-1 is problematic for humans because the enzyme confers antibiotic resistance to any cell expressing it, whether in genome or in plasmid. Past outbreaks have stated blaNDM-1 to be the cause of various health issues including bloodstream infections and pneumonia (Moellering, 2010). Consequently, control of blaNDM-1 in wastewater treatment is crucial for public health.

Beta- lactamases are bacteria produced enzymes that are known to be multi-resistant to beta-lactam antibiotics including the main four- penicillin, cephalosporin, monobactam, and carbapenem- all which hold a four-atom ring molecular structure called the beta- lactam ring. This resistance is evoked by the enzymes breaking down the bonds of the antibiotic.

ARGs are considered to be detrimental due to their unique characteristics such as having the ability to transfer from one organism to another. Of the numerous genes observed and studied in the scientific community, blaNDM-1 is an ARG which continues to harm human health. blaNDM-1 was identified in 2008 in a Swedish tourist patient in India (Parola, 2010). In just the ten years since its discovery, the gene has managed to spread across the globe via a group of bacteria commonly stated as the “superbugs.”

Most commonly reported in India and Pakistan and the first three United States cases reported in mid-2010, blaNDM-1 carried bacteria is a new area study for the scientific community with strains resistant to all commonly utilized antibiotics. This superbug is not only easily spread in clinical settings but also remains challenging to treat with the medications currently available. The epidemic has serious ramifications in developing countries where water access is already difficult due to majority of the population being dependent on public water access and facilities which may not implement proper sanitation and purification.
Enterobacteriaceae, which blaNDM-1 is categorized in, is a large family of gram-negative bacteria that in the past decade has been a major global concern in regard to the quantity of infections it is known to cause and the abortive effect majority of antibiotics now have on it. blaNDM-1 is a novel gene of metallobeta-lactamases (MBLs) characterized in the Bush Jacob Group 1 and Ambler Class B. It has the capabilities of locating itself in large plasmids of various bacteria including Klebsiella pneumoniae (K. pneumoniae) and E. coli. A high risk of worldwide spread is believed for this carbapenemase due to its advantageous ability to duplicate itself into a range of bacteria which then can be carried by passengers traveling from one location to another-increasing the risk of the genes spread in whatever environment the passengers reside in.

Considering current technologies and easy availabilities to transport one-self from one location to another globally, if one is to carry bacteria with the resistant gene, a myriad of locations can be concluded in which blaNDM-1 breakout is recognized. For only one patient to be carrying the gene and infecting their environment is needed for worldwide dissemination and an unstoppable spread of clones.

Furthermore, blaNDM-1 has a unique genetic structure and characterizations which creates a greater need for studying and obtaining fundamental knowledge on the gene. With a molecular mass of 28 kDa, it is monomeric and has been stated to hydrolyze all beta-lactams with the exception of aztreonam (Yong, 2009). In regard to its structure, gene libraries and amplification indicated tighter binding to cephalosporins (group of board ranged antibiotics which resemble penicillin) but loose binding to carbapenems in comparison to other MLBs including VIM-1 and VIM-2. This loose binding provokes a low efficacy of carbapenems for the treatment of pathogenic bacteria carrying the gene.
The transmissible genetic element is also part of the first enzyme group, beta-lactamase, that has shown capabilities of destroying penicillin. The occurrence provokes a never-ending challenge of creating medications and bacteria’s increasing ability to be unaffected by them.

When blaNDM-1 was first discovered in India, it was found contained within the K. pneumoniae gram-negative bacteria. An intensive study on that K. pneumoniae multidrug resistant bacteria showcased the 180-kb transmissible genetic element which was quickly discovered to be a novel metallo-beta-lactamase consisting of a variety of determinants including genes which are inactive to erythromycin, ciprofloxacin, rifampicin, and chloramphenicol antibiotics (Moellering, 2010). The gene also encodes the assurance of transcription of its genes and transmissibility-allowing it to easily be spread amongst various strains of the Enterobacteriaceae. In addition, after only a year of conducting studies on the new organism since its first finding, 22 Enterobacteriaceae species were shown to be NDM-1 producers including not only K. pneumoniae but also E. coli, Morganella morganii and 2 other Enterobacter species.

To study the disinfection processes of blaNDM-1, encoding the gene into a bacteria cell can be a useful sample for experiments. E. coli naturally lives in the lower intestine of animals including humans. The most harmless E. coli strain, E. coli B and E. coli K-12, are commonly used as a standard for studying bacteria due to the ease of operation and inexpensiveness to grow it in the laboratory. E. coli containing the blaNDM-1 gene would also provide real-world relatable knowledge since starting in 2010, the emergence of the metallo-beta-lactamase blaNDM-1 producing resistant E. coli became detected in a range of places across the globe including Australia (Poriel, 2010). As it can be stated, the origin, spread, and characteristics of the blaNDM-1 gene emphasize why it is such an important ARG to find a sustainable solution to.
The blaNDM-1 ARG is of greater concern because of its mechanism of hydrolysis that is zinc-catalyzed (Wang, 1999). Luo (2014) looked at the proliferation of the multi-drug resistant gene in a municipal wastewater treatment plant (WWTP) of Northern China. Here, the paper addressed finding blaNDM-1 gene levels in the effluent discharge of two WWTPs which represented as 4.4% to 93.2% of all genes. Furthermore, the study also looked at soil samples and waste sludge which accounted for approximately 6.2 X 10^7 copies/gram of dry weight. Finding high gene levels in the effluent discharge that is disseminated to the environment creates a need to understand and control the propagation from plants. While much is known on the mechanism and abundance of the blaNDM-1, there is a still a great need on studying the inactivation of blaNDM-1 via conventional and advanced disinfection processes practiced in WWTPs.

2.3 Consequences of Antibiotic Resistance and Linkage to Wastewater Treatment Plants

The overuse and misuse of antibiotics over the last decades have resulted in an unforeseen consequence of growing urgency due to the rise of ARB (Alanis, 2005). Reports have shown for hospital settings and agriculture to account for the majority of sources where antibiotics are heavily misused. In many developing nations such as China and India, unprescribed antibiotics account for 20 to 100% of the total antibiotic usage (Morgan, 2012). In the United States, agriculture and human prescriptions continue to be the top two sources (Blaser, 2014). Antibiotics usage in factory farms and crops is virtually a necessity due to crowded and unsanitary conditions. Antibiotics are also widely utilized in subtherapeutic concentrations in animal feed in order to promote animal growth. Various practices such as these have led to the high rate of resistance observed today (Allen, 2010).
Antibiotic resistance compromises the effectiveness of pathogenic bacterial prevention and treatment and has been observed for nearly all antibiotics that have been developed (Blair, 2015). Notably, the antibiotic resistance crisis is in broad scope as illustrated by the mounting publications emphasizing the thrive of ARB and ARGs in natural and medical settings all around the world (Taylor, 2014; Gandra, 2014). A recent report estimated cumulative economic losses of $100 trillion and 300 million premature deaths world-wide by 2050 due to substantial rises in rates of antibiotic resistance (O’Neill, 2014). Therefore, antibiotic efficiency is of utmost importance and thus finding a solution to the ever-spreading antibiotic resistance is vital as it can cause detrimental public health issues.

While in recent years research focusing on ARBs and ARGs has grown, a complete understanding on the growth, dissemination, and unique characteristics of resistant bacteria and genes are still needed (Araujo, 2010; Boczek, 2007; Ferreira, 2007). In fact, the World Health Organization (WHO) has labeled the antibiotic resistant phenomenon as one of the leading risks to human life (WHO, 2014). A previous study focusing on removal efficiency in WWTPs concluded a removal of 20.7% of ARGs from sludge with at least 78 different subtypes of antibiotic resistance continuing to persist (Araujo, 2010). The amount of ARB regularly being discharged into outlet waters is abundant. In final effluent streams, water can hold roughly $10^{10}$ Colony Forming Units (CFU) of ARB daily. A study conducted in Germany tested wastewater to find 140 different genes, many showing rapid spread into wastewaters, which resisted a wide range of antibiotics including aminoglycosides, chloramphenicol, and fluoroquinolone (Martins, 2006; Sabate, 2008; Baquero, 2008). Therefore, it is important to not only find an effective disinfection method but one which can remove ARB and ARGs that prevail through various mechanisms (Kummerer, 2009).
ARB are also emerging as waterborne contaminants (Pruden, 2006; Sapkota, 2007; Li, 2009). As the main wastewater collecting ground for communities and ARG sources such as hospitals and agricultural land, WWTPs world-wide have been proven to serve as breeding grounds and point sources of ARB and ARGs. Even genes resistant to last-resort utilized antibiotic carbapenems such as the New Delhi metallo-beta-lactamase-1 has been found present in significant levels (1316 to 1431 copies/mL) in the effluent discharge stream (Luo, 2014). Such numbers convey the abundance of ARGs found in municipal WWTP and the need for effective WWTP treatment processes. Currently, a majority of WWTPs do not have the adequate technology required for the successful removal of ARBs and ARGs from water, leading to such bacteria and genes disseminating into the environment (Jaeger, 2018). Consequently, there is an enormous impetus to develop effective approaches for the disinfection of ARBs and ARGs during water treatment and reuse.

2.4 Disinfection Processes

2.4.1 Chlorination

Due to its easy application and moderate cost, chlorine is a widely utilized disinfectant for drinking water treatment since the early 1900s (Rusin, 2001). In a traditional wastewater treatment plant, chlorine treatment is usually one of the initial stages of water filtration. Free chlorine disinfectant (Cl, (aq), HOCl, and OCl) works as an oxidant that affects subcellular compounds, fragment proteins, the permeability of membranes, and reacts with nucleotides (Albrich and Hurst, 1982). Free chlorine can be used as both a primary disinfectant (in order to inactivate microorganisms) and secondary disinfectant (in order to maintain a certain level of residual in the network). The general chlorine chemistry is shown below. The chlorine gas rapidly hydrolyzes to hypochlorous acid (HOCl) and can disassociate to OCl since it’s a weak acid.
Cl₂ (g) ⇌ → Cl₂ (aq)  
Cl₂ (aq) + H₂O ⇌ → H⁺ + Cl⁻ + HOCl
HOCl ⇌ → H⁺ + OCl⁻

(if reacted with sunlight) 2HOCl ⇌ → 2Cl⁻ + 2H⁺ + O₂

Previous studies have also shown for free chlorine to be sensitive to numerous factors such as concentration, pH levels, and temperature in regard to reaction rates and disinfection efficiency (Li, 2012). For example, an increase in concentration of free chlorine can increase the ability of chlorine to oxidize and possibly also form toxic disinfection byproducts (DBPs) by reacting with natural organic matter (NOM) (Wang, 2007; Wu, 2009). pH levels also affect the inactivation process since HOCl (the stronger and more effective oxidant) dominates at lower pH and OCl⁻ dominates at higher pH. Typically, in a pH range of 6.5-8.5, the reaction is not complete and therefore both species (OCl⁻ and HOCl) are present. A 50:50 ratio equilibrium of both species is generally around a pH of 7.5.

Chlorine inactivates bacteria cells by reacting with components of cell walls, breaking down the enzymes, and causing DNA damage. Its effectiveness is measured commonly by the Ct value which is the product of concentration and time. For water treatment, tables showcase the required Ct values to achieve specific levels of microorganism of interest’s removal from the water.

\[ \text{Ct} = \text{concentration of chlorine} \times \text{operating time} \]  

Due to chlorine being a highly reactive chemical, measuring chlorine demand is vital. In this case, a breakpoint level or curve is utilized for reference. Before the breakpoint, chlorine is primarily reacted with other species such as bromide, sulfur compounds, and ammonia active in the water. Beyond this breakpoint, the concentration of chlorine added to the system is known as
free chlorine. There are numerous microbial inactivation patterns found for chlorine disinfection processes including pseudo first order, a shoulder effect, and tailing effect. There are various kinetic models which showcase different trends of inactivation such as the Rennecker-Marinas, Collins-Selleck, and Hom-Haas models. However, the most common disinfection kinetic model is the Chick-Watson model and is presented below.

The Chick-Watson law follows a first order reaction in regard to the number of microbes as a function of time. This model is accurate for ideal conditions in which all cells of the single species are uniformly spread out in the system of water, are equally susceptible, and are in the presence of the disinfectant for the same contact time period.

\[
\text{Chick’s Law: } \ln \left( \frac{N}{N_0} \right) = -k \cdot t \quad (6)
\]

\[
\text{Chick-Watson’s Law: } \ln \left( \frac{N}{N_0} \right) = -k_0 \cdot C^n \cdot t \quad (7)
\]

where, \(-k_0 \cdot C^n = k\) \quad (8)

and when \(n = 1\), \(k_0 = -\frac{\ln\left( \frac{N}{N_0} \right)}{C \cdot t}\) \quad (9)

where, \(N = \) concentration of organisms \([\text{CFU/mL}]\), \(N_0 = \) initial concentration of organisms \([\text{CFU/mL}]\), \(C = \) disinfection concentration \([\text{mg/L}]\), \(n = \) coefficient of dilution (assumed \(n = 1\) for linear model), \(t = \) time \([\text{min}]\), \(k_0 = \) inactivation rate constant \([\text{L/mg\cdotmin}]\), \(k = \) rate constant \([\text{min}^{-1}]\)

Previous studies have reported the inactivation effect of chlorination for a variety of microorganisms. Results of experiments using municipal water from WWTPs and cultured samples in a laboratory have shown different data. Macauley, et al. (2006) determined the disinfection potential of chlorine against swine lagoon bacteria. Their results indicated a maximum
of 2.4 log bacteria reduction from lagoon samples. However, it is important to note that the study’s chlorine concentrations were in the range of 0 mg/L to 30 mg/L and repeated experiments has chlorine concentrations in the range of 50 mg/L to 500 mg/L. Compared to the Environmental Protection Agency (EPA) standards which report 4.0 mg/L to be the maximum recommended chlorine concentration to have in water treatment, this study’s dosages were higher.

Some studies have also looked at a specific microorganism for desired inactivation. Rice (1999) analyzed isolates of *E. coli* O157:H7, a common cause of waterborne outbreaks, in order to determine their sensitivity to chlorine. Their study showcased a 1.4 log removal after a 2-minute exposure time. Furthermore, Huang et al. (2011) demonstrated the effects of a constant Ct value but with different concentration and operating times in secondary effluents of a municipal WWTP. The study used a Ct of 50 mg * min/L for all experiments and found for an exposure of 25 mg/L Cl₂ for an exposure time of 2 minutes was led to a higher log removal than did 2 mg/L Cl₂ dose for 25 minutes. This finding emphasizes that disinfection is more advantageous with a shorter contact time but higher concentration than vice-versa.

Few research studies have focused on chlorine’s effect specifically on ARB and ARGs. Huang et al. (2011) looked at the disinfection of chloramphenicol-resistant bacteria and found for it to be the dominant species in the microbial community after chlorine disinfection. The study also showed for reactivation of some types of ARB (chloramphenicol-, ampicillin-, and penicillin-resistant bacteria) post-chlorination for the experiments that used a low chlorine dosage. This evokes a need to greater understand the factors that can influence the selection of ARB by chlorination in effluents including the type of antibiotic resistance and mode of operation. Research studying the effect of treating similar types of ARB through chlorination have been conflicting. Grabow (1976) studied a sample of ampicillin-resistant bacteria in sewage water and
found the percentage of ARB to decrease post-chlorination. Conversely, Templeton (2009) studied ampicillin-resistant *E. coli* which showcased a higher inactivation than the antibiotic-sensitive *E. coli* however, trimethoprim-resistant *E. coli* was more resistant to chlorine than the antibiotic sensitive *E. coli*. While both studies portray selection of ARB via chlorination, there are limited studies experimenting same types of bacteria (resistant and non-resistant) to prove as such. Also, other studies have further suggested the genome size of different strains to impact the overall efficiency of water treatment. Prutz (1996) and Yoon (2017) found for chlorine to have a higher reactivity to toward G-C base pairs and is less reactive towards A-T base pairs when comparing inactivation effects of different bacteria cells. Therefore, depending on the type of gene tested with, there will be a variation in inactivation. This also suggests for the chlorination process to be more complex than current understandings, especially for microbial disinfection.

2.4.2 Ultraviolet Light- A with Titanium Dioxide Photocatalysis

The usage of advanced oxidation processes (AOPs) in regard to UV irradiation with titanium dioxide (TiO$_2$) photocatalyst for water treatment has been growing in the past decade. UV/TiO$_2$ involves the production of highly reactive hydroxyl radicals which has shown to degrade a wide range of contaminants. The interaction of photons from the UV light at a specific energy level with the catalyst undergoes a photocatalytic oxidation. Of the numerous photocatalysts, TiO$_2$ is promising in decomposing bacteria in water and as an alternative to disinfection with chemicals that have the potential to produce DBPs (Richardson, 1996). TiO$_2$ was first utilized by Frank and Bard (Frank, 1977) in order to decompose cyanide- a study which showcased the potential of photocatalyst for degradation. The general mechanism and charge transfer of UV/TiO$_2$ photocatalysis is as follows (Thiruvenkatachari, 2008):

1. A photocatalytic surface is illuminated by light
2. This excites the electrons in the valance band to the conduction band

3. This leads to the formation of a positive hole ($p^+$) in the valance band and an electron in the conduction band.

4. The positive hole then directly oxidizes pollutants and produces hydroxyl free radicals while the conduction band electrons reduce oxygen adsorbed to the TiO$_2$ photocatalyst.

Summary:

\[
\text{TiO}_2 \xrightarrow{hv} e^- + p^+ \quad (10)
\]
\[
e^- + O_2 \rightarrow O_2^- \quad (11)
\]
\[
p^+ + \text{Organic} \rightarrow \text{CO}_2 \quad (12)
\]
\[
p^+ + H_2O \rightarrow \text{HO (radical)} + H^+ \quad (13)
\]
\[
\text{HO (radical)} + \text{Organic} \rightarrow \text{CO}_2 \quad (14)
\]

It is important to note the consumption of electrons in order to achieve photocatalytic oxidation. In the case of equations 12 and 13 not occurring, an abundance of electrons can be present in the conduction band which lead to a recombination of positive holes and electrons. Furthermore, because TiO$_2$ absorbs radiation below the visible light range, only a maximum light wavelength of around 340 nm can absorbance be reached. Overall, UV light ranges between 100nm to 400 nm. UVA is considered a long wavelength between 320 and 400nm whereas UVC is the shorter wavelength ranging from 200 to 280 nm. Generally, 200 to 300nm is considered the germicidal range for disinfection. UV’s mechanism of inactivation involves damage to the nucleic acids via the high energy photons. Damage to the DNA is done by dimerization of T-T, C-T, and C-C and breakage of DNA standards. With the addition of TiO$_2$, the catalyst is adsorbed onto the cell wall surface and reacts with UV light to destruct the bacterium’s cell membrane. However, it
is important to note that light intensity through the depths of water decreases and so as does the photocatalysis reaction rate. Due to this, the average reaction rate can be calculated using the quantum yield [chemical reaction rate per unit of light that is absorbed].

Previous studies show interesting findings on the inactivation effect of photocatalysis for a range of types of bacteria. Nyangaresi (2019) compared UV and UV/TiO₂ for the disinfection of *E. coli* in water. The study showed the addition of TiO₂ (1.0 g/L) significantly increased bacteria reduction than UV did alone. Kuhn (2003) showed the effectiveness of the same disinfection process for *E. coli* and *pseudomonas aeruginosa* which assumed hydroxyl free radicals make an initial attack from the outside of the bacteria cell membrane. Overall, their findings showed a 4-log removal with an operation time of 60 minutes and a first-order dependence following the Chick-Watson model: \((\log_{10}(N_t/N_0)) = -kt\).

Furthermore, studies focusing on disinfection for water treatment such as Kim’s (2013) study aimed to learn the effects in a liquid culture of *E. coli* O157:H7. Through scanning electron microscopy and confocal laser scanning microscopy, it was observed that the UV/TiO₂ treatment led to genomic DNA structural changes and visible cell damage after an operation time of 30 minutes. The studies mentioned and others (Dadjour, 2006; Ashikaga, 2000; Dunlop, 2008) prove that reactive oxygen species successfully disrupt and damage the overall cell structure and its functions.

In regard to ARB and ARG inactivation specifically, Rizzo (2014) focused on the disinfection of a multi-drug resistant *E. coli* strain. For an operation period of 60 minutes, 2 log removal was achieved and suggested for TiO₂, photocatalysis to be effective in controlling the release of ARB and ARGs from urban WWTPs. However, there are very few studies focusing on ARB and ARGs
effect by photocatalysis and hence a need for understanding the direct effect of UV/TiO₂ for the inactivation of resistant genes.

### 2.4.3 Ultraviolet Light - C

Similar to chlorine, UV is also a common disinfection method used for water treatment, however, with a low DBP produced potential and leaving behind any residue (Zhang, 2015). UV is absorbed directly by DNA, which may damage DNA by breaking down the molecular bonds and producing thymine dimers that then disable the operations of the microorganism (McKinney, 2012). This method is effective for bacteria and protozoa however, not for all viruses. Furthermore, dosages of UV to achieve a certain level of viruses’ disinfection is also usually 1 to 2 orders higher than susceptible bacteria such as *E. coli*. The current EPA standard for proper UV disinfection if 3-log bacteria reduction.

The UV light is an electromagnetic radiation with wavelengths shorter than visible (UVC being the shortest) and is mutagenic to bacteria and various other microorganisms. The inactivation kinetics involve a direct correlation between the concentration of microorganisms and light intensity used, as shown below. Similar to the Ct concept of chlorine, UV can be measured in the form of \( I_t = \text{light intensity} \times \text{operating time} \).

\[
\left( \frac{dN}{dt} \right)_\lambda = -(k_\lambda \times I_\lambda)N \quad (15)
\]

\[
\ln \frac{N}{N_0} = -(k_\lambda \times I_\lambda \times t) \quad (16)
\]

Where, \( N \) = concentration of microorganisms, \( \lambda \) = wavelength of the light source [nm], \( k \) = coefficient (function of \( \lambda \)), \( I(\lambda) \) = light intensity.

The determination of \( I(\lambda) \) can be achieved using the Beer- Lambert law.
\[ Abs = - \log \left( \frac{I}{I_0} \right) = \varepsilon(\lambda)xC \quad (17) \]

\[ \ln \left( \frac{I}{I_0} \right) = -\varepsilon'(\lambda)xC \quad (18) \]

Where, \( \varepsilon(\lambda) = \text{at}(\lambda) \), 10-based absorptivity of light \([\text{L/mole*cm}]\), \( \varepsilon'(\lambda) = \text{e-based absorptivity of light} \) \([\text{L/mole*cm}]\), \( x = \text{length of the light path [cm]} \), \( C = \text{concentration of the light that is absorbing the solute [mole/L]} \).

Many studies have been conducted for the inactivation credit of UV radiation for pollutants in water. Hijnen (2006) states the numerous studies which show an increase in UV resistance of bacteria- leading to the need for a higher UV dosage in order to achieve the same levels of inactivation. Reported studies that a UV fluence of 5 mJ/cm\(^2\) in wastewater is able to attain 3.1 log removal of \textit{E. coli} O157:H7 (Wilson, 1992) but only 1.9 log removal at UV fluence of 4.9 mJ/cm\(^2\) of \textit{E. coli} O157:H7 (Sommer, 2000). Guo (2013) looked at UV disinfection for bacteria with antibiotic resistances to erythromycin and tetracycline in the effluents of WWTP streams at a UV dosage of 5 mJ/ cm\(^2\) and had achieved bacteria reduction in the range of 1.0 to 2.4 log. These studies presented and others (Al-Gabr, 2012; Cheng, 2013; Ma, 2011) highlight the abundance of bacteria and ARGs in WWTPs and that UV disinfection achieves bacteria reduction for both resistant and non-resistant bacteria. However, the method also shows microbial selectivity to ARB and ARGs.

Studies have also shown for genome size encoded in the bacteria cell to be a determining factor on the inactivation effect of UV oriented disinfection processes. McKinney (2012) states for bacterial cells of various genome sizes showcasing different susceptibility to UV. His study tested with four different types of ARGs, mec(A), van(A), tet(A), and amp(C). It is believed the total genome sizes and amount of adjacent TT pairs the gene holds is a vital factor in bacteria disinfection to consider since it’s related to either higher or lower potential pyrimidine dimer
targets. McKinney found a strong correlation between the inactivation effect of UV and the total genome size and its adjacent TT pairs of each of the four ARGs. Such studies portray for UV disinfection to showcase different inactivation efficiencies depending on the bacterial species and the genes that it encodes.

3. **Inactivation of antibiotic resistant bacteria and genes by conventional and advanced disinfection methods.**

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Abstract

The abuse and misuse of antibiotics in human medicine and agriculture has led to the prevalence of antibiotic resistant bacteria (ARB) and genes (ARGs) in numerous environments including wastewater treatment plants (WWTPs). While previous studies have experimented a variety of disinfectants for the removal of ARB, the efficiency of WWTP disinfection methodologies for the inactivation of resistant and non-resistant bacteria of the same strain has yet to be established. This research compares the efficiency of three disinfection methodologies on the destruction of the multi-drug resistant New Delhi metallo-beta-lactamase-1 (blaNDM-1) gene and the inactivation of *E. coli* BL21 carrying the resistant gene. The disinfectants include chlorination, ultraviolet-C (UVC) irradiation, and titanium dioxide photocatalysis (UVA/TiO$_2$). In order to compare resistant and non-resistant data with the same operation parameters, experiments were performed with both the resistant *E. coli* BL21 blaNDM-1 sample and susceptible non-resistant *E. coli* BL21. Our results show that compared to *E. coli* BL21, the *E. coli* BL21 blaNDM-1 ARB was significantly more resistant to all three disinfection methods, especially UVA/TiO$_2$ photocatalysis (p<0.05). *E. coli* BL21 blaNDM-1 reached a maximum log (C/C$_0$) bacterial removal of -2.1±0.10, -2.6±0.03, and -3.6±0.25 for chlorine, UVA/TiO$_2$, and UVC whereas non-resistant *E. coli* BL21 achieved -2.7±0.10, -3.4±0.01, and -3.9±0.03. This extended persistence of virulent ARB can be related to resistance genes holding greater protective mechanisms such as cell envelope synthesis, self-repair, and response to oxidative stress. Results from this study can inform the optimization of chlorine, UV, and photocatalysis-based disinfection systems to achieve ARB and ARG reduction.
1. Introduction

The overuse and misuse of antibiotics over the last few decades have resulted in an unforeseen consequence of growing urgency due to the rise of antibiotic resistant bacteria (ARB) (Alanis, 2005). ARB have acquired the ability to not only survive in the presence of antibiotics but also proliferate due to mutations and horizontal gene transfer. Due to antibiotic resistant genes (ARGs) being able to be up-taken by other microorganisms via transformation, transduction, and conjugation mechanisms (Alekshun, 2007), it is important to not only inactivate the ARB but also the encoded ARGs (Molton, 2013; Wang, 1999; Worthington, 2013). Furthermore, the antibiotic resistance crisis is in broad scope as illustrated by the mounting publications emphasizing the thrive of ARB and ARGs in natural and medical settings all around the world (Taylor, 2014; Gandra, 2014). A recent report estimated cumulative economic losses of $100 trillion and 300 million premature deaths world-wide by 2050 due to substantial rises in rates of antibiotic resistance (O’Neill, 2014). Therefore, antibiotic efficiency is of utmost importance and thus finding a solution to the ever-spreading resistance is vital.

Notably, of the numerous ARGs detected in the past several decades, the New Delhi metallo-beta-lactamase-1 (blaNDM-1) gene encoding the novel NDM-1 enzyme has been of growing concern. First discovered in 2009, blaNDM-1 causes beta-lactam antibiotics to be ineffective by its zinc-catalyzed mechanism and hydrolyzation of beta-lactam rings (Luo, et al., 2014). blaNDM-1 has been found on every continent except Antarctica, is plasmid borne, and has shown to disseminate rapidly. Therefore, multidrug resistant bacteria such as those that encode blaNDM-1 poses an even greater threat to human health.

Moreover, as the main wastewater collecting ground for communities, wastewater treatment plants (WWTPs) have been proven to serve as breeding grounds and point sources of ARB and ARGs
(Czekalski, 2014; Luo et al., 2014). However, a majority of water plants currently do not have the adequate technology required for the successful removal of ARB and ARGs (Munir, 2011; Rizzo, 2013). Wastewater effluent has also been a pointed source of finding the proliferation of ARGs in the form of extracellular DNA (eDNA) in sludge and receiving waters (Martins, 2006; Zhang, et al., 2013; Storteboom, 2010; Marti, 2014). Consequently, there is an enormous impetus to develop effective approaches for the disinfection of not just ARB, but also ARGs during water treatment.

While previous studies have looked into the inactivation of ARGs using current WWTP methodologies (Al-Jassim, 2017; Huang, 2011; Yoon, 2017; Zhang, 2015, Alexander, 2016; McKinney, 2012), experiments providing insight on the inactivation of resistant and non-resistant bacteria of the same strain and eDNA still thoroughly needs to be established.

This study investigated three conventional and advanced oxidation disinfection processes which consisted of chlorination, ultraviolet light-C (UVC), and ultraviolet-A light with titanium dioxide photocatalysis (UVA/ TiO$_2$), for the removal of ARB and ARGs. Here, the two main objectives of the study were to investigate if ARB achieves similar concentration reduction as non-resistant bacteria for similar operation parameters and what levels of ARG reduction are reached for the same three disinfection methodologies. The ARB sample used for experiments consisted of an engineered blaNDM-1 plasmid encoded in a competent E. coli BL21 strain. In order to compare reduction patterns between resistant and non-resistant bacteria of the same strain, experiments were repeated for a non-resistant bacteria sample, E. coli BL21 (without the encoded ARG). Furthermore, since the E. coli BL21 sample is a competent strain used mostly in laboratories, a few of the experiments were repeated with a wild type K12 strain. Similarities between E. coli K12 and E. coli BL21 proved the accuracy of the BL21 results for real world applications. Lastly, due to the proliferation of genes in the form of eDNA, disinfection processes were repeated for an
extracted ARG sample, which consisted of only the blaNDM-1 gene. Successful removal of ARB and ARGs can provide an improved understanding of efficient disinfection process kinetics for bacterial inactivation and the resistance mechanisms of ARGs.

2. Materials and methods

2.1. Materials: One Shot E. coli BL21 competent cells (ThermoFisher Scientific, Catalog No. C600003) and E. coli K12 (ATCC 10798) were purchased and the blaNDM-1 ARG was received from Baylor College of Medicine, TX, USA. Bacteria cells (both resistant E. coli BL21 blaNDM-1 and non-resistant E. coli BL21) were inoculated using lysogeny broth (LB) and were washed with 0.1 M phosphate- buffered saline (PBS), both received from Fisher Scientific, before experimentations. For all chlorination experiments, sodium hypochlorite was used as the free chlorine source and sodium thiosulfate was used as the chlorine neutralizer post- experiment. Hach DR/4000 spectrophotometer and Hach power pillow packets were used to achieve the desired chlorine concentrations. For the photocatalysis experiments, Evonik Degussa P-25 TiO₂ was utilized as the catalyst with Hitachi 8W UVA lamps in a pre-stabilized photoreactor (Luzchem research, Inc., Ottawa). UVC experiments used solely UVC lamps in the same photoreactor. The QIAprep Spin Miniprep extraction kit (Qiagen, Valencia, CA) was used for plasmid extraction and ARGs testing. To quantify ARGs post- experiments, 1uL of primer solution (covering 200 base pairs), 5.5uL of DNA- free water, and 7.5uL of SYBR- Green dye was utilized. The primer sequence was designed according to the published NDM-1 sequences (GenBank entry FN396876).

2.2. Gene Preparation and Bacteria Preparation: The blaNDM-1 plasmid was engineered by cloning the blaNDM-1 gene onto a commercially bought pET29a (+) vector plasmid using Ndel/Xhol restriction sites. For the expression of the NDM-1 enzyme, the recombinant plasmid was transformed into the competent E. coli BL21 strain, which then served as the ARB sample for
all experiments. To cultivate bacteria cells, 4.5 grams of agar, 7.5 grams of LB medium, and 300 mL of deionized (DI) water solution was placed onto sterile petri dishes. Bacteria cells were freshly streaked from the overnight LB agar plates into 5 mL of LB broth containing 1.0 mg/L aztreonam, which was then cultivated at 37 °C for 12 hours to the stationary phase. 100 μL of the cultured solution was then transferred into 5 mL of fresh LB broth (containing 1.0 mg/L of aztreonam) and incubated for 8 hours to reach a mid-exponential phase. The cells were then washed by centrifuging at 4,000 rpm for 10 minutes and had the broth discarded, rinsed three times with 0.1 M PBS (pH 7) and re-suspended in the PBS solution. The initial bacterial cell concentration in the primary stocks for all experiments was in the range of ~10^9 CFU/ml, which was determined by the plate count method after serial dilution. In order to compare the inactivation of ARB (E. coli BL21 blaNDM-1) with non-resistant bacteria, E. coli BL21 was cultivated and prepped in a similar manner and underwent the same disinfection processes. Furthermore, in order to study the inactivation of solely ARGs, the plasmid from the ARB sample was extracted using a plasmid extraction kit before following similar disinfection experiments.

2.3. Free Chlorine Disinfection: Sodium hypochlorite was added to the bacteria suspension at concentrations ranging from 1.0 to 3.0 mg/L. The Hach DR/4000 Spectrophotometer and Hach pillow packets were used to determine the initial chlorine concentration by the DPD free chlorine method. The initial chlorine concentrations were then diluted with DI water to get the desired dosages (1.0- 3.0 mg/L). The testing set-up included a sterile vial that consisted of the specific chlorine dosage, 0.1 M PBS, DI water, and bacteria sample (ARB or non-resistant bacteria). At predetermined times, samples were collected, immediately neutralized using sodium thiosulfate, and analyzed after 24-hour cultivation at 37°C for live bacteria concentration.
2.4. UVA/TiO. Photocatalysis: Carried out in a pre-stabilized photoreactor fitted with 8W UVA lamps, the photocatalysis experiments involved mixing the bacteria suspension with 0.1 M PBS, DI water, and 100 mg/L of TiO₂ in a petri dish under different irradiances at 350 nm. The light intensity was measured with a UV radiometer by placing the probe in the middle of the photoreactor. In order to confirm radiometer readings, chemical actinometry was also applied (Appendix B). The sample was uniformly vibrated in the dark for 30 minutes and then illuminated under the UVA light. The irradiance values of the UVA lamps varied between the range of 2.0 - 8.0 mW/cm². The petri dish was then uncovered and placed on a stirrer. Similar to the chlorine experiment, at predetermined times, samples were withdrawn and underwent 10-fold serial dilution and placed on sterile petri dishes to be cultivated overnight at 37°C and analyzed for live bacterial concentration by plate counting.

2.5. UVC Disinfection: UVC disinfection experiments were carried out in a photoreactor similarly as the UVA/ TiO. experiments but solely equipped with UV-C germicidal lamps (245 nm) with an irradiation intensity range of 0.5 -1.0 mW/cm², measured by a radiometer. The number of lamps utilized were altered with the range of intensity levels desired. The bacteria suspension consisted of the *E. coli* BL21 cells, 0.1M PBS, and DI water. The petri dish was then uncovered and placed on the stirrer. The mixture was stirred in the dark for 30 minute and then illuminated in the reactor at certain light intensity. Similar to previous experiments, samples were withdrawn to determine each batch’s final concentration by plate count.

2.6. ARG Experiments: In order to study the inactivation of ARGs, similar operation parameters were used as that for the resistant and non-resistant bacteria experiments. However, here, the isolated blaNDM-1 disinfection experiments represented the inactivation of extracellular genes. After plasmid extraction and undergoing disinfection, at pre-determined times, 100 μL of the
testing sample mixture was injected into a well of a sterile 96-well plate. Real time (RT) quantitative polymerase chain reaction (qPCR) was performed on a CFX96™ Real-Time System and utilized to determine gene removal. For this SYBR Green qPCR reaction, the reaction mixture contained 1 μL of DNA, 1μL of primer solution, 7.5 μL of 2× SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), and DNA-free water, yielding a total volume of 15 μL. The temperature program for SYBR Green qPCR is the following: 95 °C for 10 minutes, 45 cycles of 95°C for 15 seconds and annealing temperature for 1 minute. The primer utilized covered the following sequence: forward- GGAATTGCCCAATATTATGC and reverse- CGCAGCTTGTGGCCCATG; covering 200 base pairs. The standard curve to determine gene numbers used the cycle threshold (CT) values (obtained from qPCR as the independent variable) and the following standard curve equation: CT = 48.99 – 3.47log(CFU), which showed an R² value of 0.998.

3. Results and Discussion

3.1 ARB and Non-Resistant Bacteria Inactivation

The effect of chlorine, UVA/ TiO₂, and UVC on the inactivation of the ARB, E. coli BL21 blaNDM-1, and non- resistant bacteria, E. coli BL21, is showcased in figures 3.1.1-3.1.8. During all three experiments, especially UVA/ TiO₂, ARB achieved a lower bacteria reduction than the susceptible sample. In addition, since the non- resistant sample is a strain utilized mostly in laboratories and not found in outside environments, a non-resistant wild type E. coli K12 strain was also experimented on and compared with BL21. These experiments conveyed the similarity of inactivation levels achieved for both a laboratory strain and wild- type strains. Results showcased that for all three disinfectants, non-resistant K12 achieved comparable inactivation
levels as the non-resistant competent BL21. Of all three processes, UVA/TiO₂ achieved the highest level of bacteria reduction, followed by UVC then chlorine.

*Chlorine:* The inactivation of *E. coli* BL21 and *E. coli* BL21 blaNDM-1 by free chlorine disinfection is compared in Fig. 3.1.1. Bacteria reduction was determined in relation to CT values (CT = concentration of chlorine X operating time [mg*min/L]). The maximum obtained bacteria reduction (log C/ C₀) at chlorine doses of 1.0, 2.0 mg/L, and 3.0 mg/L was -1.62±0.03, -2.06±0.05, and -2.70±0.10 for *E. coli* BL21 and -1.43±0.25, -1.72±0.05, and -2.10±0.06 for *E. coli* BL21 blaNDM-1. Moreover, in comparison to the non-resistant *E. coli* BL21 bacteria results, the ARB disinfection process achieved a lower bacterial removal for the same chlorine dosages, especially at the highest tested chlorine dosage level. Removal efficiency was lower (p < 0.05) for resistant than non-resistant trials. Similar trends were shown for the removal of ARB at all tested chlorine concentrations. A traditional linear trendline model was used to characterize the survival curves of both susceptible and non-resistant bacteria samples. Both *E. coli* BL21 and *E. coli* BL21 blaNDM-1 samples exhibited linear lines with a high regression value and fit to the Chick model:

\[
\ln\left(\frac{N}{N_0}\right) = -k \cdot t \quad \text{Eq. (A.1)}
\]

In which, \(N\) = concentration of organisms [CFU/mL], \(N_0\) = initial concentration of organisms [CFU/mL], \(t\) = operating time [min], \(k\) = inactivation rate constant [min⁻¹]. The \(k\) values were determined by the non-linear regression method.

As expected, the inactivation rate was greater with the increase of chlorine concentration. The lower removal efficiency for ARB suggests tolerance of the bacteria at these chlorine concentrations. This was also observed by Akhtar, et al. (2016) where they compared high resistance and completely susceptible samples of *E. coli* O26 and O103. Likewise, their results showed the antibiotic profile to affect chlorine resistance, where the susceptible strain of both O26
and O103 serogroups had less resistance to chlorine compared to the resistant sample. It is known that chlorination’s role as an effective oxidant that exerts inactivation by penetrating through the cell wall, reacting with various intracellular components and effecting the metabolic processes and vital functions of the bacterium (Venkobachar, 1977). However, it is important for the disinfectant bactericide to attain a certain level of concentration at the target sites of the bacterium in order to properly apply its antimicrobial operation (Courvalin, 2005). Therefore, it is possible that self-repair and/or chlorine concentrations not reaching specific target sites of the ARB sample took place. Notably, it is observed that cell survivability in the presence of free chlorine is dependent not only on type of species (Helbling 2007; Virto 2005) but also the genes that the cell encodes. Moreover, it is likely that ARB holding ARGs such as blaNDM-1 possess a higher antioxidant capacity leading to its survival compared to susceptible bacteria.
Fig. 3.1.1) Effect of chlorine on resistant (*E. coli* BL21 blaNDM-1) and non-resistant (*E. coli* BL21) bacteria. Data points are quantified from plate counts that were run three times (Mean ± standard deviation).

<table>
<thead>
<tr>
<th></th>
<th>Chlorine [mg/L]</th>
<th>CT [mg*min / L]</th>
<th>Average K [min⁻¹]</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td><em><em>ARB (<em>E. coli</em></em> BL21 blaNDM-1)</em>*</td>
<td>1.0</td>
<td>1.5</td>
<td>0.39</td>
<td>0.98</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>3.0</td>
<td>0.85</td>
<td>0.94</td>
</tr>
<tr>
<td></td>
<td>3.0</td>
<td>6.0</td>
<td>1.45</td>
<td>0.94</td>
</tr>
<tr>
<td><strong>Non-resistant Bacteria (<em>E. coli</em> BL21)</strong></td>
<td>1.0</td>
<td>1.5</td>
<td>0.55</td>
<td>0.90</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>3.0</td>
<td>1.07</td>
<td>0.91</td>
</tr>
<tr>
<td></td>
<td>3.0</td>
<td>6.0</td>
<td>-1.89</td>
<td>0.92</td>
</tr>
</tbody>
</table>

Figure 3.1.2) Chick Model kinetics of chlorine experiments.
Fig. 3.1.3) Comparison of competent *E. coli* BL21 and wild-type *E. coli* K12 strains after chlorine dose of 3.0 mg/L. Data points are quantified from plate counts that were run three times (Mean ± standard deviation).

3.2. **UVA/ TiO$_2$ Photocatalysis:** Fig. 3.1.4 compares the inactivation of the non-resistant *E. coli* BL21 with the *E. coli* BL21 blaNDM-1 by UVA/ TiO$_2$ photocatalysis. The irradiance of 350 nm UVA lamps was varied between the range of 2.0 - 8.0 mW/cm$^2$. ARB results did reach a lower removal than non-resistant bacteria (p < 0.05). For all ARB results, the observed trend can be described as two separate phases: pre-shoulder (0-180 minutes) and post-shoulder (180- 240 minutes) effects. A lag phase was observed for all irradiances at an operating time below 180 minutes. This is attributed to only slight damage of the bacteria cells during the first phase of the experiment, and therefore it is likely that self-repair occurred. In this moment, the auto-repair enzymes were consistent with the adsorption of UV photons and the production of the hydroxyl free radicals. This induction period where the active species are beginning the attack on the membrane but not causing sufficient damage has also been observed in previous studies (Ricon 2003; Nyangaresi, 2019).

At an operating time greater than 180 minutes, the rate of microbial inactivation is found increasing. During this second phase of the ARB data results, higher irradiance values achieved a greater bacterial reduction in shorter time intervals. This is due to the greater flow of photons that directly attack the cells and the repeated hydroxyl free radicals attack on the cell membrane which left the bacterium’s auto-repair mechanisms and anti-stress enzymes to be not as significant in protecting the cell and thus, leading to membrane perforation. Also, it is possible that during this second phase of higher inactivation, ferrous and ferric ions are released and react in order to form
additional hydroxyl free radicals which increase the level of inactivation (Benabbou, 2007). The phenomenon is shown by the following equations:

\[
\begin{align*}
\text{Fe}^{2+} + \text{H}_2\text{O}_2 & \rightarrow \text{Fe}^{3+} + \text{OH}^- + \text{OH} \cdot \\
\text{Fe}^{3+} + \text{OH}^- &= \text{Fe(OH)}^{2+} \\
\text{Fe(OH)}^{2+} &\rightarrow \text{hv} \rightarrow \text{Fe}^{2+} + \text{OH} \cdot
\end{align*}
\]

Moreover, the figure demonstrates a significant difference in reduction levels of *E. coli* BL21 and *E. coli* BL21 blaNDM-1 for the various UV exposures. After the total 240 minutes operation time on the resistant bacteria sample, the irradiances of 2.05, 4.70, 6.80, and 8.00 mW/cm² achieved an ARB log inactivation (C/ Co) of -1.51±0.2, -3.02±0.21, -3.50±0.06, and -3.60±0.03. Our results of ARB achieving a lower removal compared to susceptible bacteria differs from the work conducted by Giannakis, S., et al (2018). In this study, the solar photo-fenton disinfection of 11 different ARB was studied, yet, they did not find a significant inactivation difference between antibiotic- resistant and antibiotic- susceptible bacteria. This difference could be attributed to multiple reasons including (1) the different *E. coli* strains studied, (2) the ARGs utilized (our study concentrating solely on the blaNDM-1 gene), (3) catalyst of choice, and (4) the experimental conditions. Some differed experimental conditions include their lower initial cell concentration (10⁶ CFU/mL) and higher UV irradiance of 75 mW/cm² used compared to the maximum irradiation in our study being almost 10xs lower (8.0 mW/cm²). In addition, the type of catalyst utilized for UV disinfection is also an important factor for the efficacy of inactivation levels. For example, fenton is a homogenous process bulk photocatalyst whereas TiO₂ undergoes a heterogenous process as a surface photocatalyst (Aljubourya, 2016). In a photo-fenton process, the hydroxyl radicals are products on the reaction of Fe²⁺ and H₂O₂ when exposed to irradiation. On the other hand, when TiO₂ is exposed to light, an electron in the conduction band and a hole in the valence
band is produced which causes the oxidation of the hydroxyl ions that then produce hydroxyl radicals at the surface of TiO₂. This leads to the ratio of the TiO₂ concentration and bacteria cell concentration effecting the overall disinfection efficiency. Due to both catalyst processes having different first steps, our study’s disinfection process’s dependence on the adsorption to the surface of TiO₂ and the utilization of a much higher initial bacterial cell concentration (10⁹ CFU/ mL) are reasons for the different results between both studies.

Fig. 3.1.4) Effect of UVA/ TiO₂ on resistant (E. coli BL21 blaNDM-1) and non-resistant (E. coli BL21) bacteria. Irradiance values = mW/cm² units, data points are from plate counts that were run three times (Mean ± standard deviation).
Fig. 3.1.5) Comparison of competent *E. coli* BL21 and wild-type *E. coli* K12 strains after UVA/ TiO₂. Data points are from plate counts that were run three times (Mean ± standard deviation). Experimental conditions: UVA irradiance = 8.0 mW/cm², TiO₂ concentration = 100 mg/L.

3.3 *UVC*: Compared to chlorine and photocatalysis data, results of UVC disinfection showed a smaller difference between the resistant and susceptible samples for the irradiance range of 0.5 – 1.0 mW/cm² (Fig. 3.3.1). Similar to using CT values to state chlorine dosages for water treatment, disinfection by UVC can be stated through IT values (IT = irradiance * operation time [J/cm²]). UVC results showcased lower ARB reduction than non-resistant bacteria (p < 0.05). Moreover, a notable shoulder effect (during the IT range of 0 J/cm² to 1.7 J/cm²) was observed for both the resistant and non-resistant strains, but more severe for the *E. coli* BL21 blaNDM-1. At an IT value greater than 1.7 J/cm², a linear model was observed. The trend pattern provides a good fit and regression line of data for which the inactivation follows the rules of the kinetics model, the Chick linear equation, as also shown in previous bacteria removal by UVC studies (Yoon, 2017; Bhullar, 2018). Observing the linear decrease in fig. 3.1.6 for all four UVC experiments implies that the reactor and stirring used provides consistent mixing for the solution such that each fluid obtained
adequate and uniform exposure to UV. In general, increasing the UV dose increased the amount of bacterial inactivation for all experiments. Overall, at an irradiance value of 1.0 mW/cm² (IT = 10.8 J/cm²), -2.70±0.25 log(C/C₀) ARB reduction was achieved. Moreover, UVC is known to cause direct damage to DNA which is resulted from the formation of pyrimidine dimers. However, when testing for ARB and ARGs reduction via UV disinfection, the McKinney (2012) study also suggested for this phenomenon to not be the only mechanism of UV inactivation and that there are more complex responses which lead to inactivation by UV light. Indirect mechanisms consist of photosensitizers absorbing UV light which then generate reactive oxidative species (ROS) that cause damage to a number of cellular components including nucleic acids, proteins, and the cell membrane. Though a limited amount, studies have shown differences in inactivation kinetics for different bacteria strains after UV-oriented disinfection (Al-Jassim, 2017; Fernandez, 2006). Therefore, it is likely that the observed difference in levels in the present study are due to the variant genomic content, size, and nucleotide base composition.
Fig. 3.1.6) Effect of UVC on resistant (*E. coli* BL21 blaNDM-1) and non-resistant (*E. coli* BL21) bacteria. Irradiance values = mW/cm², data points are from plate counts that were run three times (Mean ± standard deviation).

<table>
<thead>
<tr>
<th></th>
<th>UVC Irradiance [mW/cm²]</th>
<th>IT [J/cm²]</th>
<th>Average K [min⁻¹]</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ARB (E. coli BL21 blaNDM-1)</strong></td>
<td>0.5</td>
<td>5.4</td>
<td>0.41</td>
<td>0.98</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>10.8</td>
<td>0.96</td>
<td>0.95</td>
</tr>
<tr>
<td><strong>Non-resistant Bacteria (E. coli BL21)</strong></td>
<td>0.5</td>
<td>5.4</td>
<td>0.52</td>
<td>0.96</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>10.8</td>
<td>1.22</td>
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</tr>
</tbody>
</table>

Figure 3.1.7) Chick Model kinetics of UVC experiments.
Fig. 3.1.8) Comparison of non-resistant *E. coli* competent BL21 and *E. coli* wild-type K12 strains after UVC. Data points are from plate counts that were run three times (Mean ± standard deviation), UVC irradiance = 1.0 mW/cm².

3.2 ARG: Due to concerns of gene transfer and the prevalence of eDNA in the sludge of wastewater systems, ARG inactivation for chlorine, UVA/TiO₂, and UVC were conducted and are shown in Fig. 3.2.1. qPCR assays and a representative standard curve was developed to identify initial and final gene numbers. UVA/TiO₂ and UVC methods were able to achieve more than 95% destruction of the blaNDM-1 gene on the extracellular plasmid. After an operating time of 240 minutes, UVA/TiO₂ and UVC achieved a roughly close inactivation of -1.36±0.02 log (C/C₀) and -1.49±0.01 log (C/C₀). The similar trendline pattern and generally close inactivation level could mean that hydroxyl free radicals play a minor role specifically for the inactivation of ARGs, as also suggested by Yoon (2017). However, further studies need to be conducted in order to confirm. In addition, ARG inactivation for chlorine achieved a -1.20±0.10 log (C/C₀) removal (the least effective disinfection process of the three), respectively. All ARG destruction values detected are lower than bacterial inactivation observed for the *E. coli* BL21 blaNDM-1 ARB. The phenomenon of low inactivation levels for ARG occurring compared to ARB inactivation for chlorine and UV has also been observed in a previous study (Yoon, 2017). It is likely that cellular components play an active role in protecting intracellular DNA from disinfectant damage. This plasmid extracted- ARG experiment supports the hypothesis that active ARGs can still be persist.
Fig. 3.2.1) Effect of 3.0mg/L chlorine dose on isolated ARG (blaNDM-1). Data points are quantified from qPCR and standard curve (Mean ± standard deviation).

Fig. 3.2.2) Effect of UVA/TiO$_2$ on isolated ARG (blaNDM-1). Irradiance values = mW/cm$^2$, data points are quantified from qPCR and standard curve (Mean ± standard deviation).
Fig. 3.2.3) Effect of UVC on isolated ARG (blaNDM-1). Irradiance values = mW/cm^2, TiO$_2$ = 100 mg/L, data points are quantified from qPCR and standard curve (Mean ± standard deviation).

3.4 Discussion Summary: Our study showed a lower bacteria reduction of the resistant- gene carrying E. coli BL21 blaNDM-1 sample compared to the non-virulent E. coli BL21 for chlorine, UVA/TiO$_2$, and UVC disinfection processes. Such phenomena when comparing resistant to non-resistant bacteria (but of different strains) has been observed by other studies (Akhtar, 2016; Al-Jassim, 2017) for a variety of strains, operation parameters, and bactericidal usage (chlorine, UVA, and/or UVB). The extended persistence of virulent ARB such as blaNDM-1 E. coli BL21 can be related to greater protective mechanisms such as cell envelope synthesis, self-repair and response to oxidative stress. Some studies have further suggested that the genome size of different strains impact the overall efficiency of water treatment. For example, Prutz (1996) and Yoon (2017) found for chlorine to have a higher reactivity toward G-C base pairs and is less reactive towards A-T base pairs. Therefore, depending on the type of gene tested with, there will be a variation in
inactivation. Our study agrees that while such treatment processes can reduce the total number of bacteria cells, viable \textit{E. coli} and their expressed genes still remain. It is likely that the blaNDM-1 ARG possesses advantageous and/or self-repair mechanisms. This further creates a need to perform in-depth sequence analysis to allow for a comparative study on diverse gene expressions at the different stages of each sample’s inactivation.

4.0 Conclusion: Both UV experiments achieved at-least a -2.70 log C/C, ARB reduction unlike the chlorine experiment. UVC and chlorination portrayed the first order Chick model whereas the UVA/ TiO\textsubscript{2} data results showcased a two-phase pattern consisting of a shoulder effect. Comparing highest log removal values of all three experiments, photocatalysis appeared to be the most effective in the removal of both susceptible bacteria and ARB. Data also proved that ARB reduction is significantly lower for all three experiments in comparison to non-resistant bacteria. Observing differences in inactivation levels of ARB versus non-resistant bacteria implies that the strain-to-strain variability between the two samples effects survivability to disinfectant exposure and has to be considered for inactivation kinetics and the monitoring of water quality. These results also emphasize the importance of the inactivation of the specific resistance gene, along with the bacterium cell. Similar to ARB experiments, ARG chlorination resulted in the lowest reduction whereas ARG UVA/TiO\textsubscript{2} achieved the highest. Overall, ARG experiments showed a much lower level of inactivation than ARB. Performing transcriptomic analysis consisting of RNA-sequence in order to examine the molecular responses for chlorine, UVA/ TiO\textsubscript{2}, and UVC processes can provide insight into reasoning behind the different susceptible bacteria, ARB, and ARG inactivation levels observed. Such analysis can also reveal possible upregulation in protective and repair mechanisms of resistant \textit{E. coli} strains that leads to a lower damage response. It could also be possible that the blaNDM-1 gene encodes for more than just the NDM-1 enzyme and holds
additional protective mechanisms. Expanding efforts to learn the specific functions and repair capabilities of resistant genes in ARB can also lead to better disinfection. Our findings suggest that monitoring the presence and abundance of ARB and ARGs is crucial in order to treat wastewater.

**Conclusion and Future Research**

The objective of this study is to investigate the impact of chlorine, UV-A/ TiO, photocatalysis, and UV-C on the inactivation of the ARG, blaNDM-1, ARB, blaNDM-1 E. coli, and to compare bacteria reduction levels to the non-resistant sample, E. coli K12. Bacteria plate counts and qPCR experiments were performed to achieve bacteria reduction values and the following conclusions were reached.

UV/TiO2 was the most effective process, followed by UVC and then chlorine. ARG bacteria reduction was much lower than that of ARB, which was also found in previously as well (Yoon 2017). ARB experiments showcased the Chick-Watson first-order model for chlorine and UVC and a two-phase shoulder effect for UVA/ TiO2, which has also been observed by previous studies but for different bacteria samples and operation parameters (Bhullar, 2018; Nyangaresi 2018; Yoon, 2017).

For all three disinfection methods, ARB showed a lower overall bacteria removal than the non-resistant sample. Previous studies have found similar findings and stated possible reasonings such as different genome sizes showcasing higher or lower reactivity levels, specific target sites, and the disinfectants process to be more complex than originally believed. Yet, there is still a need for more in-depth analysis on the mechanisms of genes during different stages of a disinfection process. Analysis such as RNA-seq can reveal possible upregulation in protective and repair
mechanisms of resistant E. coli strains. Expanding efforts to learn the specific functions and repair capabilities of resistant genes in ARB can lead to better disinfection.

**Appendix**

### A. Data Tables for ARB and Non-Resistant Bacteria

<table>
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<tr>
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<th><strong>blaNDM-1 E. coli</strong></th>
<th><strong>E. coli K12</strong></th>
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<td>CT [mg*min/L]</td>
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<tr>
<td></td>
<td>6</td>
<td>-2.10</td>
</tr>
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</table>

**Figure A1:** ARB and Non-Resistant Bacteria Chlorine Data

<table>
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<tr>
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<th><strong>blaNDM-1 E. coli</strong></th>
<th></th>
<th><strong>E. coli K12</strong></th>
<th></th>
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<tbody>
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<td>log (C/Co)</td>
<td>Std.</td>
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<td>240</td>
<td>-3.88</td>
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</tbody>
</table>

**Figure A2:** ARB and Non-Resistant Bacteria UVA/ TiO₂ Data
In order to estimate the light intensity inside of the petri dish, the potassium ferrioxalate chemical actinometry was applied for UVA/TiO₂. Two actinometry experiments were conducted and the intensity values resembled those shown by the radiometer. The process follows the steps of the Lee (2018) study. Here, 0.2M of ferric sulfate solution in 2N H₂SO₄ (Solution A), 1.2M potassium oxalate solution (Solution B), 0.6 M sodium acetate buffer solution (Solution C), 0.2%
1,10-phenanthroline solution (Sol D), 2 N H₂SO₄ solution (Sol E), and 0.4 mM FeSO₄ in 0.1 N H₂SO₄ (Sol F) were individually prepared in amber flasks under red light. In order to determine the irradiance available in the petri dish, the photocatalysis experiment was carried out in a similar manner but solely with DI water. Absorbance values at 510nm were determined for each sample and control, which were then used to achieve irradiance values [mW/cm²] by the following equations:

\[
\text{Moles of Fe}^{2+} = \frac{A_{510}(\text{sample}) - A_{510}(\text{blank}) \times 10 \times V}{11,110 \times V1}
\]

**Equation B.1**

Where, \(A = \) absorbance value, \(V = \) total irradiated volume [mL], \(V1 = \) volume withdrawn from the solution [mL], and 11,100 M⁻¹ cm⁻¹ is the molar absorption coefficient of the Fe(II)–o-phenanthroline complex.

\[
\text{Einstein of UV absorbed} = \frac{\text{Moles of Fe}^{2+}}{\text{Quantum Yield}}
\]

**Equation B.2**

Where, quantum yield = 1.26 moles of Fe²⁺/Einstein for wavelength < 365.5nm

\[
\text{Photon Flux} = \frac{\text{Einstein of UV Absorbed}}{\text{Operating Time} \times \text{Surface Area}}
\]

**Equation B.3**

\[
\text{Intensity} \left[ \frac{\text{mW}}{\text{cm}^2} \right] = \frac{\text{Photon Flux} \times h \times c \times N_A}{\text{Wavelength}}
\]

**Equation B.4**

Where, \(h = \) Plank's Constant \((6.63 \times 10^{-34} \, \text{J}\cdot\text{s})\), \(c = \) speed of light \((3.0 \times 10^8 \, \text{m/s})\), \(N_A = \) Avogadro’s Number \((6.022 \times 10^{23} \, \text{1/mole})\), UVA wavelength = 350*10⁻² m.

Results for the radiometer and actinometry comparison are shown below.
### Photocatalysis Irradiance Measurement

<table>
<thead>
<tr>
<th>Radiometer [mW/cm²]</th>
<th>Ferrioxalate Chemical Actinometry [mW/cm²]</th>
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</thead>
<tbody>
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</tr>
</tbody>
</table>

**Figure B. 5: Irradiance values of UVA Radiometer versus Chemical Actinometry**

**References**


44. Li, D., et al. (2009), Antibiotic-resistance profile in environmental bacteria isolated from penicillin production wastewater treatment plant and the receiving water. Environmental Microbiology, 1 (6), 1506-1517.


84. Wilson, B.R., et al. (1992), UV water disinfection efficacy test surrogate for bacterial and viral pathogens, Proceedings of AWWA WQTC.


