ANTIBIOTIC RESISTANT BACTERIA AND GENES IN DRINKING WATER

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ABSTRACT

Antimicrobial resistance is now considered a growing public health challenge around the world. This research aimed to investigate the presence and identity of antibiotic-resistant bacteria (ARBs) and antibiotic resistance genes (ARGs) in drinking water supplies and to better understand the ability of water treatment processes to control ARBs and ARGs, as well as the potential reactivation of antibiotic resistance following disinfection.

In the first part of this research, a screening survey for ARBs and ARGs was carried out in tap waters across London, one of the first such studies to do so. For this purpose, a sampling programme focusing on the resistance against six antibiotics was conducted via six sampling rounds of tap water sampled from residential properties. Subsequently, sets of culture-based (viable bacterial counts on nutrient media supplemented with antibiotics) and molecular tools (detection and quantification of ARGs from environmental DNA using quantitative real-time PCR) were applied, followed by species identification using a biochemical method (API 20NE identification). Bacteria that were resistant to vancomycin, erythromycin, amoxicillin, ciprofloxacin, tetracycline, and trimethoprim were found at all sampling points, with the percentage of the bacterial populations displaying trimethoprim resistance the highest throughout all sampling rounds. Seasonal differences did not affect the percentage of resistant bacteria significantly, although the percentage of resistant bacteria tended to elevate during winter (i.e. December to January). Antibiotic resistance genes *bla*-$_{\text{TEM1}}$, *tet*(A), *sul*1, *mph*(A) and *dfrA*7 were commonly present, with the *sul*1 gene the most common. This study was the first to find the presence of the resistance gene *mph*(A) in municipal drinking water. Also, antibiotic-resistant opportunistic pathogen species, including *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Stenotrophomonas maltophilia* and *Burkholderia cepacia,* were detected.

A series of laboratory experiments to evaluate the impact of chlorine and UV disinfection on ARBs and ARGs and their regrowth/reactivation was conducted in the second part of this research. ARGs inactivation required much higher UV and chlorine doses than ARB inactivation. The *bla*-$_{\text{TEM1}}$, *tet*(A), *sul*1, and *mph*(A) genes could not be completely inactivated under the typical disinfection doses applied in water treatment. On the other hand, the application of sequential UV disinfection followed by chlorination at typically applied doses significantly reduced the studied ARGs and had synergistic effects compared to single disinfectant use. Antibiotic-resistant *Escherichia coli* and *Pseudomonas aeruginosa* demonstrated the ability to regrow in phosphate buffer saline after chlorine disinfection of 1 to 5 mg/L and UV dose of 3 to 10 mJ/cm^2. However, it is noteworthy that this UV dose is much lower than that normally applied in water treatment.

Overall, the research has highlighted the great challenge posed by antimicrobial resistance in indigenous microbial populations commonly found in water supplies. It is evident that UV and chlorination are limited in their potential to control ARBs and ARGs on their own, though synergies were shown in their

effectiveness as disinfectants when applied sequentially. The finding that some of the ARBs commonly found in tap waters are also opportunistic pathogens adds weight to the general advice that is currently given to those who are immune-compromised/suppressed (e.g. infants, hospital patients) that they should provide supplemental treatment to tap water before use (e.g. boil or filter it). Water companies should apply multiple disinfection barriers in drinking water treatment plants where possible, as well as maintain sufficient chlorine residuals in drinking water distribution networks to minimise re-growth of ARBs and spreading of ARGs during distribution.

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GLOSSARY

16S rRNA	16S ribosomal ribonucleic acid, component of the 30S small subunit of prokaryotic ribosomes
API 20NE	Standardised system for the identification of non-fastidious, non-enteric Gram-negative rods
ARB	Antibiotic resistant bacteria
AR	Antibiotic resistant
AMR	Antimicrobial resistance
ARG	Antibiotic resistance genes
AOC	Assimilable organic carbon
ATCC	American Type Culture Collection
AWWA	American Water Work Association
Aac6'lb'cr'	Name of a gene coding for plasmid-mediated quinolone resistance
Bla-$_{TEM1}$	Name of a gene coding for plasmid-mediated beta-lactams resistance
BDOC	Biodegradable dissolved organic carbon
CFU	Colony forming Unit
CLSI	Clinical and Standard Laboratory Institute
DBPs	Disinfection by-products
*dfrA*7	Name of a gene coding for trimethoprim resistance
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPD	N,N-diethyl-p-phenylene diamine
DWI	Drinking water inspectorate
DWTP	Drinking water treatment plant
EPA	Environmental Protection Agency
EUCAST	The European Committee on Antimicrobial Susceptibility Testing
FAS	Ferrous ammonium sulfate
GC-MS	Gas chromatography mass spectrometry
HGT	Horizontal gene transfer
HPC	Heterotrophic plate count
HPLC	High pressure liquid chromatography
H_2O_2	Hydrogen peroxide
LB	Luria Broth
LED	Light-emitting diode
MIC	Minimum inhibition concentration
Mph(A)	Name of a gene coding for macrolides resistance

mJ	Millijoules
ml	Millilitre
NCTC	National collection type culture
OD	Optical density
O_3	Ozone
PCR	Polymerase chain reaction
pH	Logarithm (base 10) of the hydrogen ion concentration
qPCR	Quantitative polymerase chain reaction
RPM	Rotation per minute
R2A	Medium used for heterotrophic bacteria enumeration
*Sul*1	Name of a gene coding for plasmid-mediated sulfonamide resistance
SYBR	Cyanine dye used as nucleic acid stain
TOC	Total organic carbon
Tet-A	Name of a gene coding for plasmid-mediated tetracycline resistance
TSA	Tryptic soy agar
TSB	Tryptic soy broth
UV	Ultraviolet
US-EPA	United State Environmental Protection Agency
WHO	World health organization
WWTP	Wastewater treatment plant
WTP	Water treatment plan

CHAPTER 1

INTRODUCTION

The modern era of antibiotics started in 1928 when Sir Alexander Fleming discovered penicillin. Since then, antibiotics have transformed modern medicine and saved millions of lives. However, by the 1950s, penicillin resistance became a significant clinical problem. Nowadays, antibiotic resistance has escalated into a major health issue. It is estimated that there are annually 400,000 cases of reported antibiotic-resistant infection with 25,000 deaths each year in the European Union. The World Health Organization (WHO) has placed antimicrobial resistance (AMR) as one of its top priorities to tackle. In 2015, a global action plan on antimicrobial resistance was established with five strategic objectives: (1) to improve awareness and understanding of antimicrobial resistance; (2) to strengthen knowledge through surveillance and research; (3) to reduce the incidence of infection; (4) to optimise the use of antimicrobial agents; and (5) to ensure sustainable investment in countering antimicrobial resistance. In addition, many countries and regions have developed their own antimicrobial resistance action plans (European Commission 2011, Department of Health/DEFRA 2013).

The continuous overuse and misuse of antibiotics have increasingly led to the development of antibiotic resistance. Microorganisms have developed a range of mechanisms to render antibiotics ineffective. Susceptible populations of bacteria may become resistant to antimicrobial attacks through mutation or selection or by acquiring resistance from other bacteria that possess the genetic information that encodes resistance. The latter can occur through one of several genetic mechanisms including transformation, conjugation and transduction.

Despite the widespread and growing evolution of AMR, there has been limited research into the role of environmental factors in spreading the resistance. Only recently has the environment been recognised for its role in the global spread of clinically related antibiotic resistance. Aquatic environments, specifically, may receive water for urban sewage and runoff from agricultural facilities. The occurrence of antibiotic resistance in aquatic environments has been reported since the 1970s, when *Enterobacteriaceae* was observed in rivers in the USA (Feary *et al.*, 1972). Furthermore, it has been

demonstrated that rivers and lakes that are contaminated with wastewater effluent also contain ARBs (Iwane *et al.*, 2001; Czekalski *et al.*, 2012).

The application of antibiotics in human medicine, veterinary medicine and agriculture for more than 60 years has had a major impact on bacterial communities, resulting in various resistances to antibiotics. Nowadays, antibiotics are considered an emerging class of micro-pollutants in the aquatic environment (Jones *et al*. 2001). Some removal of antibiotic compounds has been observed in wastewater treatment plants (WWTPs); however, WWTPs are not designed for the removal of micro-pollutants such as drugs (Jansen *et al.,* 1997). Furthermore, many countries currently practise water re-use, either directly, i.e. through the treatment of wastewater into a potable form, or indirectly, i.e. through the discharge of wastewater effluent into water bodies that are subsequently used for drinking water supply. In addition, a small number of previous studies has shown that some water treatment processes can contribute to selection for antibiotic-resistant organisms, meaning that the proportion of the microbial community that is antibiotic resistant following treatment is higher than the proportion before treatment (Armstrong *et al.*, 1982; Xi *et al.*, 2009; Shi *et al.*, 2013).

Recent studies have shown that conventional wastewater disinfection cannot completely remove ARGs from wastewater effluent (Zhang *et al.*, 2015; Guo *et al.*, 2015; Zhuang *et al.*, 2015). Further, antibiotic resistance can be disseminated through drinking water produced from surface water sources, which provide the route for introducing resistant genes into the natural ecosystem (Fletcher, 2015). The improvement of drinking water disinfection for the complete or maximum destruction of ARBs and ARGs therefore has an urgent importance.

Antibiotic resistance studies on drinking water have focused on culture-based approaches, considering only faecal or pathogen indicators. However, it is well known that the majority of environmental bacteria are not easily cultivable. Nevertheless, culture-based tools allow, to a certain extent, for enumeration of the viable resistant bacteria present in an environmental sample. On the other hand, molecular tools, for instance, polymerase chain reaction (PCR) and quantitative polymerase chain reactions (qPCR) are culture independent, accounting for the entire microbial community by detecting and quantifying the particular gene of interest. Several studies have detected antibiotic resistance in

drinking water by means of molecular techniques (Xi *et al.*, 2009; Bergeron *et al.*, 2015; Khan *et al.*, 2016). However, there have been no studies examining seasonal and spatial variation in antibiotic resistance, particularly in drinking water systems. Additionally, considering the growing evidence that clinical resistance is closely associated with environmental ARBs and ARGs, it is quite clear that research activities need to be expanded to include non-pathogenic or environmental microorganisms.

1.1 Thesis Outline

The thesis comprises seven main chapters consisting of: Introduction, Literature review, and Materials and Methods, followed by the Results chapter, which analyses all the experimental results and summarises the main findings. The penultimate chapter is the Discussion chapter, which integrates the key findings from the various aspects of this research and presents recommendations for water utilities and for future research. The closing chapter presents the Conclusions and summarises the contributions to knowledge of this research.

During this study, three peer-reviewed papers, along with two oral presentations and one poster presentation at international conferences, were published.

Peer review papers:

(1) Destiani, R., Templeton, M., Kowalski, W. (2018). The relative UV Sensitivity of Selected Antibiotic Resistance Genes in Waterborne Bacteria. Environmental Engineering Science. Vol. 35, No. 7. https://doi.org/10.1089/ees.2017.0179.

(2) Destiani, R and Templeton, M. (2018). The Antibiotic Resistance of Heterotrophic Bacteria in Tap Waters in London. Water Science and Technology: Water Supply. Vol. 19, No. 1. https://doi: 10.2166/ws.2018.065.

(3) Destiani, R and Templeton, M. (2019). Chlorination and ultraviolet disinfection of antibiotic resistant bacteria and antibiotic resistance genes. AIMS Environmental Science (Under review).

Conference:

(1) **252nd American Chemical Society (ACS) National Meeting and Exposition, Oral Presentation**: Destiani, R. and Templeton, M. Antibiotic-Resistant Bacteria and Genes in Drinking Water. Philadelphia, 2016.

(2) **7th IWA-ASPIRE conference and Water Malaysia Exhibition, Oral Presentation**: Destiani, R. and Templeton, M. Chlorination and Ultraviolet Light Disinfection of Antibiotic Resistant Bacteria: Survival and Potential Repair. Kuala Lumpur, 2017.

(3) **10th IWA Micropol and Ecohazard Conference, Poster Presentation**: Destiani, R. and Templeton, M. The potential Synergistic Effect of Chlorine and UV in Inactivating Antibiotic Resistance Genes in Waterborne Bacteria. Vienna, 2017.

CHAPTER 2

AIMS AND OBJECTIVES

The overall aim of this study is to determine the presence and persistence of antibiotic-resistant bacteria (ARBs) and antibiotic resistance genes (ARGs) in drinking water supplies and to better understand the ability of water treatment processes to control ARBs and ARGs. The specific objectives of this research were:

1. Determine the prevalence of antibiotic-resistant bacteria (ARBs) in heterotrophic organisms commonly present in tap water samples in London.
2. Determine and quantify antibiotic resistance genes' (ARGs) prevalence in drinking water samples in London.
3. Identify the diversity of antibiotic-resistant heterotrophic bacteria commonly found in drinking water and examine whether opportunistic pathogen bacteria are present in drinking water samples.
4. Determine the effectiveness and kinetics of chlorine with regard to the inactivation of ARBs (compared to non-resistant bacteria) and its ability to inactivate ARGs.
5. Determine the effectiveness and kinetics of UV disinfection with regard to the inactivation of ARBs (compared to non-resistant bacteria) and its ability to inactivate ARGs. To also compare the relative order of sensitivity of different ARGs to UV and attempt to explain this order.
6. Examine the repair and/or regrowth of ARBs following UV and chlorine disinfection and their potential spreading following UV and chlorine disinfection.
7. Explore the potential use of sequential applications of UV and chlorine to inactivate ARGs and to compare the effectiveness of single disinfection and sequential disinfection.

CHAPTER 3

LITERATURE REVIEW

3.1 Antibiotics and Antibiotic Resistance

Antibiotics are defined as low-molecular-weight microbial metabolites that at low concentrations inhibit the growth of other microorganisms. Antibiotics are unique among pharmaceutical treatments in that they do not direct their action towards our own cells but selectively target foreign cells.

Table 3.1 Major antibiotic categories and mechanisms of action adapted from Kohansi *et al.,* (2010)

Mechanisms of action	Antibiotic categories
Interference with cell synthesis	penicillin, cephalosporins, carbapenems, monobactam, vancomycin, teicoplanin
Protein synthesis inhibition	Tetracyclines, aminoglycosides, Oxazolidonones, streptogramins, ketolides, macrolides, lincosamides
Interference with nucleic acid synthesis	Fluoroquinolones
Inhibition of metabolic pathway	Sulphonamides, trimetrophim
Disruption of bacterial membrane structure	polymyxins, daptomycin

The history of antibiotic resistance can be tracked back to the 1940s when penicillin-resistant *Staphylococcus* was detected even before penicillin was prescribed to treat serious infections in 1943 (Ventola, 2015). Since then, the development of antibiotic-resistant bacteria has occurred successively with the discovery of antibiotics. **Figure 3.1** illustrates the development of antibiotic resistance from the 1940s until 2010.

Antibiotic resistance is the ability of microbes to withstand the effect of antibiotics. This may be due to intrinsic or natural resistance, for instance the absence of an antibiotic target. Antibiotic resistance may also be acquired, which means that previously sensitive bacteria develop tolerance to the presence of an antibiotic. Resistance of antibiotics occurs through four general mechanisms: target modification, efflux, immunity and bypass (Walsh, 2003). Further, the antibiotic can be enzymatically modified or degraded to an inactive derivative. Target modification can occur through mutation of the targets

themselves or by the production of enzymes that modify antibiotic targets. Antibiotic targets and mechanisms of resistance are summarised in **Figure 3.2.**

The acquisition of new genetic material by antimicrobial-susceptible bacteria from a resistant strain of bacteria may occur through conjugation, transformation or transduction (Wright, 2010). In addition, transposons often facilitate the incorporation of multiple-resistance genes into host genomes or plasmids (McManus, 1997). Gene fragments then can be transported via plasmids, viruses or as a bare DNA fragment, and human health risks arise when these genes are able to be incorporated into human pathogens. Also, horizontal gene transfer (HGT) is considered to be a major mechanism for sharing ARGs between microbes. Several studies have shown that gene transfer could occur between non-pathogens, pathogens and even between Gram-positive and Gram-negative bacteria (Courrvalin, 1994; Anderson *et al.*, 1999).

Antimicrobial resistance, often referred to as AMR, became a concern for public health worldwide, consequently limiting the treatment options for bacterial infections and increasing treatment costs and mortality (Cosgrove, 2006). The Centers for Disease Control and Prevention (CDC) have estimated that antimicrobial resistance has added $20 billion in excess direct healthcare costs, with additional costs to society related to lost productivity as high as $35 billion a year (CDC, 2013). According to a report entitled “Antimicrobial Resistance: tackling a crisis for health and wealth of nations” by the United Kingdom Government in 2014, it is predicted that by 2050, 10 million people will die every year due to antimicrobial resistance. Furthermore, the WHO recently published a global priority list for ARBs, comprising the 12 families of bacteria that pose the greatest threat to human health, as can be seen in **Table 3.2.**

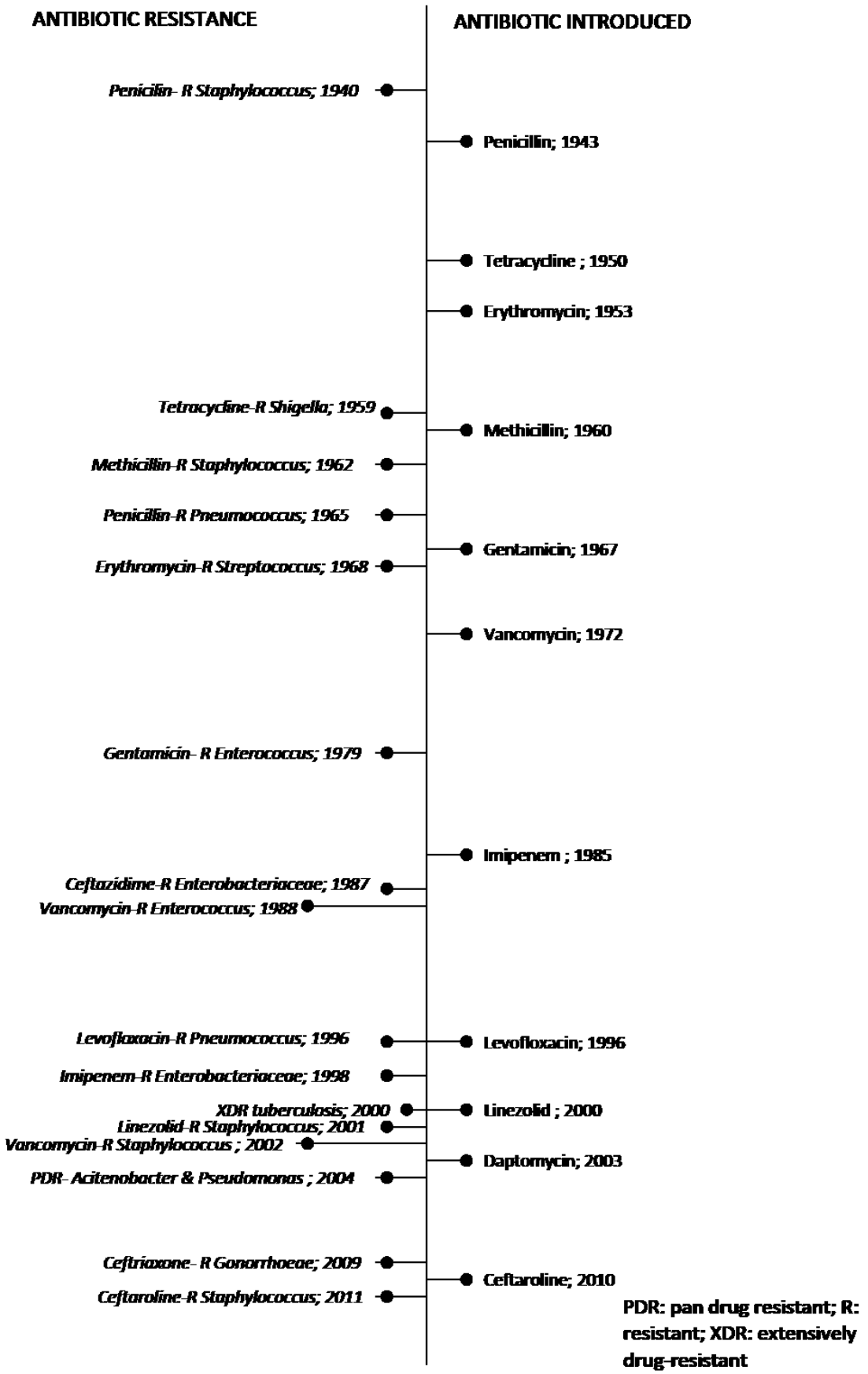


Figure 3.1 Developing antibiotic resistance (Figure reproduced from Ventola, 2015)

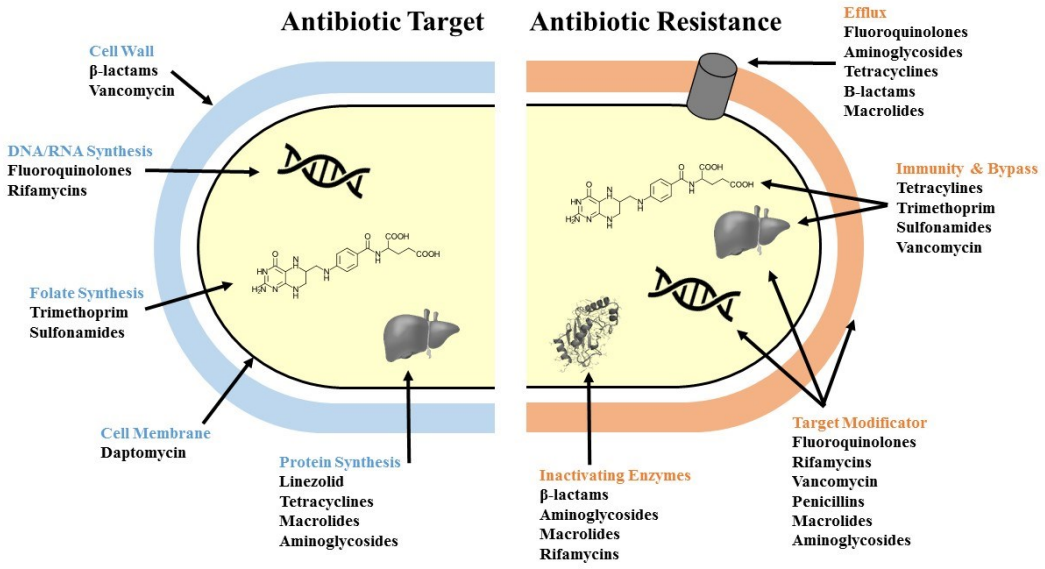


Figure 3.2 Antibiotic targets and mechanisms of resistance (Figure reproduced from Wright, 2010)

Table 3.2. WHO list of priority antibiotic-resistant bacteria (WHO, 2017)

Priority	**Pathogen**	**Resistant to**
Critical	*Acinetobacter baumannii*	Carbapenem
	Pseudomonas aeruginosa	Carbapenem
	Enterobacteriaceae	Carbapenem + 3rd generation cephalosporin ESBL-producing
High	*Enterococcus faecium*	Vancomycin
	Staphylococcus aureus	Methicillin + vancomycin
	Helicobacter pylori	Clarithromycin
	Campylobacter spp	Fluoroquinolone
	Salmonellae	Fluoroquinolone
	Neisseria gonorrhoeae	3rd generation cephalosporin + Fluoroquinolone
Medium	*Streptococcus pneumoniae*	Penicillin-non-susceptible
	Haemophilus influenzae	Ampicillin
	Shigella spp.	Fluoroquinolone

3.2 Selected Classes of Antibiotics and Resistance Genes

3.2.1 Sulphonamides

Sulphonamides were the first antibiotics developed for large-scale introduction into clinical usage. The selective effectiveness of sulphonamides against bacteria is due to their inhibiting effect on the formation of folic acid. Historically, sulphonamides were used worldwide as cheap and effective remedies against bacterial infection. Today, however, the use of sulphonamides is limited. This is due to the development of allergic side effects from the blood-forming organs and the skin of many patients (Skold, 2011). Further, resistance towards sulphonamides emerged rapidly in many human pathogens.

This rapid development of sulphonamide resistance occurred in many pathogens, for instance, streptococci, meningococci, and gonococci. Resistance nowadays is also very common among Gram-negative Enterobacteria that infect the urinary track. Resistances to sulphonamide are often encoded by mutations located on highly conserved areas of dihydropteroate synthase (DHPS) genes (*sul*). Four types of *sul* genes (*sul*1, 2, 3 and A) have been found in bacteria of environmental origin. *Sul*1 and s*ul*2 have been detected in various environmental samples, for example in the faecal slurry of dairy farms (Srinivasan *et al.*, 2005), sediments (Huerto *et al.*, 2012) and even from river or sea water with no evidence of it being polluted (Lin and Biyela, 2005; Mohapatra *et al.*, 2008).

3.2.2 Fluoroquinolones

Fluoroquinolones (FQ) are a group of broad-spectrum synthetic antibiotics. They are a by-product of the industrial synthesis of the malaria drug; chloroquine. This antibacterial group consists of ciprofloxacin, norfloxacin, ofloxacin, levofloxacin and moxifloxacin. Fluoroquinolones have a selective bactericidal effect by interfering with the synthesis of DNA (Zhao *et al.*, 1997). The fluoroquinolones are widely used to treat bacterial infection of the respiratory, gastrointestinal and urinary tracts, as well as sexually transmitted diseases.

Two mechanisms of resistance to quinolones are currently recognised: mutations that alter the drug targets and alterations that limit the permeation of the drug into the target (Fluit *et al.*, 2001). Fluoroquinolone resistance is mostly due to mutations in the quinolone-resistance-determining regions,

particularly the *gyr*A gene (Fluit *et al.*, 2001). There have been at least seven resistance genes related to fluoroquinolones, namely *gyr*A, *gyr*B, *par*A, *par*C, *qnr*A, *qnr*S, and *qnr*B.

3.2.3 Tetracycline

The mechanism of action of tetracyclines is to inhibit peptide synthesis. They bind to the 70S ribosome and inhibit the binding of the amino acid carrying the tRNA molecule to the ribosome (Skold, 2011). Four tetracycline derivatives are the most commonly used in clinical settings: tetracycline, oxytetracyline, doxycycline and lymecycline. Tetracyclines have been used widely in both humans and animals due to their effectiveness, their moderate side effects and their low cost.

Three different specific mechanisms of tetracycline resistance have been identified so far: limiting the access of tetracycline to the ribosomes, altering the ribosome to prevent effective binding of tetracycline and producing tetracycline-inactivating enzymes (Speer *et al.*, 1992). There are 38 identified tetracycline resistance (*tet*) genes and three oxytetracycline resistance (*otr*) genes (Roberts 2005; Thompson *et al.*, 1997). Among them, more than 22 *tet* or *otr* genes have been found in bacterial isolates from water treatment plants (Zhang *et al.*, 2009). Agerso and Peterson (2007) found that the *tet*E gene is often located on large horizontally transferable plasmids of *Aeromonas spp*. and the gene has been proved to be capable of interspecies transfer to *Escherichia coli*. Akinbowale *et al.* (2007) also discovered that *tet*(A), *tet*D and *tet*M can be transferred horizontally by the oxitetracycline resistance plasmid from environmental microorganisms to a non-resistant *E. coli*, which suggests the potential environmental hazard posed by *tet* ARGs.

3.2.4 Macrolides

Macrolides belong to one of the most commonly used families of clinically important antibiotics used to treat infections caused by Gram-positive bacteria. Erythromycin is a well-known example of the macrolide group. It was discovered in 1952 as an antibiotic product by *Streptomyces erythreus*. Erythromycin selectively inhibits bacterial growth by binding to bacterial ribosomes, where it reversibly inhibits protein synthesis (Skold, 2011).

Resistance to macrolides is generally mediated by the methylation of a specific adenine residue in 23S rRNA and it is correlated with *erm*(B) genes, which encode rRNA methylase. Among the macrolide resistance determinants, *erm*B is considered the most prevalent gene in environmental microorganisms, especially in the *Enterococcus* strain (Hayes *et al.*, 2005).

3.2.5 β-Lactams antibiotics

β-lactams are among the most commonly prescribed antibiotics. β-lactam antibiotics are a broad class of antibiotics, consisting of all antibiotic agents that contain a beta-lactam ring in their structure. The antibacterial mechanism of beta-lactams is to inhibit cell wall synthesis. In detail, beta-lactams antibiotics interfere with cell wall synthesis by binding to penicillin-binding-proteins (PBPs), which are located in cell walls. This inhibition of PBPs leads to the inhibition of peptidoglycan synthesis, leading to cell death.

The most common form of bacterial resistance against beta-lactams is that the bacterium produces a beta-lactamase, an enzyme cleaving the beta-lactam bond and thus inactivating the ability of the beta-lactam to interfere with the bacterial cell synthesis (Skold, 2011). More than 400 different β-lactamases encoded by hundreds of ARGs (*bla*) have been identified, and the enzymes are divided into four molecular classes (A-D), mediating resistance to a broad range of β-lactams (Li *et al.*, 2007).

3.3 Presence of Antibiotic-resistant Bacteria and Antibiotic Resistance Genes in the Aquatic Environment

National surveillance data and independent studies show that drug-resistant diseases have caused bacteria to multiply and spread at alarming rates in recent decades. The World Health Organisation has recently published an Antimicrobial Resistance Global Report on Surveillance (2014), in which one of the most important findings was that resistance is now commonly global among pathogens such as *Escherichia coli*, *Klebsiella pneumonia* and *Staphylococcus aureus*. Furthermore, many studies have reported the presence of ARBs and ARGs in soil (Popowska *et al.,* 2011; Pruden *et al.,* 2006), groundwater (Sanford *et al.,* 2001), surface water (Huerta *et al.,* 2013), wastewater effluent (Caplin *et al.,* 2008; Iwane *et al.,* 2001) and drinking water (Amstrong *et al.,* 1981; Schwartz *et al.,* 2003; Xi *et al.,* 2009).

The human health risk of resistant bacteria has been clearly illustrated in hospital environments, where MAR bacteria occur and threaten human health. Bacterial resistance often results in treatment failure, which can have serious consequences, especially in already critically ill patients. However, in the hospital setting the cycle of resistance is rather short, between patients and/or the contaminated room or equipment and antibiotic usage. On the other hand, in the wider environment (e.g. soil, water) there is a extensive range of species of bacteria. Studies have shown that the continuous consumption of antimicrobial agents by society leads to the higher prevalence of resistant genes for various antibiotic classes in the surrounding environmental gene pool. The selection of resistance then continues as antibiotic usage keeps growing and these antibiotics persist in the environment, as illustrated in **Figure 3.3**.

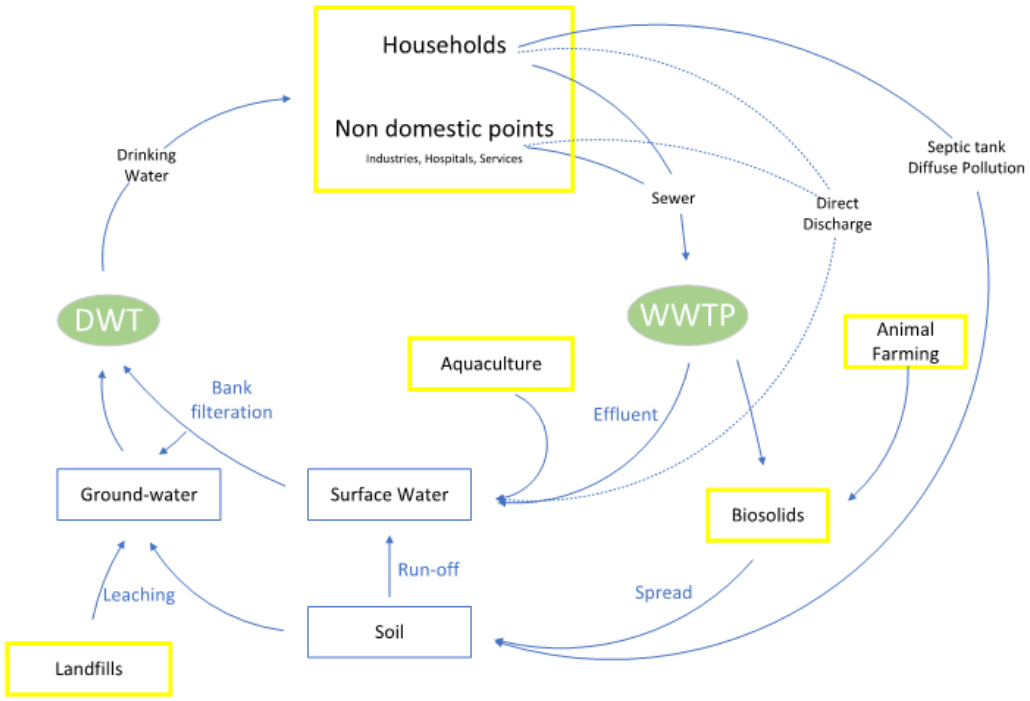


Figure 3.3 Antibiotic cycle and persistence in environments (Figure reproduced from Petrovic *et al.,* 2003).

Several works have highlighted the presence of ARBs and ARGs in microbial communities in natural waters that were not polluted or only slightly polluted (Sanford *et al.*, 2001; Pruden *et al.*, 2006). For example, studies have shown that several ARGs commonly occur in rivers and sediments, namely *sul*1, *sul*2 and *tet (W)* and *tet (O)* for sulphonamides and tetracycline resistance respectively. A study conducted by Sanford *et al.* (2001) found eight classes of tetracycline-resistant genes

[*tet*(O), *tet*(Q), *tet*(W), *tet*(M), *tetB*(P), *tet*(S), *tet*(T), and *otrA*] in water samples collected from two swine lagoons. Further, these resistance genes were found to be seeping into the underlying groundwater and could be detected as far away as 250 metres downstream from the lagoon.

Various studies have been conducted to assess both the prevalence and presence of ARBs in drinking water treatment and distribution systems. An early study of resistant bacteria in drinking water treatment was conducted by Armstrong *et al.* (1982), showing that standard plate count bacteria isolated from a flash mixing tank possessed resistance to streptomycin and kanamycin. This study also discovered that there was a significant increase in the proportion of the bacterial population that was multi-drug-resistant (MAR) following flash mixing with chlorine. Another study later revealed that the proportion of heterotrophic ARB and ARG is greater in tap water than in finished water and source water (Xi *et al*., 2009). The increased proportions of ARBs and ARGs in tap water compared to those in finished water indicate that water treatment may contribute to elevating the antibiotic resistance of surviving bacteria and induce transfer of ARGs among particular bacterial populations, or that ARBs are inherently more resistant to water treatment methods than non-AR bacteria. However, the mechanisms for this phenomenon are still not well understood. Nonetheless, the prevalence and resistance patterns of several microbial genera isolated from drinking water distribution have been reported (Koksal *et al.*, 2007; Ram *et al.*, 2008). These studies observed multiple-antibiotic-resistant bacteria in tap water, distribution systems and water storage tanks. **Table 3.3** shows selected ARGs found in drinking water and their biological sources.

Studies have also indicated that bottled mineral water could be a possible source of ARBs. Mary *et al.* (2000) reported that 70% of the total isolates of HPC bacteria from bottled mineral water in Italy were resistant against up to four antibiotics. Overall, the frequency of resistance of isolates from bottled mineral water in Italy is in the following order: nalidixic acid > ampicillin > streptomycin > tetracycline > gentamycin > chloramphenicol > rifampin. Other studies have revealed that most of the bacteria colonies found in French and Portuguese bottled water showed resistance to three or more classes of antibiotics, with *Variovorax*, *Bosea*, *Ralstonia*, *Curvibacte*r and *Afipia* as predominant genes (Dias *et al.*, 2012).

Table 3.3. Selected antibiotic resistance genes found in drinking water

Gene	Biological source	References
*Tet(*A)	*Aeromonas spp*, *Alcaligenes*, *Pseudomonas spp*, *E. coli*	Cernat *et al.* (2007), Shi *et al.* (2012), Bergeron *et al.* (2015).
*Sul*1	*Actinobacteria*, *Aeromonas spp*, *E. coli*	Xi *et al.* (2009), Shi *et al*. (2012), Khan *et al.* (2015), Adesoji *et al*. (2016), Bergeron *et al.* (2015).
Bla-TEM1	*E. coli, Pseudomonas spp.*	Cernat *et al.* (2007), Xi *et al*. (2009).
*dfr*A	*Alcaligenes faecalis*	Cernat *et al.* (2007), Adesoji *et al.* (2016).
Mph(A)		No references found

Table 3.4 summarises studies on the presence of ARBs and ARGs in drinking water that are most relevant to the present study.

Table 3.4. Summary of relevant papers on ARB and ARG presence in drinking water systems

Authors	Paper description
Armstrong *et al.* (1981)	This is one of the first studies to report the presence of ARB in drinking water. They identified Gram-positive multiple resistant bacteria (Staphylococcus) and Gram-negative resistant bacteria including *Pseudomonas, Alcaligenes* and *Acitenobacter* from treated drinking water. This paper also revealed that the treatment of raw water and its subsequent distribution select for standard plate count bacteria exhibiting the MAR phenotype.
Xi *et al.* (2009)	This paper examined the prevalence of ARBs and ARGs in drinking water systems in Michigan, USA. They observed that the quantities of *bla*-TEM, *cat*, *sul*1 and *sul*2 genes were greater in tap water than in finished water from water treatment plants. They also observed the elevated prevalence of ARBs in tap water compared to finished drinking water. This paper is among the first to prove that water treatment might select antibiotic resistance. However, their study did not include seasonal variation or the identification of the resistant bacteria.
Khan *et al.* (2016)	This paper aimed to determine the prevalence of bacteria and resistance genes in drinking water. Assays included genes responsible for resistance to quaternary ammonium compounds (*qac* genes) and sulphonamide antibiotics (*sul*1 and *sul*2 genes). Mobile genetic integrons were detected via their

	integrase gene intI1 and intI2. This is one of the first studies to determine the presence and prevalence of ARGs and ARB in drinking water in the UK, particularly in Glasgow, Scotland. The presence of both Gram-negative and Gram-positive bacteria was confirmed in the tap water, some of which can be pathogenic. Species of *Bacillus*, *Paenibacillus*, *Brevibacillus*, *Kocuria*, *Staphylococcus*, *Arthrobacter*, *Comamonas*, *Acidovorax*, *Blastomonas*, *Variovorax*, *Escherichia* and pathogenic *Burkholderi*a were found in the water distribution system. Their results showed that *sul*1 and s*ul*2 genes were detected in 12 out of 148 isolates. The *Sul*1 gene was detected more frequently than the *sul*2 gene, while the *qac* gene was not found in all isolates. This study confirmed the presence of ARBs and ARGs in the UK drinking water distribution system. This study was conducted and published concurrently with this dissertation's research. Several aspects have been improved and extended in the present study, including the sampling frequency and the diversity of the antibiotics tested.

3.4 Methods for Detecting and Quantifying Antibiotic Resistance in Environmental Samples

3.4.1 Culture-based techniques

The culture-based method is the earliest method to detect the presence or absence of ARBs, and *in vivo* examinations can be achieved using this technique. Selected nutrient media might be used with or without the addition of antibiotics. The European Committee on Antimicrobial Susceptibility Testing (EUCAST) and the Clinical and Standard Laboratory Institute (CLSI) provide information on the minimum inhibitory concentrations of various antibiotics. Microbial communities from environmental samples are inoculated into nutrient media with antibiotics added, and bacteria will grow and be visible as colonies if ARBs are present.

However, most bacteria present in environmental samples are non-culturable or of low or difficult cultivability. In addition, chlorine used in most tap water disinfection systems may cause sub-lethal injury to some bacteria, thereby rendering them non-culturable (Oliver, 2000). Therefore, the culture-based technique may produce incomplete results regarding the actual presence of ARBs.

3.4.2 Molecular techniques

Analytical techniques targeting 16S rRNA of functional genes have been widely used for microbial quantification. These comprise hybridisation-based techniques (Raskin *et al.*, 1995), fluorescence *in situ* hybridisation (FISH) (Okabe *et al.*, 1999) as well as PCR-based techniques, including conventional PCR and real-time PCR and DNA microarrays (Gilbride *et al.*, 2006). DNA can be extracted directly from environmental samples, for instance water, soil and sediment, and analysed using these molecular techniques. Molecular hybridisation has been used to detect the presence/absence of ARGs for almost 30 years, and it is considered to be the oldest molecular technique (Mendez, 1980).

The most efficient method of detecting resistance in environmental samples with non-culturable bacteria is by the specific detection of genes of interest from DNA obtained from the environmental sample. The basic principle of PCR is to produce copies of DNA from one DNA molecule. Each PCR cycle consists of three steps: denaturation of the double-stranded DNA templates, specific annealing of primers to the single-stranded DNA template, and the extension of the primer and template defined sequence length by the enzyme polymerase. A specific protein known as polymerase, an enzyme that is able to string together individual DNA molecules to form a long molecular strand, accomplishes the continuous doubling of the DNA. A small fragment of DNA known as a primer is also required for the PCR reaction; the primer serves as the starting point for DNA synthesis.

There are two types of PCR analyses, namely conventional PCR and real-time PCR. Conventional PCR measures the amount of accumulated PCR product at the end of the PCR cycle in a gel. The precision of conventional PCR may be compromised due to a number of factors, including reagent depletion and the loss of polymerase activity. On the other hand, quantitative PCR, also known as qPCR, measures PCR amplification as it occurs. The data are collected during the exponential growth phase when the quantity of the PCR product is directly proportional to the amount of template nucleic acids, measured by the fluorescence generated during the reaction. Two detection chemistries commonly used for qPCR application are: (1) use of an intercalating dye such as SYBR® Green I dye; and (2) use of primer or short oligonucleotide specific to the target of interest such as TaqMan® probes, Molecular beacon, or Scorpion primer. SYBR® Green is a non-specific dye that binds to all doubled-stranded DNA and will

emit green fluorescence when bound to dsDNA. The dye will exhibit a small fluorescence when unbound but will produce a strong fluorescent signal when bound to the dsDNA. The signal will increase during polymerisation and decrease when DNA is denatured. SYBR green dye by far is the most common method used to quantify the ARGs from environmental samples (Zhang *et al.*, 2009). It is relatively cheap, and it can be used with any primers for any target. However, the presence of any non-target dsDNA or primer-dimer might decrease the specificity of the dye.

Table 3.5 shows the general advantages and disadvantage of the culture-based method and the molecular-based method.

Table 3.5. Comparison between the molecular-based method and the culture-based method (Rizzo *et al.*, 2013)

Molecular-based method	**Culture-based method**
Advantages	
High specificity and sensitivity	Direct proof of living and progeny capacities
Detection independent from physiological status of the bacteria	Comparable results over longer time scales and among different laboratories
Direct taxonomical affiliation coupled with functional genes	Easy to perform, robust and reliable
Direct quantification of DNA targets in whole communities	Low cost
Diagnostic without any enrichment feasible	
Detection almost independent from other microflora	
Disadvantages	
Free DNA and DNA from dead microorganisms are detected in parallel	Physiological factors influence the quantification and detection
Low or no information about microbial progeny	Viable but not culturable bacteria and injured bacteria are not detected
Cross reaction with uncharacterised microflora is possible	Laborious and time consuming
Inhibitors might influence the detection of the target DNA	Isolation bacteria from complex habitats are difficult
High cost and complex procedure	Detection of virulence factor is missing
No standardised protocols	Cultivation and detection of some microorganisms are time consuming
Restricted to small sample volumes, otherwise time-consuming sample preparations are needed	

3.5 Disinfection Studies on Antibiotic-resistant Bacteria and Antibiotic Resistance Genes

3.5.1 General

Disinfection is the inactivation of pathogenic microorganisms, used to reduce their number below the threshold safe level. Disinfection is most commonly accomplished using (1) chemical agents; (2) physical agents; (3) mechanical means; or (4) radiation. Reducing the number of microorganisms in the water treatment process is achieved by two strategies: physical removing and inactivating. The term disinfection is commonly used to refer to two processes: (1) primary disinfection – the inactivation of microorganisms during water treatment; and (2) secondary disinfection – maintaining a disinfectant residual along the distribution system up to the consumer's tap. Therefore, disinfection does not necessarily start and end at the inlet and outlet of the chlorine contact tank in a drinking water treatment plant; other parts of the treatment process may provide disinfection by removing microorganisms, as well as ensuring that the water is suitable for the final disinfection process.

3.5.2 Chlorine

Chlorination is still the primary disinfection method used in drinking water treatment due to its low cost, relative ease of application and ability to inactivate a wide range of pathogens. It is a nonselective oxidant that is employed due to its vigorous reactions with different cellular components and its ability to interrupt bacterial metabolic processes (Virto *et al.*, 2005). It also provides additional protection for the water after it leaves the treatment works. Hypochlorous acid and hypochlorite ions are formed when chlorine is introduced into water. The pH and temperature of the water play a role in the distribution between HOCl and OCl-. Generally, HOCl is a stronger chemical oxidant and therefore a better disinfectant than hypochlorite ions. Hypochlorous acid is a weak acid, and the extent of disassociation is pH dependent. The pKa for HOCl is 7.6 at 20°C. Hypochlorous acid (HOCl) is the predominant form below this pH value and hypochlorite ion (OCl-) is the main form above this value (Crittenden *et al.*, 2012). The dissociation of chlorine can be seen in Equations 1 and 2. The forms of chlorine most often used in the treatment of drinking water are chlorine gas and sodium hypochlorite solution.

$$Cl_2 + H_2O \leftrightarrow HOCl + H^+ + Cl^- \qquad \text{(Equation 1)}$$

$HOCl \leftrightarrow H^+ + OCl^-$ (Equation 2)

Advantages of chlorination include (Crittenden *et al.* 2012):

- It is a potent germicide: chlorine is a strong oxidising agent against a wide spectrum of pathogens.
- Chlorine is more cost-effective than other disinfection technologies, e.g. ozone.
- Biological growth control: chlorine helps eliminate many bacteria, viruses, algae and protozoa in water distribution systems by providing a longer disinfectant effect in the form of chlorine residuals.
- Chemical control: chlorine removes hydrogen peroxide, ammonia and other nitrogenous compounds that are responsible for an unpleasant taste and odour in water.

Disadvantages of chlorination (Crittenden *et al.* 2012):

- Chlorine can oxidise certain types of organic matter in water to create disinfection by-products, such as trihalomethanes (THMs) and haloacetic acids (HAAs).
- All forms of chlorine are toxic and corrosive. Therefore, it might pose a risk during shipping, handling, and storage.
- Chlorine is not effective in killing some pathogens such as *Cryptosporidium parvum* cysts and *Giardia lamblia*.

The dominant hypochlorous acid form should be sustained during water treatment – this is because bacterial pathogen surfaces typically carry a natural negative electrical charge. They are therefore more readily penetrated by the uncharged, electrically neutral hypochlorous acid than by negatively charged hypochlorite ions. Free chlorine attacks the cytoplasmic membrane of Gram-negative bacteria to produce a cellular lesion or injury that results in an elevated sensitivity to surfactants (Zaske *et al.*, 1980).

3.5.3 UV light

The discovery of chlorination by-products and chlorine-resistant organisms has led to a revaluation of the use of chlorine as a primary disinfectant agent. Ultraviolet light is a non-chemical disinfectant that will not produce as many known disinfection by-products. The first modern installation of an ultraviolet

disinfection system in water treatment dates back to 1955 in Switzerland and Austria. Advantages of UV disinfection include (Bolton and Cotton, 2008):

- It is a very effective disinfection technology for *Cryptosporidium* and *Giardia*.
- It does not significantly alter the water quality; that is no change in total organic carbon, pH, corrosivity, or DBP formation potential.
- The technology is relatively inexpensive with low capital and operating costs, compared to other disinfection options for protozoa.
- It is relatively easy to operate the UV equipment based on changes in water flow, water quality, etc.
- The UV equipment has a relatively small footprint and it is usually amenable to being retrofitted into existing water treatment plants.
- No chemicals are needed for UV disinfection.
- Disinfection is very fast. Contact times are in the range of a few seconds.

Disadvantages of UV disinfection include (Bolton and Cotton, 2008):

- There is no residual disinfection capacity. Therefore, some level of chlorine or chloramines is usually added to maintain disinfection residual in the distribution system.
- At present, it is not possible to directly monitor the UV dose in a continuous fashion, therefore operators have to rely on secondary measurements (sensor readings, UV transmittance, water flow rates, etc.).
- Most reactors contain mercury lamps; therefore simultaneous breakage of multiple UV lamps represents a possible mercury hazard.
- There are times when water could be under disinfected because of power interruptions or lamp warm-up.

The UV wavelength inactivates microorganisms by attacking their DNA or RNA. Deoxyribonucleic acid (DNA) is a large molecule compound formed of subunits named nucleotides, which consist of deoxyribose, phosphate, and nucleic acid bases often called nucleobases. The four components of nucleic acid bases are thymine (T), adenine (A), guanine (G) and cytosine (C). These four bases always

form a base pair, often abbreviated as bp. either thymine bonded to adenine or cytosine bonded to guanine. Thymine forms two hydrogen bonds with adenine, while cytosine forms three hydrogen bonds with guanine. Therefore, thymine and adenine bonds represent the weakest connection in the structure.

UV light damages DNA bases by many different mechanisms, including the production of oxygen radicals, which lead to oxidative damage and the formation of dimers between adjacent pyrimidine bases by addition across their hydrogen double bond, as shown in **Fig.3.4** (Crittenden *et al.* 2012). The primary dimers produced by UV light are thymine dimers. The dimerisation of thymine bases can lead to the inactivation of the DNA or RNA, resulting in cells that are unable to reproduce, as shown in **Fig. 3.4** below.

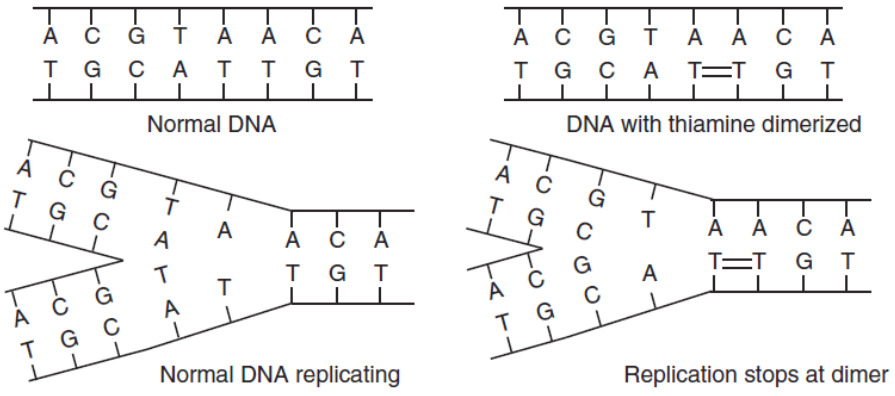


Figure 3.4 Formation of thymine dimers by UV light interferes with normal replication of microorganism (Crittenden *et al.* 2012)

Bacteria and viruses are able to reactivate/repair damage done by UV to their DNA and RNA. There are two main mechanisms of DNA repair: by photoreactivation through simultaneous or subsequent exposure to near-UV and visible light (300-500 nm), often called light repair, or by nucleotide or base excision repair pathways often referred to as dark repair (Kowalski, 2009). There are two major steps involved in the DNA repair process: the first is the formation of a complex between a photoreactivation enzyme (PRE) and the dimer, and the second is photolytic reactions in which light energy is absorbed and the lesion is repaired (Fletcher, 2013). The restoration of the dimer to its original monomerised form is completely dependent upon light energy intensity. The reaction occurs in less than a millisecond; consequently, the limiting step of the whole reactivation process is the formation of the

PRE-dimer complex. An extended period of exposure to photo reactivating light would enable the release of PRE, which would then be available to form new complexes (step 1).

Treated water in drinking water distribution systems are subjected to a long detention period before reaching the consumer's tap. During this time, UV-irradiated microorganisms may have the opportunity to carry out dark repair and potentially regrow within the system, although no studies to-date have shown that this occurs when the UV doses that are typically applied in drinking water treatment (e.g. 40 mJ/cm^2 and above) are used.

3.6 Antibiotic Resistance and Water Treatment

Recent studies have shown that a variety of ARBs and ARGs occur in drinking water treatment around the world (Li *et al.*, 2015). Wastewater and drinking water treatment processes are unable to effectively remove ARBs and ARGs in water. It is also known that chlorination might alter the structure of bacteria communities in drinking water systems. Moreover, several studies have demonstrated the resistance of *Escherichia coli* to relatively low doses of chlorine and UV disinfection (Rizzo *et al.*, 2013; Oh *et al.*, 2014).

Armstrong *et al.* (1981) found that, according to a standard plate count, bacteria isolates after the water treatments had a greater proportion of multiple-antibiotic-resistant (MAR) bacteria, which had 67.8% resistance compared to those in raw water at 18.6% resistance. These bacteria were isolated from the flash mix tank, where chlorine, alum and lime were added to the water. The authors also observed the enrichment of resistance to specific antibiotics such as carbenicillin, chloramphenicol, kanamycin, streptomycin and tetracycline after water treatment. However, this study concluded that the reason for the survival of MAR bacteria within the water treatment facility is not yet understood.

Several studies observed that chlorination and UV could not eliminate ARB and ARG in wastewater treatment plants (Huang *et al.*, 2011; Guo *et al.*, 2013; Zhang et al,. 2015; Zhuang *et al.*, 2015). Several studies also suggest that ARGs are more difficult to remove than ARBs (McKinney and Pruden, 2013; Yoon *et al.*, 2017). Intact remnants of DNA may confer resistance to downstream bacteria by

transformation or transduction; additionally, injured ARB can be fully activated and retain their resistance.

Studies exploring new disinfection technologies to remove antibiotic resistance have been conducted. For instance, Oh Junsik *et al.* (2014) conducted a study on the effectiveness of common disinfection agents including chlorine and ozone, compared to e-beam (i.e. electron beam) disinfection, in the removal of MAR *E. coli* DH5α and its resistance genes. They found that more than 30 mg/L of chlorine and 3 mg/L were needed to inactivate over 90% of ARB and ARG. They concluded that ozone and e-beam disinfection are more effective ways to control antibiotic resistance compared to chlorination. A recent study by Guo *et al.* (2017) explored the use of TiO_2 photocatalysis for the removal of MRSA *S. aureus* and *P. aeruginosa* along with *mec*A and *amp*C resistance genes. The results showed that approximately 4.5- to 5.8-log inactivation of ARBs was achieved with TiO_2 under UV fluence of 6-12 mJ/cm^2, while to achieve the same inactivation for ARGs required TiO_2 under UV fluence of 120 mJ/cm^2. **Table 3.6** below summarises the papers related to antibiotic resistance reduction in wastewater and drinking water that are most relevant to the current study.

Table 3.6. Summaries of papers related to antibiotic resistance inactivation in wastewater and drinking water

Authors	**Paper description**
McKinney and Pruden (2012)	This paper described UV disinfection as a treatment technology for ARG inactivation in water and wastewater under laboratory conditions. They tested resistance genes harboured in Gram-positive and Gram-negative bacteria. They explained the relationship between the pyrimidine adjacency number and the ARG inactivation rate. The results showed that damage of ARGs required much greater UV doses than ARB inactivation and the proportion of amplifiable ARGs following UV treatment showed a strong negative correlation with the number of adjacent thymine bases. This paper is the first comprehensive study about the efficiency of UV disinfection in the removal of ARGs involving the counting of adjacent thymine and cytosine bases. It covered a wide range of ARGs and employed the latest qPCR technology.
Shi *et al.*	This paper assessed the effect of chlorination in a water treatment plant located in China on the diversity and abundance of ARBs and ARGs. It is the first study to

Authors	Paper description
(2013)	employ a combination of next generation sequencing and metagenomic analysis to analyse the resistant bacteria communities following chlorination. Their results suggested that chlorination in drinking water altered bacterial communities. After chlorination, a higher proportion of the surviving bacteria was resistant to chloramphenicol, trimethoprim and cephalothin.
Zhang *et al.* (2015)	This is the first study to investigate the sequential disinfection of UV/chlorination to remove ARGs from municipal wastewater. Four ARGs were tested: *sul*1, *tet*X, *tet*G and *int*l1. Their results showed that sequential UV/chlorination application achieves higher ARG inactivation, with synergy values observed between 0.006 and 0.31. However, since their study was focused on wastewater, the chlorine doses that they used were relatively high (i.e. 5 mg/L to 30 mg/L). Therefore, it cannot represent chlorine doses in drinking water. They also did not cover a wide range of ARGs.
Huang *et al.* (2011)	This study examined the effect of chlorination on ARBs in the secondary effluent of a wastewater treatment plant. They also estimated the microbial health risk from ARBs by investigating the regrowth and reactivation of bacteria resistant to penicillin, ampicillin, tetracycline, chloramphenicol, and rifampicin following chlorination (incubation time was 22 hours after chlorination). The results indicated that 10 mg/L of chlorination resulted in a higher proportion of chloramphenicol-resistant bacteria post-chlorination. They did not observe any reactivation of heterotrophic bacteria and ARBs when the chlorine dose reached 5 mg/L for 10 min contact time. However, some reactivation occurred when a low chlorine dose was used (i.e. 2.5 mg/L). There are several factors that could have been improved in this study, including applying a lower chlorine dose but for a longer contact time, to represent conditions in water supply systems.
O'Flaherty *et al.* (2017)	This is the first study to examine the human health consequences from ARBs (*E. coli*) through drinking water consumption. The effect of different drinking water treatment on antibiotic-resistant *E. coli* concentration was also evaluated. The results showed that exposure to antibiotic-resistant *E. coli* were between 3.44×10^{-7} and 2.95×10^{-1} CFU/day. UV disinfection achieved the largest inactivation of antibiotic-resistant *E. coli*.

3.7 Bacteria Regrowth in Drinking Water Systems

Brazos and O'Connor (1985) proposed a definition of 'regrowth' and 'aftergrowth'. Regrowth can be defined as the recovery of any disinfectant-injured cells that have entered the water distribution network from the water treatment source. Meanwhile, aftergrowth is the multiplication of native microorganisms in the water distribution system. Also 'breakthrough' is sometimes used as the term to describe the increase in bacterial counts in distribution networks resulting from viable bacteria that pass through the disinfection process, and 'growth' is the increase in living bacterial numbers in the distribution system resulting from bacterial growth downstream of the disinfection treatment (McFeters, 1990).

Bacterial regrowth is a potential issue faced by the drinking water industry. The major concern involving regrowth is the increase in numbers of potentially pathogenic and/or opportunistic pathogen microorganisms. However, some degree of bacterial regrowth in drinking water distribution systems is inevitable due to the presence of organic content, such as assimilable organic content (AOC) and total organic carbon (TOC). Escobar *et al.* (2001) observed that the bacterial regrowth in drinking water distribution networks was associated with AOC concentrations rather than biodegradable dissolved organic compounds (BDOC). There are also several other factors that can be important in bacterial regrowth in drinking water distribution systems, such as pipe age, pipe materials and the diameter of the pipe (LeChevallier, 2003).

Many microorganisms detected in drinking water distribution systems might have the characteristics of opportunistic pathogens, which can cause disease to individuals with a weak immune system. This is supported by various studies that have shown the presence of potentially opportunistic pathogens in drinking water systems (Adesouji *et al.*, 2016; Khan *et al.*, 2016). For example, it has been demonstrated that *Pseudomonas aeruginosa*, a common bacteria in water, can cause infectious diarrhoea in immunodeficient or antibiotic-treated individuals (Adlard *et al.*, 1998). **Table 3.7** shows the typical heterotrophic bacteria and opportunistic pathogen bacteria commonly found in drinking water distribution systems.

Table 3.7. Heterotrophic bacteria and opportunistic pathogen bacteria commonly found in drinking water (Allen *et al*. 2004)

Typical heterotrophic bacteria isolated from drinking water distribution systems	**Opportunistic bacterial pathogens commonly found in drinking water**
Aeromonas spp	*Acinetobacter* spp
Acinetobacter spp	*Achromobacter xylosoxidans*
Actinomycetes spp	*Aeromonas hydrophila*
Alcaligenes spp	*Bacillus* spp
Bacillus spp	*Campylobacter* spp
Citrobacter freundii	*Chromobacter violaceum*
Corynebacterium spp	*Citrobacter* spp
Enterobacter agglomerans	*Enterobacter aerogenes*
Escherichia coli	*Enterobacter agglomerans*
Flavobacterium meningosepticum	*Enterobacter cloacae*
Micrococcus luteus	*Flavobacterium meningusepticum*
Moraxella spp	*Hafnia alvei*
Mycobacterium spp	*Klebsiella pneumoniae*
*Plesiomona*s spp	*Legionella pneumoniae*
Pseudomonas alcaligenes	*Moraxella* spp
P. cepacian	*Pseudomonas* spp
P. flourescens	*Serratia fonticola*
P. mallei	*S. liquefaciens*
Stenotrophomonas maltophilia	*S. marcescens*
Staphylococcus aureus	*Staphylococcus* spp
S. epidermidis	*Vibrio fluvialis*
Yersinia enterocolitica	

3.7.1 Factors Promoting Bacterial Regrowth in Drinking Water Distribution Systems

(1) Assimilable organic carbon (AOC)

Carbon is one of the most important requirements for microbial growth. In fact, half of the dry weight of a bacterial cell is carbon (Jeffrey, 2013). Organic compounds provide energy sources for heterotrophic bacteria that use organic carbon for new cell production. Organic substrates in drinking water can be quantified as assimilable organic carbon (AOC). Assimilable organic carbon is a fraction of the total organic carbon (TOC) that can be utilised by a specific strain of bacteria or a mixed bacteria community. The lower the AOC concentration, the less bacterial regrowth will take place, all else being equal. Liu *et al*. 2002 demonstrated that AOC levels in water distribution networks were influenced by seasonal changes, explained by the fact that chlorine reacts more rapidly with organic matter at higher temperatures resulting in lower AOC levels. It is also known that AOC consumption in distribution systems is influenced by other factors such as the pipe material (Liu *et al.,* 2002).

(2) Temperature

Temperature might be the most important factor affecting microbial growth in drinking water distribution systems, since microbial processes tend to be faster at warmer temperatures. Temperature also influences other physical and chemical characteristics of water, either directly or indirectly; for example, chlorine is slightly more effective at higher water temperatures. The correlation between temperature and bacterial regrowth in drinking water distribution systems has been widely studied. Lechevallier (1996) observed significantly increased coliform growth at temperatures higher than 15°C, and Sakyi and Asare (2012) reported that higher water temperature (in the absence of nutrients) was an important factor in increasing the survival of total coliform, *E. coli* and heterotrophic plate count (HPC). The minimum temperature at which microbial activity was observed has varied from one study to another.

(3) Disinfectant type and residual disinfectant level

The type of disinfectant used and its dose in water treatment plants influence bacterial regrowth in water distribution networks. Chlorine certainly has a higher ability to inactivate microorganisms, whereas chloramines may be better at penetrating distribution system biofilms and inactivating attached bacteria

than chlorine. Furthermore, the chemical reaction of disinfectant with organic matter leads to an increase in assimilable organic matter, and chlorine can react faster with dissolved organic carbon than chloramine (Hwang *et al.*, 2012). On the other hand, Gram-negative bacteria are slightly more resistant to chlorine than Gram-positive bacteria. This is thought to be due to the outer membrane of Gram-negative bacteria, which consists of phospholipid and lipopolysaccharides, limiting the entry of antibacterial agents (McDonnel and Russel, 1999).

Furthermore, insufficient disinfectant doses result in incomplete inactivation of organisms, resulting only in injury instead of death of organisms, which might increase the ability of microorganisms to regrow, although it is still unclear whether bacteria will be able to retain their pathogenicity after the injury. The maintenance of residual disinfectant throughout the drinking water distribution network is critical to create unfavourable conditions for bacterial regrowth. Systems that maintained free chlorine at concentration <0.2 mg/L or monochloramine <0.5 mg/L have substantially more coliform occurrences than systems maintaining higher chlorine and monochloramine residuals (LeChevallier, in WHO, 2003). In the UK currently, there are no regulations for the minimum disinfectant residual concentration; on the other hand, the WHO recommend maintaining disinfectant residuals higher than 0.5 mg/L.

Disinfectant type has been shown to influence microbial communities in drinking water systems. Hwang *et al.* (2012) observed a selection of microbial populations in chlorinated and chloraminated systems. *Cyanobacteria*, *Methylobacteriaceae*, *Sphingomonadaceae*, and *Xanthomonadaceae* were more abundant in chlorinated water, and *Methylophilaceae*, *Methylococcaceae*, and *Pseudomonadaceae* were more abundant in chloraminated water.

3.8 Controlling Microbial Activity in Drinking Water Distribution Systems

Water treatment should be designed so as to achieve and maintain biologically stable water. This can be achieved by applying the most suitable water treatment process, including biological, physical and chemical treatments (World Health Organization, 2003). Drinking water treatment in the UK typically involves screening, clarification, filtration, granular activated carbon, ozone, and disinfection (typically chlorination) (DWI, 2015). Additionally, new technologies in water treatment processes and appropriate

pre-treatment depending on the raw water quality or the source of raw water must be considered on a site-by-site basis.

The characteristics of finished water rely on the treatment processes and technology that are applied in a water treatment plant. Biodegradable organic carbon (BDOC) and assimilable organic carbon (AOC) have been used to characterise the biostability of drinking water. Various studies have shown that GAC and BAC can significantly reduce AOC concentrations with a removal rate range from 60% to 80% (Lou *et al.*, 2014; Chien *et al.*, 2007). On the other hand, nanofiltration did not significantly remove AOC from raw water and a switch from chlorination to ozonation significantly increased the AOC of the plant effluent and distribution system, which might be due to oxidation from large organic matter (Escobar *et al.*, 2001).

It is argued that residual chlorine concentrations are one of the main factors controlling bacterial regrowth in drinking water distribution systems. Volk *et al.* (1994) determined minimum values of 0.15 mg/L at 20°C and 0.30 mg/L at 15°C for achieving biological stability in the distribution systems of the Paris suburbs. It is also suggested that large pipe diameters tend to favour suspended planktonic bacteria, while smaller diameter pipes favour biofilm development (Lechevallier, 2003).

The fact that environments facilitating dissemination of resistant bacteria, where resistance genes might transferred between non pathogen to pathogen or opportunistic pathogen bacteria. Additionally, still much remain to be understood in terms of dissemination routes, environmental survival and resistance selection. Furthermore, the incomplete understanding on how antibiotic resistant bacteria and genes respond to disinfections treatment indicated further research on this issue is immediately required.

CHAPTER 4

MATERIALS AND METHODS

4.1 The Antibiotic Resistance of Heterotrophic Bacteria in Tap Waters in London

4.1.1 Summary

This chapter will discuss the materials and methodologies used and conducted in the entire project. Antibiotic resistance has been the focus of research mainly in clinical settings. However, the significance of environmental microbes as a reservoir of resistance in the environment was not considered until quite recently. Fresh water, including rivers and lakes, is still relied upon as a drinking water source, and this water environment is one of the reservoirs of resistance in the environment. In the present study we aimed to determine the presence and profile of ARBs and ARGs in London tap water and to better understand the ability of water treatment processes to control ARBs and ARGs.

4.1.2 Study area and sample collection

Tap water samples were collected from residential properties at nine locations across London, United Kingdom. The locations were selected randomly to capture a geographical spread across the city. A total of four replicate water samples were collected from each location six times between January 2015 and July 2016. The majority of the faucets were metallic mixer taps. Prior to sample collection, each faucet was wiped with 70% ethanol to ensure no contamination entered the water samples. Tap water was then allowed to run for two to three minutes before sample collection. To quench any residual chlorine, 100 µl of 2 g/l of sodium thiosulphate (Sigma Aldrich, Missouri, US) was added to the sterile Schott sampling bottles. Each water sample (100 ml) with some space left for air on the top of the bottle was collected. Samples were stored in a cool box during transportation to the laboratory and processed within 2 to 4 hours of collection.

4.1.3 Water quality measurement

Tap water samples' physical and chemical characteristics were analysed, including pH, temperature, TOC and chlorine residual concentration. Total organic carbon, also known as Non-Purgeable Organic Carbon (NPOC), was analysed by the 'acidify and sparge' method, using the Shimadzu TOC analyser

model 5000A (Shimadzu Corporation, Kyoto, Japan). First of all, tap water samples were filtered through a 0.45-µm pore size membrane. The samples were then poured into a TOC bottle (average 20 ml per bottle). Chlorine residual concentration was determined by the (N, N-diethyl-p-phenylenediamine) DPD ferrous titration method (AWWA, 2005). Briefly, 5 ml of DPD solution and 5 ml of phosphate buffer solution were added to a 125 ml conical flask, and 10 ml of the tap water sample were then also added. The mix was then left for two minutes to allow a complete reaction. The solution was then titrated with FAS titrant.

4.1.4 Isolation and quantification of heterotrophic bacteria and antibiotic-resistant bacteria

The presence of heterotrophic plate count (HPC) bacteria and antibiotic-resistant heterotrophic plate count bacteria in the water was determined using R2A agar, as per the American Public Health Association and the American Water Works Association (AWWA, 2005). The membrane filter (MF) method was used to enumerate the HPC bacteria from the tap water samples.

Tap water samples (each 100 ml) were filtered through a 0.45µm pore size mixed cellulose ester filter membrane (Pall Laboratory, New York, USA). The membrane was then aseptically removed with sterile forceps and placed on R2A (Fluka Analytical, Busch, Switzerland) agar with and without antibiotics. Plates were incubated at 25°C for 72 hours. Three replicates for each water sample were tested. Six different antibiotics were chosen: (1) tetracycline (15 mg/L), (2) amoxicillin (10 mg/L), (3) ciprofloxacin (5 mg/L), (4) trimethoprim (5 mg/L), (5) erythromycin (5 mg/L) and (6) vancomycin (8 mg/L). The concentrations of antibiotics were chosen according to the MICs (minimum inhibitory concentrations) recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2012).

The number of CFU/100ml was compared between the plates with added antibiotics and the control plates without antibiotics. The proportion (%) of ARBs was determined with the following formula:

$$ARB\ (\%) = \frac{Number\ of\ each\ ARB\ (\frac{CFU}{100\ ml})}{Total\ HPC\ count\ (\frac{CFU}{100\ ml})} \qquad \text{(Equation 3)}$$

4.1.5 Identification of antibiotic-resistant Heterotrophic Plate Count (HPC) bacteria using API 20NE system

Representative antibiotic-resistant colonies were isolated from the plates for identification. These colonies were picked based on the dominant colony, i.e. the dominant colour and shape of the colonies found on every antibiotic plate. The representative colonies were then purified onto new plates and incubated at 35±2°C overnight for further tests.

The API 20NE system covers 61 non-enterobacterial Gram-negative taxa. The API® strip consists of micro-tubes (cupules) containing dehydrated substrates to detect the enzymatic activity or the assimilation/fermentation of sugar by the inoculated microorganisms. Selected isolates were initially screened for Gram-positive/ negative characteristics. Bacteria were grown in selective media for Gram-negative MacConkey agar (Oxoid, Basingstoke, UK). Further, a cytochrome oxidase test was performed to determine whether the bacteria were able to produce cytochrome c oxidase. Only Gram-negative and oxidase positive isolates were then identified with the API 20NE identification system.

Briefly, there were three steps that needed to be completed. The first was the preparation of the bacteria culture: bacteria were grown in R2A agar for 24 h prior to identification. Further, one loop of the bacteria culture was added to 2 ml of API NaCl 0.85%. The bacteria inoculums were then adjusted to a Biomerieux 0.5 McFarland solution. The second step was the inoculation of the strip: bacteria inoculums in saline solution were distributed into the tubes NO_3, TRP, GLU, ADH, URE, ESC, GEL and PNPG. The rest of the bacteria inoculum (~200 µl) was then transferred to an API AUX medium, followed by inoculation into the rest of the tubes (as shown in **Figure 4.1**). The strip was then incubated at 29°C ± 2°C for 24 h (± 2 h). During incubation, the metabolism produces colour changes that are either detected directly or revealed by the addition of reagents. The third and final step was reading and interpreting the strip: after the incubation period, the strip was read by referring to the reading table. All spontaneous reactions except for NO_3 and TRP tubes were recorded on the result sheet. One drop of API NIT 1 and API NIT 2 reagent were added to the NO_3 cupule, while for the TRP tubes, one drop of API James reagent was added to the cupule. Identifications were obtained as 7-digit numerical profiles,

and these profiles were then analysed with Apiweb™ identification software. **Figure 4.2** shows the API® 20NE strip.

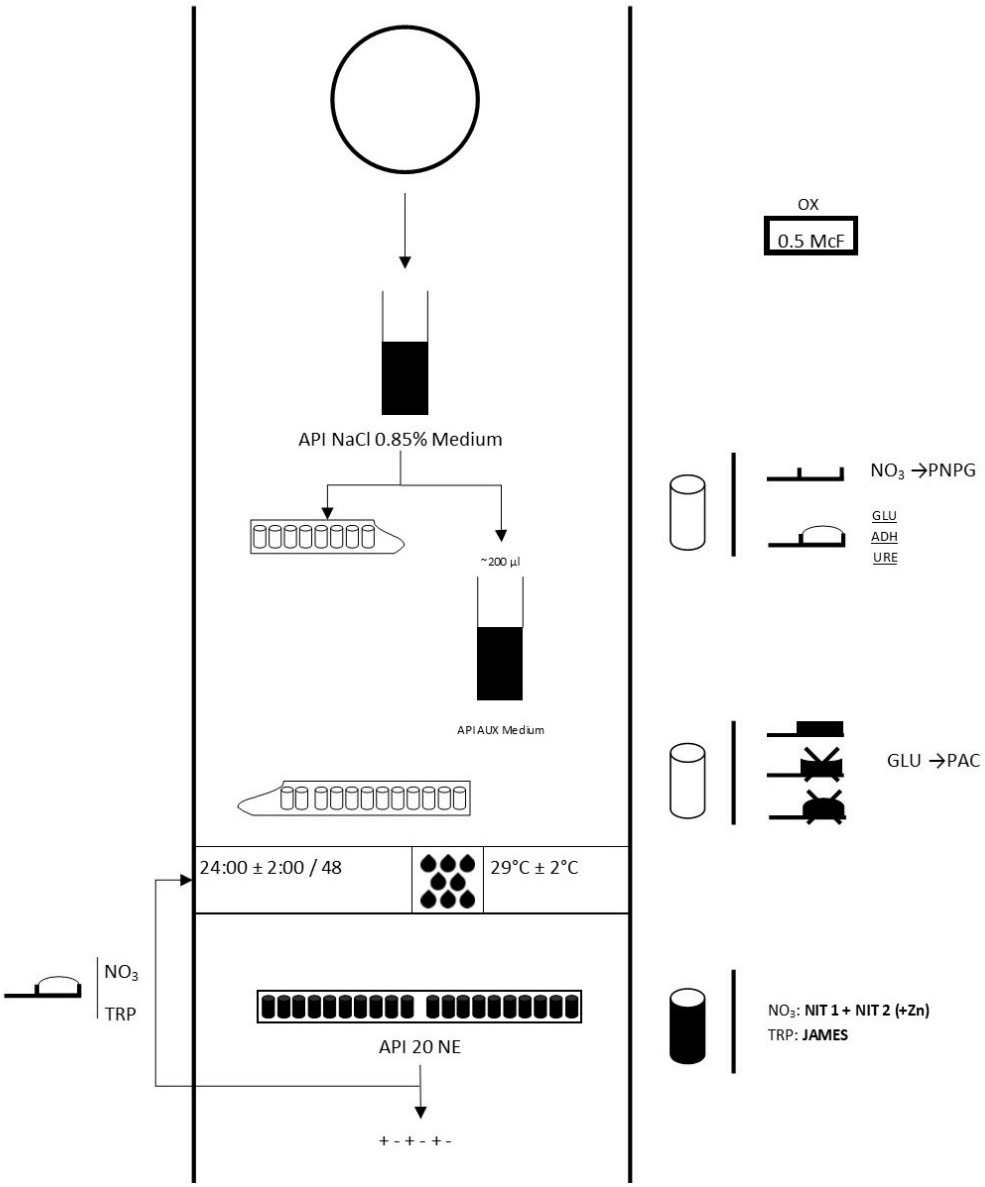


Figure 4.1 Bacteria identification protocol with the API 20NE identification system

There are four possible identification results: excellent, very good, good, acceptable identification and low discrimination, defined as ≥ 99.99, ≥99, ≥90, ≥80 and <80% certainty of identification, respectively.

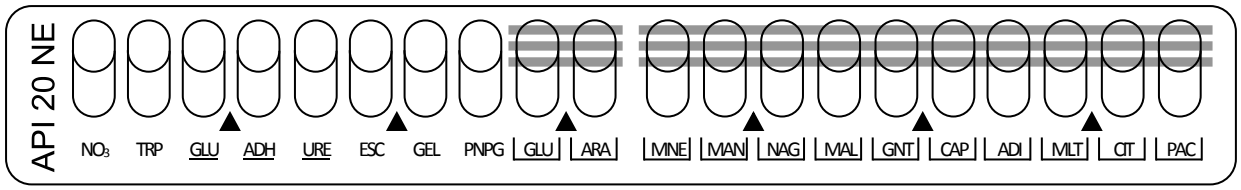


Figure 4.2 API® 20NE strip (Biomeriux.com)

4.2 Detection and Quantification of Antibiotic Resistance Genes

4.2.1 DNA isolation

Relatively large water volumes were necessary to obtain a measurable amount of DNA; thus, 2-3 litres of tap water sample were collected for DNA extraction each time. Genomic DNA was isolated and purified using a commercial DNA isolation kit, the PowerWater® DNA isolation kit (Mobio Company, San Diego, California, USA) according to the manufacturer's recommendations as illustrated in **Figure 4.3**. Briefly, water samples were filtered through a 0.45-µm pore size filter membrane (Pall Corporation, New York, USA). The membrane was then placed into a bead beating tube, followed by vortex mixing whereby cell lysis occurred. The next step was the removal of proteins and inhibitors, then the total genomic DNA was captured on a silica spin column. A Tris buffer was then used to elute the DNA from the spin column. DNA quantity and quality were measured using a UV spectrophotometer at wavelengths 260 nm and 280 nm, and the DNA was then stored at -20°C until further analysis.

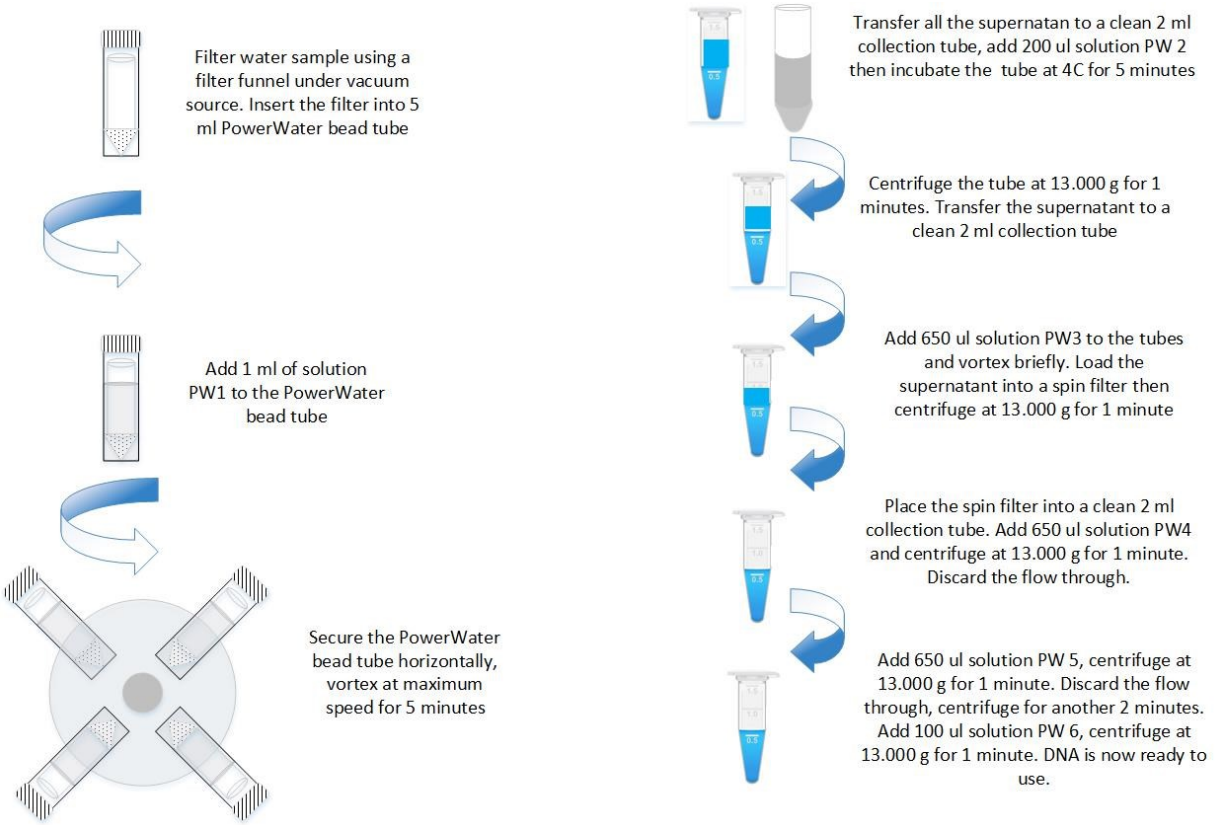


Figure 4.3 DNA isolation protocol using PowerWater DNA isolation kit

4.2.2 Quantitative Polymerase Chain Reaction (qPCR)

A Quantitative Polymerase Chain Reaction (qPCR) detection assay was used for broad-scale screening of the presence or absence of five resistance genes and to quantify their copy number. These genes were: *tet*-A for tetracycline resistance genes, *sul*1 for sulphonamides resistance genes, *bla*-$_{\text{TEM1}}$ for beta-lactams resistance genes, *mph*(A) for macrolides (including erythromycin) resistance gene and *dfr*A7 for trimethoprim resistance gene. A universal primer targeting Eubacterial 16S rRNA was used to quantify total bacteria populations. By quantifying the 16S rRNA gene, it was then possible to compare ARGs proportionally between samples of different overall population sizes. **Table 4.1** summarises the primers and reaction conditions used during the qPCR analyses. All the primers used in this study were obtained from Thermo Fisher Scientific (Waltham, Massachusetts, USA).

Table 4.1. Primer sequences and reaction conditions

Target gene	Primers	Sequence	Condition	Ref
16S rRNA	27f	AGAGTTTGATCATGGCTCAG	Annealing temperature 68°C	(Hoefel *et al.*, 2015)
	1492r	GGCTACCTTGTTACGACTT		
tet-A	*tet*-A FW	GGTCATTTTCGGCGAGGATC	Annealing temperature 68°C	This study
	tet-A RV	GAAGGCAAGCAGGATGTAGC		
mph(A)	*mph*(A) FW	ACCATCGCAGTCGAGTCTTC	Annealing temperature 68°C	This study
	mph(A) RV	GCCGATACCTCCCAACTGTA		
bla-$_{TEM1}$	*bla*-$_{TEM1}$FW	GCGCCAACTTACTTCTGACAACG	Annealing temperature 68°C	(Xi *et al.*, 2009)
	bla-$_{TEM1}$RV	CTTTATCCGCCTCCATCCAGTCTA		
*sul*1	*sul*1 FW	CGCACCGGAAACATCGCTGCAC	Annealing temperature 70°C	(Xi *et al.*, 2009)
	*sul*1 RV	TGAAGTTCCGCCGCAAGGCTCG		
*dfr*A7	*dfr*A7 FW	CAACGATGTTACGCAGCAGG	Annealing temperature 68°C	This study
	*dfr*A7 RV	GGACCACTACCGATTACGCC		

(1) Quantification method and PCR reactions

The resistance genes (*tet*(A), s*ul*1, *bla*-$_{TEM1}$, *mph*(A), and *dhfr*A7) and bacterial 16S rRNA gene fragments were quantified from the environmental DNA extract using quantitative real-time SYBR green-PCR. Reactions were run in volume 20µl for all resistance genes and bacterial genes using Dynamo Flash SYBR green master mix (Thermo Fisher, Waltham, Massachusetts, USA). **Tables 4.2** and **4.3** show the reaction set-up for the qPCR analysis and qPCR conditions, respectively.

Table 4.2. qPCR reaction set-up

Component	Volume	Final concentration
2x master mix	10 µl	1X
Primer mix	2 µl	10 mM
50x ROX reference dye	0.4 µl	1X
Template DNA	2 µl	
H_2O	Add to 20 µl	

Table 4.3. qPCR conditions

Step	Temperature	Duration
Initial denaturation	95°C	15 minutes
Denaturation	95°C	10 seconds
Annealing	70°C (*sul*1)	30 seconds
	68°C (*dfr*A7, *bla*-$_{TEM1}$, *mph*(A), *tet*(A), 16S rRNA)	
Final extension	60°C	30 seconds
Melting curve	60-95°C	
Hold	20°C	20 seconds

All samples and standards were run in triplicate in 96-well plates on a PikoReal time PCR system (Thermo Fisher, Waltham, Massachusetts, USA) and analysed using Thermo Scientific PikoReal software version 2.2 (Thermo Fisher, Waltham, Massachusetts, USA). Standard curves were prepared from a serial dilution of the plasmid, serving as a positive control for each resistance gene. Dilution series were prepared as recommended by the Applied Biosystems tutorial 'Creating Standard Curves with Genomic DNA or Plasmid DNA for use in quantitative PCR'. A No-Template Control (NTC), which consists of all reaction components except the DNA sample, was included on every plate for every primer set, in order to check for contamination.

To confirm the presence of the resistance genes and primer specificity, PCR products were analysed using gel electrophoresis. SYBR safe DNA stain (Thermo Fisher, Waltham, Massachusetts, USA) was used to stain the DNA. Briefly, 0.7µl of PCR product was mixed with 0.3µl of SYBR safe DNA stain then loaded into 1.3% electrophoresis gel (Bio-Rad Laboratories, Hercules, California, USA). The electrophoresis process was conducted at 70 volts for 60 minutes, then the gel was viewed using an UV transilluminator.

4.2.3 Quality control and data analysis

When working with qPCR, it is important to have control over the steps that are included in the process. There are five factors that require attention to ensure a valid and reliable qPCR analysis.

(1) qPCR efficiency and the r^2 value of the standard curve

A robust and efficient qPCR assay is normally expressed with qPCR efficiency close to 100%. A good PCR reaction should have 90% to 100% efficiency. In this study, the typical qPCR efficiency obtained for each reaction ranged from 85% to 110%.

The r^2 value of the standard curve represents how well the experimental data fit to the regression line, i.e. data linearity. Data linearity gives variability measured across the assay replicates. A significant difference in observed Cq values between replicates will lower the r or r^2 value. An r with an absolute value >0.990 or an r^2 value >0.980 is desirable for RT-qPCRs**. Figure 4.4** illustrates the typical standard curves obtained in this study; the r^2 values were always in the range of 0.99 to 0.98, which showed good linearity between the reference genes and the samples throughout the study.

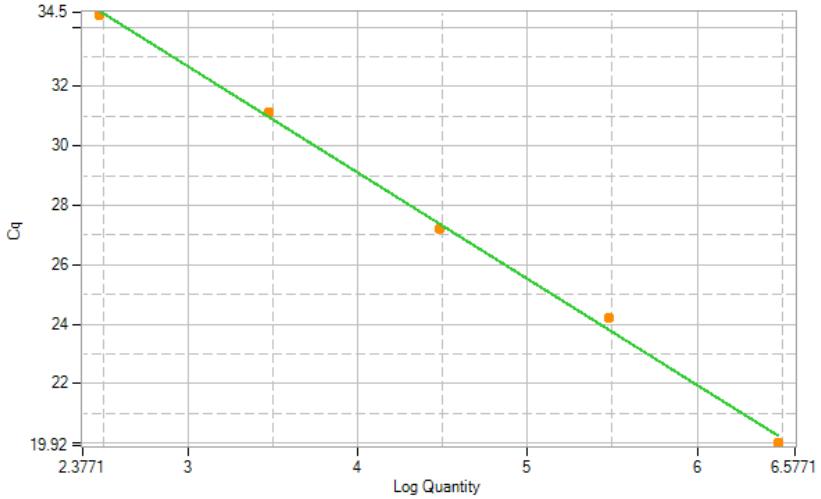


Figure 4.4 Typical standard calibration curve obtained from the qPCR analysis in this study

(2) Melt curve

A melting curve is observed when double-stranded DNA combined with fluorescent dye is dissociated from a single-stranded DNA. A specific PCR reaction will be distinguished by a single sharp peak of a specific Tm (melting temperature). On the other hand, a non-specific PCR reaction will normally exhibit more than one peak of Tm or a broad peak, which indicates the formation of a primer-dimer. **Figure 4.5** shows the typical melting curve obtained from the qPCR analysis in this study.

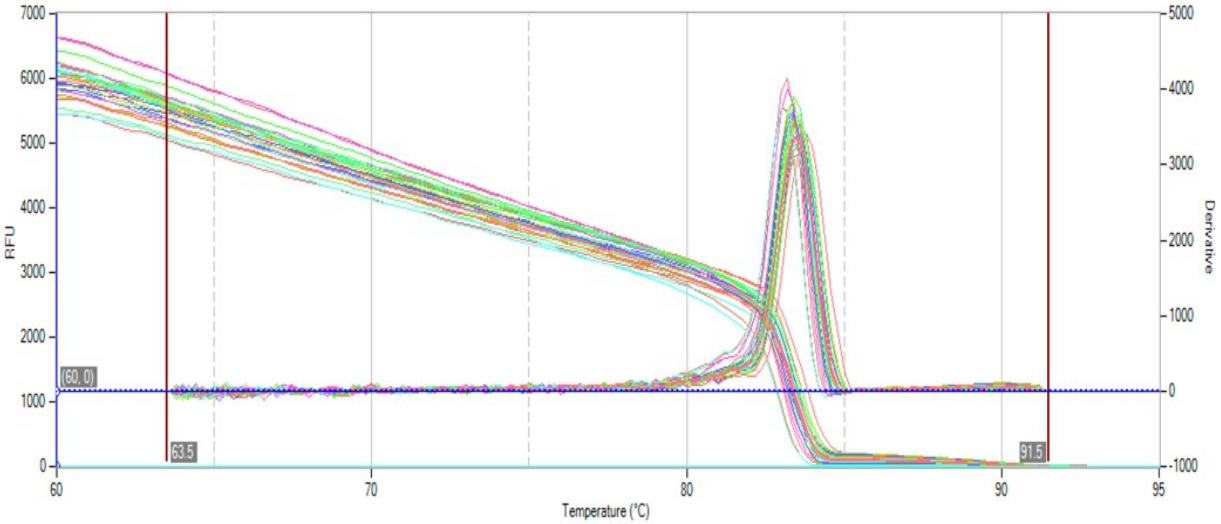


Figure 4.5 Typical primer melting curve obtained from the qPCR analysis in this study

(3) Control

A no-template control (NTC), i.e. containing all the PCR reagents except the DNA sample template, should be incorporated into each qPCR reaction. The NTC will determine whether there is any cross-DNA contamination during reaction set-up. Several studies have stated that if the Cq value between the NTC and the sample with the highest Cq is significantly large, then the signal can be ignored.

4.3 Statistical Analysis for Antibiotic-resistant Bacteria and Antibiotic Resistance Genes

All statistical analyses were conducted in SPSS version 24.0 and Microsoft Excel 2016. Analysis of Variance was used to determine the effect of different sampling periods and sampling times. To describe the relationship between antibiotic-resistant rates and tap water quality parameters, Spearman's rank correlations were used for each antibiotic. Pearson's chi square test was used to evaluate the association between different antibiotic-resistant bacteria or genes.

4.4 Effectiveness of Chlorine and UV in Inactivating Antibiotic-Resistant Bacteria and Antibiotic Resistance Genes in Waterborne Bacteria

4.4.1 Medium preparation

Tryptone soya broth medium was prepared by dissolving 30 grams of medium (Fluka Analytical, Busch, Switzerland) in 1 litre of distilled water. The prepared medium was then sterilised at 121°C for 20 minutes. The medium was stored at 4°C for a maximum of two weeks until further use. Tryptone soy agar was made by dissolving 20 grams of medium (Fluka Analytical, Busch, Switzerland) in 1 litre of distilled water. The medium was autoclaved at 121°C for 20 minutes. The sterile medium was then allowed to cool to hand-hot, i.e. approximately 50°C, and antibiotics were then added to the medium.

4.4.2 Phosphate buffer saline solution

The phosphate buffer saline was prepared with 8 grams sodium chloride (Sigma Aldrich, Missouri, USA); 0.2 grams potassium chloride (Sigma Aldrich, St Louis, Missouri, USA); 1.44 grams sodium phosphate dibasic (Sigma Aldrich, St Louis, Missouri, USA) and 0.24 grams monopotassium phosphate (Sigma Aldrich, St Louis, Missouri, USA). These were dissolved in 800 ml of distilled water, and the volume was then adjusted to 1 litre by adding more distilled water. 1 M hydrochloric acid (Sigma Aldrich, St Louis, Missouri, USA) or 2 M sodium hydroxide were used to adjust the pH to 7.4 using a pH meter, and 200 ml of PBS were dispensed into 250 ml Schott Duran® reagent bottles and then autoclaved at 121°C for 20 minutes. Further, PBS stock (pH 7.4) was dispensed into 9 ml culture tubes, then autoclaved at 121°C for 20 minutes.

4.4.3 Bacteria strains and preparation

In this experiment, a MAR *Escherichia coli* (NCTC 13400), an antibiotic-sensitive *Escherichia coli* (NCTC 12241) and a MAR *Pseudomonas aeruginosa* (NCTC 13437) were used. All of the bacteria were purchased from a national collection of type cultures (Public Health England, UK): *E. coli* NCTC 13400, the 117kb plasmid pEK499 carrying ten resistance genes, including *tet*(A), *bla*-$_{TEM1}$, *su*l1, *mph*(A), *bla*$_{CTX-M-15}$, *bla*$_{OXA-1}$, *aac*6'-Ib-cr and *dfr*A7. *Pseudomonas aeruginosa* NCTC 13437 is resistant towards β-lactams antibiotics including amoxicillin and amikacin. In addition to the bacterial

strain obtained from NCTC, an environmental strain bacterium isolated from tap water was also used. The environmental strain was identified using API 20NE system as described in section 4.1.5.

Bacteria were grown on tryptic soy broth (TSB) medium at 37°C with 130 rpm shaking. An overnight culture of the microorganisms was harvested by centrifugation at 3000 rpm for 10 minutes. The harvested bacteria were washed three times then re-suspended in 50 ml sterile phosphate buffer saline in a 50 ml Falcon tube. A bacteria stock solution was prepared daily and stored at 4°C.

4.4.4 Minimum inhibition concentration (MIC) for environmental strain bacteria

Antibiotics stock were prepared at the following concentrations: (1) tetracycline 1000 μg/ml, (2) trimethoprim 1000 μg/ml, (3) amoxicillin 1000 μg/ml.

Bacteria inoculum was grown in TSB at 35°C for 24 hours. Bacteria were then harvested with centrifugation at 3000 rpm for 10 minutes. The supernatant was discarded, the bacteria was then washed with sterile PBS (pH 7.2) three times. Bacteria concentration was determined using a UV-VIS spectrophotometer, to obtain a density of ~10^8 CFU/ml (i.e. OD value 0.25-0.3 at 600 nm). Subsequently, this bacterial solution with 10^8 CFU/mL was diluted to a concentration of 10^5 CFU/ml by suspending 2 mL 10^8 CFU/ml solution in exactly 38 ml sterile PBS in 50 ml sterile centrifuge tubes. The bacterial solution with 10^5 CFU/ml was used in MIC experiments conducted in a biosafety cabinet.

Sterile 96-well tissue culture plates with a low evaporation lid from Falcon® were used in the MIC experiments. The first and last rows were sterile control; 50 μL of the sterile PBS was added to the sixth and seventh row, and 50 μL of 10% DMSO to the fifth row, then 50 μL of antibiotics stock was added from the second row to the fourth row by using multichannel pipettes. Twofold serial dilution was conducted by mixing and extracting 50 μL solution from the second row to the third row until the last row, and 50 μL solution at the last row was discarded. Afterwards, 30 μL of Mueller Hinton broth and 10 μL of resazurin dye were added to all wells in the culture plates in order. Finally, 10 μL 10^5 CFU/ml bacteria solution were added, except two sterile PBS columns where 10 μL PBS was added instead. Two or three culture plates were prepared for each MIC experiments and incubated at 35°C for 24 hours. Results were recorded after 24 hours incubation.

4.4.5 Bacteria growth curves

Bacteria growth curves were prepared by streaking each bacterium strain onto new prepared TSA plates and incubating them overnight at 37°C. A single colony was inoculated into 25 ml of TSB then incubated overnight in a shaker incubator at 37°C with 125 rpm shaking. Following incubation, 100 µl of suspension was transferred into a 500-ml flask, which contained 100 ml of TSB. Samples were placed in the shaker bath at 37°C and 125 rpm. At 0, 2, 4, 6, 8, 10 and 12 hours after the incubation, 3.5 ml of this new growth suspension was removed, then placed in a disposable cuvette (Brand, Wertheim, Germany) and measured for optical density at 600 nm using a UV spectrophotometer.

4.4.6 Chlorination

4.4.6.1 N.N-diethyl-p-phenylenediamine (DPD) ferrous titrimetric method

The DPD ferrous titration method was used to determine the total and combined chlorine residual concentration. The method is based on the Standard Method for the Examination of Water and Wastewater (American Water Work Associations, 2008).

A phosphate buffer was prepared by dissolving 24 grams anhydrous disodium hydrogen phosphate (Na_2HPO_4) and 46 grams potassium dihydrogen phosphate (KH_2PO_4) in distilled water. The solution was combined with 100 ml distilled water in which 800 mg disodium EDTA had been dissolved, then the volume was adjusted to 1 L with distilled water.

A DPD indicator was made by dissolving 1.1 g of anhydrous DPD sulphate in distilled water containing 8 ml (1 + 3) sulphuric acid and 200 mg disodium EDTA. The solution was adjusted to 1 L with distilled water and stored in a brown glass stoppered bottle in the dark. The solution was discharged when the absorbance value at 515 nm was more than 0.002 cm^{-1}.

A standard FAS titrant was prepared by dissolving 1.106 grams ferrous ammonium sulphate in 1 ml of (1 + 3) sulphuric acid and adjusting the volume to 1 L with distilled water. The stock was diluted ten-fold for use in all the titration methods.

For the chlorine residual analysis: 5 ml of phosphate buffer and 5 ml of DPD indicator solution were added to a 100 ml Erlenmeyer flask. The solutions were mixed thoroughly then 10 ml of sample was added. The solution was left to stand for two minutes to allow for a complete reaction between the

sample and the buffer. The solution was then titrated using FAS titrant until the red colour disappeared, and the volume of FAS titrant required was then recorded.

4.4.6.2 Chlorine stock solution

A chlorine stock solution was prepared by dosing sodium hypochlorite (Sigma Aldrich, St Louis, Missouri, USA) (1.206 mg/L and 10% as free chlorine) in deionised water (free chlorine demand-water) and this was diluted to ~100 mg/L. The chlorine concentration in the stock was periodically determined by the DPD ferrous titration method. The stock solution was titrated against FAS to standardise it by diluting 0.1 ml of stock solution with 14.9 ml of phosphate buffer solution and 5 ml of DPD indicator. 1 ml of FAS titrant was equivalent to 1 mg/l of Cl. The stock concentration was checked daily. The stock solution was prepared weekly and stored in a 500-ml volumetric flask covered with aluminium foil at 4°C.

4.4.6.3 Antibiotic-resistant bacteria inactivation trial

Experiment was conducted in sterile 250 ml Schott Duran® reagent bottles. The bacteria stock solution was added to 200 ml sterile phosphate buffer saline (pH 7.4) to obtain a bacteria concentration of ~10^7 CFU/ml. This suspension was then stirred with a magnetic flea at 20 ± 2°C. Chlorine was accurately dosed in two concentrations: 1 mg/L and 2 mg/L. After 2, 5, 8, 11 and 15 minutes, 5 ml of sample was withdrawn then added to culture tubes that contained 1 ml of sodium thiosulphate (2 g/L). In addition, the free chlorine residual measurement was also taken at each contact time.

4.4.6.4 Antibiotic resistance genes inactivation trial

An experiment was conducted in sterile 250 ml Schott Duran® reagent bottles. The bacteria stock solution was added to 200 ml sterile phosphate buffer saline (pH 7.4) to obtain a bacteria concentration of ~10^7 CFU/ml. This suspension was then stirred with a magnetic flea at a speed of 200 rpm; the experiment was conducted at room temperature (20±2°C). Chlorine was accurately dosed in three different concentrations: 1 mg/L, 2 mg/L, and 5 mg/L. Two contact times were employed during the experiment: 15 and 30 minutes.

The residual chlorine concentration was determined as follows: 5 ml of phosphate buffer solution was added with 5 ml of DPD indicator to a 100 ml Erlenmeyer flask. 10 ml of solution was then added to the mix. The suspension was titrated with FAS solution until the red colour disappeared.

4.4.6.5 Antibiotic-resistant bacteria analysis

At the following times – 0, 2, 5, 8, 11 and 15 minutes – 5 ml of sample was taken using a sterile pipette and transferred into culture tubes containing 1 ml of sodium thiosulphate as a quencher. The solution was then thoroughly mixed using a vortex. Serial decimal dilutions were performed for each time sample, to obtain 10-200 colonies per 55 mm plate. One ml of sample was poured sequentially into several culture tubes containing 9 ml of PBS pH 7.4. The solutions were then mixed by inverting the tubes several times.

The membrane filtration method was used to enumerate the bacteria. The serial dilutions were each poured into a 100-ml funnel with a 0.45 μm pore 47 mm sterile membrane filter (PALL Corporation, New York, USA) under vacuum conditions. The membrane was then transferred onto 55 mm TSA plates with and without antibiotics for the control experiment. The plates were then incubated overnight at 35 ± 2°C. The number of colonies were recorded for each plate and expressed as CFU/ml. Each inactivation trial was conducted under the same circumstances in three analytical replicates.

4.4.6.6 Antibiotic resistance genes sample analysis

PBS containing bacteria inoculum (200 ml) was filtered through 0.45 μm sterile membrane filter (PALL Corporation, New York, USA). DNA was extracted as described in section 3.2.1.

Quantitative PCR analysis was used to quantify four antibiotic genes, namely: *tet*-A, *sul*1, *bla*-$_{TEM1}$, and *mph*(A). All samples were run in triplicate in a 96-well plate in a PikoReal time PCR system (Thermo Fisher, Waltham, Massachusetts, USA) and analysed using Thermo Scientific PikoReal software version 2.2 (Thermo Fisher, Waltham, Massachusetts, USA). Standard curves were prepared from the serial dilution of the plasmid serving as the positive control for the resistance gene. Dilution series were prepared as recommended by the Applied Biosystems tutorial 'Creating Standard Curves with Genomic DNA or Plasmid DNA for use in quantitative PCR' (Thermo Fisher, Waltham, Massachusetts, USA). Reactions were run in a volume of 20μl using Dynamo Flash SYBR green master mix (Thermo Fisher,

Waltham, Massachusetts, USA). Each 20µl volume consisted of 10µl 2x master mix, 10 mM forward and reverse primer, 2 µl of DNA template, 0.4 µl ROX™ passive reference dye, and sterile RNAse/DNAse-free water.

4.4.6.7 Control experiment

The effectiveness of sodium thiosulphate ($Na_2S_2O_3$) in quenching chlorine residuals was tested. 1 mg/L of chlorine (2 ml of 100 mg/L Cl_2 stock solution) was added to a Schott Duran® reagent bottle containing 200 ml sterile PBS at pH 7.4. 40 ml of 2 g/L sodium thiosulphate was subsequently added, and the solution was stirred with a magnetic flea. The concentration of the residual chlorine was then determined. The chlorine present in the PBS solution was completely quenched by sodium thiosulphate as the sample solution in the conical flask remained colourless.

The viability of bacteria in PBS was also determined. For each bacteria type, the sample stock was added to a Schott Duran® reagent bottle containing 200 ml sterile PBS at pH 7.4 to achieve a concentration of ~10^6-10^7 CFU/ml. At 1, 5, 10, 15, and 25 minutes, 1 ml of the sample was added into culture tubes containing 9 ml sterile PBS at pH 7.4. Serial dilutions were performed and the resulting sample was then plated according to the membrane filtration method.

The stability of free chlorine residuals in the absence of bacteria was determined by dosing 1 and 2 mg/l of Cl_2 into 200 ml sterile PBS at pH 7.4. 10 ml samples of the solution were taken over a 40-minute period, and the concentration of the residual chlorine was determined using the DPD ferrous titrimetric method. The free chlorine decay in the presence of bacteria was also evaluated. Once bacteria had been added to 200 ml sterile PBS at pH 7.4, Cl_2 was added at different concentrations i.e. 1 mg/L, 2 mg/L, and 3 mg/L. A free chlorine residual analysis was determined over 25 minutes using the DPD ferrous titrimetric method.

4.4.7 UV disinfection experiment

4.4.7.1 UV exposure protocol

A UV disinfection experiment was conducted using a bench-scale UV collimated beam system. The beam consisted of a low-pressure UV lamp emitting nearly monochromatic light at a wavelength of 254 nm. The diameter of the tube was 14 cm and the tube length was 35 cm.

The microbial solution was added to a sterile pH 7.4 PBS solution to obtain a density of ~10^7 CFU/ml. An experiment was performed in a 6.5 cm diameter Pryex® glass beaker and the depth of the sample was 3 cm. The glass beaker containing the sample was shielded using cardboard and placed on a stir plate under the UV lamp. Samples were stirred by a magnetic flea at room temperature: 20±2°C.

Standard methods for UV dose measurement and calculations for collimated beam experiments were followed (Bolton and Linden, 2003). A radiometer (model ILT1700, International Light, Peabody, Massachusetts, USA) was used to measure the UV intensity across the exposure surface and a UV spectrophotometer accounted for the UV absorbance at 254 nm of the samples. The UV irradiance at the centre of the water samples was 0.2 mW/cm^2. Room lights were switched off when running the experiments and a piece of cardboard was used to cover the collimated beam outlet to ensure that no UV overexposure occurred. The beaker was placed under the collimating tube and slowly mixed while exposed to UV radiation for the selected time periods. Samples were exposed to different UV doses: 3, 5, 7 and 10 mJ/cm^2 for ARB inactivation and 20, 50, 100 and 200 mJ/cm^2 for ARG inactivation.

4.4.7.2 Antibiotic-resistant bacteria analysis

A membrane filtration method was used for bacteria enumeration. Following UV exposure, 1 ml of sample aliquot was transferred to 9 ml sterile PBS. Serial dilutions were performed for each UV dose, with the aim of obtaining 20-100 colonies per plate. The appropriate serial dilutions were poured onto a 0.45 μm pore, 47 mm diameter sterile membrane filter under vacuum conditions. The membrane was then transferred onto 55 mm diameter TSA plates (containing antibiotics or no antibiotics accordingly). The TSA plates were then incubated at 35±2°C overnight, and the number of colonies were tallied and recorded as CFU/ml.

4.4.7.3 Antibiotic resistance genes sample analysis

DNA extraction protocol and qPCR analysis were performed as described in section 3.5.5.6.

4.5 Potential Repair of Antibiotic-resistant Bacteria Following Chlorination and UV Disinfection

4.5.1 Chlorination experiment

Chlorination and chlorine residual measurements were conducted as described in section 3.5.5.1. Two sets of chlorination were conducted: 1, 2 and 5 mg/L with 30 minutes contact time, and 1 mg/L and 2 mg/L with continuous chlorination.

For the first set of the experiment, subsequently to 30 minutes of chlorination, 40 ml of 2 g/L sodium thiosulphate (Sigma Aldrich, Waltham, Missouri, USA) was added to the samples to stop the chlorine reaction. The samples were then incubated inside a dark laboratory cabinet, where the temperature was set at 20°C. Samples were analysed at 24 hours, 48 hours, 72 hours and one week after the chlorination.

The second set of the experiment was conducted with continuous chlorination (e.g. sodium thiosulphate was not added to the sample). Two chlorine doses were applied: 1 mg/L and 2 mg/L. The samples were stored inside a dark incubator and analysed at 24 hours, 72 hours, one week and two weeks after the chlorination. During sample collection, the chlorine residual was also analysed using the DPD titration method as described in section 3.5.5.1.

4.5.2 UV disinfection experiment

UV exposures were conducted in Pryex® glass beakers. 50 ml of phosphate buffered saline containing ~ 10^7 CFU/ml of microbial solution samples were then exposed to UV doses of 3, 5 and 10 mJ/cm^2, the doses applied in this study were lower than the doses used in treatment practice but were chosen to allow an examination of potential dose-response profiles for the tested bacteria, since higher UV doses resulted in complete inactivation (no survivors). The UV exposure protocol was performed as described in section 3.5.7.1.

4.5.2.1 Dark repair experiment

After UV exposure, beakers were placed in a dark laboratory incubator and covered using sterile Petri dishes to avoid any external contaminations. The beakers were then positioned in a magnetic stirrer and

gently stirred, with the temperature set at 20 ± 2°C. Samples were taken at the following times: 3 hours, 15 hours and 24 hours after UV exposure.

4.5.2.2 Light repair experiment

After exposure to UV light, the aliquot of the samples was placed in a covered sterile beaker. These beakers were then positioned in a block magnetic stirrer under daylight simulation. A desk lamp with an adjustable flexible neck and an LED daylight bulb (270 lumen, 4.5w ≈ 25w Eq, E14/SES) were used for photo repair trials. The vertical distance between the lowest part of the bulb and the top surface of the sample contained in the beakers was 18.5 cm. The temperature inside the laboratory incubator was set to 20±2°C. Samples were taken at the following times: 3 hours, 15 hours and 24 hours after UV exposure. Both positive (i.e. bacteria suspension that had not undergone UV exposure) and negative controls (i.e. sterile PBS) were incorporated on each run. Each repair trial was conducted under the same conditions in three analytical replicates.

The repair (%) was calculated using the equation from Koivunen and Heinonen (2005) below:

$$\%repair = \frac{N_t - N_0}{N_{initial} - N_0} \qquad \text{(Equation 4)}$$

Where Nt is the concentration of bacteria at exposure time *t*, to repair conditions (log CFU/ml), N_0 is the concentration of bacteria immediately after chlorine or UV disinfection (log CFU/ml), and $N_{initial}$ is the number of bacteria before chlorine or UV treatment (log CFU/ml).

4.6 The Potential Synergistic Effect of Chlorination and UV Disinfection in Inactivating Antibiotic Resistance Genes in Waterborne Bacteria

Firstly, UV experiments were carried out according to the description in section 4.5.2.1. The fluence rate was 0.2 mW/cm^2 measured by a radiometer (model ILT1700, International Light, Peabody, Massachusetts, USA). 100 mL PBS containing bacteria suspension was added to the beaker and mixed gently with a magnetic stirring bar; the water depth was 3 cm. Before initiating the experiment, the apparatus was allowed to warm up for 15 minutes and it was stirred for approximately 10 seconds to ensure stable UV fluence rate and a well-mixed solution. In this study, the exposure time for UV disinfection alone was from 0 to 24 minutes, i.e. samples were irradiated for 6 mins, 12 mins and 24 mins, and the corresponding UV doses were 50 mJ/cm^2, 100 mJ/cm^2 and 200 mJ/cm^2, respectively. The

dose was calculating by multiplying the irradiation time and UV lamp fluence rate (Bolton and Linden, 2003).

Secondly, in the following chlorination experiments, chlorine was added to the UV-irradiated samples in doses of 1 mg/L and 2 mg/L. After chlorination of 15 minutes, $Na_2S_2O_3$ solution was added to stop the chlorination for the subsequent ARGs analysis. All disinfection treatments were conducted at room temperature (20±2 °C). Each disinfection trial was conducted under the same conditions in three analytical replicates and control beakers (i.e. beakers without exposure to chlorine and UV) were incorporated on each experiment. The ARGs analyses (i.e. DNA extraction and qPCR) were the same as those described in sections 4.2.1 and 4.5.5.6. The synergy value of the sequential UV/chlorination treatment was calculated by the following equation (Koivunen and Heinonen-Tanski, 2005):

Synergy (log units) = log reduction by combined UV/chlorination disinfection − (log reduction by UV disinfection + log reduction by chlorine disinfection) (Equation 5)

Synergy exists from the combined disinfection treatment when the value of the synergy is positive, while a negative value represents an antagonistic effect. A value of zero indicates that the efficiency of the combined treatment is the same as the sum of the two individual treatments.

4.7 Statistical Analysis for Disinfection Studies

Log_{10} (N/N_0) was used to evaluate the disinfection effectiveness, where N = number of bacteria or genes after the disinfection treatment and N_0 = the number of bacteria or genes before the disinfection treatment.

Statistical analysis was performed using Microsoft Excel and SPSS version 24. The Student T test was used to assess statistical differences between disinfection treatments. The null hypothesis that the log reduction was not different between different treatments was rejected at a p-value less than or equal to 0.05.

4.8 Determination of Oxidative DNA Damage from Chlorination and UV Disinfection by HPLC-UV

First of all, DNA from *E. coli* NCTC 13400 was extracted following the methodology described in section 4.2.1. The concentration of DNA was then determined using a UV spectrophotometer at 260 nm; A_{260} is equals to 50 µg of DNA/ml. Subsequently, DNA was diluted to 100 µg/ml using molecular-grade water. The diluted DNA was then treated with 10 mg/L of chlorine for 15 minutes contact time. The chlorine concentration was accurately measured using the DPD ferrous titration method as previously described in section 4.4.6.1. Another set of the diluted DNA was exposed under a UV lamp at 300 mJ/cm^2 UV dose, with the UV exposure was conducted as described in section 4.5.2.1.

Aliquots of the DNA solution were then hydrolysed according to the method by Kaur & Halliwell (1996). Briefly, DNA aliquots (1 ml of 50 mg DNA each) were hydrolysed with 0.5 ml of formic acid (60%, v/v) in a sealed tube for 45 minutes at 150°C. Formic acid was then removed by the freezing-drying method. Before analysis by high-performance liquid chromatography (HPLC), DNA was re-dissolved in HPLC-grade water with a final volume of 1.5 ml.

All the standards used in this study, including 8-oxo-7, 8-dihydro-2'-deoxyguanosine (8-oxoGuo) and 5-hydroxyuracil (isobarbituric acid, 5-OHU), were purchased from Sigma Aldrich in HPLC grade (>98%).

The DNA was then separated using Waters 2695 HPLC coupled with a photodiode array detector (Waters, Milford, USA). All of the HPLC profiles for the chlorinated DNA, UV-irradiated DNA and individual standards were carried out using the following chromatography conditions:

Column : Luna 5µ C18 (2) 100A (2.1 x 260mm)
Flowrate : 0.20 ml/min
Injection Volume : 10 µl
Detection : Absorbance, UV at 280 nm
Colum temperature : 25°C
Eluents : (A) 2mM ammonium formate (B) 2 mM ammonium formate and 10% acetonitrile
System backpressure : 1900~2100 psi during the gradient

Gradient conditions were as described in **Table 4.4** below:

Table 4.4. Gradient mode for the HPLC separation

Time (min)	Eluent A (%)	Eluent B (%)
0	75	25
10	75	25
15	75	25
30	0	100
40	0	100
65	75	25

CHAPTER 5

RESULTS

5.1 The Antibiotic Resistance of Heterotrophic Bacteria in Tap Waters in London

This study assessed the occurrence and prevalence of ARBs and ARGs in tap water sampled across London, United Kingdom. Sampling was conducted seasonally from nine locations spread geographically across the city.

In recent years, studies have demonstrated the emergence of antibiotic-resistant heterotrophic bacteria in drinking water in various parts of the world (Ribeiro *et al.*, 2014; Bergeon *et al.*, 2015; Khan *et al.*, 2016), and the World Health Organization (WHO) recently published 12 species of antibiotic-resistant waterborne bacteria, which they consider may pose a threat to human health (WHO, 2017). Drinking water regulations in most countries do not regulate the number of heterotrophic plate count (HPC) bacteria in finished water; however, the United States Environmental Protection Agency (EPA) states that the HPCs numbers in drinking water should be below 500 colony-forming units per millilitre (CFU/ml) (EPA, 2012). The presence of HPCs may not be related to faecal contamination; however, they can be monitored to assess treatment efficiency, overall cleanliness, and the integrity of distribution systems. The presence of persistent heterotrophic bacteria in drinking water distribution systems is inevitable, since even properly operated drinking water treatment processes do not completely sterilise the water. Conditions in drinking water distribution systems such as low (or lack of) disinfectant residuals, pipe corrosion and biofilm presence can also lead to elevated microbial content in tap water. Some of these heterotrophic bacteria that are present might also be opportunistic pathogens. Some studies have shown that drinking water treatment processes might increase the percentage of bacteria that are resistant to antibiotics in some cases (Armstrong *et al.*, 1982; Xi *et al.*, 2009; Guo *et al.*, 2013; Jia *et al.*, 2015). However, there is limited information available regarding the typical occurrence levels of ARBs and the genes that impart antibiotic resistance in tap waters, whether this varies significantly temporally and spatially within a water network, and the most common types of ARBs and ARGs present.

5.1.1 Study area

Tap water samples were collected from residential properties at nine locations across London, United Kingdom. The locations were selected randomly to capture a geographical spread across the city. A total of four replicate water samples were collected from each location six times between January 2015 and July 2016.

Most of the population of London is serviced by Thames Water, with six water treatment plants serving Greater London, including Ashford Common, Hampton and Kempton Park water treatment works in west London, and Walthamstow, Desborough Island and Hornsey water treatment works in north London (DWI, 2015). Most of the drinking water for Greater London originates from surface water, abstracted from the Thames River; the rest of the water supply is from groundwater (DWI, 2015). The Lower Thames reservoir serves as the main water source for water treatment plants (WTPs) located in the west London area, while WTPs in the north London area abstract their water from Lee Valley reservoir and groundwater (DWI, 2015). Typical surface water treatment processes employed include screening, slow sand filtration, clarification, aeration, ozonation, granular activated carbon filtration, and chlorination (DWI, 2015).

Figure 5.1 illustrates the location of water treatment plants in Greater London and the nine sampling points used in this study. The sampling locations were differentiated by postcodes, as follows: sampling point 1: SE (South East), sampling point 2: NW (North West), sampling points 3 and 9: E (East), sampling points 4 and 7: SW (South West), sampling points 5 and 6: W (West), and sampling point 8: N (North) of London.

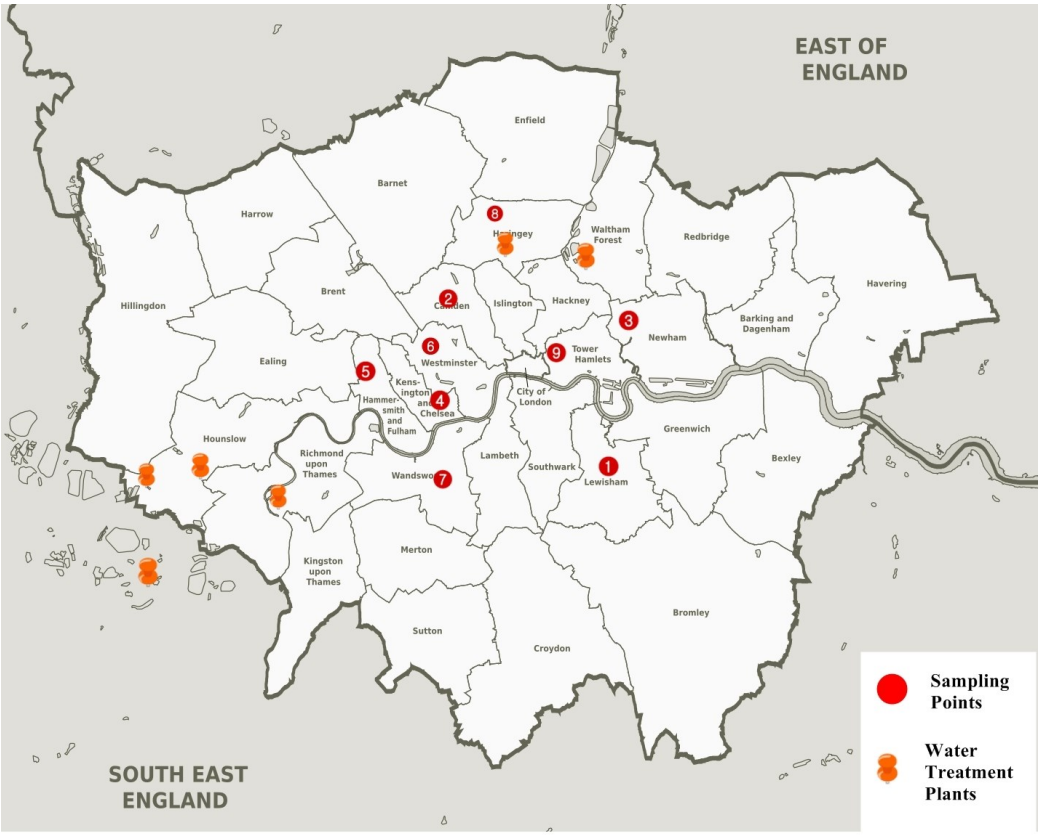


Figure 5.1 Sampling points and water treatment plant locations in the Greater London area

5.1.2 Water quality characteristics of drinking water in London

Tap water quality data can be found in Appendix B. Tap water temperature during the January 2015 sampling time was in the range of 7 to 8.7°C, with an average of 8.2°C. The water temperature increased slightly to 9.7°C in the April 2015 sampling period, with the highest temperature (11°C) observed at sampling point 1, while the temperature in the April 2016 sampling round was 0.5°C higher, with an average of 10.2°C. The average temperature in the July 2015 and 2016 sampling was 19.8°C, with sampling points 1, 4, 6 and 7 showing the highest value at 20°C.

Free chlorine residual concentrations were in the range of 0.15 – 0.5 mg/L, with the average being 0.29 mg/L. In warmer months (i.e. sampling time July 2015 and July 2016), the chlorine residuals were lower compared to other sampling times, with an average of 0.21 mg/L. The highest free chlorine residual value was found to be 0.41 mg/L and was found at sampling point 9. The lowest chlorine residual concentration obtained during the sampling programme was 0.22 mg/L and was found at sampling point 5.

TOC concentrations in tap water ranged from 0.8 to 3.2 mg/L. It appears that the seasonal differences did not affect TOC concentrations; however, it was observed that the average TOC concentrations were higher in the October 2015 sampling round than in the others. It was not possible to measure AOC or BDOC in this study, but this will be discussed later as a recommendation for potential future research.

5.1.3 Heterotrophic plate count bacteria and universal 16S rRNA gene in tap water samples

Figure 5.2 shows the number of total cultivable HPCs in the water samples.

The HPC number ranged from 10 to 500 CFU/100ml. Higher HPC numbers were generally observed during warmer months (i.e. April to July); nevertheless, the seasonal difference was not statistically significant ($p>0.05$). However, there were significant differences between the six sampling rounds at each location, except for one sampling point (number 8), which was reasonably consistent between rounds. The highest HPC number was observed in the April and July sampling times at sampling points 1, 4, 5 and 8 and sampling points 2, 3 and 7, respectively. Sampling point 9 had consistently the lowest HPC number in all sampling rounds, with an average of 23 CFU/100ml, while sampling point 5 was the highest with an average of 270 CFU/100ml.

Spearman's test was conducted to analyse the correlations between water quality parameters and total bacteria numbers. The results indicated that pH, residual chlorine and TOC were significantly correlated with the total bacteria numbers in tap water samples ($p<0.01$). The strong negative correlation between HPCs and chlorine residual shows that the disinfectant residual is an important factor in controlling the regrowth of bacteria in bulk drinking water. It has been shown that the disinfectant residual decreases with water age (assuming no chlorine boosting in the water network) because of oxidant-demanding reactions, which means that bacteria can grow more easily. Additionally, at low chlorine doses, bacteria often become injured rather than permanently inactivated (Rizzo *et al.,* 2007), and under suitable conditions the injured bacteria can repair cellular damage and become infective once more, and thus to be detected on culture media.

Furthermore, TOC (of which AOC accounts for 0.1 - 9% (Kooij, 1990), also plays an important role in the growth of bacteria (i.e. Spearman's value 0.353). Zhang and DiGiano (2002) also demonstrated a

correlation between HPC numbers and AOC concentration. Organic carbon provides an energy source for the heterotrophic bacteria, which use the carbon to produce new cellular materials and an energy source. In the UK, there are no regulatory limits on TOC concentrations. Thames Water utilities reported that their average TOC value in tap water in London was 2.2 mg/L (Thames Water, 2017), which falls within the same range as this study. Temperature, however, did not show any correlation with the total HPC bacteria. The temperature range in this study was 9 - 20°C, suggesting that the lowest temperature is still suitable for the growth and activity of heterotrophic bacteria.

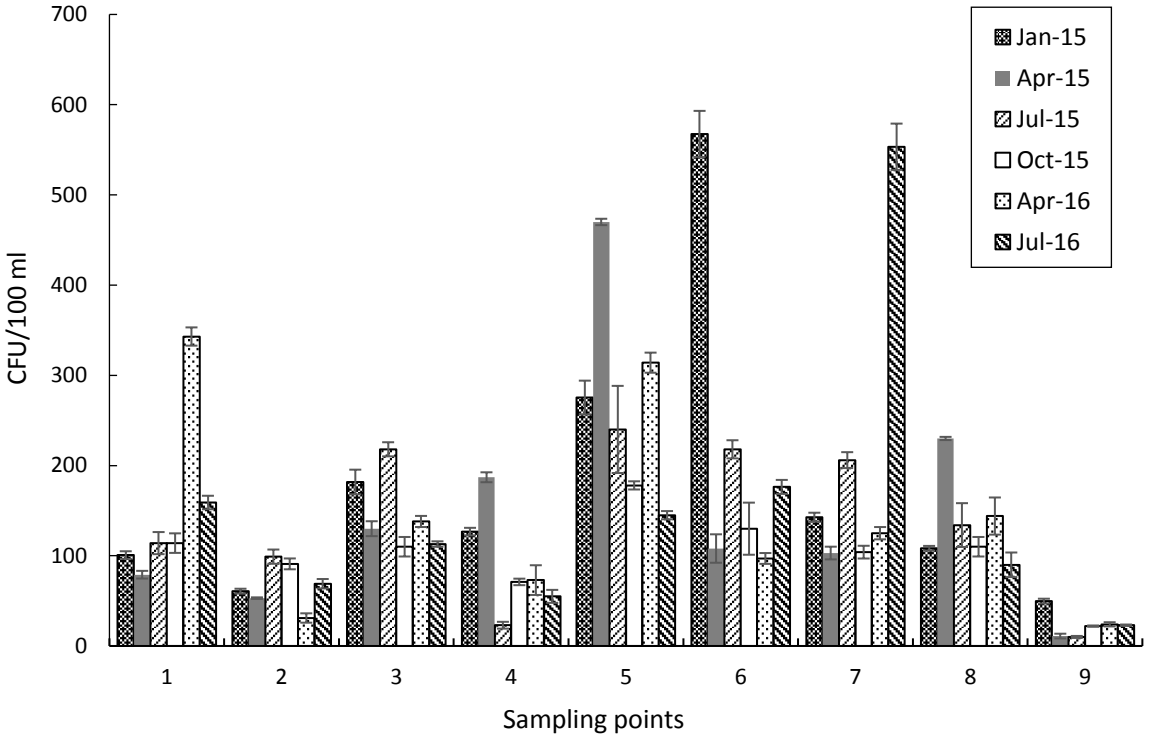


Figure 5.2 Total HPC bacteria in nine sampling points over an 18-month period. Sampling points indicate different areas across the city of London, as shown in Figure 5.1.

Table 5.1. Spearman's rank correlation coefficient between heterotrophic bacteria and water quality parameters

Water quality	**HPC (CFU/100ml)**
pH	**0.383***
Residual chlorine (mg/L)	**-0.569***
TOC (mg/L)	**0.353***
Temperature (°C)	0.019

Bold indicates the relationships are significant statistically
$0.01 \leq p \leq 0.05$
** $0.001 \leq p \leq 0.01$*

Tap water samples during the sampling period April to July 2016 were analysed using quantitative polymerase chain reaction (qPCR). **Figure 5.3** shows the number of universal gene 16S rRNA and HPC bacteria measured in the water samples. The copy numbers of 16S rRNA were in the range of $2.2x10^2$ to $2x10^7$ copies/100 ml water. The value is higher by 2- to 4-log compared to the HPC numbers measured. This suggests that cultivable bacteria in tap water only account for a small percentage of the total bacteria biomass. Studies have shown that only 5-10% of total bacteria in wastewater and soil can cultivated and identified using culture-based method (Hiraishi, 1988, Xi *et al.*, 2009). Furthermore, many bacterial species in drinking water endure in a viable but non-culturable (VBNC) state (Byrd *et al.*, 1991). On the other hand, the molecular-based method that was used cannot distinguish between viable or dead cells and so would overestimate the viable microbial content of water samples if used on its own. Therefore, the combination of culture-based and molecular-based analysis is more suitable to determine the total biomass in drinking water.

The average copy numbers of 16S rRNA in the April sampling period were significantly higher by 1.7-log than in the July sampling time ($p<0.05$). On the other hand, the total HPCs numbers between these sampling times were similar (i.e. log-2 CFU/100ml).

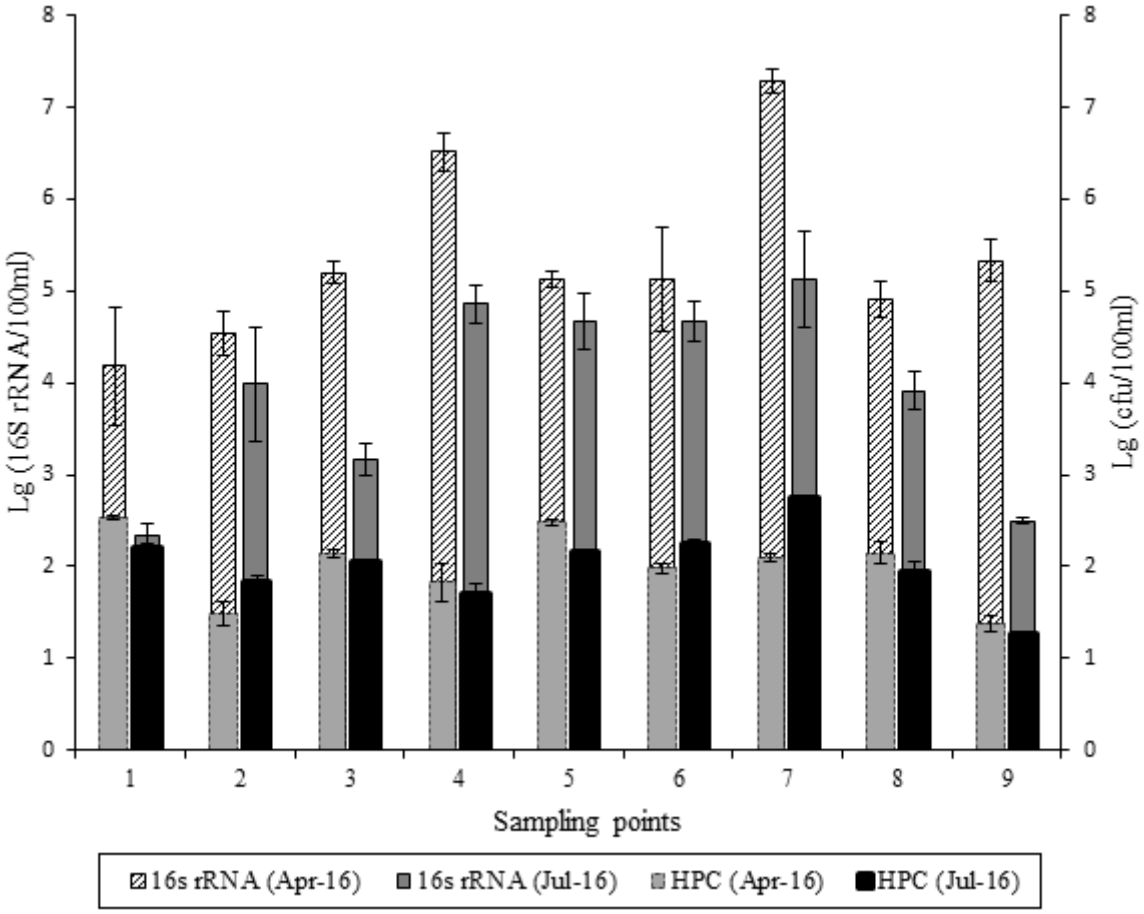


Figure 5.3 Copy numbers of the 16S rRNA in 100ml water samples from April and July 2016 sampling times compared to the total number of HPC bacteria. Sampling locations are shown in Figure 5.1.

5.1.4 Antibiotic-resistant bacteria in the water samples

Figure 5.4 illustrates the percentage of bacteria in the sampled tap waters in London that were found to be antibiotic resistant. The percentage of resistant bacteria was calculated from the number of each ARB divided by the total HPC. The resistance fluctuated considerably between the sampling rounds, from January 2015 to July 2016. There were significant differences in erythromycin, amoxicillin, ciprofloxacin, tetracycline, and trimethoprim resistance across the sampling locations. The percentage of resistant bacteria tended to be higher in colder months, i.e. during the December to April sampling period, however the number were not statistically different.

Vancomycin-resistant bacteria were detected in all locations and sampling rounds. The average ranged from 16% to 24% throughout the sampling period. The highest resistant was found in the October 2016 sampling time with an average of 27%, whilst the January sampling round observed the lowest resistant

number, with an average of 16%. In general, vancomycin resistance was found to be slightly higher during the warmer months, i.e. from July to October. Resistance to vancomycin is due to the presence of operons that encode the enzymes responsible for modifying the vancomycin-binding target (Arthur *et al.*, 1996). Caplin *et al.* (2008) reported the occurrence and diversity of vancomycin-resistant enterococci; 71% of the bacteria recovered from an urban wastewater in Brighton UK, were resistant to vancomycin.

Erythromycin belongs to the macrolides antibiotic group, which has been used widely to treat primary respiratory disease caused by Gram-positive and fastidious Gram-negative pathogens. Erythromycin-resistant bacteria were also detected in all sampling points and sampling rounds. In this study, the bacteria resistant to erythromycin fluctuated considerably during the six sampling rounds, with the values ranging from 12% to 35% and the difference between sampling times (i.e. July 2015 vs July 2016 and April 2015 vs April 2016) was found to be statistically significant ($p<0.0001$). The lowest resistance was found at sampling point 9, with an average of 13% throughout the study and the highest resistance was observed at sampling point 6 with an average of 38%. Antibiotic removal efficiencies by wastewater treatment are less effective for macrolides, which are relatively persistent in water treatment (Gulkowska *et al.*, 2008). According to Public Health England, macrolide antibiotics were among the most frequently prescribed in England in 2015 (Public Health England, 2015).

Amoxicillin-resistant bacteria were detected in all sampling locations and the percentage of resistant bacteria across the sampling locations and sampling times varied between 8% and 43%. There was no significant difference between sampling times, with the average resistance being 25%, 23%, 26%, 14%, 22% and 18% for January 2015, April 2015, July 2015, October 2015, April 2016 and July 2016 sampling times respectively. The occurrences of amoxicillin resistance have been previously reported in drinking water distribution networks (Xi *et al.*, 2009), tap water (Vaz-Moreira *et al.*, 2012; Khan *et al.*, 2016) and even in bottled mineral water (Falcone-dias *et al.*, 2011).

Ciprofloxacin- and tetracycline-resistant bacteria were the lowest in all sampling points, ranging from 1.5% to 14%. The trend was similar to that reported by several studies that also stated relatively low tetracycline- and ciprofloxacin-resistant bacteria in drinking water samples, with Xi *et al.* (2009)

reporting 10%-13% of bacteria being ciprofloxacin resistant and 0.04%-3.78% of bacteria being tetracycline resistant in tap water samples in Michigan, USA. The low level of evidence for ciprofloxacin- and tetracycline-resistant bacteria in tap water samples might be due to the fact that wastewater treatment can eliminate nearly 80% of fluoroquinolones and tetracycline, and they are also susceptible to photo-degradation (Sukul and Spitteler, 2007; Gulkowska *et al.*, 2008).

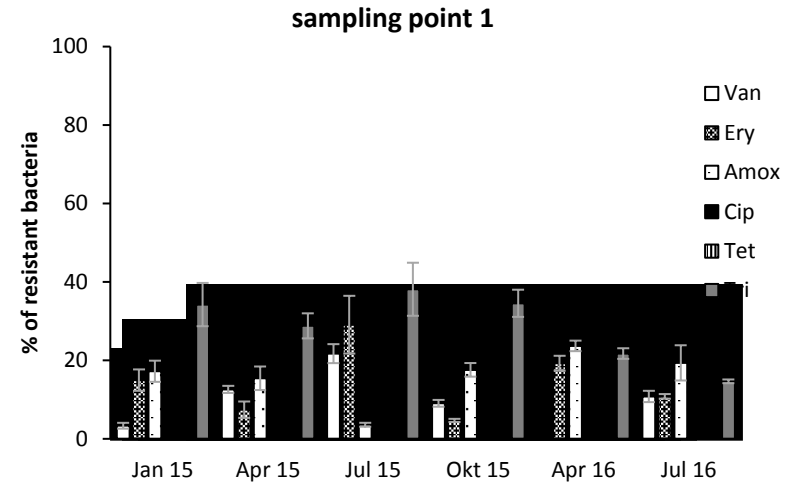
The percentage of trimethoprim-resistant bacteria was in the range of 30% to 47%, higher than the other antibiotics. The highest resistant was observed in the October 2016 sampling period, with an average of 47% and the lowest resistance was found in the July 2016 sampling round with an average of 30%. In terms of sampling location, trimethoprim resistance was found to be consistently lower at sampling point 9 and higher at sampling point 5. Resistance to trimethoprim may be either intrinsic or acquired by horizontal acquisition via plasmid or conjugation (Eliopoulos and Huovinen, 2001). The occurrences of trimethoprim resistance in drinking water and drinking water distribution systems have been previously reported (Shi *et al.*, 2013; Ribeiro *et al.*, 2014).

Amoxicillin and trimethoprim were observed to be the most prescribed antibiotics in England, with an average of 11,872 and 3,784 items dispensed per month in 2015 (Public Health England, 2015). However, despite the high consumption of amoxicillin and trimethoprim, studies have also found that the removal rate of these two antibiotics in drinking water treatment plants can be higher than 95% (Utrilla *et al.,* 2013). The high resistance of trimethoprim observed here is most likely due to the single use of the drug in this study – trimethoprim is known to have a synergistic effect when applied together with sulfamethoxazole. Trimethoprim is usually used in the treatment of urinary tract infections and in combination with other kinds of antibiotics to treat certain types of pneumonia (Huovinen *et al.*, 1995).

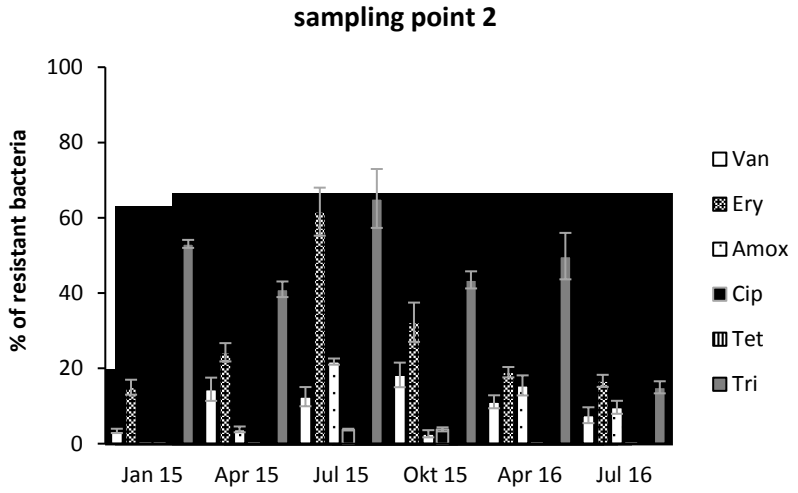
In terms of temporal variations from year to year, amoxicillin, vancomycin, ciprofloxacin, tetracycline and trimethoprim resistances were higher in July 2015 compared to July 2016. The prevalence of trimethoprim- and erythromycin-resistant bacteria in July 2015 was statistically higher by 12% and 21% respectively than at the July 2016 sampling time. Additionally, only erythromycin-resistant bacteria were present in significantly higher numbers between the April 2015 and April 2016 sampling times.

The resistance patterns of the other tested antibiotics did not vary significantly between the same sampling times in different years, i.e. April 2015 versus April 2016 or July 2015 versus July 2016.

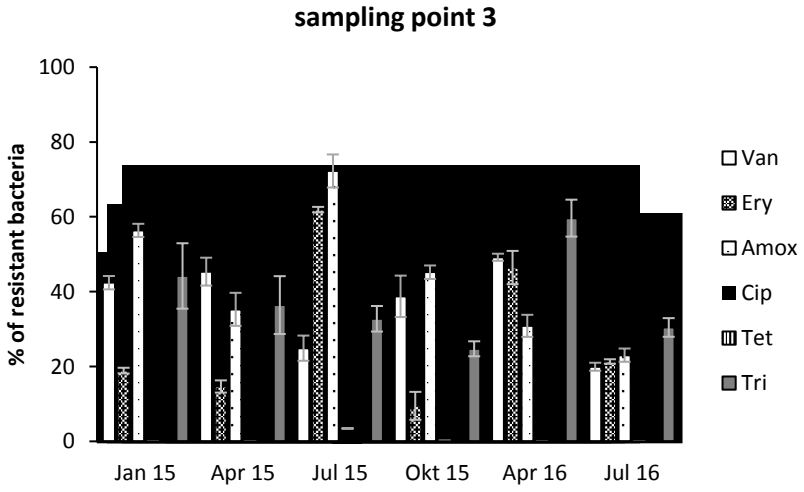
Mohanta and Goel (2014) previously reported that the occurrences of multiple-antibiotic-resistant bacteria in two rivers in India were higher post-monsoon, followed by winter then summer. Furthermore, Cauicci *et al.* (2016) observed higher antibiotic resistance genes abundance in wastewater treatment plant in autumn and winter than other seasons, which coincided with the higher rate of overall antibiotic prescriptions observed in their study. Nonetheless, no studies have reported the seasonal effect on antibiotic resistance in drinking water system. Seasonal variations in antibiotic prescription levels have been observed previously (Public Health England, 2016), with increased consumption of antibiotics in colder months due to seasonal illness and higher antibiotic misuses (e.g. treating flu with antibiotics). Here, however, no relationship between season and prevalence of antibiotic resistant bacteria was found in drinking water.


sampling point 1
% of resistant bacteria
100
80
60
40
20
0
Jan 15
Apr 15
Jul 15
Okt 15
Apr 16
Jul 16
Van
Ery
Amox
Cip
Tet

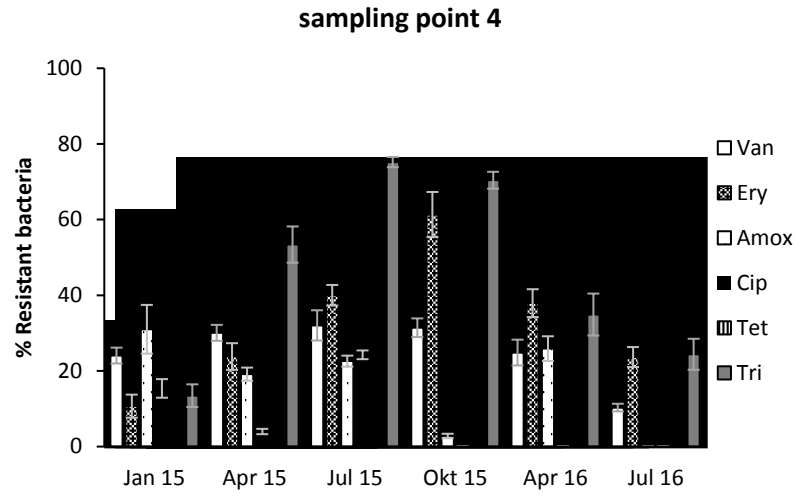
(a)


sampling point 2
% of resistant bacteria
100
80
60
40
20
0
Jan 15
Apr 15
Jul 15
Okt 15
Apr 16
Jul 16
Van
Ery
Amox
Cip
Tet
Tri

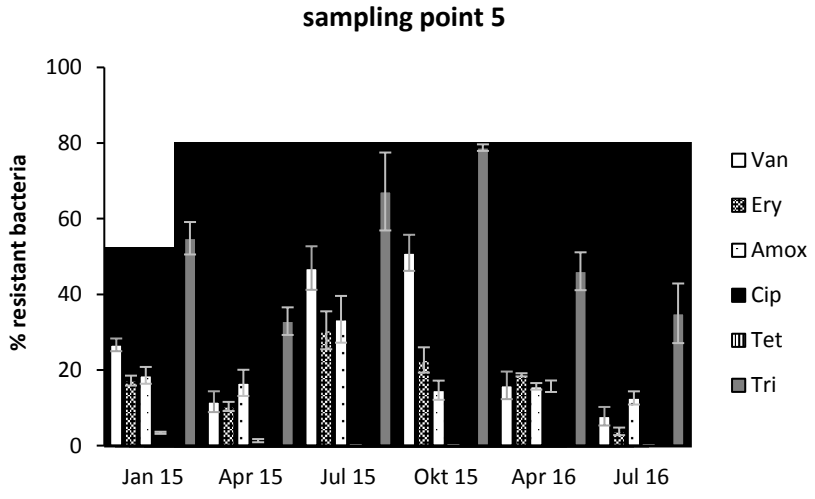
(b)


sampling point 3
% of resistant bacteria
100
80
60
40
20
0
Jan 15
Apr 15
Jul 15
Okt 15
Apr 16
Jul 16
Van
Ery
Amox
Cip
Tet
Tri

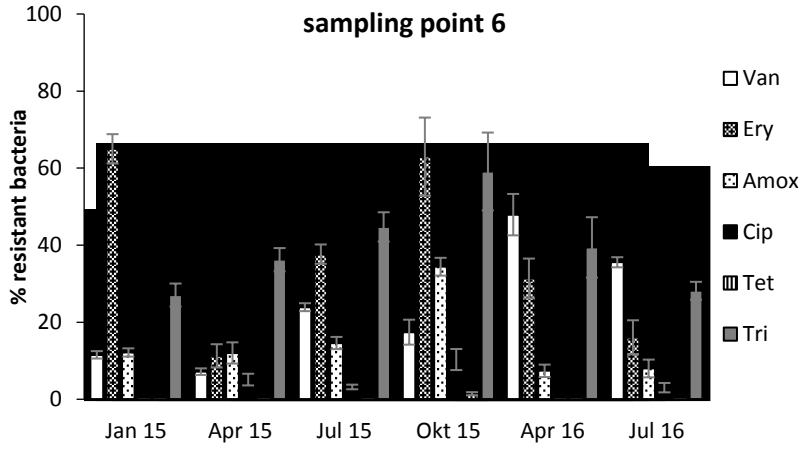
(c)


sampling point 4
% Resistant bacteria
100
80
60
40
20
0
Jan 15
Apr 15
Jul 15
Okt 15
Apr 16
Jul 16
Van
Ery
Amox
Cip
Tet
Tri

(d)


sampling point 5
% resistant bacteria
100
80
60
40
20
0
Jan 15
Apr 15
Jul 15
Okt 15
Apr 16
Jul 16
Van
Ery
Amox
Cip
Tet
Tri

(e)


sampling point 6
% resistant bacteria
100
80
60
40
20
0
Jan 15
Apr 15
Jul 15
Okt 15
Apr 16
Jul 16
Van
Ery
Amox
Cip
Tet
Tri

(f)

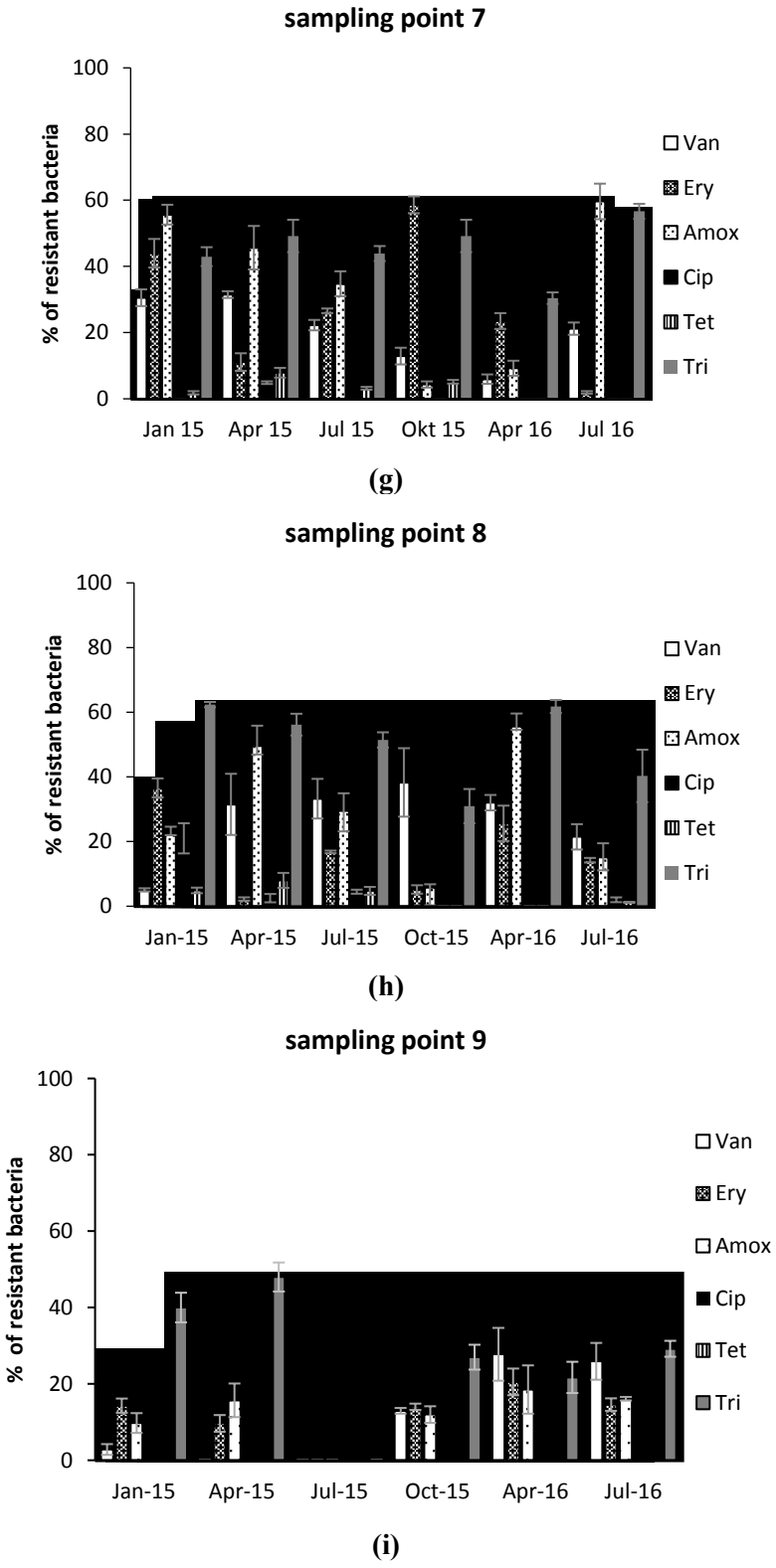


Figure 5.4: Percentage of bacteria found in London tap water samples that were antibiotic resistant. Data are from nine sampling points, six sampling times, and four replicates per sampling time. Sampling points indicate different areas across London, as shown in Figure 5.1.

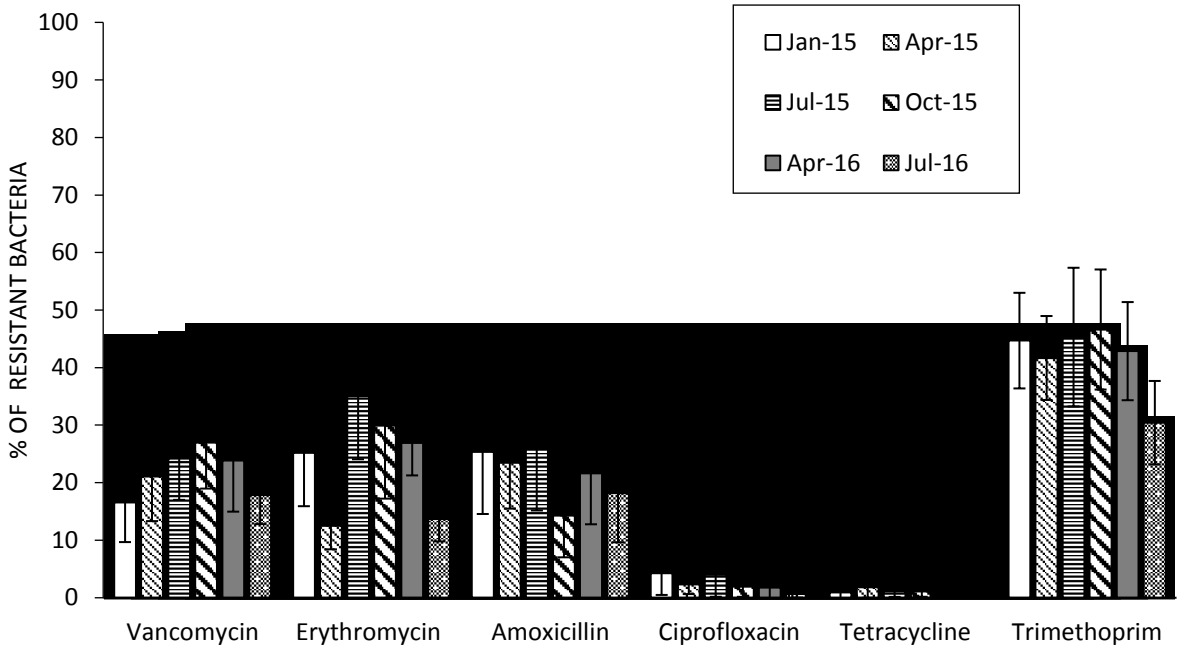


Figure 5.5: Summary of resistant bacteria found in London tap water samples across the sampling periods. Data are from nine sampling points, six sampling times, and four replicates per sampling time. Sampling points indicate different areas across London, as shown in Figure 5.1.

Spearman's rank correlation analysis was conducted to determine the correlations between the prevalence of ARBs and drinking water quality parameters. The relationship between certain water qualities and antibiotic resistant bacteria have been reported by several studies such as, water depth, temperature, river flow, precipitation, ammonium, total dissolved solid and dissolve oxygen (Maal-Bared *et al.* 2013; Middleton and Salierno, 2013; Staley *et al.* 2015). As shown in **Table 5.2**, the results demonstrated that water quality parameters including pH and temperature were not correlated with antibiotic resistance rates, while residual chlorine and TOC demonstrated significant correlation with tetracycline ($p<0.05$) and vancomycin ($p<0.01$) resistance, respectively. Blanco *et al.* (2009) observed that the addition of nutrients might enhance the horizontal transfer of genetic resistance elements. In their study, *E. coli* isolates carrying genetic resistance elements had much stronger viability than sensitive isolates under high nutrient concentrations, which led to increasing resistance. In the present study, few statisitically significant correlations were observed between the resistance presence and any of the measured water quality parameters. This may be partly explained due to the small variation apparent in some of the water quality parameters measured between sampling rounds and locations, e.g. chlorine residual was fairly constant. Nonetheless, the significant negative correlation with TOC

suggested that susceptible heterotrophic bacteria may outcompete resistant bacteria; in other words, resistant bacteria may require more nutritional resources to survive compared to non-resistant bacteria. It has been suggested that chlorination and further residual chlorine in drinking water might enhance the abundance of class I integrons and the diversity of gene cassettes which contain various ARGs, including tet genes (Krige, 2009; Shi *et al.,* 2013). This might explain the positive significant correlation between residual chlorine and *tet*-A gene observed in the current study.

Table 5.2. Spearman's rank correlation coefficient between antibiotic resistance rate and water quality parameters.

Water quality	**Resistant to antibiotics**					
	VAN	ERY	AMOX	CIP	TET	TRI
pH	-0.189	0.027	0.043	0.009	0.131	0.008
Residual chlorine (mg/L)	0.094	0.209	-0.034	0.202	**0.325***	0.263
TOC (mg/L)	**-0.391****	-0.073	-0.240	-0.090	-0.188	-0.190
Temperature (°C)	0.144	0.014	-0.108	0.217	0.011	0.100

Bold indicates the relationships are significant statistically
**0.01≤ p ≤ 0.05*
*** 0.001≤ p ≤ 0.01*

Pearson's analysis was conducted to assess the association between antibiotic resistances in the tap water samples, as shown in **Table 5.4**. The results indicated that resistance to vancomycin showed an association with resistance to amoxicillin and ciprofloxacin (*0.001 ≤ p ≤ 0.01*). There was a strong correlation between erythromycin and trimethoprim resistance (0*.001 ≤ p ≤ 0.01*), whereas a weak correlation was observed between amoxicillin and tetracycline resistance (*0.01≤ p≤ 0.05).* These correlations may be the result of cross-selection and or co-selection (Dang *et al.* 2006) despite the antibiotics used in this study belonging to different classes, e.g. tetracycline, macrolide, beta-lactams, quinolone and glycopeptide. Significant correlations between antibiotics have been previously reported, and the correlations between tetracycline, chlortetracycline, and quinolone resistance have been reported the most (Maal-Bared *et al.* 2013; Zhang *et al.* 2014). However, in this study, no correlation between tetracycline and ciprofloxacin was observed, regardless of the fact that tetracycline efflux

proteins have similar amino acid with other efflux protein including quinolone (Chopra and Roberts, 2001).

Table 5.3. Association between antibiotic resistances in tap water samples

	Antibiotics					
	VAN	ERY	AMOX	CIP	TET	TRI
VAN	1					
ERY	0.161	1				
AMOX	**0.415****	0.083	1			
CIP	-0.07	0.095	0.017	1		
TET	0.143	0.006	**0.280***	0.147	1	
TRI	**0.400****	**0.524****	0.226	0.227	0.248	1

*The level of significance was assessed by Pearson's test: -, $p \geq 0.05$; *, $0.01 \leq p \leq 0.05$; ** $0.001 \leq p \leq 0.01$*

5.1.5 Antibiotic resistance genes in the water samples

Antibiotic resistance genes and total bacteria genomes from two sampling periods (April and July 2016) were detected and quantified using real-time qPCR. The copy number of each ARG in 100 ml water was normalised to the copy number of the total 16S rRNA gene to obtain the relative abundance of the resistance genes. **Table 5.5** shows the occurrence of ARGs during the sampling periods of April and July 2016. **Figures 5.6** and **5.7** summarise the proportion of ARGs and 16S rRNA genes in the 100 ml water sample and the copy number of each resistance gene in 100 ml water samples in the April and July 2016 sampling times, respectively.

At the April 2016 sampling time, all ARGs tested were detected in all sampling points, except for *mph*(A) and *bla*-$_{TEM1}$ genes, which were not detected in sampling points 4 and 7, respectively. At the July sampling time, all the tested genes were only detected in sampling points 1, 8 and 9, with *sul*1 and *dfr*A7 genes being found at all sampling times. However, in sampling points 2, 3, 4, 6 and 7 only *dfr*A7 and *sul*1 genes were detected. In general, the copy numbers of the resistance genes were lower at the July 2016 sampling time than at the April 2016 sampling time and they were significantly different for all studied genes (p<0.05). Meanwhile, the relative abundance of *bla*-$_{TEM1}$, *dfr*A7 and *mph*(A) genes was

significantly higher at the April sampling time. **Table 5.4** summarises the occurrence of the tested ARGs in the nine sampling points during the April and July sampling times.

Table 5.4. The occurrences of tested ARGs during two sampling periods. Sampling points indicate different area across London, as shown in Figure 5.1.

Sampling points	Resistance genes									
	***tet*(A)**		***bla*-$_{TEM1}$**		***dfr*A7**		***mph*(A)**		***sul*1**	
	Apr	**Jul**	**Apr**	**Jul**	**Apr**	**Jul**	**Apr**	**Jul**	**Apr**	**Jul**
1	+	+	+	+	+	+	+	+	+	+
2	+	-	+	-	+	+	+	-	+	+
3	+	-	+	-	+	+	+	-	+	+
4	+	+	+	-	+	+	-	-	+	+
5	+	+	+	-	+	+	+	+	+	+
6	+	+	+	-	+	+	+	-	+	+
7	+	+	-	-	+	+	+	-	+	+
8	+	+	+	+	+	+	+	+	+	+
9	+	+	+	+	+	+	+	+	+	+

The occurrences of the *sul*1 gene were relatively high, as the gene was detected in all sampling points both in April and July. The relative abundance of the *sul*1 gene ranged from 1.44-log to 4.67-log in the April period and 1.11-log to 5.66-log in the July period. Meanwhile, the copy number of the gene ranged from 0.3-log to 5.7-log per 100 ml of water sample.

The highest relative abundance of the *sul*1 gene was observed at location 5 both in April and July, with an average of 1.24-log. The lowest abundance of *sul*1 in April sampling was observed at sampling point 9, with an average of 4.67-log. However, in the July sampling time, the abundance was significantly increased to 1.75-log. In terms of the copy number, the highest was found at sampling point 8 with an average of 3.5-log and 1.3-log for April and July sampling times, respectively. The possible reason for the high abundance of the s*ul*1 gene is that the gene is located in a mobile genetic element, known as the class I integron, making it possible to transfer the gene between bacteria. Previous studies have also shown the high presence of the *sul*1 gene in drinking water samples (Shi *et al.,* 2013; Adesoji *et al.,* 2016).

The second most frequently detected gene in this study was *dfr*A7, which was found at all sampling locations during both the April and July sampling times. The copy number of the gene ranged from 0.75-log to 2.31-log in the April sampling round and 1.15-log to 1.95-log in the July sampling period. The copy number of the gene was found to be higher by 0.73-log in the April sampling time and the difference was statistically significant ($p<0.05$). The highest relative abundance of *dfr*A7 genes was observed at sampling point 7, for both April and July sampling times. Meanwhile, the highest gene copy number was detected at sampling point 2, with an average of 2.31-log and 2.73-log for the April and July sampling times, respectively. *dfr* genes encode the modification of the target enzyme dihydrofolate reductase (dfr), which is responsible for most of trimethoprim resistance. Adesouji *et al.* (2016) detected *dfr*A15, *dfr*7, and *dfr*A1 resistance genes in drinking water in southwestern Nigeria. Most of the *dfr*A7 gene is located in the integron cassette, which can be transferred horizontally (Blahna *et al.*, 2006). Studies have also proposed that trimethoprim resistance genes can be associated with other resistance determinants, such as sulfamethoxazole.

Tet(A) gene, which encodes resistance towards tetracycline, was detected in all sampling locations during the April period and in seven locations during the July sampling time. The gene was not detected at sampling points 2 and 3 in the July sampling time. *Tet*(A) gene copy numbers ranged from 0.86- to 2.54-log in April and 1.26-log to 0.43-log in the July sampling time. The relative abundance of the gene was found to be the highest at sampling point 7 for both April and July sampling times and the lowest at sampling point 1. The *tet*(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E), *tet*(G), *tet*(H), *tet*(I), *tet*(J), *tet*(Y), *t*et(30), and *tet*(31) genes are found exclusively in Gram-negative genera (Chopra & Roberts, 2001). The majority of *tet* genes are associated with mobile gene elements, which may explain the wide distribution of *tet* (A) genes in this study.

Bla-$_{\text{TEM1}}$ is the most common gene coding β-lactamases and extended spectrum beta-lactamase, which is responsible for resistance towards β-lactam antibiotics by hydrolysing the β-lactam ring. In this study, the *bla*-$_{\text{TEM1}}$ gene was detected in eight of the nine locations during the April sampling period. In the July sampling time, the occurrence of the gene was considerably less frequent, as it was only detected in two locations. The *bla*-$_{\text{TEM1}}$ gene was found in the range of 1.04-log to 2.07-log gene copy numbers per

100 ml sample. The highest gene copy numbers were detected at sampling point 1 and 8, for the April and July sampling period, respectively. These results suggest that the spread of the *bla*-$_{TEM1}$ gene occurs in the water distribution system, due to the fact that the proportion of the gene was relatively higher in tap water compared to treated water. Several different β-lactamases have been identified and classified into different sub-groups based on their functional and structural characteristics. It has been reported that TEM-, SHV, and OXA- type β-lactamases are the most predominant (Skold, 2011). ARGs coding β-lactams, sulphonamides, aminoglycoside, tetracycline and quinolone were detected in a chlorinated drinking water system in China (Jia *et al.*, 2015), with the relative abundance of the *sul1* gene being the highest. It has been suggested that chlorine might enhance the expression of ARGs in drinking water by pumping out the disinfectant agent along with the antibiotic via the efflux pump (Xi *et al.*, 2009).

The *mph*(A) gene was found in eight of the nine locations, though the copy numbers of *mph*(A) were relatively lower than the other tested genes. The highest abundance of the gene was observed at sampling point 2 in the April sampling time; however, at the same location the gene was not detected in the July sampling time. Macrolide resistance is becoming more common, with several genes encoding its resistance, including *erm*(A), *erm*(B), *mph*(A), *mph*(B) and *mef*(A). The occurrence of the *mph*(A) gene in drinking water has not been reported, to the best of our knowledge. However, other types of macrolide resistance genes, for instance, *erm*(A) and *erm*(B), were previously detected in treated sewage water in Germany (Hess and Gallert, 2014), and a drinking water reservoir in Spain (Huerta *et al.*, 2013).

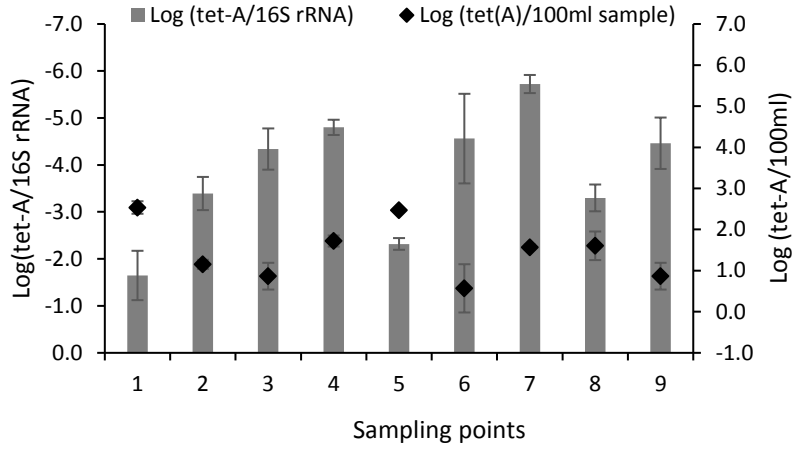
Table 5.5 shows the correlations between tested ARGs. There are significant correlations between *tet*(A) and *bla*-$_{TEM1}$; *tet*(A) and *dfr*A7; *tet*-A and *su1*1. A strong correlation was observed between *dfr*A7 and *mph*(A) genes ($0.01 \leq p \leq 0.05$). Correlation was also observed between *dfr*A7 and *mph*(A) ($0.01 \leq p \leq 0.05$); *dfr*A7 and *sul*1 ($p<0.05$). Resistance to different classes of antibiotics are often located on the same plasmid, giving selective advantage to the bacterial recipient when several antimicrobials are simultaneously administrated. Trimethoprim and sulphonamides resistance are often located on the incA/C plasmid, which also show a very broad host range, including *Pseudomonas* and Enterobacteriaceae (Colinon *et al.* 2007). It has been reported that the *tet*(A) gene in *Salmonella* isolates

is located in the same mobile genetic element as the *bla*-TEM1 and *sul*1 genes (Lugo-Melchor *et al.,* 2010). Interestingly, there was no correlation observed between the *sul*1 gene and *bla*-TEM1 gene, although they are normally situated in the same mobile element, in an integron of the Tn21 type.

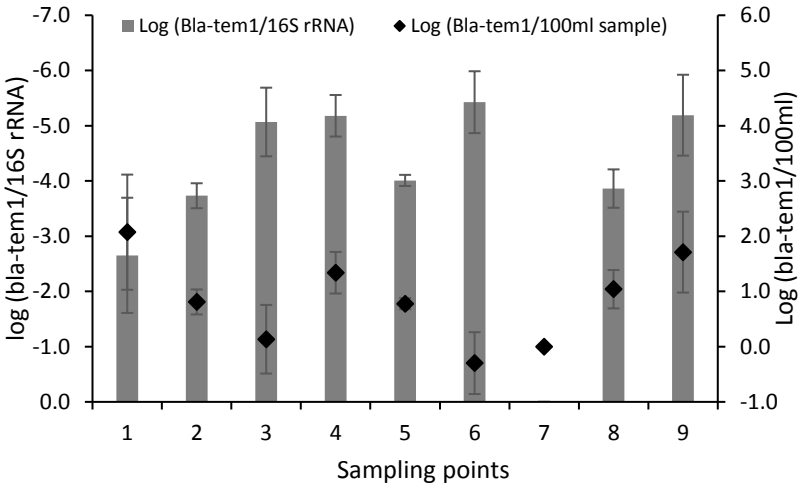
Table 5.5. Correlations between the presence of the studied antibiotic resistance genes in water samples

	***Tet(*A)**	***Bla*-TEM1**	***Dfr*A7**	***Mph*(A)**	***Sul*1**
*Tet(*A)	1				
Bla-TEM1	**0.537****	1			
*Dfr*A7	**0.404****	0.202	1		
Mph(A)	0.218	**0.289***	**0.353****	1	
*Sul*1	**0.676****	0.185	**0.281***	-0.004	1

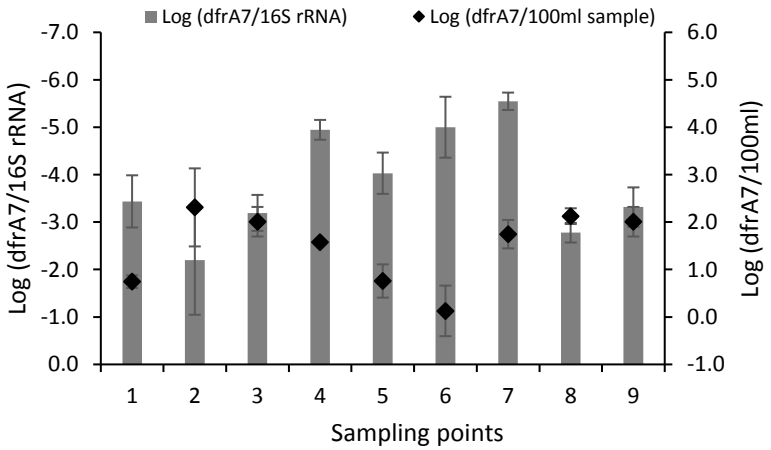
*The level of significance was assessed by Pearson's test: -, $p \geq 0.05$; *, $0.01 \leq p \leq 0.05$; ** $0.001 \leq p \leq 0.01$*


Log (tet-A/16S rRNA)
Log (tet(A)/100ml sample)
Log(tet-A/16S rRNA)
Log (tet-A/100ml)
-7.0
-6.0
-5.0
-4.0
-3.0
-2.0
-1.0
0.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
-1.0
1
2
3
4
5
6
7
8
9
Sampling points

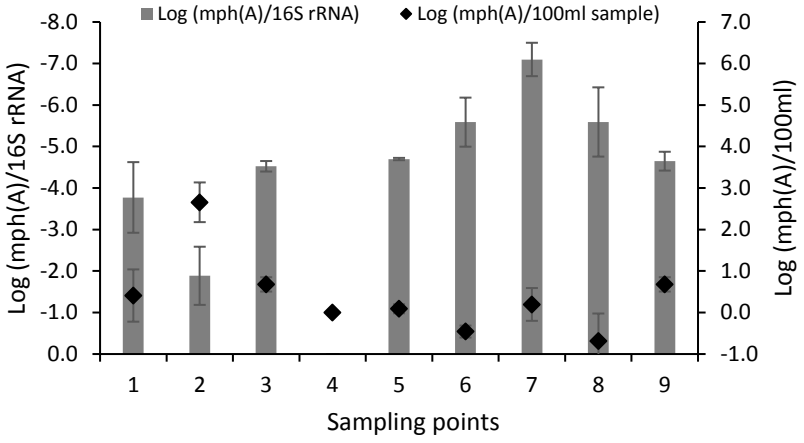
(a)


Log (Bla-tem1/16S rRNA)
Log (Bla-tem1/100ml sample)
log (bla-tem1/16S rRNA)
Log (bla-tem1/100ml)
-7.0
-6.0
-5.0
-4.0
-3.0
-2.0
-1.0
0.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
-1.0
1
2
3
4
5
6
7
8
9
Sampling points

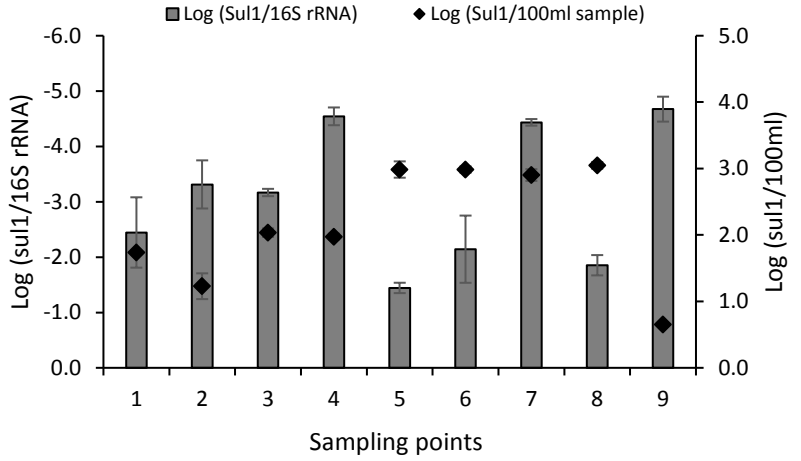
(b)


Log (dfrA7/16S rRNA)
Log (dfrA7/100ml sample)
Log (dfrA7/16S rRNA)
Log (dfrA7/100ml)
-7.0
-6.0
-5.0
-4.0
-3.0
-2.0
-1.0
0.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
-1.0
1
2
3
4
5
6
7
8
9
Sampling points

(c)

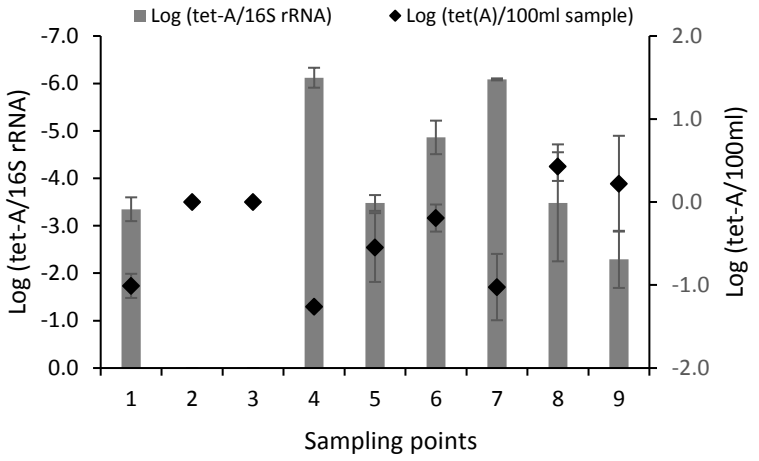


(d)

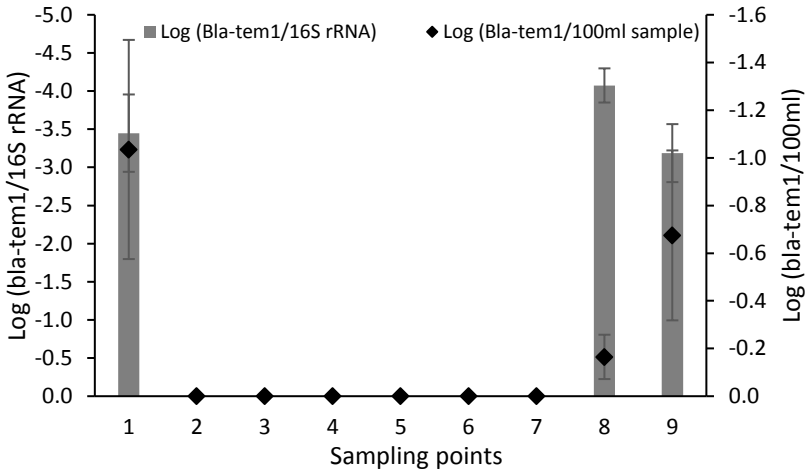


(e)

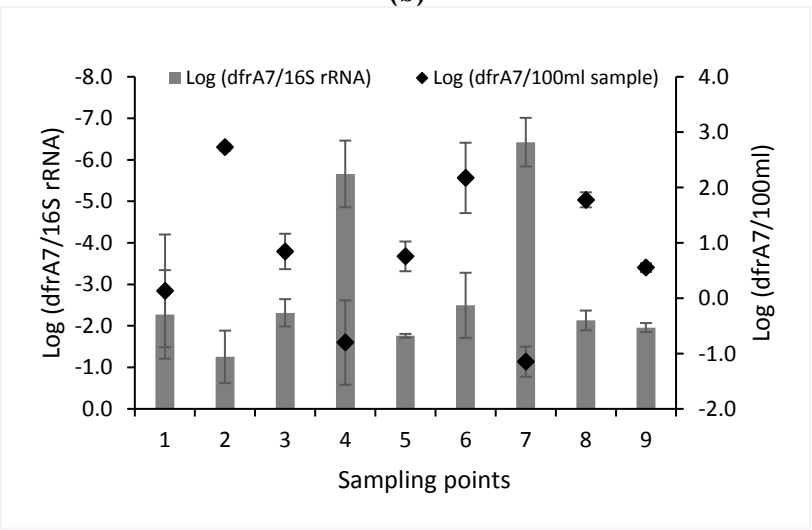
Figure 5.6: Quantities of antibiotic resistance genes (a) *tet*(A) (b) *bla*-$_{\text{TEM1}}$ (c) *dfr*A7 (d) *mph*(A) and (e) *sul*1 from nine London tap water sample locations in the April 2016 sampling time. The data represent the copy number of the genes normalised to 16S rRNA and the copy number of resistance genes in a 100 ml water sample. Sampling points indicate different areas across London, as shown in Figure 5.1.


Log (tet-A/16S rRNA)
Log (tet(A)/100ml sample)
Log (tet-A/16S rRNA)
Log (tet-A/100ml)
-7.0
-6.0
-5.0
-4.0
-3.0
-2.0
-1.0
0.0
2.0
1.0
0.0
-1.0
-2.0
1 2 3 4 5 6 7 8 9
Sampling points

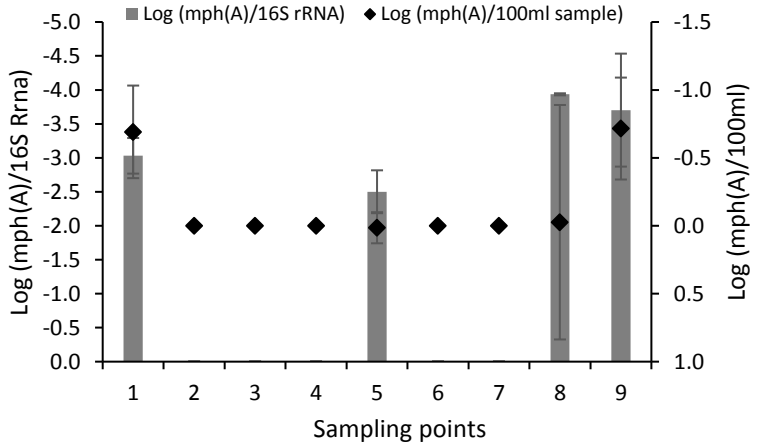
(a)


Log (Bla-tem1/16S rRNA)
Log (Bla-tem1/100ml sample)
Log (bla-tem1/16S rRNA)
Log (bla-tem1/100ml)
-5.0
-4.5
-4.0
-3.5
-3.0
-2.5
-2.0
-1.5
-1.0
-0.5
0.0
-1.6
-1.4
-1.2
-1.0
-0.8
-0.6
-0.4
-0.2
0.0
1 2 3 4 5 6 7 8 9
Sampling points

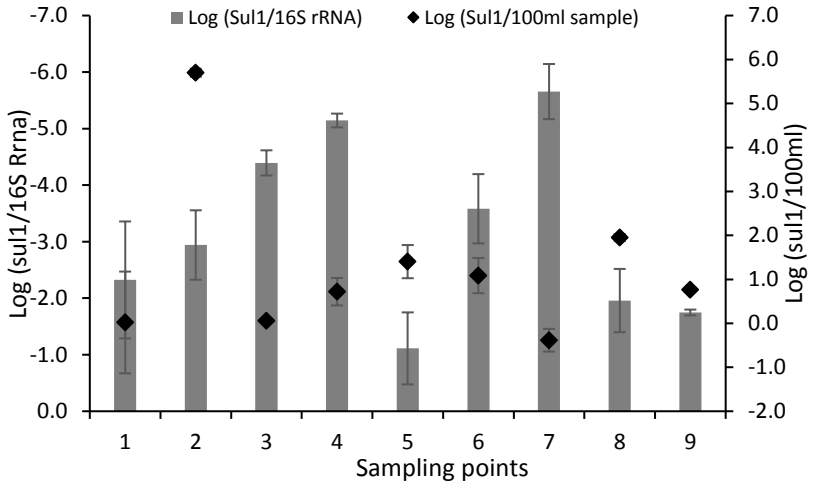
(b)


Log (dfrA7/16S rRNA)
Log (dfrA7/100ml sample)
Log (dfrA7/16S rRNA)
Log (dfrA7/100ml)
-8.0
-7.0
-6.0
-5.0
-4.0
-3.0
-2.0
-1.0
0.0
4.0
3.0
2.0
1.0
0.0
-1.0
-2.0
1 2 3 4 5 6 7 8 9
Sampling points

(c)



(d)



(e)

Figure 5.7: Quantities of antibiotic resistance genes (a) *tet*(A) (b) *bla*-$_{TEM1}$ (c) *dfr*A7 (d) *mph*(A) and (e) *sul*1 from nine London tap water sample locations in the July 2016 sampling round. The data represent the copy number of the genes normalised to 16S rRNA and the copy number of resistance genes in a 100 ml water sample. Sampling points indicate different areas across London, as shown in Figure 5.1.

5.1.6 Identification of antibiotic-resistant HPC by API 20NE identification system

Tables 5.6 to **5.10** show the identification results of antibiotic-resistant HPC bacteria from selected sampling points. The data presented here are characterised as ≥ 90% identification or very good identification – data with less than 90% identification were not included. In total, 64 antibiotic-resistant colonies were identified as very good to excellent identification. The API 20NE identification system consists of a microtube containing dehydrated substrates to detect enzymatic activity or assimilation of sugars by the inoculated organisms. The generated profiles were then compared against the API 20NE online database system. There are seven major genera identified from the tap water sample, including *Aeromonas, Burkholderia*, *Delftia*, *Ochrobactrum Pseudomonas*, *Rhizobium*, *Sphingomonas,* and *Vibrio*.

The dominant antibiotic-resistant colonies were identified as *Pseudomonas spp* – they were found in all sampling points. Five Pseudomonas species were detected, including *P. flourescens*, *P. luteola*, *P. alcaligenes*, *P. putida* and *P. aeruginosa*. The occurrence of *P. flourescens* was more frequent than other Pseudomonas species, being detected 16 times during all of the sampling times. *Pseudomonas flourescens* was found in seven out of nine locations; it was not found at sampling points 4 and 9. The second highest occurrence was *P. alcaligenes*, which was detected four times throughout the sampling period. Meanwhile, *P. putida* and *P. luteola* were identified three and two times, respectively. *Pseudomonas aeruginosa* was found at sampling points 1, 3 and 4.

Pseudomonas spp. resistant to trimethoprim were found in all sampling points, except at sampling point 9, with *P. flourescens* being the most frequently detected. In terms of occurrence per sampling time, trimethoprim-resistant *P. flourescens* was consistently found at sampling points 5 and 1 during the April 2015, July 2015, and April 2016 sampling times. Amoxicillin-resistant *Pseudomonas* was identified in all sampling times, with *P. flourescens* again being the most frequent. Interestingly, amoxicillin-resistant *P. aeruginosa* was found at sampling points 1 and 3 during the October 2015 and July 2016 sample times. *P. aeruginosa* is considered to be one of the most important opportunistic pathogens and therefore the presence of the bacterium in drinking water is rare. *Pseudomonas spp*. resistant to erythromycin was detected in seven locations, except at sampling points 1 and 6. Again, resistant *P.*

aeruginosa was found at sampling points 3 and 4. No ciprofloxacin- or tetracycline-resistant *Pseudomonas spp*. were identified in the entire study. *P. aeruginosa* is notorious for its resistance to many antibiotics, due to the permeability barrier supported by its outer membrane lipopolysaccharides.

Given the metabolic versatility of *Pseudomonads* and their ability to survive different forms of stress, their presence in treated water, including drinking water, is not unexpected. Some studies have reported that antibiotic-resistant *Pseudomonas* species are more resistant to chlorination than other species and might possess selection for chlorine (Shrivastava *et al.,* 2004). Furthermore, increases in the abundance of antibiotic-resistant *Pseudomonas* and *Sphingomonas* have been observed after chlorination (Jia *et al.*, 2015). Several species of *Pseudomonas spp.* also have a tendency to colonise the distribution pipe surface in a biofilm form, making the cells impermeable to any disinfectant agents. Moreover, multi-resistant *P. aeruginosa* were frequently found in hospital wastewaters and in low abundances in wastewater from housing areas, indicating hospitals as one hotspot for the dissemination of antibiotic-resistant clinically-relevant bacteria (Schwartz, 2012).

Two *Aeromonads* species were detected in this study: *Aeromonas salmonicida* and *Aeromonas hydrophila*. These two species were found at sampling points 1, 2, 7, 8 and 9, with nine occurrences throughout the sampling periods. Amoxicillin-resistant *Aeromonas salmonicida* were found at sampling points 1, 2, 7 and 9. The highest occurrence was at sampling point 9, detected during the sampling periods of April and July 2015. Erythromycin-resistant *A. hydrophila* was only found at sampling point 7 during April 2016. The presence of *A. salmonicida* resistant to trimethoprim was detected at sampling locations 1, 8 and 9, with the occurrence at sampling point 9 being the most frequent (detected during April and July 2015 sampling times). Ciprofloxacin- and tetracycline-resistant *Aeromonas spp* was not found during the study. The presence of resistant *Aeromonas* in drinking water has been reported previously – Koksal *et al.* (2006) found 41% amoxicillin resistance among *Aeromonas* strains in the drinking water system in Istanbul, Turkey. Several species of *Aeromonads* are linked with gastroenteritis, muscle infection and skin disease (Igbinosa *et al.*, 2012).

Stenotrophomonas maltophilia resistant to amoxicillin were detected at two sampling points – 4 and 8. The bacteria were found in the April 2015 sampling time at location 8 and in April 2016 at location 4.

Stenotrophomonas maltophilia is an aerobic Gram-negative bacillus that is found in various aqueous environments. One of the important characteristics of this bacterium is the ability to form biofilm in water-associated environments; various studies have shown that *S. maltophilia* contaminate sinks, faucets and taps in hospitals (Cervia *et al.*, 2008) and in water treatment plants (Hoefel *et al.*, 2005). *Stenotrophomonas maltophilia* has emerged as an important opportunistic pathogen, particularly among hospitalised patients, causing pulmonary and bacteraemia infection (Brooke, 2012).

Another important opportunistic pathogen found in the London tap water samples was *Burkholderia cepacia.* It was detected at three locations and five times throughout the sampling time. Erythromycin-resistant *B. cepacia* was found at sampling point 5 at the April and July 2015 sampling times. At sampling point 1, amoxicillin- and tetracycline-resistant *B. cepacia* were found at the July 2015 and April 2016 sampling times, respectively. *Burkholderia cepacia* that is resistant to trimethoprim was detected only at sampling point 3 during the April 2015 sampling time.

Sphingomonas paucimobilis was identified at two locations, sampling points 1 and 2. Amoxicillin- and tetracycline-resistant *S. paucimobilis* was found at sampling point 1 during the July and October 2015 sampling times. Ciprofloxacin-resistant *S. paucimobilis* was identified at sampling point 2 in July 2015. The occurrence of trimethoprim-resistant *Ochrobactrum anthropi* was detected at sampling points 1, 3 and 8, while erythromycin-resistant *Delftia acidovorans* was found at sampling points 1, 4 and 7 at the April 2015 and July 2016 sampling times.

Non-fermenting Gram-negative bacilli, including *Acinetobacter baumannii*, *Burkholderia cepacia*, *Pseudomonas aeruginosa*, *Ralstonia pickettii* and *Stenotrophomonas maltophilia* are known as major opportunistic pathogenic bacteria. In this study, we identified *Pseudomonas aeruginosa*, *Burkholderia cepacia* and *Stenotrophomonas maltophilia.* The presence of these bacteria in drinking water systems have been previously reported; for instance, Zanetti *et al.* (2000) found *B. cepacia* in public drinking water in Italy. Nevertheless, studies focusing on antibiotic-resistant opportunistic pathogens in drinking water are still rare. Vaz-Moreira *et al.* (2012) found *Pseudomonas aeruginosa* that was resistant to fosfomycin, rifampicin, and cotrimoxazol in tap water. Similar to this study, they also observed no occurrence of *Pseudomonas spp.* resistant to ciprofloxacin and tetracycline.

In terms of the diversity of bacteria species that have been identified throughout the study, the largest number of ARBs were detected during the April sampling event. The number of bacteria identified was nine species, across all sampling times in 2015 and 2016, while six and seven species were identified in July 2015 and 2016, respectively. The risk to the general population of infections caused by heterotrophic plate bacteria is low (Rusin *et al.*, 1997). However, this study found a number of opportunistic pathogenic species of HPCs in tap water, which were also antibiotic resistant. This suggests that further purification of tap water before consumption by individuals considered having an elevated risk of opportunistic infections is important and that further research into methods for reducing the occurrence of antibiotic-resistant opportunistic pathogens in distribution system is warranted.

Table 5.6. Erythromycin-resistant HPC, sampling points indicate different area across London as shown in Figure 5.1. 'n/a' signifies that the identification results were < 80%.

Sampling Points	Sampling Time				
	April 2015	**July 2015**	**October 2015**	**April 2016**	**July 2016**
1	n/a	*Burkholderia cepacia*	n/a	n/a	*Delftia acidovorans*
2	n/a	n/a	n/a	n/a	*Pseudomonas flourescens*
3	n/a	n/a	n/a	*Pseudomonas flourescens*	*Pseudomonas aeruginosa*
4	*Delftia acidovorans*	n/a	n/a	*Pseudomonas aeruginosa*	n/a
5	*Burkholderia cepacia*	*Burkholderia cepacia*	n/a	n/a	n/a
6	n/a	n/a	n/a	n/a	n/a
7	*Delftia acidovorans*	n/a	n/a	*Aeromonas hydrophila*	n/a
8	*Pseudomonas flourescens*	n/a	n/a	n/a	*Pseudomonas flourescens*
9	*Pseudomonas luteola*	*Pseudomonas putida*	*Pseudomonas putida*	n/a	n/a

Table 5.7. Ciprofloxacin-resistant HPC, sampling points indicate different area across London as shown in Figure 5.1. 'n/a' signifies that the identification results were < 80%.

Sampling Points	Sampling Time				
	April 2015	**July 2015**	**October 2015**	**April 2016**	**July 2016**
1	n/a	n/a	n/a	n/a	n/a
2	n/a	*Sphingomonas paucimobilis*	n/a	n/a	n/a
3	n/a	n/a	n/a	n/a	n/a
4	n/a	n/a	n/a	n/a	n/a
5	n/a	n/a	n/a	n/a	n/a
6	n/a	n/a	n/a	n/a	n/a
7	n/a	n/a	n/a	n/a	n/a
8	n/a	n/a	n/a	n/a	*Vibrio metschnikovii*
9	n/a	n/a	n/a	n/a	n/a

Table 5.8. Amoxicillin-resistant HPC. Sampling points indicate different areas across London as shown in Figure 5.1. 'n/a' signifies that the identification results were < 80%.

Sampling Points	**Sampling Time**				
	April 2015	**July 2015**	**October 2015**	**April 2016**	**July 2016**
1	*Aeromonas salmonicida*	*Sphingomonas paucimobilis*	*Pseudomonas aeruginosa*	*Burkholderia cepacia*	*Delftia acidovorans*
2	*Pseudomonas alcaligenes*	*n/a*	n/a	*Aeromonas salmonicida*	n/a
3	*Pseudomonas alcaligenes*	*Pseudomonas alcaligenes*	n/a	*Pseudomonas flourescens*	*Pseudomonas aeruginosa*
4	n/a	n/a	n/a	*Stenotrophomonas maltophilia*	n/a
5	*Pseudomonas flourescens*	n/a	n/a	*Pseudomonas fluorescens*	n/a
6	n/a	n/a	n/a	*Rhizobium radiobacter*	n/a
7	*Pseudomonas flourescens*	n/a	n/a	n/a	*Aeromonas salmonicida*
8	*Stenotrophomonas maltophilia*	n/a	n/a	*Pseudomonas fluorescens*	*Pseudomonas flourescens*
9	*Aeromonas salmonicida*	*Aeromonas salmonicida*	n/a	n/a	n/a

Table 5.9. Trimethoprim-resistant HPC. Sampling points indicate different areas across London as shown in Figure 5.1. 'n/a' signifies that the identification results were < 80%.

Sampling Points	Sampling Time				
	April 2015	**July 2015**	**October 2015**	**April 2016**	**July 2016**
1	*Ochrobactrum anthropi*	*Pseudomonas flourescens*	n/a	*Pseudomonas flourescens*	n/a
2	*Pseudomonas alcaligenes*	*Pseudomonas flourescens*	n/a	*Aeromonas salmonicida*	*Shewanella putrefaciens* group
3	*Burkholderia cepacia*	n/a	*Pseudomonas aeruginosa*	*Ochrobactrum anthropi*	*Pseudomonas aeruginosa*
4	*Pseudomonas luteola*	n/a	n/a	n/a	n/a
5	*Pseudomonas flourescens*	*Pseudomonas flourescens*	n/a	*Pseudomonas fluorescens*	n/a
6	n/a	n/a	n/a	n/a	n/a
7	*Ochrobactrum anthropi*	n/a	n/a	n/a	*Pseudomonas flourescens*
8	*Aeromonas salmonicida*	n/a	n/a	*Ochrobactrum antrophi*	*Pseudomonas putida*
9	*Aeromonas salmonicida*	*Aeromonas salmonicida*	n/a	n/a	n/a

Table 5.10. Tetracycline-resistant HPC. Sampling points indicate different areas across London as shown in Figure 5.1. 'n/a' signifies that the identification results were < 80%.

Sampling Points	Sampling Time				
	April 2015	**July 2015**	**October 2015**	**April 2016**	**July 2016**
1	n/a	*Burkholderia cepacia*	*Sphingomonas paucimobilis*	n/a	n/a
2	n/a	n/a	n/a	n/a	n/a
3	n/a	n/a	n/a	n/a	n/a
4	n/a	n/a	n/a	n/a	n/a
5	n/a	n/a	n/a	n/a	n/a
6	n/a	n/a	n/a	n/a	n/a
7	n/a	n/a	n/a	n/a	n/a
8	n/a	n/a	n/a	n/a	n/a
9	n/a	n/a	n/a	n/a	n/a

5.2 Effectiveness of Chlorine in Inactivating Antibiotic-resistant Bacteria and Antibiotic Resistance Genes

5.2.1 Bacteria growth curve

In addition to operational and environmental factors, physiological features, adaptation or genetic changes of different bacterial strains can play a role in diverse resistance towards disinfectants (Wojcicka *et al.,* 2007). Also, microorganisms have different morphological and physiological features at different growth stages, and some microorganisms exhibit higher resistance to antibacterial agents with increasing growth rates (Cherchi and Gu, 2011). Therefore, it is important to use bacteria for disinfection experiment purposes that are at the same growth stage. In this study, the stationary growth stage was used throughout the experiment to ensure the consistency of each experiment. Bacteria were known to exhibit highest resistance toward antibiotics and disinfectants during the stationary growth stage.

The lag phase for all the bacteria studied occurred between 0 and 2 hours after the incubation, as shown in **Figure 5.8.** The exponential growth rate for *E. coli* NCTC 13400, *E. coli* NCTC 12441 and environmental strain trimethoprim-resistant bacteria was observed between 2 and 8 hours of incubation**.** The stationary phase of *E. coli* NCTC 13400, *E. coli* NCTC 12441 and environmental strain *P. luteola* was observed between 8 and 10 hours of incubation (data subsequent to 10 hour were not collected)**.** On the other hand**,** *Pseudomonas aeruginosa* NCTC 13437 experienced a longer exponential phase, up to 10 hr of incubation.

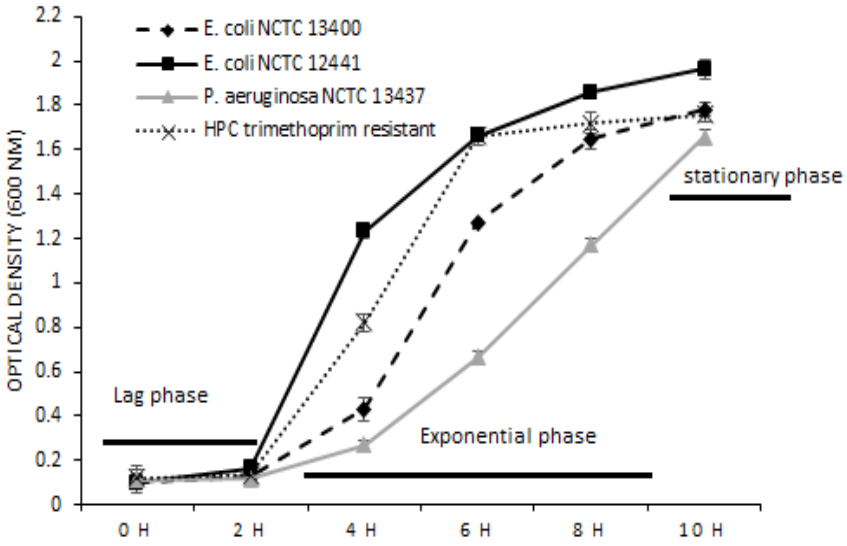


Figure 5.8 Studied bacteria growth characteristics after 10-hour incubation time

5.2.2 Effectiveness of chlorination in inactivating antibiotic-resistant bacteria

Studies on the effect of chlorination on ARBs can be tracked back to the 1980s, when chlorination was observed to influence the proportion of MAR bacteria in drinking water and wastewater (Armstrong *et al*. 1980). Inactivation rates of antibiotic-resistant *E. coli*, antibiotic-sensitive *E. coli*, antibiotic-resistant *P. aeruginosa* and environmental strain resistant bacteria (*P. luteola*) by chlorination are shown in **Figures 5.9** and **5.10.**

The bacterial suspension exerted a high chlorine demand, which resulted in a decrease in chlorine residual concentrations to 0.3 to 0.5 mg/L after 2- 5 minutes trial (at 1 mg/L chlorine dose); the residual chlorine then remained stable at 0.2 mg/L. The residual chlorine concentrations remained stable at 0.3 mg/L (at chlorine dose 2 mg/L), except for *P. luteola*, which exhibited lower chlorine demand than any other tested bacteria. Studies that investigated chlorine demand have shown that different organisms have unique chlorine demands, even at the same bacteria concentration (Shang and Blatchley, 2001). Chlorine demand also depends on the initial bacteria number; in this study ~10^6 to 10^7 CFU/ml was used as the initial bacteria number.

The inactivation of antibiotic-resistant *E. coli* was in the range of 4.3- to 5.6-log and 5.3- to 7.2-log for chlorination 1 mg/L and 2 mg/L, respectively. On the other hand, antibiotic sensitive *E. coli* showed 4.3- to 5.4-log reduction and 4.9- to 6.8-log reduction for the same chlorination dose. The log inactivation results which are expressed relative to their Ct (i.e. the integrated product of chlorine

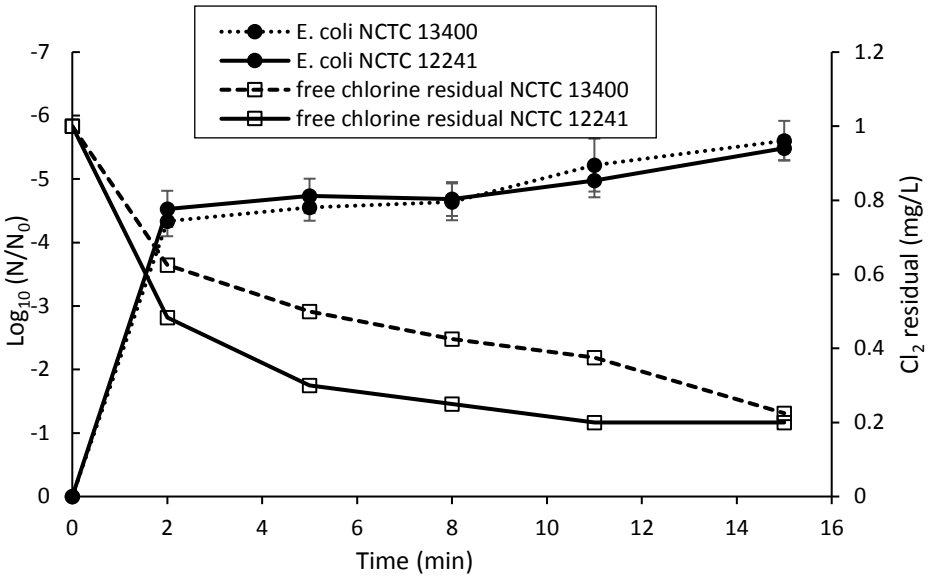
residual and contact time) value is shown in **Figure 5.11**. It can be seen that the inactivation of antibiotic-resistant *E. coli* and sensitive *E. coli* was similar ($p=0.48$). This result contradicts Templeton *et al.* (2009), whose results showed that ampicillin-resistant *E. coli* 145 had less tolerant to chlorination than an antibiotic-sensitive one, though only slightly. The different susceptibility of *E. coli* to chlorine could be attributed to different experimental conditions (e.g. initial bacteria number, bacteria growth culture conditions) and different *E. coli* strains might also exhibit different susceptibility to chlorination. Genetic differences among bacterial strains may lead to differences in the ability of the *E. coli* species to adapt to various environmental conditions or stresses (Cherchi and Gu, 2007). Chlorination affects bacteria's cell membrane, causing changes to its vital functions and eventually lysis. It has been widely researched that many bacteria, including *E. coli*, acquires antibiotic resistance through loss or functional changes in porins, an outer membrane pathway for antibiotics (Nikaido, 2003; Delcour, 2009) such as tetracycline, and β-lactams antibiotics. Loss in porins translates to reduce cell permeability; hence, this could potentially be a reason for the increased resistance towards chlorine in this study.

The environmental strain bacteria isolated from tap water were identified using the API 20NE system, and the bacteria were identified as *Pseudomonas luteola* with >90% (i.e. very good) identification. The minimum inhibition concentration (MIC) test was conducted in order to determine the resistance profile of the bacteria. The results showed that the MIC value for trimethoprim was 250 μg/ml, the MIC for tetracycline was 0.97 μg/ml, and the MIC for amoxicillin was 7.81 μg/ml (details can be found in Appendix A). The apparent inactivation of the laboratory culture *P. aeruginosa* NCTC 13437 was significantly higher than that of the environmental strain resistant *P. luteola* obtained from tap water ($p<0.05$). The bacteria isolated from tap water could have been in the water distribution system for several days; therefore it could also be hypothesised that the physical aggregation of the cells, and the subsequent formation of biofilms, may have caused the increased resistance to chlorination.

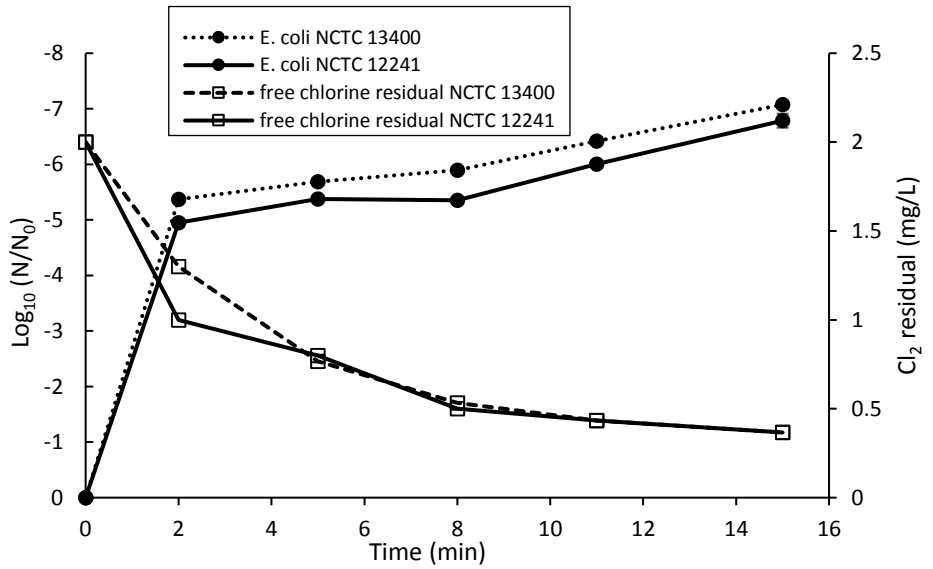
According to Shi *et al.* (2013) and Khan *et al.* (2016), ARBs' response to chlorination varies depending on concentration levels; responses could range from complete inhibition at high concentrations to a biochemical stress response at low chlorine concentrations. The bacteria that were isolated from tap water would have undergone and survived a chlorination process during the water treatment process.

Surviving bacteria may intrinsically have an increased resistance (Khan *et al.,* 2016). This was evident in the study by Jia *et al.* (2015), who observed an increase in antibiotic-resistant *Pseudomonas* after treatment with chlorine as a disinfectant. It is possible that the sub-inhibitory chlorine concentrations that were employed during the experiment could increase the risk of selection of ARB through chemical stress (Huang *et al.*, 2013). While this was not tested in the experiment, the cause of this may be attributed to a stress-response mechanism that the bacteria undergoes, whereby it is enriched with plasmids and integrons involved in the transfer of resistant markers among bacteria (Shi *et al.*, 2013). This could potentially explain the low inactivation rate constant that was experienced by *P. luteola*, i.e. its previous exposure to chlorine disinfection causes its current rate of inactivation to diminish.

Tailing of dose response was observed in both *P. aeruginosa* NCTC 12437 and *P. luteola*. However, it was not observed in antibiotic-resistant and sensitive *E. coli*. Tailing is a common phenomenon in disinfection studies, which might be attributed to several factors such as clumping of the organisms, decrease in germicidal properties and increasing resistance to disinfectants with increasing exposure (Crittenden *et al.*, 2012).

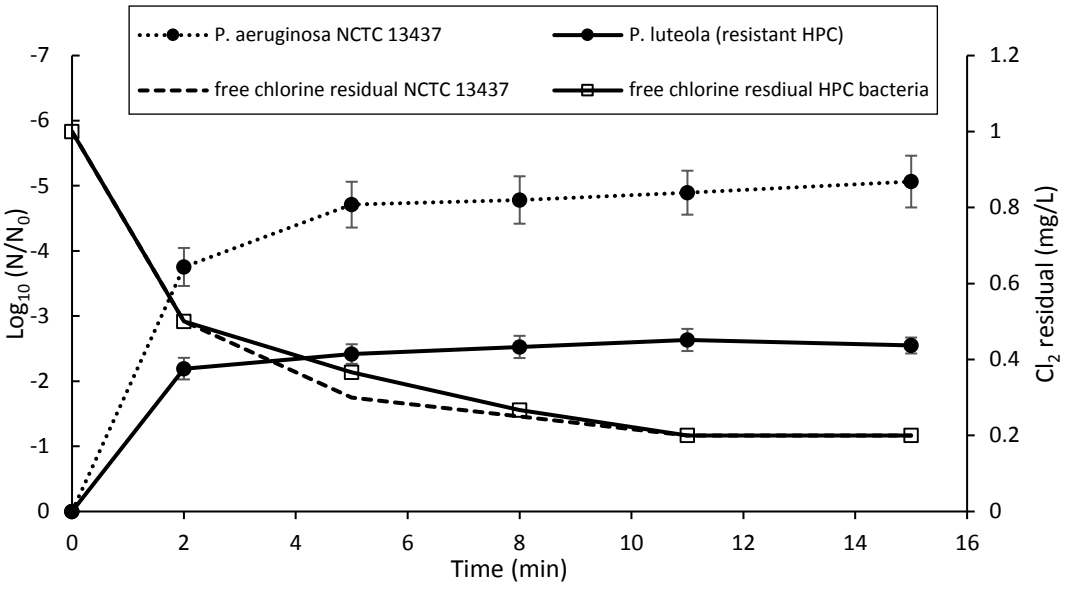


(a)

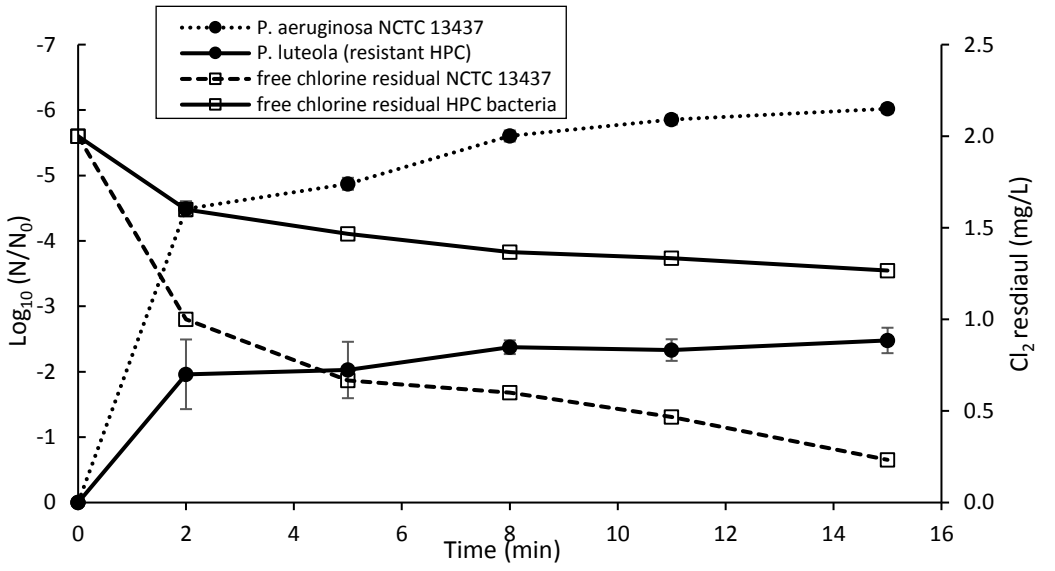


(b)

Figure 5.9 Log inactivation data and free chlorine residual of resistant *E. coli* NCTC 13400 and sensitive *E. coli* NCTC 12247 with (a) representing chlorine dose 1 mg/L and (b) representing chlorine dose 2 mg/L. The error bar represents standard deviation from three replicates.

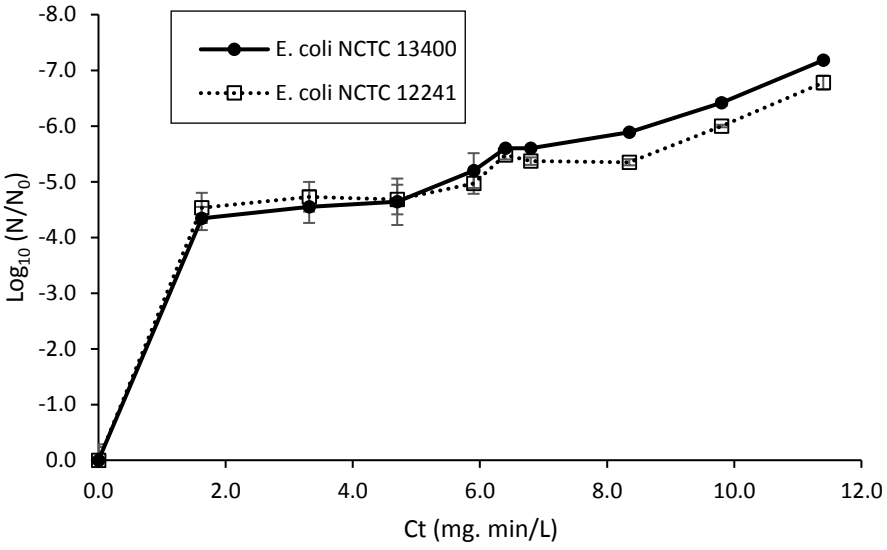


(a)

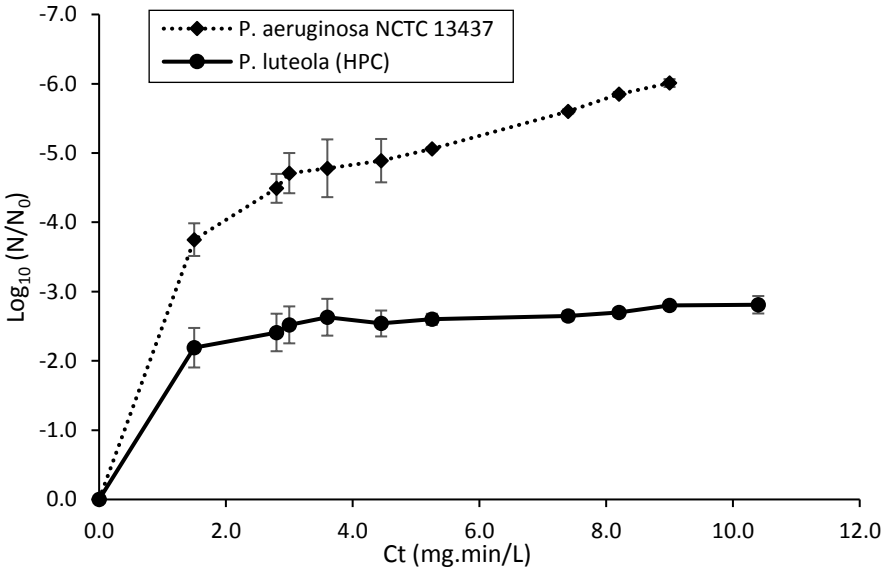


(b)

Figure 5.10 Log inactivation data and free chlorine residual of resistant *P. aeruginosa* NCTC 13437 and HPC resistant *P. luetola* with (a) representing chlorine dose 1 mg/L and (b) representing chlorine dose 2 mg/L. The error bar represents standard deviation from three replicates.



(a)



(b)

Figure 5.11 Comparison of chlorine disinfection results, expressed relative to Ct value **(a)** antibiotic resistant and sensitive *E. coli* and **(b)** antibiotic-resistant *P. aeruginosa* and *P. luteola*

5.2.3 Effectiveness of chlorination in inactivating antibiotic resistance genes

This study investigated the effectiveness of chlorine in inactivating four resistance genes harboured in *Escherichia coli* NCTC 13400. **Figure 5.12** illustrates the influence of chlorine dose and contact time on the inactivation of the studied ARGs, namely, *bla*-$_{TEM1}$, *tet*(A), *mph*(A) and *sul*1. As expected, greater free chlorine dosage and longer contact time lead to higher inactivation for all the resistance genes. A contact time of 30 minutes resulted in up to 0.94-log higher inactivation compared to 15 minutes of contact time.

It was noted that *bla*-$_{TEM1}$ and *tet*(A) gene inactivation showed significantly different inactivation rates with 15 and 30 minutes of contact time at chlorine doses of 1 and 2 mg/L. However, no significant difference was observed at 5 mg/L chlorination. Meanwhile, the *sul*1 and *mph*(A) genes only exhibited significantly different inactivation at chlorination of 2 mg/L. The maximum inactivation was achieved with a chlorine dose of 5 mg/L and 30 minutes of contact time, which resulted in 3.6-log reduction of *mph*(A) and *sul*1 genes. The inactivation of *tet*(A) and *bla*-$_{TEM1}$ genes at the same chlorine dose was 0.2-log lower. Several studies have reported that higher chlorine doses were required to achieve such log inactivation. For instance, Zhang *et al.* (2015) reported that the maximum inactivation of *sul*1, *tetG*, and *tetX* genes in municipal wastewater treatment plant was achieved at a chlorine dose of 30 mg/L, resulting in 1.3 to 1.49-log reduction for all studied genes. Real wastewater samples with potentially interfering factors such as high-suspended solids and organic compounds were used in their experiments, which might explain the difference inactivation observed in the current study. The log inactivation of *tet*(A) gene at 1 and 2 mg/L was significantly higher than that of the *sul*1 gene ($p<0.05$); however, the inactivation of these genes was observed to be similar at 5 mg/L. Interestingly, the *tet*(A) gene concentration decreased rapidly by 1-log at a low chlorine exposure of 1 mg/L, and then decreased much more slowly following first order kinetics with respect to the chlorine dose.

Chlorine Ct value (the integrated product of chlorine residual multiplied by chlorine contact time) is normally used to express the disinfectant dosage for chlorination. A Ct value of 179 mg Cl_2.min/L did achieve significantly more inactivation for all tested ARGs; a value of 179 mg Cl_2.min/L achieved significant inactivation of ARGs, 3.2- to 3.6-log inactivation, for *tet*(A), *bla*-$_{TEM1}$, *sul*1 and *mph*(A)

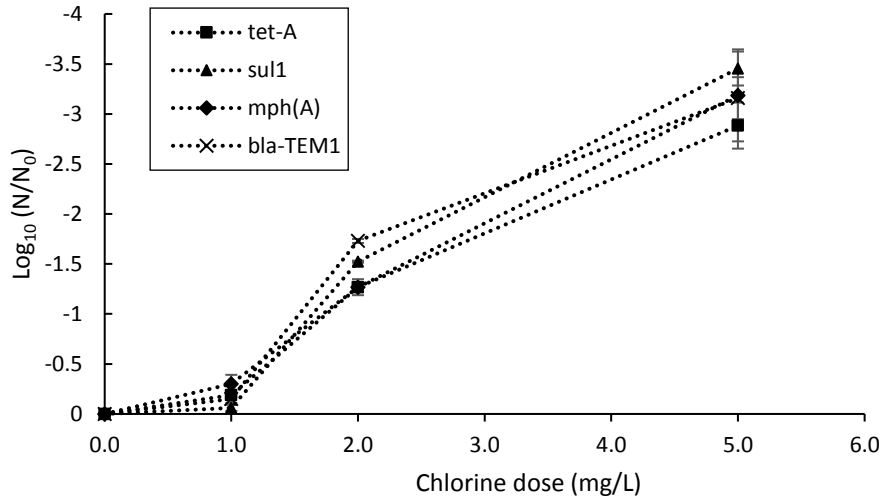
genes, respectively as shown in **Figure 5.13**. *tet*(A) and *bla*-$_{TEM1}$ genes had lower inactivation compared to other tested genes, and this result is in agreement with Guo *et al.* (2015), who also observed low inactivation of *tet*(A) gene during wastewater chlorination.

It was noted that the *bla*-$_{TEM1}$ and *tet*(A) genes showed significant inactivation differences at a chlorine dose of 2 mg/L ($p<0.05$), while *bla*-$_{TEM1}$, *sul*1 and *mph*(A) showed no difference in their inactivation rates at all chlorine doses. This result suggested that β-lactams, sulfonamide, and macrolide resistance genes exhibit similar susceptibility to chlorination. However, despite the lower log inactivation, *bla*-$_{TEM1}$ and *tet*(A) gene inactivation occurred at a higher rate compared to *mph*(A) and *sul*1 genes. Meanwhile, Yoon *et al.* (2017) observed higher inactivation than in this study for *amp* and *kan* resistance genes and found that intracellular resistance genes were more sensitive to chlorination. Dodd (2012) explained that free chlorine is highly reactive with free thymidine and 2'-deoxyguanosine-5-monophosphate. Additionally, chlorine shows a high reactivity with guanosine- and thymidine-based nucleotides but only moderate or low reactivity with cytosine- and adenine-based nucleotides (Prütz, 1996). *Tet*(A) gene contains a higher number of chlorine reactive G -C (i.e. 803 bp) than *bla*-$_{TEM1}$, *mph*(A), and *sul*1 gene, this might explain the sensitivity of *tet*(A) to chlorination.

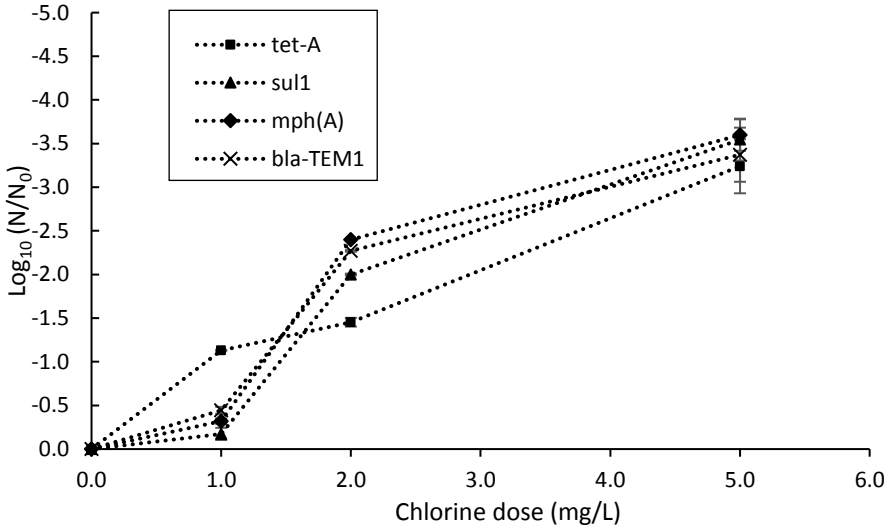
It has been suggested that chlorination might select some ARBs and ARGs. This idea dates back to a study conducted by Armstrong *et al.* (1981), who observed an elevated percentage of MAR in the bacteria following water treatments. A recent study by Shi *et al.* (2012) found that chlorination caused enrichment of *ampC*, *aphA*2, *bla*-$_{TEM1}$, *tet*(A), *tet*(G), *ermA*, and *ermB* resistance genes. Additionally, extracellular stress can promote the replication of plasmids in bacterial cells. Metagenomic analysis has confirmed that chlorination of drinking water can concentrate ARGs, plasmids, insertion sequences and integrons involved in ARG horizontal transfer (Shi *et al.*, 2013). Furthermore, tetracycline resistance genes harbouring inside the *E. coli* NCTC 13400 used in this study encode efflux protein, which could pump out several antibiotics as well as chlorine. Tandukar *et al.* (2013) showed that quaternary ammonium compound-resistant isolates are resistant to antibiotics because they possess one or more efflux pumps, which efflux quaternary ammonium disinfectants and antibiotics simultaneously.

Various studies have been conducted to assess the effectiveness of chlorine in removing ARGs; however, most of these studies were performed in real or artificial wastewater. Therefore, the applied chlorine doses were relatively high, i.e. more than 20 mg/L. In this study, we used typical chlorine doses that are normally applied in drinking water (i.e. 1 to 5 mg/L). Hence, the results from this study are more able to accurately predict ARG reduction in drinking water treatment systems. Moreover, a high chlorine dose in general is not desirable due to the operational cost and the potential formation of disinfection by-products. In this study, 5 mg/L of chlorination with 30 minutes of contact time was sufficient to inactivate *sul*1, *tet*(A), *bla*-$_{TEM1}$ and *mph*(A) genes up to 3.5-log. However, at more practical chlorine dose (i.e. 2 mg/L) the reduction was only 1.5- to 2.2- log.

Overall, the study showed that typically applied chorine doses in drinking water treatment do not achieve complete inactivation of ARGs in waterborne bacteria.



(a)



(b)

Figure 5.12 Influence of different chlorine doses on inactivation rate of antibiotic resistance genes **(a)** contact time 15 minutes **(b)** contact time 30 minutes. The error bar represents standard deviation from three replicates.

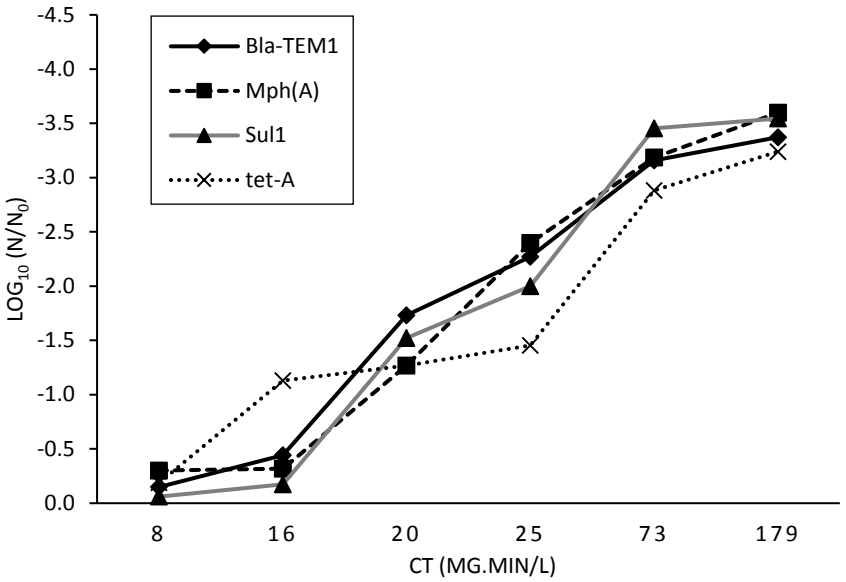


Figure 5.13 Comparison of chlorine disinfection results, expressed relative to Ct.

5.3 Potential Regrowth of Antibiotic-resistant *Escherichia coli* and *Pseudomonas aeruginosa* Following Chlorination

After chlorination of drinking water or wastewater, a microbial health risk might still exist. Several studies have demonstrated that bacteria are able to reproduce in drinking water distribution systems despite the presence of a disinfectant residual. However, only a few studies have investigated the reactivation of ARBs after chlorination. The reproduction of bacteria after chlorination could be the sum of the reactivation of inactivated bacteria (i.e. bacteria that were injured) and the regrowth of reactivated bacteria. The reactivation of bacteria in this study includes the reactivation of injured bacteria, the regrowth of reactivated bacteria and regrowth of surviving bacteria following chlorination.

Therefore, in this chapter the reactivation of antibiotic-resistant *E. coli* NCTC 13400 and *P. aeruginosa* NCTC 13437 under various chlorination doses and incubation times will be discussed. Regrowth and/or reactivation experiments were conducted to simulate the conditions in which treated drinking water might remain in distribution systems or collection storage, for up to two weeks. In this experiment, two sets of scenarios were employed: (1) conditions without the presence of residual chlorine, a situation that can represent water distribution systems in some European countries that do not apply secondary

disinfection (e.g. Austria, Netherlands); and (2) conditions with the presence of residual chlorine throughout the experiment (as in UK distribution networks).

The number of viable bacteria (*E. coli* and *P. aeruginosa*) significantly decreased following 30 minutes of chlorination at 1, 2 and 5 mg/L, as anticipated. After quenching the chlorine residual and 24 hours of dark incubation, antibiotic-resistant *P. aeruginosa* had increased following chlorine doses of 1, 2 and 5 mg/L. However, it was less than 20% for both *E. coli* and *P. aeruginosa*. Meanwhile, further bacteria decay of 5.7% was observed in resistant *E. coli*, although the decay was only observed at a 2 mg/L chlorine dose. As expected, dark incubation for 1 week (i.e. the longest incubation period in this study) resulted in higher recovery of bacteria counts, with the percentage of reactivation ranging from 38% to 77% for all doses of chlorine used. In contrast, Huang *et al.* (2011) observed no significant regrowth of total heterotrophic bacteria and ARBs in wastewater secondary effluent. Moreover, they also observed that reactivation and decay of ARBs occurred when the dosage of chlorine was lower than 2 mg/L with 10 minutes' contact time. The discrepancy observed between these two studies might be explained by different protocols; for instance, in this study the contact time was longer (i.e. 30 minutes) and the initial bacterial number were higher than in the study by Huang *et al*.; also, the fact that they used a mixed bacterial culture (i.e. more than one species) might also affect the bacterial repair ability.

A higher chlorine dose led to lower recovery in bacteria counts in both studied bacteria. Chlorination of 5 mg/L resulted in 37% recovery of counts of resistant *E. coli*, 26% lower than with at 1 mg/L chlorination. The same phenomenon was also observed in resistant *P. aeruginosa*. As can be seen in **Figure 5.14,** the bacteria count recovery at a chlorine dose of 5 mg/L was slightly lower by 15%. When a high dose of chlorine is used, the increased absorption of chlorine into the cell might inhibit the process of reactivation. Nevertheless, Li *et al.* (2013) observed regrowth of *Salmonella* and *Enterococcus* after chlorination of 69 mg.min/L.

Resistant *P. aeruginosa* showed significantly higher reactivation than resistant *E. coli* in all chlorine doses applied ($p<0.05$). This can also be seen from the higher number of viable bacteria subsequent to varied incubation periods. *Pseudomonas aeruginosa* is well known to have minimal nutrient requirements for survival and a notable ability to adapt to a variety of different environments.

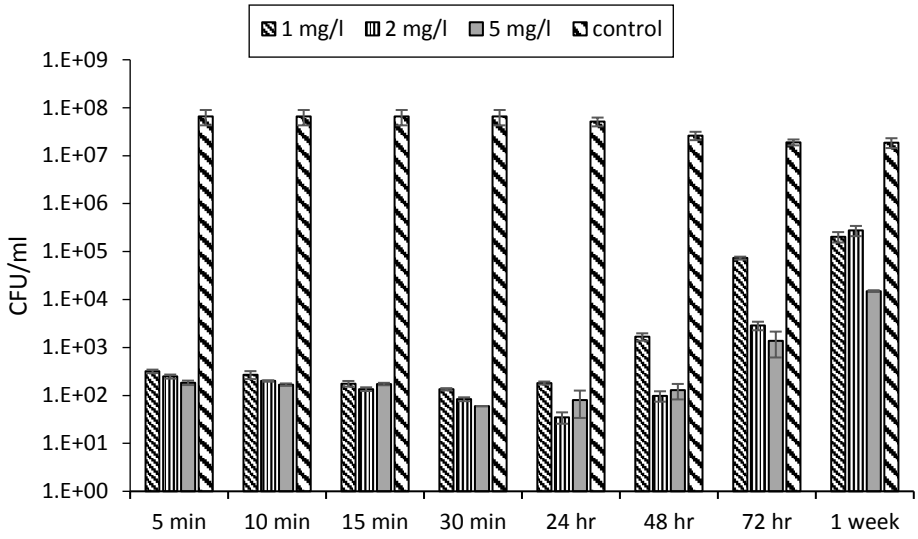
Moreover, this species can survive and multiply in certain disinfectants, particularly several types of quaternary ammonium compounds (Palleroni, 1984).

As shown in **Figures 5.14** and **5.15** following 1 mg/L of chlorination, the viable bacteria number after one week incubation increased 3.2-log and 4.3-log for resistant *E. coli* and resistant *P. aeruginosa*, respectively. The bacteria number decreased gradually with an increasing chlorine dose. At a chlorine dose of 5 mg/L, the resistant *E. coli* number was 4.2-log CFU/ml, while the number of resistant *P. aeruginosa* was 0.6 log-higher. However, it is noted that the bacterial recovery never reached its initial number. The ability of resistant *E. coli* and *P. aeruginosa* to regrow following chlorination might cause dissemination of the resistance gene to non-resistant bacteria along the distribution pipe. Additionally, it has been reported that *E. coli* can undergo a viable but non-culturable (VBNC) state during water treatment. If this phenomenon occurs, the number of bacteria obtained in this study may be underestimated. The VBNC bacteria cannot grow in conventional bacteriological mediums but can be reactivated in certain conditions, rendering them culturable once more (Higgins *et al.*, 2007).

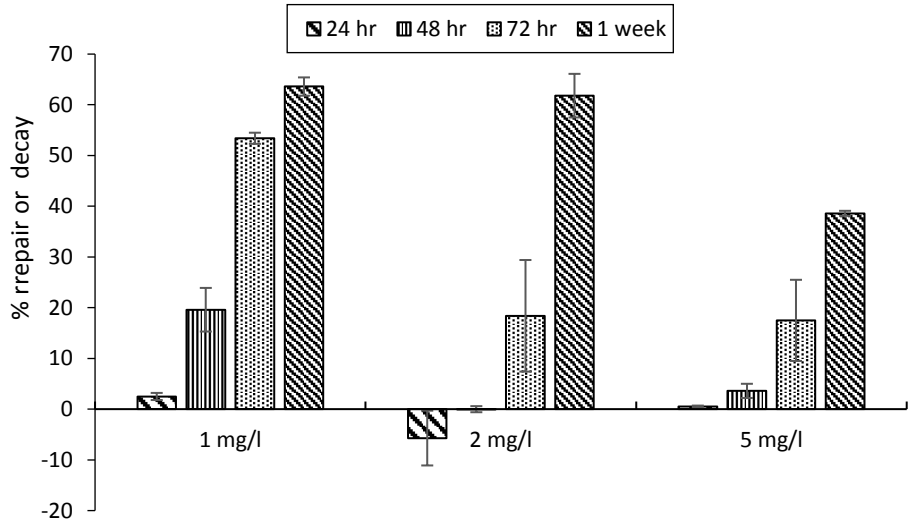
The fact that viable bacteria were still present after chlorination, as seen in **Figures 5.14** and **5.15,** might enhance the repair or regrowth ability of the studied bacteria. Milbauer and Grossowicz (1959) suggested that the existence of living cells could also improve the recovery of nutritionally defective cells through the syntrophism (i.e. interactions between different or the same bacteria species with complimentary methods of energy metabolism) mechanism. Furthermore, the presence of dead bacteria might also enhance bacterial growth to some extent by providing additional nutrients (Garvie, 1955). In this study, the living bacteria were still present even after 30 minutes of chlorination. This might explain the rapid recovery of resistant bacteria, particularly in lower chlorine doses, as surviving bacteria existed in higher numbers.

No recovery in bacteria counts was observed in the continuous chlorination experiment, even at the lowest chlorine dose (data can be found in Appendix F). This result emphasises the importance of maintaining adequate chlorine residual in distribution and storage systems. However, it has to be noted that in this experiment only two bacteria species were used, in contrast to the complex bacteria community normally found in real drinking water distribution systems. Studies have been shown that

interactions between different species of bacteria might enhance the regrowth process in water distribution systems (Berry *et al*. 2006).

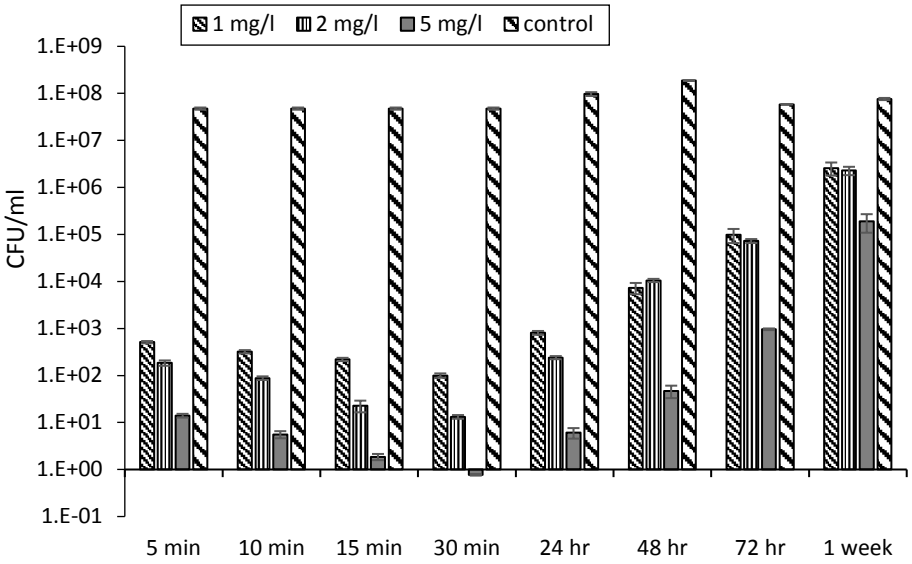


(a)

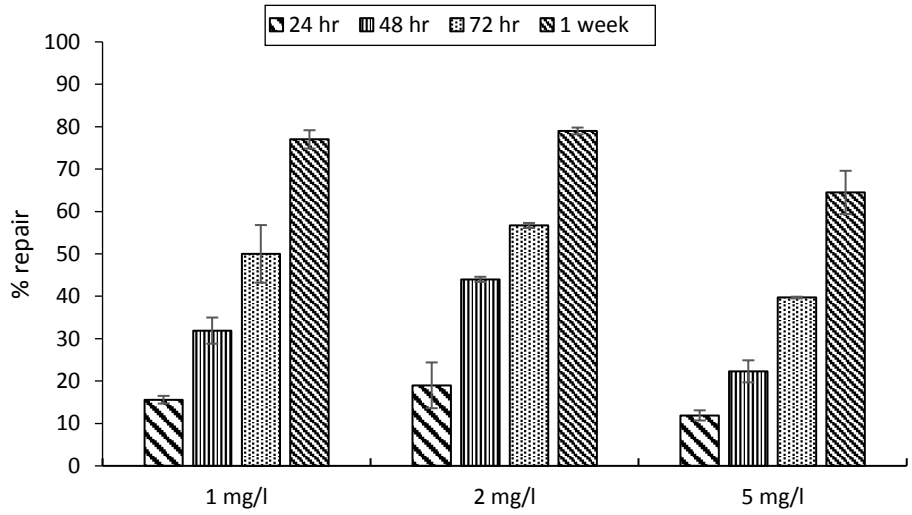


(b)

Figure 5.14 (a) Regrowth of *E. coli* NCTC 13400 as a function of chlorine dose following various incubation times **(b)** Reactivations of *E. coli* NCTC 13400 following various incubation times. The error bar represents standard deviation from three replicates.



(a)



(b)

Figure 5.15 (a) Regrowth of *P. aeruginosa* NCTC 13437 as a function of chlorine dose following various incubation times **(b)** Reactivations of *P. aeruginosa* NCTC 13437 following various incubation times. The error bar represents standard deviation from three replicates.

5.4 Effectiveness of UV in inactivating antibiotic-resistant bacteria and antibiotic resistance genes

5.4.1 The effectiveness of UV in the inactivation of antibiotic-resistant bacteria

Various studies have been conducted to assess the efficiency of UV disinfection in inactivating ARBs. However, most of these studies have focused on the wastewater application (Guo *et al*. 2013; Rizzo *et al.* 2013; Oh *et al.* 2014; Huang *et al*. 2016). Only two studies have examined ARBs' and ARGs' inactivation using UV in a drinking water application (McKinney and Pruden, 2012; Yoon *et al*. 2017). In this experiment, sensitive *E. coli* NCTC 12241, resistant *E. coli* NCTC 13400, resistant *P. aeruginosa* NCTC 13437 and trimethoprim-resistant heterotopic bacteria were used as model bacteria. Four UV doses (1, 3, 5 and 7 mJ/cm^2) were applied to examine the dose-response curves of the studied bacteria. It must be noted that these UV doses are significantly lower than the minimum UV dose of 40 mJ/cm^2 that would be commonly applied in drinking water treatment. Only low doses of UV were used because of reaching the countable limit of surviving bacteria in the experiments.

As expected, UV disinfection resulted in an increasing inactivation of the bacteria in the sample with increasing UV dose. Log reduction in antibiotic-resistant *E. coli* was in the range of 2.42-log to 6.64-log across the applied UV doses **(Figure 5.16).** Application of a UV dose of 1 mJ/cm^2 rendered 2.5-log inactivation of resistant *E. coli*. Exposure to a UV dose of 3 mJ/cm^2 resulted in 5.6-log bacteria inactivation, while a plateau curve was observed between UV doses of 5 to 7 mJ/cm^2, with log inactivation of 6.3- to 6.6-log. The inactivation values obtained in this study were generally higher than in related UV disinfection studies. For instance, McKinney and Pruden (2012) found that the inactivation of tetracycline resistant *E. coli* SMS 3-5 at UV fluence of 5 mJ/cm^2 was 2-log. In comparison, Rizzo *et al.* (2013) observed a 3-log reduction of indigenous resistant *E. coli* from a wastewater treatment plant effluent following UV radiation at 5 mJ/cm^2.

The inactivation rate of sensitive *E. coli* (NCTC 12241) at a UV dose of 1 mJ/cm^2 was 1.1-log, 1.4-log lower than resistant *E. coli*. The inactivation of sensitive *E. coli* between UV doses of 3 and 7 mJ/cm^2 yielded a linear decline in bacteria numbers, ranging from 1.7-log to 5.5-log. The UV dose response of

the bacteria generally agreed with previous UV disinfection kinetics for the same *E. coli* strain, ATCC 11229 (Chang *et al.*, 1985; Harris *et al.*, 1987; Claus *et al.*, 2005).

Antibiotic-resistant *E. coli* appear to be less resistant to UV than antibiotic-sensitive *E. coli* ($p<0.05$). This result differs from Templeton *et al.* (2009), who observed similar inactivation behaviour between trimethoprim-resistant *E. coli* and antibiotic-sensitive *E. coli*. The contradictory results might arise from the different *E. coli* strains used in the studies. Cherchi and Gu (2011) reported that genetic diversities among the same bacterial strains might result in the different adaptive capabilities of the *E. coli* species in response to various environmental conditions and stresses.

At UV dose 1 mJ/cm^2 the reduction of antibiotic-resistant *P. aeruginosa* NCTC 13437 was achieved at 1.45-log. The inactivation then increased to 3.78-log at a UV dose of 3 mJ/cm^2; however, the inactivation rate was observed to only slightly increase to 3.82-log when a UV dose of 5 mJ/cm^2 was applied **(Figure 5.17).** The application of a UV dose of 7 mJ/cm^2 resulted in 4.2-log inactivation. The dose-response curve of resistant *P. aeruginosa* was found to be similar to that in a study by McKinney and Pruden (2012), who also used resistant *P. aeruginosa* as a model bacteria. In the UV disinfection test, resistant *E. coli* had a lower inactivation rate (by 1.6 to 3.5-log) than *P. aeruginosa*, and the difference was statistically significant ($p\leq0.05$). Antibiotic-resistant *P. aeruginosa* was observed to undergo a tailing effect at higher UV doses i.e. >5 mJ/cm^2. Tailing can occur at high inactivation levels due to resistant strains, aggregation or the particle-association of organisms and might be due to mathematical artefact (i.e. when the experiment reached the maximum countable limit of bacteria). The initial bacteria number used in this study was ~10^6 CFU/ml, which was relatively higher than the typical bacteria number found in real drinking water treatment plants. The high colony counts used in this study might increase the likelihood of dead bacterial cells shielding live ones from UV light.

Trimethoprim-resistant HPC bacteria presented the lowest inactivation against UV disinfection across all UV doses applied. When UV doses of 1 mJ/cm^2 were applied the inactivation achieved was only 1-log. The inactivation gradually increased with the UV dose; the dose response was shown to be linear. Interestingly, unlike in *P. aeruginosa*, the tailing effect was not observed in *P. luteola*, suggesting that the mechanism of tailing in *P. aeruginosa* was not applicable in *P. luteola*.

All in all, heterotrophic resistant bacteria were found to be more resistant towards UV disinfection compared to lab-cultured *P. aeruginosa*, with the difference in log inactivation ranging from 0.5 to 1.7 log units. However, the variances observed between the two bacterial strains did not differ statistically ($p>0.05$). Environmental isolates that have been previously exposed to other disinfection methods such as chlorine and monochloramine may develop higher resistance towards disinfectants or any environmental stresses. The characteristic behaviour of an organism in its natural environment may alter during isolation, purification and maintenance in laboratory culture media. Furthermore, Mechin *et al.* (1999) found that the sub-culturing process of *P. aeruginosa* resulted in more resistant bactericidal compounds.

Among all the studied bacteria, MAR *E. coli* showed the highest UV-sensitivity, with up to 6-log inactivation at a UV dose of 7 mJ/cm^2. Heterotrophic resistant *P. luteola* was revealed to be the most resistant towards UV disinfection. The results appear to indicate widely varying tolerances or sensitivity features of ARBs to UV treatment, and differences between environmental isolates and type culture strains.

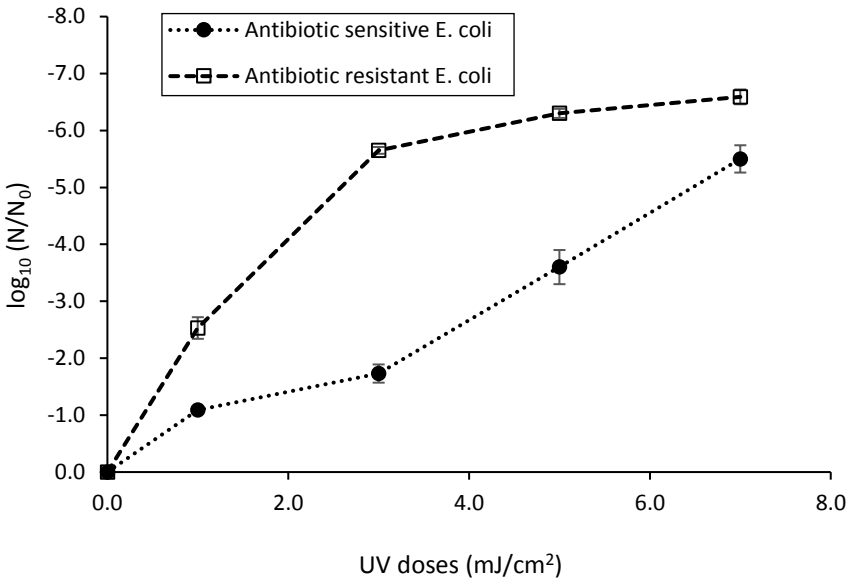


Figure 5.16 Comparison of UV disinfection result of antibiotic-sensitive *E. coli* NCTC 13400 and multiple-antibiotic-resistant *E. coli* NCTC 12241. The error bar represents standard deviation from three replicates.

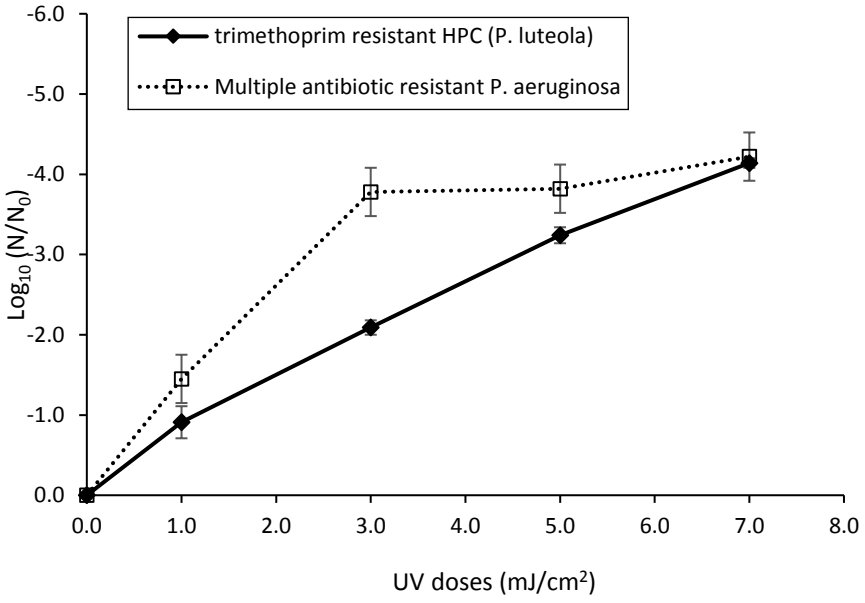


Figure 5.17 Comparison of UV disinfection result of trimethoprim-resistant HPC (*P. luteola*) and multiple-antibiotic-resistant *P. aeruginosa* NCTC 13437. The error bar represents standard deviation from three replicates.

5.4.2 The effectiveness of UV in the inactivation of antibiotic resistance genes

Disinfection efficiency was evaluated by measuring the remaining fraction (N/N_0) of four resistance genes (*tet*(A), *mph*(A), *bla*-$_{TEM1}$ and *sul*1). Experiments were conducted using UV doses of 20, 50, 100 and 200 mJ/cm^2, to cover the range of typical UV doses applied in water and wastewater disinfection.

Figure 5.18 shows the log inactivation of ARGs after UV disinfection. The *mph*-A gene inactivation was observed to be the lowest among all the tested resistance genes. At UV dose 20 mJ/cm^2, the inactivation was only 0.05-log, whilst at 200 mJ/cm^2, the highest UV dose applied, the inactivation was 0.42-log. The *bla*-$_{TEM1}$ gene was the least resistant to UV treatment, compared to the other tested genes. The log inactivation gradually increased along with an increase in UV dose. The inactivation achieved 1.18-log at a UV dose of 200 mJ/cm^2, the highest inactivation among all the tested genes. The difference was statistically significant with all of the studied genes ($p<0.05$)**.** The *tet*(A) gene achieved 0.05-, 0.36-, -0.38 and 0.74-log inactivation at UV doses of 20, 50, 100 and 200 mJ/cm^2, respectively.

All the studied genes, except *bla*-$_{TEM1}$, showed similar inactivation behaviour at a low UV dose (20 mJ/cm^2), with 0.05-log inactivation. The data indicates that four of the studied genes are far more UV resistant than the host microbe, *E. coli*, which has a well-established D90 (i.e. dose required to inactivate 90% of the bacteria) in water of approximately 2.6 mJ/cm^2 (Kowalski 2009). The inactivation of *sul*1 and *tet*(A) was not significantly different at UV doses of 50 ($p=0.23$), 100 ($p=0.31$) or 200 mJ/cm^2 ($p=0.26$). There was no significant difference between *tet*(A) and *bla*-$_{TEM1}$ at a UV dose of 20 ($p=0.5$) and 50 mJ/cm^2 ($p=0.13$), although the inactivation was found to be significantly different at UV doses of 100 *($p=0.009$)* and 200 ($p=0.01$) mJ/cm^2. The inactivation of *bla*-$_{TEM1}$ was always higher than that of the *sul*1, *tet*(A) and *mph*(A) genes and the difference was statistically significant for UV doses of 20, 50, 100 and 200 mJ/cm^2 ($p < 0.05$).

In this experiment, intracellular ARGs (e.g. the genes within the *E. coli* host) were used. This might explain the low inactivation achieved in this study compared to if extracellular ARGs had been considered. Intracellular DNA is protected by cell walls and bacteria cytoplasm, making it more difficult to attack with UV disinfection. That said, a previous study by McKinney and Pruden (2012) observed no significant difference in the inactivation of intracellular and extracellular ARGs. The

results might be explained by the fact that UV light can penetrate the UV-transparent structures in the cell and is mainly only absorbed by the nucleobases, comprising DNA and RNA (Dodd, 2012).

One of the interests in this study was the potential for UV to convey damage to ARGs in order to reduce the risk of them being transformed into new downstream hosts. Quantitative PCR assay was selected to detect DNA damage such as oxidised base, abasic sites and thymine dimer formation. Any DNA damage interferes with amplification by polymerase enzymes. While qPCR is not an assay that can directly determine loss of DNA function, several studies have shown that UV-damaged genes, including ARGs, can no longer function within a host bacterial cell. However, the qPCR method might underestimate the DNA damage caused by disinfectants. Suß *et al.* (2009) reported that the longer the targeted DNA fragment, the more UV-induced DNA lesions inhibited the PCR. This however is clearly restricted, due to the principle or nature of the techniques used. For instance, SYBR green assays are restricted to a short amplicon size, ideally ranging from 100-200bp, therefore, an amplicon size greater than 700bp will strongly reduce the PCR efficiency. In this study, the amplicon lengths were 190, 162, 293, and 246 bp for the *tet*(A), *sul*1, *mph*(A) and *bla*-$_{TEM1}$ genes, respectively.

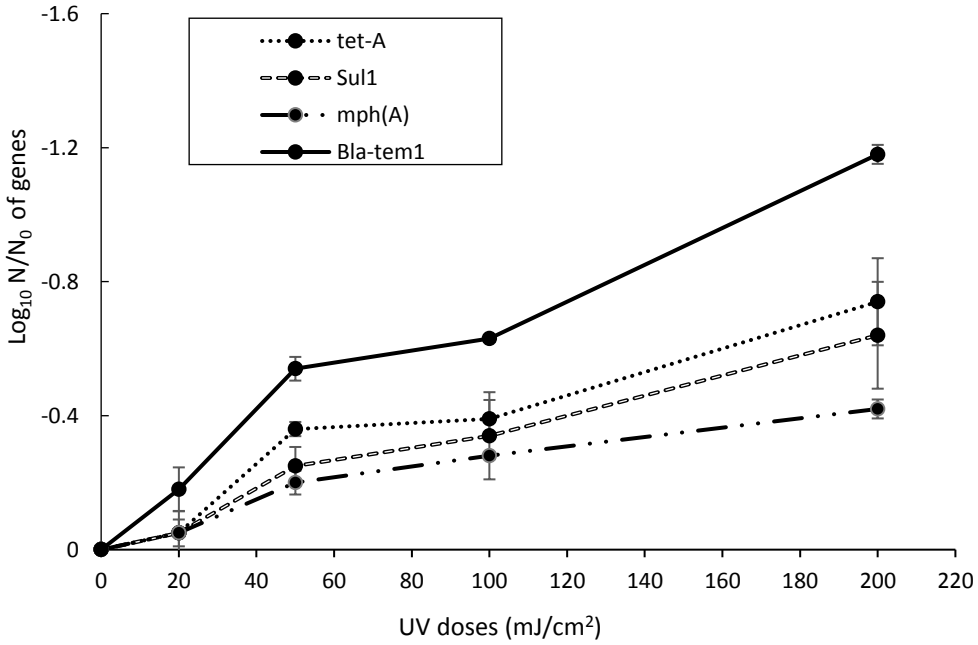


Figure 5.18 The inactivation of antibiotic resistance genes (ARGs) following UV exposure of 20, 50, 100 and 200 mJ/cm^2. The error bar represents standard deviation from three replicates.

The log inactivation achieved in this study is lower than that reported by McKinney and Pruden (2012) and Yoon *et al.* (2017). McKinney and Pruden (2012) reported that UV doses of 200-400 mJ/cm^2 could reduce *mec*A, *van*A, *tet*(A) and *amp*C by 3 to 4-log. Yoon *et al.* (2017) observed a 1.1 to 1.6-log reduction of *kan* and *amp* genes at a UV dose of 40 mJ/cm^2. However, Guo *et al.* (2013) reported that UV disinfection at a dose of 10 mJ/cm^2 completely eliminated *erm* and *tet* genes in wastewater samples. These conflicting results could be attributed to the different bacteria strains and experimental conditions (e.g. the type of qPCR applied, the initial cell concentration).

UV light is mainly absorbed by the nucleobases containing DNA and RNA, and specifically by the thymine and cytosine bonds, allowing the structure to be opened (Dodd, 2012). The two major classes of UV mutagenic products are cyclobutane-pyrimidine dimer (CPD) and 6-4 photoproducts (Sinha & Häder, 2002). Thymine dimers occur more commonly than cytosine dimers and both of these are more frequent than purine dimers. Douki and Cadet (2001) reported that TT and TC are the most photo-reactive sequences, with CT and CC dimers occurring at a lower frequency. **Table 5.11** summarises the gene sizes and the number of potential dimers that could theoretically be formed within the template strand of the studied genes. The number of potential dimers was calculated from each resistance gene sequence. The complete genome sequence of pEK499 is available at https://www.ncbi.nlm.nih.gov/nucleotide/228480645. The *mph*(A) gene contains the lowest number of thymine-thymine dimer sites, followed by *sul*1, *bla*-$_{TEM1}$ and *tet*(A). The potential dimer 'YR' represents any pyrimidine (Y) that combines with a purine (R) from photodimerisation.

According to the potential dimer counts in **Table 5.11**, the *mph*(A) gene has the fewest pairs of adjacent thymines compared to the other studied ARGs. The second most UV-resistant is the *sul*1 gene, with a reduction of 0.05, 0.2, 0.28 and 0.42 at 20, 50, 100 and 200 mJ/cm^2 respectively. Meanwhile, the application of UV doses at 50, 100 and 200 mJ/cm^2 gives *tet*(A) reduction of 0.05, 0.36, 0.39 and 0.79 respectively. In this study, *tet*(A) has the longest gene size (1275 base pairs), containing 121 TT pairs and 194 CT pairs.

Table 5.11. Genome size of four antibiotic resistance genes and the number of potential dimers

Gene	Size (Base pairs)	Potential dimers				
		TT	TC	CT	CC	YR
mph(A)	906	39	128	106	140	240
*sul*1	854	71	130	110	128	236
tet(A)	1275	121	194	192	160	358
bla-$_{TEM1}$	729	73	64	62	67	160

Based on the results of each individual ARG's reductions at each UV dose (20, 50, 100 and 200 mJ/cm^2), the order of the genes in terms of the most susceptible to UV disinfection was: *bla*-$_{TEM1}$ ≥ *tet*(A) ≥ *sul*1 ≥ *mph*(A). This order was consistent with the number of potential dimers, particularly TT dimers on each gene. The sensitivity of antibiotic resistance genes was also accurately predicted by UV genomic modelling. The model was developed and conducted by Dr Wladyslaw Kowalski by considering the number of nucleic acids that are vulnerable to UV photodimerization of each resistance gene. A two-stage decay curve was used to evaluate the survival ratio of each resistance genes obtained from the experiment in order to accurately compute the D90 dose. **Table 5.12** shows the predicted extracellular UV doses required for 90% (i.e. D90) destruction of the studied antibiotic-resistance genes.

Table 5.12. The predicted extracellular UV doses required for 90% destruction of the studied antibiotic-resistance genes

Gene	D90, mJ/cm^2			UV resistance rank
	Minimum	Maximum	Average	
mph(A)	83.5	99.2	91.4	1
*sul*1	60.8	69.5	65.2	2
tet(A)	44.8	61.2	53.0	3
bla-$_{TEM1}$	28.5	30.1	20.7	4

It can be seen that the model accurately predicted the UV sensitivity of the studied antibiotic resistance genes. However, the predicted UV doses required to achieve 90% reduction based on extracellular DNA were lower than the experimentally determined intracellular UV doses required for this level of

reduction. This may be partly because the model does not consider the UV absorbance of the bacterial cytoplasmic membrane.

This study suggested that UV disinfection retains only limited potential to damage ARGs in drinking water. To achieve the minimum log reduction set by drinking water regulators a UV dose of more than 200 mJ/cm^2 is needed.

5.5 Potential Repair of Antibiotic-resistant *Escherichia coli* and *Pseudomonas aeruginosa* Following UV Disinfection

Ultraviolet disinfection is now regarded as a viable alternative to chlorination for drinking water treatment due to its exceptional biocidal properties without the formation of harmful disinfection by-products. However, it is also known that microorganisms exposed to low UV doses (e.g. <10 mJ/cm^2) are able to reverse the UV-induced damage due to the presence of ultraviolet radiation from the natural environment and the natural defence mechanism of the bacteria itself (Sinha and Hader, 2002). In this section, the repair ability of MAR *E. coli* NCTC 13400 and *P. aeruginosa* NCTC 13437 will be discussed. Two sets of experiments were conducted: (1) light repair and (2) dark repair and as mentioned in the method section that the light and dark repair of each antibiotic-resistant bacteria at each time interval expressed in percentage repair, per unit log reduction The repair in dark and light repair conditions following exposure to various UV doses are presented in **Figure 5.20** for antibiotic-resistant *E. coli* and **Figure 5.22** for antibiotic-resistant *P. aeruginosa*.

In general, photoreactivation, i.e. lighted conditions, showed higher repair by 12% to 3% in resistant *E. coli* compared to dark conditions, while the repair for resistant *P. aeruginosa* was observed to be similar, both in dark and light conditions. In this study, the maximum level of the photo repair achieved was 74% of the total number of the inactivated bacteria at a UV dose of 3 mJ/cm^2 in resistant *E. coli*. The highest repair for dark conditions was observed at 62% repair for both *E. coli* and *P. aeruginosa* following UV exposure of 3 mJ/cm^2. The maximum repair takes place within the first three hours of incubation, followed by a levelling off during a longer incubation period. This trend is consistent with a previous report by Quek and Hu (2008) and Zimmer and Slawson (2002) who also observed high photoreactivation rates of *E. coli* ATCC 11229 within the first two to three hours after incubation.

The resistant *E. coli* undergo photoreactivation starting three hours after incubation. However, Zimmer and Slawson (2002) reported that the light repair might begin as soon as 30 minutes after UV exposure. After 3 hours of light incubation the percentage of log repair was 37%, 17% and 9% for UV doses of 3, 5 and 10 mJ/cm^2, respectively. This value was much lower than that observed by Quek and Hu (2008), who obtained > 60% repair after a 3-hour incubation time. The differences observed in this study might

be because of the presence of resistant plasmid in *E. coli* NCTC 13400. The existence of this plasmid might have some effects on the efficient repair mechanisms of the bacteria. The maximum viable bacteria number was observed at UV dose 3 mJ/cm^2 with an average of 33.4 x 10^4 CFU/ml; however, the number was still lower than the initial bacteria number prior to UV disinfection. Therefore, it can be concluded that complete repair did not occur, which means some damages caused by UV light are irreversible. Zimmer *et al.* (2002) observed that pathogenic *E. coli* O157:H7 can do photoreactivation rapidly (in less than 30 minutes) following UV doses of 5 and 8 mJ/cm^2 of UV low-pressure (LP) radiation. Their results suggested that the maximum repair occurred within 30 minutes after the UV exposure, much sooner than that observed in this study. It is possible that the self-repair mechanism for ARBs require a longer time to act than those of sensitive bacteria.

Dark repair of resistant *E. coli* following UV disinfection showed a lower viable bacteria number by 0.2 to 0.81 log compared to photoreactivation. The percentage of log repair was also observed to be lower by 5% to 18%. This is expected to occur, as photolyase enzymes work more efficiently in the presence of light. However, despite the dark conditions, the resistant *E. coli* were able to repair by up to 62% following exposure to a UV dose of 3 mJ/cm^2 and 43% repair occurred at a UV dose of 10 mJ/cm^2. The bacteria numbers increased as much as 1.13 x 10^4 CFU/ml, from 0 hour (immediately after the UV exposure) to 24 hours after incubation **(Figure 5.19).** The recA gene mainly regulates the dark repair mechanism, which is responsible for nucleotide excision repair, post-replication recombinational repair and error-prone repair (Nahrstedt *et al*. 2005). Similarly, Huang *et al.* (2016) reported that tetracycline-resistant bacteria from the secondary effluent of a wastewater treatment plant were found to be capable of undergoing dark repair with a colony-forming ability of 3-log after 20 mJ/cm^2 UV exposure. They also found that reactivation of tetracycline-resistant bacteria occurred significantly when a high UV dose was applied. However, they did not observe significant differences in the reactivation of tetracycline-resistant bacteria and normal heterotrophic bacteria.

As expected, the log repair values decreased as the UV doses increased; this is due to the greater DNA damaged caused by UV light exposure. Furthermore, there are only 20 photolyase enzymes in *E. coli* and each enzyme can repair only approximately 5 dimers per minute (Friedberg *et al.*, 1995). The

results from this experiment indicating that exposure of UV-disinfected water to light should be avoided to ensure that photoreactivation does not occur; however, this may be unavoidable in the case of wastewater disinfection where water is discharged to the environment soon after UV treatment.

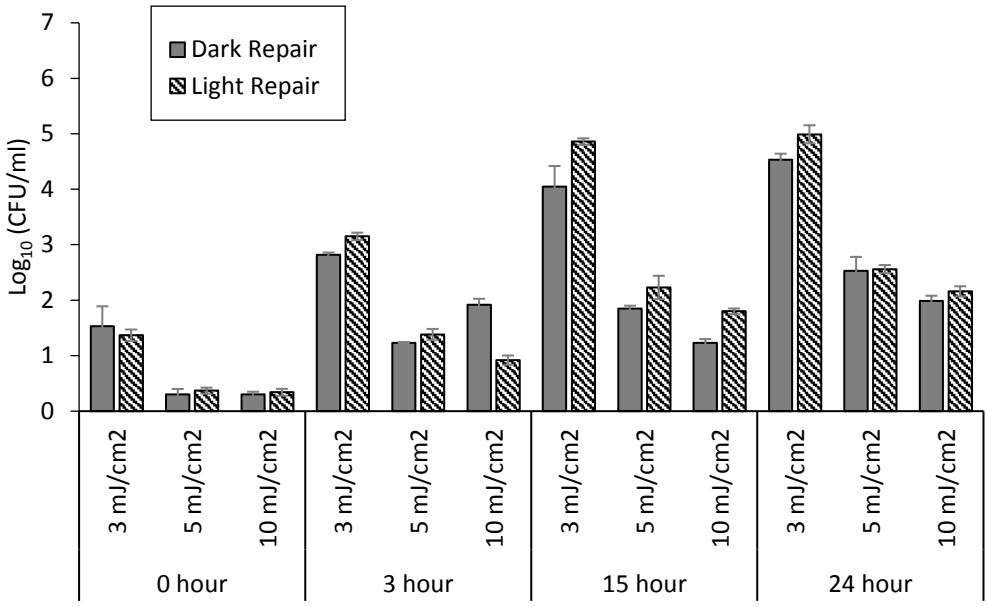
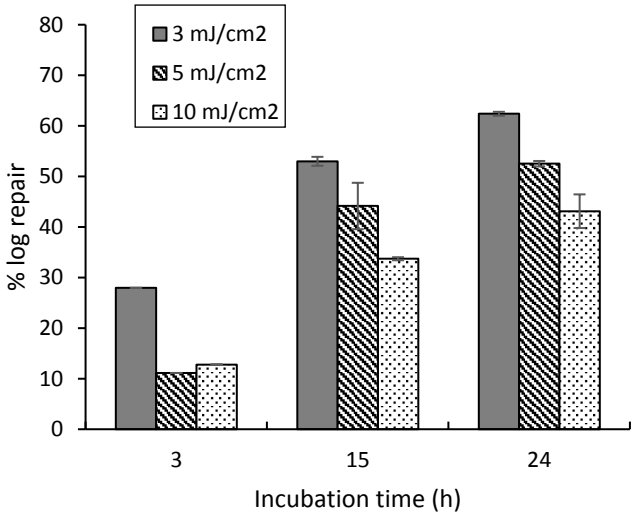
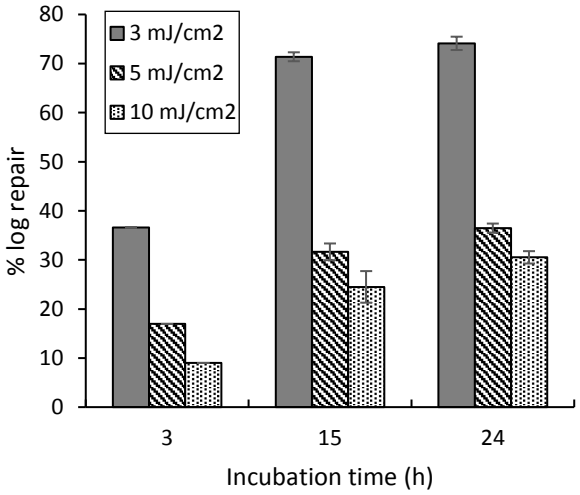


Figure 5.19 Viable number of multiple-antibiotic-resistant *Escherichia coli* NCTC 13400 after incubation in dark and light following low pressure UV disinfection. The error bar represents standard deviation from three replicates.



(a)



(b)

Figure 5.20 Percentage log repair of multiple-antibiotic-resistant *Escherichia coli* NCTC 13400 after incubation in dark **(a)** and light **(b)** following low pressure UV disinfection. The error bar represents standard deviation from three replicates.

There are only a few studies on the post-UV disinfection repair of *Pseudomonas aeruginosa* in wastewater (Zhang *et al.* 2015), and none of these studies have focused on resistant *P. aeruginosa* particularly in drinking water. In this experiment, a MAR *P. aeruginosa* NCTC 13437, which produced extended spectrum beta-lactamase, was used as the model bacteria.

The number of *P. aeruginosa* at time 0 (immediately after the disinfection) ranged from 1.16-log to 2.4-log; this initial number was higher than that of resistant *E. coli*. There was no difference in *E. coli* or *P. aeruginosa* numbers due to dark repair conditions ($p=0.35$). After 24 hours of light incubation the number of viable *P. aeruginosa* increased to 4.8-log for a UV dose of 3 mJ/cm^2 and 4.3-log for a UV dose of 5 mJ/cm^2. As shown in **Figure 5.21**, the viable number of bacteria for both light and dark incubation was similar ($p>0.05$). In the same way as was observed for resistant *E. coli*, the photoreactivation of *P. aeruginosa* also started 3 hours after the UV exposure, with 24%, 37% and 34% repair observed for UV doses of 3, 5 and 10 mJ/cm^2, respectively. Resistant *P. aeruginosa* followed the same trend as resistant *E. coli*, as the highest repair occurred within the first three hours of incubation. The maximum repair value for *P. aeruginosa* was 60% at 24 hours incubation, with a UV dose of 3 mJ/cm^2. This value is lower by 14% than resistant *E. coli*, therefore *P. aeruginosa* might have a lower photo repair capacity than *E. coli*. A study by Simonson *et al.* (1990) revealed that the existence of IncP9 plasmid in wild strain *P. aeruginosa* dramatically increased the UV resistance. Furthermore, IncP9 plasmid in *P. aeruginosa* is associated with degradation and antibiotic and toxic metal-resistance markers.

In terms of the dark incubation, high repair was observed after 24 hours incubation in all the UV doses applied. With a UV dose of 10 mJ/cm^2, from 15 hr to 24 hr incubation time, the repair increased by 24%. Interestingly, this trend was not observed in *E. coli* dark repair. Nevertheless, the maximum repair still occurred within the first three hours of incubation. Jungfer *et al.* (2007) reported that the *rec*A gene in *P. aeruginosa* was up-regulated within 2 to 6 hours of dark incubation. They also observed the activity of the *recA* gene, even after UV disinfection higher than 400 J/m^2. It has been suggested that the *recA* protein might also play an important role in dark repair regulation in *P. aeruginosa* by induction of the bacteria SOS system (Kidambi *et al.*, 1996). The number of resistant *E. coli* and *P. aeruginosa* still

increased at 24 hours of dark incubation. This trend indicates the possibility that the repair might continue for a longer time. Modifi *et al.* (2002) reported that naturally occurring heterotrophic bacteria undergo regrowth up to 1 week following treatment by low pressure UV disinfection.

In terms of comparison between antibiotic-resistant *E. coli* and *P. aeruginosa* in dark and light repair ability. The statistical analysis showed that there is no significant different in their dark repair rate ($p>0.05$), however in the lighted experiment, *P. aeruginosa* showed higher repair than *E. coli* ($p<0.05$). Several factors are known to affect bacteria repair system, including external environment of an organism and internal environment of the cell as well as horizontal gene transfer (Aravind *et al.* 1999).

As mentioned in the method section, a low-pressure UV lamp was used in this experiment. Studies have shown that compared to the medium-pressure (MP) lamp, the LP lamp may increase the ability of bacteria to repair (Quek and Hu, 2008; Zimmer *et al.*, 2007). This is due to inhibition from specific wavelengths that are present in a medium pressure lamp but not in the LP lamp. The difference in the repair ability of resistant *E. coli* and *P. aeruginosa* observed in this study might be caused by several factors, including: (1) there might be a variance in the number of initial cell densities used in the experiment for both bacteria; (2) the difference in growth characteristics between *E. coli* and *P. aeruginosa*. Besides species- or strain-specific responses, UV susceptibility and the potential to repair UV-induced damage after radiation may also be influenced by the physiological state of the cells with highest sensitivity during active growth (Malley *et al.*, 2004) as well as by temperature (Salcedo *et al.*, 2007) or by the intensity and spectra of visible light during photorepair (Bohrerova and Linden, 2007).

Typically, treated water is transported through a dark distribution pipe system; therefore, photoreactivation is normally less of a concern in drinking water treatment. However, there are potential photoreactivation issues if UV treatment occurs prior to a process unit that allows light exposure. Also, photoreactivation cannot be ruled out if water is exposed to light when it leaves the water distribution system, e.g. customers storing the water where it is exposed to the light. The fact that antibiotic-resistant *E. coli* and *P. aeruginosa* can undergo repair even in dark conditions highlights the importance of post-UV disinfection monitoring in drinking water distribution systems. Moreover, the presence of antibiotic-resistant *Pseudomonas spp.* in the tap water samples in this study, as mentioned in section

4.1.6, indicates that this group of bacteria are able to grow and strive in disinfected water. Finally, in many countries (e.g. the UK, USA) drinking water regulations would require application of a chlorine dose following UV disinfection, which might influence these UV repair processes.

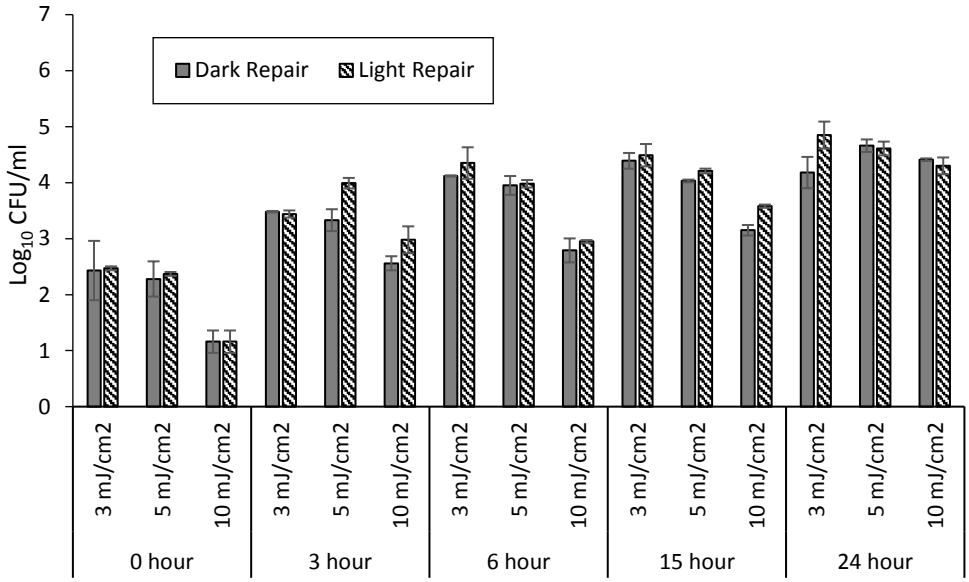
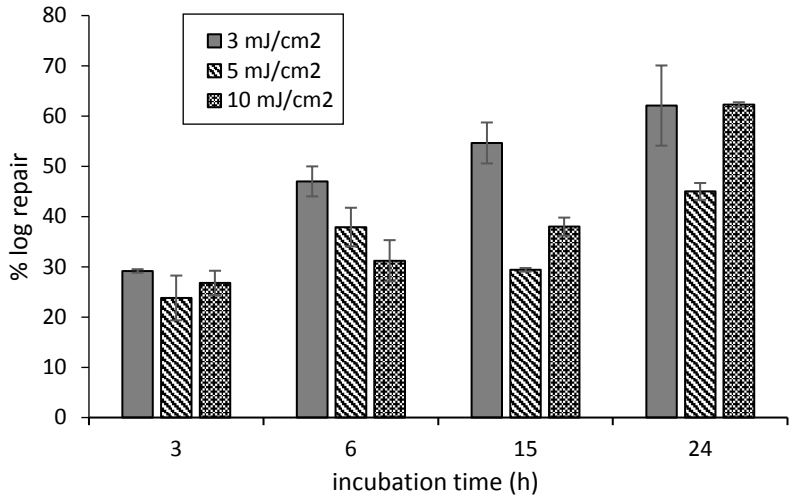
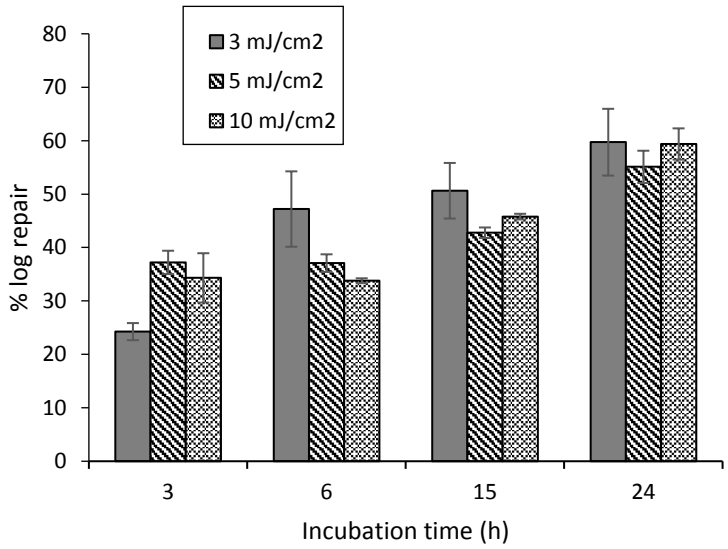


Figure 5.21 Viable number of multiple-antibiotic-resistant *Pseudomonas aeruginosa* NCTC 13437 after incubation in dark and light conditions following UV LP disinfection. The error bar represents standard deviation from three replicates.



(a)



(b)

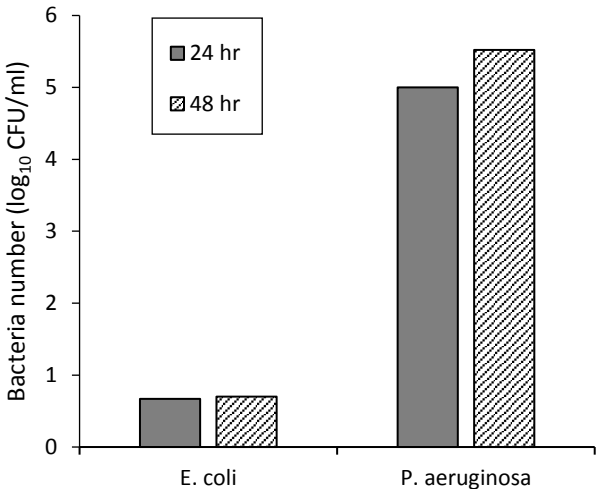
Figure 5.22 Percentage log repair of multiple-antibiotic-resistant *Pseudomonas aeruginosa* after incubation in the dark **(a)** and light **(b)** following UV LP disinfection. The error bar represents standard deviation from three replicates.

5.6 Photo-repair of Antibiotic-resistant *Escherichia coli* and *Pseudomonas aeruginosa* after High UV Dose

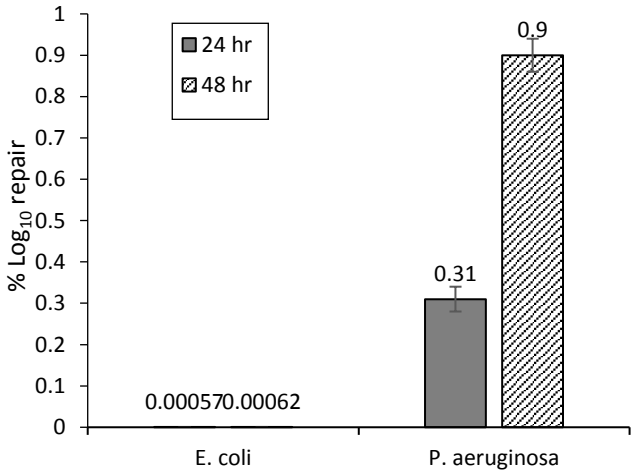
Antibiotic-resistant *E. coli* and *P. aeruginosa* were exposed to a UV dose of 40 mJ/cm^2 in order to investigate their photoreactivation ability at a typical UV dose normally applied in real drinking water treatment plants. The results are presented in **Figure 5.23**.

Photoreactivation is evident for both antibiotic-resistant *E. coli* and *P. aeruginosa*, although the numbers of viable bacteria were much lower than those with low UV dose exposure (section 5.5). The number of antibiotic-resistant *E. coli* was only 0.67-log after 24 hours and 0.7-log after 48 hours of incubation, while for resistant *P. aeruginosa* the viable number was much higher at 5-log and 5.52-log after 24 and 48 hours incubation, respectively. Nevertheless, only a small amount of repair was observed after high UV exposure, with less than 1-log repair for both bacteria. One possible explanation for this is that the signal of the *rec*A gene in *P. aeruginosa* is reported to decrease as the UV dose increases (Jungfer *et al.*, 2006) and bacteria are damaged more extensively. Nevertheless, Quek and Hu (2008) also observed photoreactivation of *E. coli* ATCC 15597 after UV exposure of 20 and 40 mJ/cm^2, while no photo repair was observed at a UV dose of 60 mJ/cm^2. Conversely, Wang *et al.* (2011) detected photoreactivation of *E. coli* DH5α following a UV dose of 60 mJ/cm^2. Thymine dimers absorb light in visible spectra that will lead to self-repair of the nucleotide bonds, furthermore photolyase enzyme which can reverses UV-induce DNA damage needs to active by energy which includes light at 300-400 nm (Kowalski, 2009).

The results indicated that ARBs, particularly *E. coli* and *P. aeruginosa*, have the capacity to photorepair themselves even at practical UV doses that are normally used in drinking water treatment plants, although normally water would be distributed in light-free piped systems and often with a chlorine residual present (e.g. in the UK and USA).



(a)



(b)

Figure 5.23 (a) Bacteria number log CFU/ml and **(b)** percentage of log repair of multiple-antibiotic-resistant *Pseudomonas aeruginosa* after incubation in light following UV LP disinfection of 40 mJ/cm^2. The error bar represents standard deviation from three replicates

5.7 The Potential Synergistic Effects of Sequential Use of UV and Chlorine in Inactivating Antibiotic Resistance Genes in Waterborne Bacteria

While chlorination disinfection alone may have a good effect on ARG removal, as described in section 5.3.3, the use of chlorine as a disinfectant is increasingly debated due to the formation of toxic, mutagenic/carcinogenic disinfectant by-products such as trihalomethane and haloacetic acids. Furthermore, it has been shown in section 5.4 that UV disinfection alone cannot achieve significant inactivation of ARGs. Therefore, in this section a combination of UV disinfection and chlorination is investigated and the potential of synergistic effects in the inactivation of four ARGs is explored. Synergy here means that the efficiency of combined disinfection methods is greater than the efficiency achieved when summing the effects of individual disinfectants.

The sequential application of two different disinfectants is a common practice in many water treatment plants. This technique fulfils the need for an efficient on-site agent and long-lasting agent to ensure a minimum residual concentration in distribution systems**.** The sequential process (UV-Cl_2) is a practical practice adopted in water treatment plants, where UV is used for pre-disinfectant and chlorine is then added to provide residual in UV-treated water. In this study, three UV dosages were used (50, 100 and 200 mJ/cm^2) and two chlorine doses (1 and 2 mg/L with a contact time of 15 minutes); these are typical UV and chlorine doses used in water treatment plants. The sequential use of UV and chlorine has been shown to be more effective in inactivating resistance genes compared to the use of chlorine or UV alone. **Table 5.13** shows the efficiency of chlorine and UV disinfection alone.

Table 5.13. Inactivation of resistance genes after treatment with chlorine or UV

Disinfectant agents	Log_{10} resistance genes (N/N_0)			
	***Bla*-$_{TEM1}$**	***Tet*-A**	***Sul*1**	***Mph*(A)**
Chlorine 1 mg/L	-0.15±0.09	-0.19±0.04	-0.06±0.03	-0.31±0.09
Chlorine 2 mg/L	-1.73±0.3	-1.14±0.14	-1.51±0.21	-1.73±0.12
UV (50 mJ cm^{-2})	-0.44±0.1	-0.26±0.2	-0.05±0.02	-0.12±0.13
UV (100 mJ cm^{-2})	-0.68±0.01	-0.33±0.1	-0.15±0.02	-0.28±0.11
UV (200 mJ cm^{-2})	-1.22±0.04	-0.74±0.07	-0.40±0.05	-0.41±0.16

Figure 5.24 summarises the effect of UV alone, chlorination and sequential UV/chlorination treatment on the inactivation of ARGs. The application of UV disinfection and a relatively low chlorine dose e.g. 1 mg/L resulted in slight inactivation, with the Δ ranging from 0.04 to 0.49-log. The highest Δ inactivation was achieved in the *mph*(A) gene with a value of 0.49-log after treatment with 100 mJ/cm^2 UV followed by 1 mg/L of Cl_2. Meanwhile, the *tet*(A) gene at a UV dose 200 mJ/cm^2 and 1 mg/L Cl_2 showed the lowest Δ inactivation by 0.04-log. A synergistic effect was observed in the inactivation of the *sul*1 gene at all UV doses, in the *bla*-$_{TEM1}$ gene at UV doses of 50 and 200 mJ/cm^2, in the *tet*(A) gene at a UV dose of 100 mJ/cm^2 only, and in the *mph*(A) gene at UV doses of 100 and 200 mJ/cm^2. The highest synergistic effect of sequential UV and chlorine at 1 mg/L was achieved in the *mph*(A) gene with a synergy value of 0.25-log.

The use of UV followed by 2 mg/L chlorine resulted in much greater reductions of all resistance genes; the inactivation was more than 1.1-log higher compared to the use of UV disinfection alone. The increase in the inactivation rate was statistically significant ($p<0.05$) for all tested genes. *Tet*(A) gene gave Δ log inactivation of 1.6 after being treated with UV fluence of 200 mJ/cm^2 followed by 2 mg/L of chlorine. The highest inactivation was achieved by the *mph*(A) gene, with Δ inactivation at a UV dose of 200 mJ/cm^2 followed by 2 mg/L of chlorine; inactivation reached 2.4-log. The inactivation of *mph*(A) was only 0.41-log after being treated with a UV dose of 200 mJ/cm^2; the reduction increased to 2.8-log with UV and chlorine treatment.

The summaries of the log synergy values achieved from combination UV/chlorine are shown in **Table 5.14**. A synergistic effect was found in the inactivation of the *tet*(A) gene in all UV doses, in the *bla*-$_{TEM1}$ gene at a UV dose of 200 mJ/cm^2, in the *sul*1 gene at UV doses of 100 and 200 mJ/cm^2, and in the *mph*(A) gene at UV doses of 100 and 200 mJ/cm^2. The maximum synergistic effect of the sequential use of UV and chlorine at 2 mg/L was found in the *mph*(A) gene with a synergy value of 0.6-log. Interestingly, only the *tet*(A) gene showed synergy when a combination of UV 50 mJ/cm^2 and chlorine 2 mg/L was used. The results suggested that the synergy occurred rapidly when high UV radiation was used, e.g. 200 mJ/cm^2. Free chlorine mainly reacts with elements of bacterial cell walls such as amino acids and membrane-bound proteins, while UV light is highly reactive towards nucleic acids (Dodds, 2012). Therefore, the synergy observed in this study may be due to the decrease in the bioactivity effected by UV irradiation, causing the chlorine to react with the cells more readily.

In terms of the relationship between the inactivation of different ARGs with chlorination and UV disinfection processes, it was found that the inactivation of the *bla*-$_{TEM1}$ gene was always higher than that of other tested genes, indicating that the *bla* genes were easier to remove. Zhang *et al.* (2015) reported that sequential UV and chlorination disinfection improved the inactivation of *sul*1, *tet*X, *tet*G and *intl*1 resistance genes in wastewater, with the highest synergy value being achieved in the *tet*G gene (0.21-log) with treatment with 249.5 mJ/cm^2 UV and 25 mg/L chlorine. Similar to this study, they also observed that the inactivation of the *tet* genes was higher than that of the *sul* genes.

Wang *et al.* (2012) revealed that the complete inactivation of HPC in wastewater was achieved by treatment with 15 mJ/cm^2 UV followed by 1.5 mg/L chlorine. The total bacteria count was completely inactivated by the sequential application of 40 mJ/cm^2 UV and 2 mg/L chlorine. Rattanakul *et al.* (2014) also observed an increasing trend of MS2 inactivation when treated with UV followed by chlorine. Interestingly, they revealed that the simultaneous use of UV and chlorine resulted in higher synergy values. This synergy might be due to the fact that the advanced oxidation process (AOP) occurred during the disinfection processes. They observed the presence of free hydroxyl radicals (OH•) and chlorine radicals (Cl•) during the treatment, while in this experiment, no hydroxyl radicals were present.

The application of pre-disinfection with an oxidant capable of yielding substantial cell envelope damage (e.g. ozone or chlorine dioxide), followed by post-disinfection with an oxidant having higher selectivity for DNA might substantially enhance the susceptibility of intracellular DNA to damage in comparison to treatment with a single disinfection (Cho *et al.,* 2006). The use of UV followed by chlorination is more favourable, because of the chlorine decay problem due to the photo-degradation caused by UV light. The synergy observed in this study may be due to the decrease in the bioactivity affected by UV irradiation, causing the chlorine to react with the cells more readily (Zhang *et al.,* 2015). The mechanism of synergy might also be explained by a multiple damage mechanism – two different disinfections might cause two distinctive types of damage or injury to microorganisms. Free available chlorine primarily damages cell walls, whilst UV irradiation mainly attacks purines and pyrimidines and nucleic acid constituents (Dodds, 2012).

In conclusion, the sequential application of UV and chlorine in water treatment appears to enhance water security and quality in terms of ARGs. The combination of a UV dose of 200 mJ/cm^2 followed by 2 mg/L chlorination resulted in more than 2-log reduction of all tested ARGs.

Table 5.14. Summary of log synergy value from different UV and chlorine dose applications

Disinfectant agents	**Log synergy value**			
	***tet*(A)**	***mph*(A)**	***sul*1**	***bla*-$_{TEM1}$**
UV (50 mJ cm^{-2}) followed by 1 mg/L Cl_2	0.03	-	0.1	0.09
UV (100 mJ cm^{-2}) followed by 1 mg/L Cl_2	0.03	0.18	0.29	0.18
UV (200 mJ cm^{-2}) followed by 1 mg/L Cl_2	-	0.17	0.25	0.23
UV (50 mJ cm^{-2}) followed by 2 mg/L Cl_2	0.13	-	-	-
UV (100 mJ cm^{-2}) followed by 2 mg/L Cl_2	0.5	0.09	0.01	-
UV (200 mJ cm^{-2}) followed by 2 mg/L Cl_2	0.61	0.62	0.29	0.09

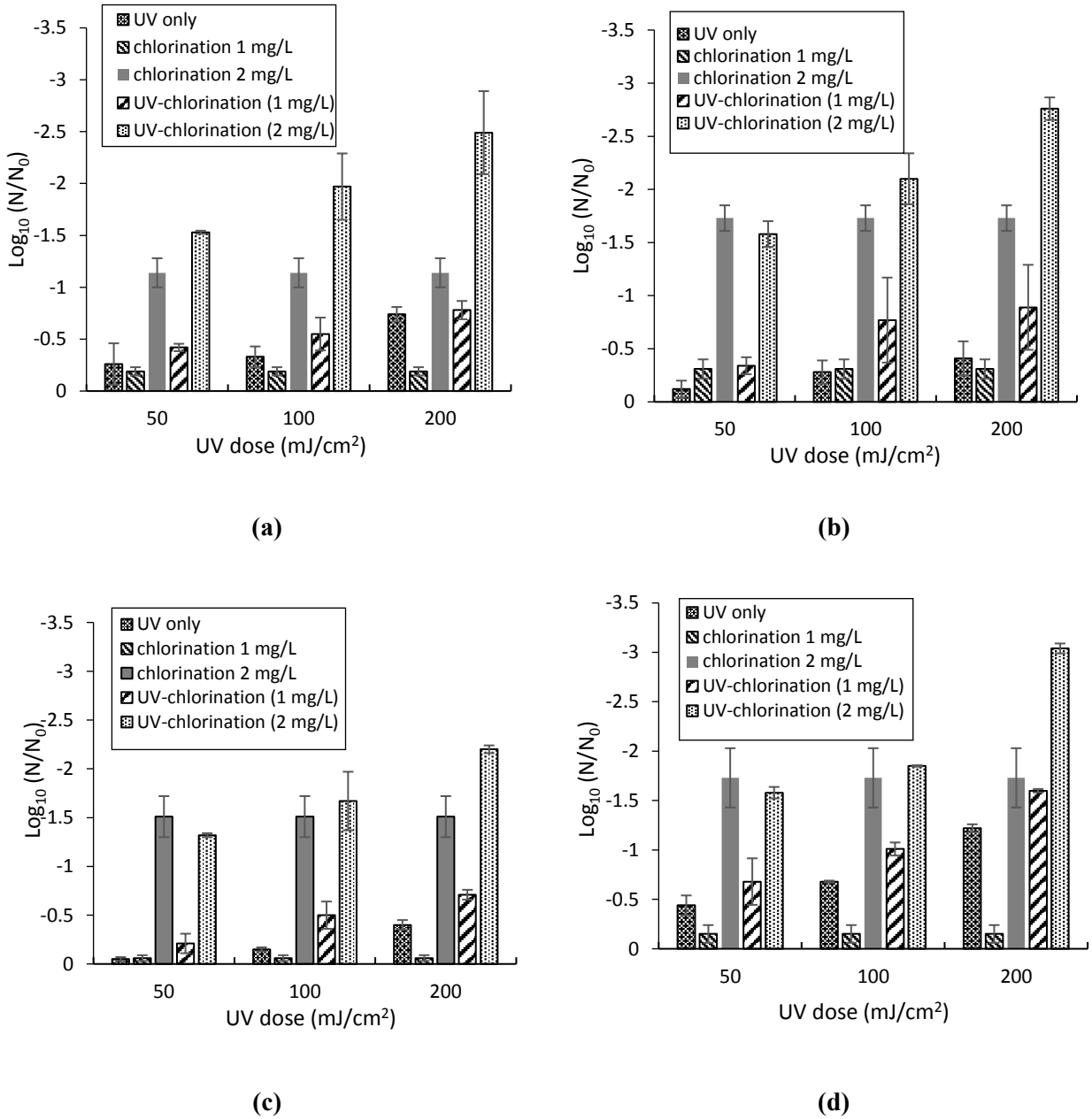


Figure 5.24. The effect of UV alone, chlorination and sequential UV/chlorination treatment on the inactivation of ARGs **(a)** *tet*(A) **(b)** *mph*(A) **(c)** *sul*1 and **(d)** *bla*-$_{TEM1}$ genes. The horizontal axis indicates three different UV dosages – 50, 100 and 200 mJ/cm^2, respectively. The error bar represents standard deviation from three replicates.

5.8 Determination of Oxidative DNA Damage caused by Chlorination and UV Disinfection by HPLC

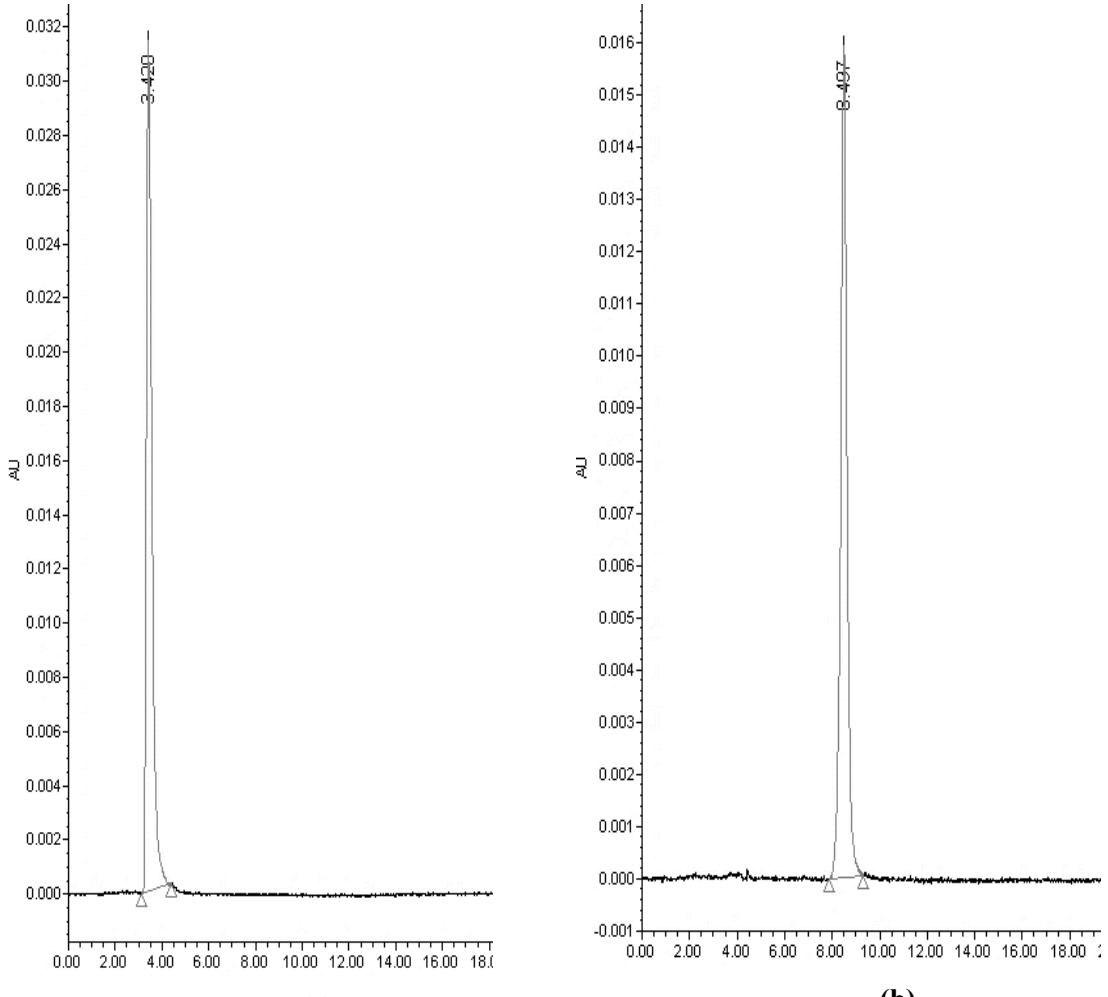
Common water disinfectants such as chlorine and UV can produce reactive oxygen species (ROS) causing oxidative damage to DNA. Chlorine is generally considered to be a highly destructive, nonselective oxidant that reacts with a variety of subcellular compounds and affects metabolic processes (Dodds, 2012). On the other hand, UVB and UVC radiation can cause the formation of DNA photoproducts such as thymine cyclobutene, and cis-syn cyclobutane-pyrimidine dimers (CPD) (Douki *et al*., 2000).

Several analytical methods have been developed to measure DNA oxidative damage, including gas chromatography-mass spectrometry (GC-MS) and HPLC coupled with ECD or MS. GC-MS is considered to be a highly sensitive method to measure oxidative DNA damage; however, the derivatisation procedures required for GC-MS are complicated and slow, and might produce additional DNA damage. In this experiment, efforts have been made to measure DNA damage products, including 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodGuo) and 5-hydroxyuracil (5-OHU) using HPLC coupled with a photodiode array detector under typical chlorination and UV disinfection doses applied in water treatment. Hydroxyl radicals, which are the most active species from the ROS, mainly react with C_4-C_5 double bonds of pyrimidines forming thymine and uracil glycol, 5-hydroxyuracil (5-OHU) and 5-hyroxycytosine (5ohC). The C_8 of purines form 8-oxoG (Evans and Cooke, 2007). The HPLC techniques that have been developed give a good separation of 8-oxodGuo and 5-OHU as shown in **Figure 5.25**. The 8-oxodGuo peak appeared after 8.4 minutes and 5-OHU was detected after 3.4 minutes.

The DNA that was treated with chlorine gave good separation; there are five peaks visible from the separation. **Figure 5.26** shows the separation of chlorinated treated DNA. UV-irradiated DNA also shows good separation, as there are four peaks visible from the separation. All of the peaks appeared within seven minutes of the HPLC running, as shown in **Figure 5.26.** Interestingly, 5-OHU was detected in both chlorinated DNA and UV-irradiated DNA samples; nonetheless, 8-oxodGuo was not

detected in either the chlorinated or UV samples. Incomplete DNA digestion by formic acid might be a plausible explanation for 8-oxodGuo not being detected in the DNA sample.

The concentration of 5-OHU was successfully generated from the standard curve calibration, resulting in a linear curve in the concentration ranging from 1 to 10 mg/L (r^2=0.99). The concentration of 5-OHU in chlorinated DNA was detected at 0.6 mg/L, while the concentration of 5-OHU in UV-irradiated DNA was 1.5 mg/L. One possible explanation for this is that UV irradiation has a higher selectivity towards DNA by absorbing thymine and pyrimidines, resulting in higher DNA oxidation products. The higher DNA photoproduct resulting from UV exposure indicating that UV attacks DNA more readily than chlorine, though it might also suggest that chlorine produces a different oxidative DNA damage product.



(a) **(b)**

Figure 5.25 HPLC elution profile of a mixture of **(a)** 5-OHU and **(b)** 8-oxodGUo. A C_{18} Luna column was used, with a gradient of acetonitrile in 2mM ammonium formate as the mobile phase.

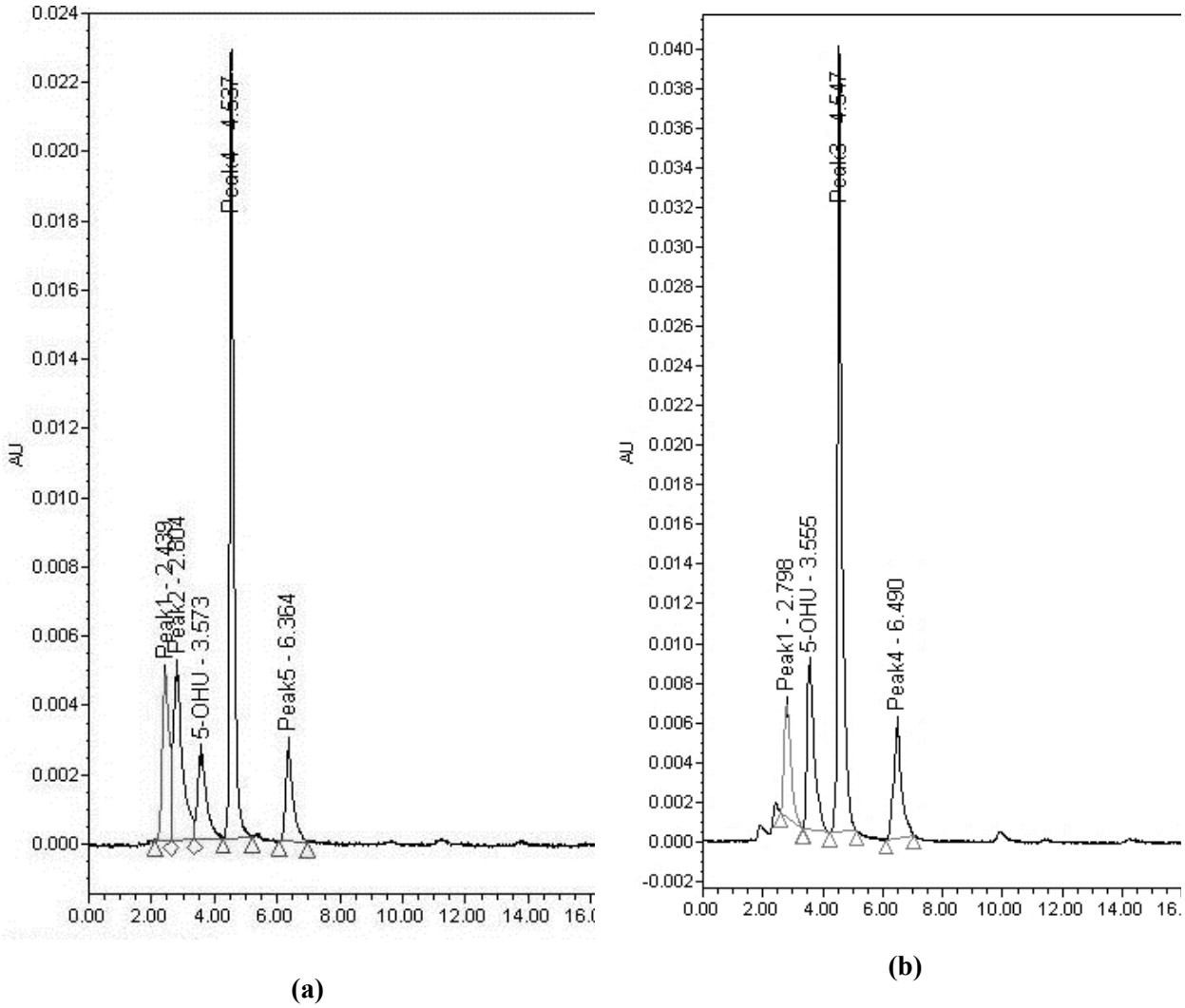


Figure 5.26 HPLC elution profile of a mixture of **(a)** chlorinated DNA and **(b)** UV-irradiated DNA. A C_{18} Luna column was used, with a gradient of acetonitrile in 2mM ammonium formate as the mobile phase.

CHAPTER 6

DISCUSSION

Antibiotic-resistant bacteria and antibiotic resistance genes are currently discussed as emerging environmental contaminants. The continuing heavy use of antibiotics in human communities, agriculture and the veterinary industry will lead to an even greater problem in future. This research was carried out to understand the occurrence and fate of ARBs and ARGs in drinking water systems as well as the behaviour of these resistances to UV and chlorine disinfection. Antibiotic resistance in wastewater effluent, rivers and other aquatic environments has been widely reported. However, it was only recently that drinking water, along with its treatment processes, began to be considered as potentially prone to antimicrobial resistance. Therefore, there was sparse information regarding the presence and prevalence of ARBs and ARGs in drinking water systems at the start of this research.

6.1 The Prevalence of Antibiotic-resistant Bacteria and Antibiotic Resistance Genes and Heterotrophic Resistant Bacteria Diversity in Drinking Water in London

The first part of this research focused on the presence of several ARBs including vancomycin, erythromycin, amoxicillin, ciprofloxacin, tetracycline, trimethoprim and ARGs, namely, *bla*-$_{TEM1}$, *sul*1, *tet*(A), *mph*(A), and *dfrA*7 for resistance to beta-lactams, sulfonamides, tetracycline, macrolides and trimethoprim antibiotics respectively, in municipal drinking water. Antibiotic-resistant bacteria were found in all tap water samples in London. There was a high prevalence of trimethoprim-resistant bacteria. The order of the percentages of ARBs in tap water was: trimethoprim > erythromycin > vancomycin > amoxicillin > ciprofloxacin > tetracycline. The results reflect the amount of antibiotic consumption in the UK. **Table 6.1** shows antibiotic consumption (as active ingredients in tonnes) in the UK for human and veterinary use, it can be seen that β-lactams antibiotics (i.e. including penicillin and amoxicillin) were the highest active ingredients used among others. Interestingly, despite the high use of tetracycline in veterinary industry, low tetracycline resistant bacteria were detected in drinking water sample in this study. This result indicates that antibiotic consumption might not be the only factor determining the presence of resistant bacteria in the environments, although it is generally accepted that antibiotic resistant bacteria and genes are ubiquitous, the selection pressure for the emergence of

antibiotic resistance is mostly anthropogenic. Even in the absence of direct pressure from an antibiotic, mobile genetic elements which contain antibiotic resistance might be favoured by co-selection by other substances such as metals and biocides (Baker-Austin *et al.* 2006; Wales and Davies, 2015). Further, Frost *et al.* 2018 showed that low antibiotic doses select for resistant strains. Another important factor that shaped the antibiotic resistance profile in environment is the existence of novel resistance genes inside the bacteria itself. However, in the current study, the aspects mentioned above were not considered, therefore the main hypothesis for the presence of antibiotic resistant bacteria and genes in this study is the pressure from antibiotics use. Antibiotic exposure has been known to increase the mutation and recombination frequencies in bacteria even at sub-inhibitory level via the SOS response (Beaber *et al.* 2004; Lopez *et al.* 2007; Blazquez *et al.* 2012). Using population and genetic methods and epidemiological observation Austin *et al.* (1999) reported that significant reductions in resistance will only occur from equally significant changes in antibiotic use pattern; furthermore, the decay in resistance after a reduction in prescribing volume will typically occur on a slower time scale than the rise in resistance once the critical threshold drug consumption is reached.

Table 6.1. Antibiotic consumption as active ingredients adapted from Public Health England (2017)

Antibiotics	Human consumption (tonnes)	Animal /veterinary consumption (tonnes)	Total (tonnes)
β-Lactams (including penicillin)	350	91	441
Tetracycline	55	182	237
Macrolides	52	43	95
Trimethoprim	18	61	79
Cephalosporin	18	5	23
Fluoroquinolones	12	3	15
Aminoglycosides	1	4	5

By comparing the presence of antibiotic resistant bacteria in relatively pristine environment (i.e. which can be translated as background levels of ARB), the effect of anthropogenic use of antibiotics to the spread of the drug resistance can be predicted. **Figure 6.1** shows the comparison of antibiotic resistant bacteria prevalence among heterotrophic bacteria from this study and Hatha *et al.* (2015), which was conducted in Kongsfjord, a relative pristine environment that offers very little selection pressure for

antibiotic resistance. It can be seen that the prevalence of amoxicillin, trimethoprim and ciprofloxacin were indeed higher in this study compared to that study by Hatha et al. The presence of antibiotic resistant bacteria has been reported in other natural environments such as caves, deep and ancient ice cores, permafrost, and arctic wetlands (Diaz *et al.,* 2017 and D'Costa *et al.,* 2011). Additionally, resistance genes are often encoded on the same gene cluster as antibiotic biosynthesis pathway genes (Martin and Liras, 1989) making it possible for resistance to be present in microbial communities without human influence. It is generally accepted that antibiotic resistant bacteria and genes are indeed present ubiquitously; the selection pressure for the emergence of antibiotic resistant mutant is mostly anthropogenic. The use and misuse of antibiotics have caused the enrichment of small subset of antibiotic resistance genes that can be found in natural microbiota even from the pre-antibiotic era (Martinez and Baquero, 2014).

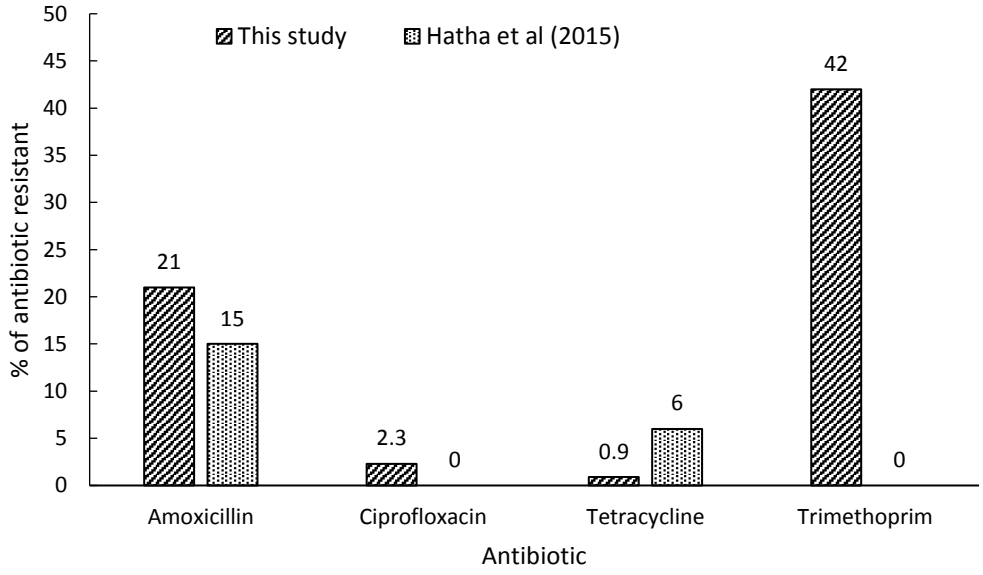


Figure 6.1 Comparison of antibiotic resistant bacteria prevalence among heterotrophic bacteria from current study and Hatha *et al.* (2015)

The prevalence of ARBs was not influenced by seasonal differences (i.e. there were no significant differences between sampling periods in the percentage of resistant bacteria). To explain this result, an analysis of the correlation between ARB and tap water characteristics was performed. It has been demonstrated that temperature, which arguably would play a role in the resistance pattern, did not show any correlation with the ARB occurrences. Additionally, a small separate sub-study was conducted to

compare ARBs and ARGs in chlorinated and chloraminated distribution systems with similar source waters (data can be found in Appendix D). The chloraminated system showed higher prevalence and abundance of ARBs and ARGs, perhaps a reflection of the weaker disinfecting power of chloramines relative to chlorine, even though a higher chloramine residual was present than the chlorine residual. The results from this study also suggest that occurrences of ARBs and ARGs tend to increase with water age in the drinking water distribution system, though some exceptions were observed. It is recommended that this small study be extended in future research to confirm these initial findings.

Quantitative real-time PCR was used to quantify ARGs including, *bla*-$_{TEM1}$, *tet*(A), *sul*1, *mph*(A) and *dfrA*7. The copy number of ARG/100 ml was then normalised with the copy number of 16S rRNA to obtain the relative abundance of each ARG in the water samples. All tested ARGs were detected in all tap water samples, except for *mph*(A), which was not found at sampling point 4. The results agreed with previous studies that also discovered *bla*-$_{TEM1}$ (Xi *et al.*, 2009; Shi *et al.*, 2013), *tet*-A (Shi *et al.*, 2013), *sul*1 (Xi *et al.*, 2009; Shi *et al.*, 2013; Adesoji *et al.*, 2016) and *dfrA*7 gene (Adesoji *et al.*, 2016) in municipal drinking water. The presence of the *mph*(A) gene in drinking water had not been previously reported. Nevertheless, the *mph*(A) gene is mostly located in the plasmid, which makes it prone to further dissemination to other non-resistant bacteria. The most abundant ARG in tap water was the *sul*1 gene, with the average quantity of 1.4 log/100 ml of water sample, higher by 0.2 – 1.3 log than the other tested genes. This result is in agreement with other studies on drinking water, which also observed the high abundance of the *sul*1 gene (Xi *et al.*, 2009; Shi *et al.*, 2013). In terms of the relative abundance of ARGs in the total bacteria population, the highest was *bla*-$_{TEM1}$ followed by *sul*1. It is noteworthy that the quantity and abundance of resistance genes was influenced by seasonal differences. The quantity of genes was higher in spring (April) than in summer (July); in contrast, their relative abundance was lower in April than in July.

Water bodies have been suggested to be important in facilitating the transport and transfer of the antibiotic resistance genes (Aminov and Mackie, 2007; Baquero *et al.,* 2008). Drinking water and wastewater treatment plants often receive bacteria from different sources, thus establishing potential compartments where environmental, human and or animal bacteria can coexist, particularly within a

biofilm system (Baquero *et al.,* 2008). Bacteria that are attached to the biofilm are known to be more resistant than the planktonic bacteria in water (Ceri *et al.,* 1999) and in one previous study a higher level of ARGs was found from the pipe than in WTP effluents due to biofilm growth in the pipeline system (Xi *et al.,* 2009). These coexistences could play a role in shaping antibiotic resistant genes and bacterial diversity in tap water samples, as it can be seen from different resistance gene types found in the current study.

Resistance is often associated with reduced bacterial fitness (Andersson and Levin, 1999). Plasmids carrying drug resistance genes usually impose a fitness cost on the bacteria, which resulting in a lowered growth rate. Studies also shown that horizontal gene transfer (HGT) as one of the main mechanisms to the spread of antibiotic resistance might reduce bacterial fitness (Elena *et al.,* 1998; Knoppel *et al.,* 2017). For this reason, it is arguable that resistant bacteria will need higher nutrition to conserve their resistance properties compared to non-resistant bacteria. However, a study by Paulander *et al.* (2009) revealed that streptomycin-resistant *Salmonella enterica* grows faster than wild type bacteria in media with poorer carbon sources, proving that RpOs, key in the regulation of stress-inducible genes, was induced in the wild type cells, but not in resistant bacteria. These phenomena certainly will affect the behaviour of antibiotic resistant bacteria in drinking water distribution systems that have limited nutrient availability. In this study, the proportion of non-resistant bacteria was found to be higher than resistant bacteria, for all antibiotics tested, suggesting that resistant bacteria might require higher nutrition than non-resistant bacteria to maintain their resistance genes and/or plasmids in drinking water distribution system.

The dominant resistant heterotrophic bacteria isolated from tap water were identified as *Pseudomonas spp*. This study highlighted the presence of potentially resistant opportunistic pathogen bacteria, including *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Stenotrophomonas maltophilia*, *Burkholderia cepacia* and *Ralstonia pickettii* in tap water samples. The occurrence of these antibiotic-resistant opportunistic bacteria are a potential threat to public health, particularly when the water is consumed by immunocompromised persons, such as persons with HIV/AIDS, patients receiving cancer chemotherapy or undergoing organ transplantation, elderly persons and infants.

Despite being recognised as an important emerging microbiological risk, the human health consequences from ARB through drinking water is currently still poorly understood. The risk of transmission of antibiotic resistant bacteria from the environment to humans must be assessed based on the frequency of ARB and ARG that are able to multiply in the human body (Manaia, 2017). In this study several opportunistic pathogen bacteria were detected as mentioned above; furthermore, some clinically important ARGs such as *bla*-$_{TEM1}$ and s*ul*1 were found. *Pseudomonas aeruginosa* is known to cause infection among patients with burn wounds, cystic fibrosis, acute leukaemia, organ transplants, and intravenous-drug addiction, therefore, it is reasonable to place *P. aeruginosa* as one of the highest risk species among antibiotic resistant bacteria found in this study. Moreover, *P. aeruginosa* is abundant in various types of moist environments in which they can multiply to large numbers, even in the presence of minimal amount of nutrients. Its polysaccharide capsule enables it to attach to surfaces and biofilm. Additionally, *P. aeruginosa* thrives best in distal parts of water distribution system, such as taps and sinks, where there is sufficient oxygen present (Walker and Hoffman, 2012). However, no data regarding the epidemiology of resistant *Pseudomonas* in the environment were found, thus it is challenging to assess the exact risk of the bacteria to human health.

A recent study by O'Flaherty *et al.* (2018) used an exposure assessment model to predict human exposure of antibiotic resistant *E. coli* through drinking water. Their results suggested that an average of between 3.44 x 10^{-7} and 2.95 x 10^{-1} cfu/day of antibiotic resistant *E. coli* was consumed through tap water. Although resistant *E. coli* contamination is not the main interest in this study and the occurrence of *E. coli* in well-treated drinking water is not expected, their results provide an insight that the exposure of resistant opportunistic pathogen bacteria through drinking water consumption might be higher. This is due to the ability of several bacteria found in this study to form biofilms, such as *Pseudomonas aeruginosa*, *Bukhloderia cepacia* and *Sphingomonas paucimobilis*.

6.2 The Effectiveness of Chlorine and UV in Inactivating ARB and ARGs As Well As Their Regrowth Potential Following Disinfection

The wide occurrences of resistant bacteria found in tap water in this study raised concerns over how effective the current disinfection technologies are in removing these bacteria. There is a potential for

antibiotic-resistant *Escherichia coli* and *Pseudomonas aeruginosa* to be present in treated water. Therefore, the second part of this study dealt with the effectiveness of chlorine and UV to inactivate ARBs and ARGs, and to control their spread following disinfection. This study presented chlorination and UV treatment under the worst-case scenario where water contained high concentrations of antibiotic-resistant pathogens (i.e. *E. coli* NCTC 13400), opportunistic pathogenic bacteria (i.e. *P. aeruginosa* NCTC 13437) and environmental strain trimethoprim-resistant bacteria (identified as *P. luteola*).

Chlorination is by far the most popular disinfection method, although some countries avoid chlorine use in distribution (e.g. Austria, Netherlands). Studies on the effect of chlorination on ARBs have been widely conducted. However, the results reported to-date are contradictory. For example, Templeton *et al.* (2009) indicated that the inactivation of ampicillin-resistant *E. coli* was greater than that of the antibiotic-sensitive one. Conversely, they also found that trimethoprim-resistant *E. coli* was more resistant to chlorine. This study showed that chlorination at relatively low doses (i.e. 1 and 2 mg/L) could effectively kill ARBs, resulting in reductions greater than 3-log. Moreover, the inactivation of antibiotic-resistant *E. coli* and antibiotic-sensitive *E. coli* was similar, with no significant differences observed. On the other hand, an environmental strain of resistant bacteria isolated from tap water was significantly more resistant to chlorine and UV disinfection than reference culture strains. Environmental strain bacteria have been previously exposed to sub-lethal disinfectant agents, such as chlorine, which might have resulted in stable higher resistance to disinfection. Chlorine inactivates the bacteria by attacking its cell wall, which leads to bacterial cell lysis then cell death. Antibiotic-resistant bacteria were readily inactivated by UV exposure of 1 to 7 mJ/cm^2. Interestingly, in UV disinfection it was observed that antibiotic-resistant *E. coli* was more susceptible than antibiotic-sensitive *E. coli*, indicating that resistant bacteria respond differently to disinfection treatments. Nevertheless, if ARB cells are inactivated by chlorine, it is possible that persisting antibiotic resistance genes may horizontally transfer to other microbial cells, giving rise to new ARB (He, *et al.,* 2019). Only two strains of *E. coli* (i.e. NCTC 12241 and 13400) were used in this research, however, there are many strains present in this group of bacteria in the environment with potentially different susceptibilities to

disinfection treatments; therefore further disinfectant studies using a wider selection of strains including wild-types is warranted.

The inactivation experiments in this study were designed to mimic disinfection systems and predict microbial responses to disinfectants in real water treatment. The microorganisms used for the experiments originated from either reference (laboratory) cultures or environmental sources. There are some advantages of using reference strains in disinfection experiments, including increased reproducibility (Gilbert & Brown 1995), limiting interferences from the complex natural water environment and to simplify the interpretation of experimental results (Shang & Blatchley 2001). Unfortunately, disinfection experiments using references culture may not adequately represent actual field conditions, such as those found in distribution systems. Laboratory results using reference cultures for inactivation experiments may overestimate the effectiveness of a given residual concentration. Therefore, precautions should be considered when translating the experiment results in this study into the real field in drinking water treatment. For instance, it has been suggested that changes in culture conditions during laboratory experiments may impact disinfection efficiency by affecting cell permeability, cell composition or growth rate. This might explain why disinfectant concentrations and contact times, determined under typical laboratory conditions, are not always sufficient to control microbial growth or survival in distribution systems. Researchers have also shown that the presence of biofilm in drinking water distribution system play important roles in microbial regrowth (LeChevallier *et al.* 1987; Berry *et al.* 2006).

As mentioned above, bacterial resistance comprises fitness; this is associated with the cost of carrying the resistance plasmid and expressing its genes, while the cost of mutations are related to decreased growth rate. This particular characteristic of ARBs certainly affects the competition between resistant and non-resistant bacteria following disinfection treatment. A recent study showed in a mixed culture, particularly during biofilm formation, the growth of susceptible cells was higher than the resistant ones (Frost *et al.,* 2018). In drinking water distribution system where bacterial communities compete for limited amount of nutrients, resistant bacteria might be outcompeted by susceptible bacteria for this reason.

The reduction in ARG levels, which was determined by qPCR, was less significant than that of ARBs. For instance, to achieve substantial ARG inactivation requires UV doses 2 log magnitude higher than for resistant bacteria. Antibiotic resistance genes, including *tet*(A), *bla*-$_{TEM1}$, *sul*1, and *mph*(A) could not be eliminated even after UV exposure of 200 mJ/cm^2, with inactivation ranging between 0.4 and 1.2-log. On the other hand, the gene inactivation at a UV dose of 50 mJ/cm^2 (the recommended UV dose is 40 mJ/cm^2) was only achieved up to 0.05-log. In terms of the relationship between different ARGs in the UV disinfection process, it was found that the inactivation efficiency of the *bla*-$_{TEM1}$ gene was higher than that of other tested genes, indicating that bla genes were easier to reduce. It is known that UV irradiation forms dimers in nucleic acids, with thymine dimers occurring more frequently than cytosine dimers. The number of potential thymine dimers of the *bla*-$_{TEM1}$ gene is slightly higher than those of the *sul*1 and *mph*(A) genes. Additionally, gene size might also play a role in ARG inactivation, as larger genomes inherently possess more targets for UV damage (McKinney and Pruden, 2012), which might explain the higher inactivation of the *bla*-$_{TEM1}$ and *tet*(A) genes observed in this study. On the other hand, a typical chlorine dose normally applied in DWTPs resulted in higher inactivation than UV disinfection, as the inactivation was more than 1.5-log for all the tested genes. Studies have shown that chlorination might promote antibiotic resistance. For instance, Jia *et al.* (2015) observed a higher abundance of resistance genes (i.e. *apm*(C), *aph*(A2), *bla*-$_{TEM1}$, *tet*(A), *tet*(G) and *erm*(B)) in drinking water after chlorination. The order of the genes from most susceptible to least susceptible to chlorination in this study was: *mph*(A) ≥*sul*1 >*bla*-$_{TEM1}$ >*tet*(A). This order was consistent with the quantity of ARGs found in tap water in London, with the *mph*(A) gene being consistently the lowest among all genes. Guo *et al.* (2015) also reported the low inactivation of the *tet*-A gene in wastewater, with 80% of the gene persisting in the wastewater after chlorination.

Considering the insufficiencies of chlorine and/or UV irradiation alone for disinfection, including incomplete ARG inactivation and increased requirements for chlorine, a sequential UV/chlorine treatment was studied. The application of subsequent UV/chlorine has been shown to be more effective in inactivating resistance genes. A UV dose of 100 mJ/cm^2 followed by 1 mg/L Cl_2 resulted in a synergy value of 0.02 to 0.29 for all tested ARGs. However, it has to be noted that the use of sequential

or combination disinfection in drinking water treatment plants may require operating costs that would limit their use in low-income settings (e.g. developing countries, or small rural water treatment plants).

Antibiotic-resistant *E. coli* and *P. aeruginosa* were able to regrow and multiply after chlorine and UV disinfection. Antibiotic-resistant *P. aeruginosa* showed a higher regrowth rate than resistant *E. coli*. For instance, after being treated with 5 mg/L followed by one week of dark incubation, the regrowth was 4.2-log and 5.3-log for *E. coli* and *P. aeruginosa*, respectively. It is noteworthy that the regrowth did not occur when continuous chlorination was performed (i.e. the chlorine residual was maintained). In terms of resistant bacteria repair/regrowth after UV irradiation, a similar trend was observed. The regrowth of antibiotic-resistant *P. aeruginosa* was higher by 2.1-log than that of *E. coli* after 10 mJ/cm^2 UV irradiation then 24 hours of lighted incubation. The UV doses used in the experiment were much lower than common dosages in drinking water treatment and the addition of chlorine after UV treatment is crucial to prevent bacterial regrowth. Additionally, in general, it is difficult to demonstrate the origin of the bacteria found in tap water, for instance to be able to tell if their occurrence results from the survival to the disinfection process or if they enter the system along the distribution network somewhere.

The selection of the method to control antibiotic resistant bacteria and genes should done on a case-by-case basis considering the general characteristics of the drinking water treatment plant, specifically the size, the operating conditions, the level of disinfection residual to be reached, and the environmental impact of the process. Treatment strategies for antibiotic resistant bacteria and non-antibiotic resistant bacteria might be considered the same, this is due to their concurrent presence in the water, and the results from this study showed no difference in recalcitrance to treatment between ARBs and non-ARBs. However, to destroy the resistance genes higher UV and chlorine doses were found to be needed. The experiments in this study have shown that higher initial chlorine concentrations and longer treatment times were required to fully inactive the ARBs. Chlorination was found to be the most effective in inactivating ARBs and ARGs compared to UV, although it has to be noted that the UV doses used in this study were lower compared to the dose levels delivered in operational drinking water treatment plant. Additionally, to effectively manage ARBs and ARGs in drinking water system and

minimise their regrowth requires careful maintenance of disinfectant residuals throughout the network. Furthermore, the multiple barrier principle is recommended at DWTPs in case a single treatment fails in the multiple processes and therefore preventing a complete breakdown of the treatment (WHO 2014), and this research has also demonstrated synergies between multiple disinfectants (e.g. UV and chlorine) when attacking ARBs. Therefore, based on the results of this study, the use of sequential UV/chlorine is more favourable to inactive ARBs and ARGs. Additionally, sequential use of UV/chlorine was recently reported to achieve higher reduction of antibiotic concentrations in samples taken from a drinking water treatment plant (Zhang *et al.,* 2019). Nonetheless, there is no collective solution; due to each of the disinfection technologies available have advantages and disadvantages as stated in the chapter 3. The capital and operation costs are important aspects to consider when the choice for a given method is required. Nevertheless, maintaining an adequate disinfection residual in distribution is still the best available technology to control and/or minimise ARB and ARG in drinking water distribution systems.

Overall, the results of this study highlight the great challenge in eliminating hazardous ARBs and ARGs from potable water, suggesting perhaps that 'end-of-pipe' water treatment solutions are never going to provide a complete solution to this problem. Instead, a greater focus on developing new antibiotics that are less persistent in the environment and for which resistance is more difficult bacteria to acquire is the only real solution.

All in all, this research has shown that water distribution networks could serve as incubators for the growth of certain ARB populations and as an important reservoir for the spread of antibiotic resistance to opportunistic pathogenic HPC bacteria. The disinfection study showed that common disinfectants could not eliminate the ARB and ARGs entirely and bacteria regrowth following disinfection was observed. While estimation of the overall human risk was not the part of this study, findings from this research suggest that there are might be elevated risk of exposure to opportunistic antibiotic resistant bacteria during tap water consumption. It can be seen from the presence of opportunistic resistant bacteria and some clinically important ARGs can survive in tap water that meets current water quality standards for potable use. The majority of previous studies were on the presence and disinfectant effectiveness of antibiotic resistance in wastewater and natural water bodies such as rivers. However, some of the bacterial groups that can be present in wastewater, surface water and drinking water often can also integrate in the human microbiome, due to their ecology and physiology (Vaz-Moreira *et al.,* 2014). The prevalence of carriage of antimicrobial-resistant *E. coli* was higher for people who consumed water contaminated with antimicrobial-resistant *E. coli* than in those using uncontaminated or adequately treated water (Coleman *et al.,* 2012). Thus, drinking water has been suggested as possible route of exposure to antibiotic resistance to humans.

Seasonal variations on the occurrence of antibiotic resistance genes and bacteria have been reported in surface waters (Yang *et al.,* 2011; Knapp *et al.,* 2012), wastewater treatment facilities (Birosova *et al.,* 2014) and most notably in hospital wastewater treatment plants (Caucci *et al.,* 2016). Understanding the main reasons for seasonal variations in antibiotic resistance in water bodies requires a comprehensive analysis of several related relevant factors. For instance, in one study of a river, ARBs were detected in higher numbers during the wet season, which was believed to be due to higher flow of the river during that season (Knapp *et al.,* 2012). On the other hand, higher antibiotic prescriptions during colder months were believed to result in higher antibiotic resistance in a wastewater in another study (Caucci *et al.,* 2016). The factors controlling ARBs and ARGs in drinking water are also complex, however, season was not found to be a significant factor influencing the prevalence or antibiotic resistant in treated potable water supplies.

The fact that antibiotic resistant bacteria were able to regrow following disinfection treatment (i.e. in this case UV and chlorine) demonstrates another aspect of the potential risk posed by ARBs and ARGs. In this study a longer observation (i.e. one week) and typical chlorine doses used in drinking water were taken into account, adding new information from previous studies. Regrowth and reactivation of resistant bacteria after chlorination (Huang *et al.,* 2011) and UV disinfection (Huang *et al.,* 2015) in wastewater effluent have been reported. Their results suggest that ARB reactivation could threaten public health during wastewater reuse. While no information on ARB regrowth in drinking water setting were reported previously, this study has provided an insight into this issue, demonstrating that this is possible under low residual conditions.

The urban water cycle is the most significant habitat and routes for the propagation of bacteria, playing a major role in the dissemination of antibiotic resistance between the environment and humans and other animals. Member of Enterobacteriaceae found in this study such as *Pseudomonas* and *Aeromonas* have been often described as opportunistic pathogens that have the ability to infect the human body. Additionally, as a carriers these bacteria might not infect humans, however, their spread and proliferation in the environment would raise the abundance and diversity of ARGs in other microorganisms. Hence, it may increase the risks of transmission of antibiotic resistance to humans. The infective dose for some bacterial groups is known, for example for *Staphylococcus aureus* (>10^5) cells), for *Mycobacterium tuberculosis* (>10 cells), or for *Escherichia coli* (>10 cells) (Schmid-Hempel and Frank, 2007). However, until now it was still unknown if the dose will be identical for antibiotic-resistant versions of the same species. Nonetheless, the values available suggest that even very low counts of some bacteria may be able cause harm to humans.

To control the spread and persistence of ARB and ARGs in the environments requires a comprehensive effort from many stakeholders. From the drinking water industry's point a view, adequate chlorine residual should always be maintained in the distribution network until consumer tap to minimise bacterial regrowth. Many experts believe that controlling antibiotic use through antibiotic stewardship programme has proven to be effective in cutting the antibiotic consumption, thus also limiting the

selection pressure for resistant pathogen (Kaki *et al.,* 2011; Lai *et al.,* 2016). Establish biological risk assessment standard for ARB and ARGs particularly from environmental sources (i.e. dose response, risk factors, and rates of transmission of environmental bacteria to human flora through ingestion and non-ingestion) should be a priority. By estimating the risk, it will be easier for decision-makers to establish policies and rules relating to antibiotic resistance in the environment and how best to counter-act them.

6.3 Implications for the Water Industry and Recommendation for Future Research

The findings of this research are that there are multiple ARB species commonly found in tap water (some of which are opportunistic pathogens), and that they and their genes are not completely inactivated by common water industry disinfectants (including one gene which was identified in tap water for the very first time in this study). Furthermore, they have the ability to recover in water distribution systems in some circumstances. The findings lend new and stronger support to the general guidance that is already given to immune-compromised individuals to boil or filter tap water before use.

The combined use of chlorine and UV treatment was shown to be more effective in eliminating ARGs than chlorine or UV alone. Therefore, adopting this practice would result in better water quality and safety in terms of antibiotic resistance**.** Ultimately, however, relying on drinking water treatment as an 'end of pipe' solution to the antibiotic resistance problem is not a solution, and society must develop new antibiotics which are less persistent in the aquatic environment and less prone to resistance.

While this study has addressed a large range of issues related to ARBs and ARGs in drinking water, there are several aspects that would benefit from further research. The human health impact from ARBs and ARGs through drinking water is currently unknown. A study like the sampling survey presented in this research could be extended to include a quantitative risk assessment examining the risk from ARBs, particularly those commonly found in drinking water (O'Flaherty *et al.*, 2017). It is also noteworthy that the application of different disinfectants (e.g. chlorine or chloramine) might affect the diversity and composition of ARB and ARGs in drinking water distribution systems. Hwang *et al.,* 2012 suggested a potential shift in the microbial communities driven by different disinfectants. Furthermore, Lin *et al.,* (2016) showed that co-selection (i.e. the tolerance of bacteria to antibiotics and other factors can increase simultaneously) might influence the enrichment of resistance genes by different factors such as disinfectant type, metals, and organic compounds. Therefore, further study exploring this issue, particularly in real drinking water systems, would be intriguing. The study of disinfection can be further improved by exploring the application of other disinfection combinations to inactivate ARGs, for instance pre-disinfection with an oxidant capable of yielding extensive cell envelope damage (e.g. ozone) followed by post-disinfection with an oxidant having higher selectivity

towards DNA (e.g. chlorine). Finally, there is more to learn about ARBs repair and regrowth, and this should be further explored in real DWTPs and drinking water distribution systems with more complex bacteria communities than the individual species considered here, and by considering all the factors that might be involved in bacteria repair (e.g. AOC / BDOC, water age, and turbidity). Real-time, high-throughput techniques such as flow cytometry may be useful in this regard, to collect more data of bacteria counts (perhaps even *in situ* in treatment plants and distribution systems) than is possible by using the culture-based techniques in the lab as were used in this study.

CHAPTER 7

CONCLUSIONS

The most important new findings arising from this research were that:

(1) The overall resistance pattern observed in London tap water samples from nine sampling points and six sampling periods was: trimethoprim resistance > erythromycin > vancomycin > amoxicillin > ciprofloxacin > tetracycline. Trimethoprim resistance was consistently the most common form of drug resistance at all sampling times and locations. Seasonal differences did not significantly affect the profile of antibiotic resistance in the indigenous bacteria populations.

(2) Resistance genes *tet*(A), *bla*-$_{TEM1}$, *sul*1, *mph*(A) and *dfr*A7 genes were detected in all water samples, with the *sul*1 gene being the most abundant. The high abundance of the *sul*1 gene in tap water samples may illustrate the future development of emerging resistance determinants for newer antibiotics such as fluoroquinolones. Interestingly, unlike the lack of a seasonal pattern of occurrence for ARBs, the ARG occurrence was seasonally influenced, with the abundance of resistance genes significantly increasing in April compared to the July sampling time.

The *mph*(A) gene in drinking water was observed for the first time, to the best of the author's knowledge. This gene is responsible for regulating macrolides' resistance by modifying phosphotransferases. It is worthy of note that the incidence of the *mph*(A) gene is supported by the high occurrence of erythromycin-resistant bacteria also found in the tap water samples in this study.

(3) The dominant resistant heterotrophic bacteria in tap water samples were *Pseudomonas spp*, followed by *Aeromonas spp, Delftia spp*, *Burkholderia spp*, and *Sphingomonas spp*. The results indicate that resistant bacteria can regrow in drinking water distribution systems, even in the presence of typically applied chlorine residual. Several of the identified bacteria were opportunistic pathogens; therefore the results support the general medical advice to disinfect water at the point of use to protect the health of individuals who may be immunocompromised/suppressed (CDC, 2003).

(4) As expected, antibiotic-resistant *E. coli* and *P. aeruginosa* were readily inactivated by chlorine and UV disinfection. However, the use of chlorine and UV disinfection at typical water industry doses could not completely eliminate the resistance genes. Using 30 mg·min/L of chlorine, the inactivation of *tet*(A), *bla*-$_{TEM1}$, *sul*1, *mph*(A) was approximately 1.7-log, while a UV exposure of 200 mJ/cm^2 only resulted in a reduction of up to 1.2-log of these genes. This suggests that these genes continue to be present at high numbers in distribution systems even following water treatment.

(5) Antibiotic-resistant *E. coli* and *P. aeruginosa* demonstrated the potential for regrowth following chlorination and UV disinfection. The maximum regrowth of *E. coli* and *P. aeruginosa* was observed after 1 mg/L of chlorination followed by one week of dark incubation in phosphate buffer saline, being 3.1 log and 3.4 log, respectively. The maximum light repair following UV exposure to 10 mJ/cm^2, the highest UV dose applied in this study was 1.8 log and 3.1 log for resistant *E. coli* and *P. aeruginosa*, respectively. The light repair at 40 mJ/cm^2 of UV dose (i.e. the minimum recommended UV dose in drinking water treatment), was lower than 1-log for both studied bacteria.

It is ultimately important to prevent prolonged light exposure following UV disinfection and maintain residual chlorine concentrations in drinking water distribution systems at an adequate level in order to reduce the repair or regrowth of ARBs (and indeed non-AR bacteria).

(6) The combination of two disinfection methods (i.e. UV followed by chlorine) was observed to have synergistic benefits for controlling dissemination of some ARGs, with a resulting synergy in the inactivation achieved (log units) ranging between 0.01 and 0.62-log for all the tested ARGs. The synergy indicates that the overall log inactivation of UV followed by chlorine is greater than the sum of that achieved by UV and chlorine on their own.

(7) Finally, this research has shown a potentially elevated risk of antibiotic resistance from drinking water consumption due to the presence of antibiotic resistant opportunistic pathogen bacteria such as *P. aeruginosa, B. cepacia* and *S. paucimobilis* and clinically important resistance genes in the drinking water. Furthermore, the evidence of resistant bacteria being able to undergo regrowth and repair following disinfection adds more risk to the spreading of and exposure to ARBs to society

through drinking water. The use of multiple disinfectants in drinking water treatment plants (e.g. UV/chlorine) and maintaining sufficient chlorine residual in drinking water provide an effective strategy to minimise survival and regrowth of ARBs and ARGs.

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APPENDICES

APPENDIX A. Supplementary Information about the Materials and Methods

A.1 Antibiotic stocks preparation

All antibiotic stocks were prepared in 10 g/L with appropriate solvent for each antibiotic. Amoxicillin was prepared in 10 g/L stock in deionised water. Trimethoprim was prepared by dissolving 10 mg in 10 ml of 50:50 ethanol-chloroform. Tetracycline stock was prepared by dissolving 10 mg in 10 ml of 10% DMSO. Ciprofloxacin was prepared by dissolving 10 mg of antibiotic in 10 ml of 0.1N hydrochloric acid. Erythromycin was prepared by dissolving 10 mg of antibiotic in 10 ml of 10% ethanol. Vancomycin was prepared in 10 g/L stock in deionised water.

A.2 Sodium Thiosulfate Quencher Solution

The quencher solution was prepared by adding 2000 mg of Sodium thiosulfate >98% (Sigma Aldrich, Missouri, US) into 1 litre of sterile distilled water.

A.3 DNA concentration measurement

The equation below was used to measure the DNA concentration:

Concentration (µg/ml) = (A_{260} reading – A_{320} reading) × dilution factor × 50µg/ml

Total yield was obtained by multiplying the DNA concentration by the final total purified sample volume.

DNA Yield (µg) = DNA Concentration × Total Sample Volume (ml)

A.4 Minimum inhibition concentration (MIC) experiment for environmental strain bacteria

Figure A.1 shows the MIC result for environmental strain bacteria which was used in chlorination and UV experiment. Wells in blue colour indicates no grow and wells in pink colour indicates bacteria grow. As expected the wells that only contain PBS or DMSO turned pink, meaning that PBS and DMSO did not interrupt the bacterial growth. On the other hand, the sterile wells (i.e. without bacteria addition) maintain its blue colour. The results showed that the MIC for tetracycline was 1.9 µg/ml, amoxicillin 15.6 µg/ml and trimethoprim 125 µg/ml.

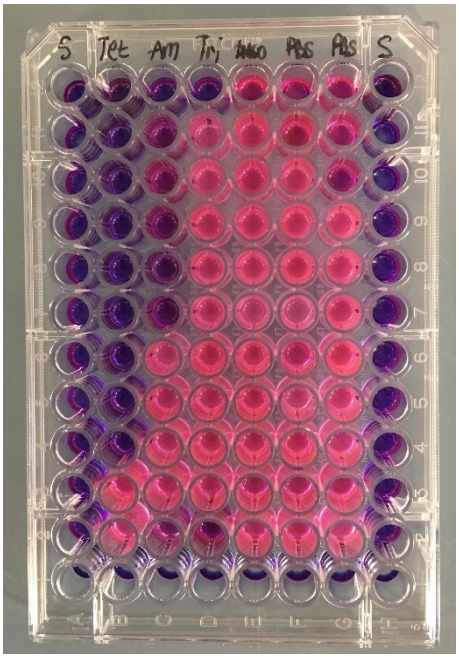


Figure A.1: Minimum inhibition concentration of the environmental strain bacteria, S (sterile), tet (tetracycline), am (amoxicillin), tri (trimethoprim), DMSO (dimethyl sulfoxide) and PBS (phosphate buffer saline)

A.5 Standardisation of UV Collimated Beam System using MS-2 Phage

Bacteriophage is a well-known standard UV surrogate organism in UV disinfection studies was used as benchmark to confirm the UV doses that were applied by UV collimated beam system.

E. coli ATCC 15977-B1 (phage) and *E. coli* ATCC 15977–B1 was used on the experiment, both *E. coli* was purchased from American Type Culture Collection (ATCC). *E. coli* ATCC 15597 (host) were grown overnight in tryptic soy broth medium at temperature 35°C. On the next day, 100 ml fresh TSB was inoculated with 1 ml of overnight host *E. coli*, followed by incubation at 37°C with 100 rpm shaking for 5-6 h. Further, 100 µl of *E. coli* 15597 B1 was added into the culture, incubated and shaking overnight (±16 h) at 37°C. The overnight culture was then centrifuged at 2000 rpm for 30 minutes. The supernatant was decanted using a sterile pipette then filtered using a 0.22 mm membrane filter (Whatman International Ltd., UK) into a sterile container. The stock phage is now ready to use and stored at 4°C refrigerator for maximum 2 weeks.

The MS2 inactivation was carried out as described in the section **3.5.7.1**, the UV dose applied was 40 mJ/cm^2, with the phage concentration was approximately 10^{6}pfu/ml. Following UV exposure, the MS2 coliphages were enumerated using a standard overlay agar assay method, Standard Method 9211-D (AWWA, 2005). One hundred microliters of *E. coli* (host) was added to 900 microliters of sample, the mixture was the added to 5 ml of modified trypticase soy agar (MTSA) and mixed thoroughly. The solution was then poured into 100 mm petri dishes containing TSA. The dishes were covered, and the

agar was allowed to gel. The plates were then inverted and incubated at 35°C overnight**,** on the following day plaque was counted.

Figure A.2 shows the log plaque forming unit number (pfu/ml) before and after UV exposure of 40 mJ/cm^2, it can be seen that the UV exposure resulted in 2.1-log phage reduction. A UV dose of 40 mJ/cm^2 should result in approximately 2 to 2.5-log reduction.

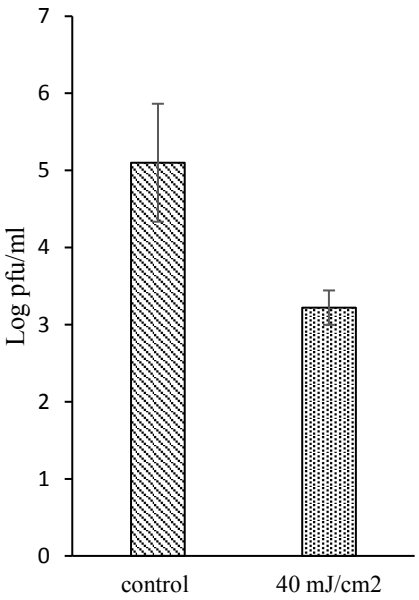


Figure A.2: Bacteriophage (MS2) inactivation by UV exposure of 40 mJ/cm^2

Appendix B. Tap Water Characteristics

Table B.1: Temperature (°C). Sampling points indicate different areas across London as shown in Figure 5.1

sampling points	Sampling time					
	Jan-15	Apr-15	Jul-15	Oct-15	Apr-16	Jul-16
1	9	11	20.1	17.1	11	20.1
2	7.8	10.5	19.8	16.6	11.8	19.8
3	8.3	10.3	19.6	16.5	10	19.6
4	7.0	9.0	20.1	16.4	9	20.2
5	8.7	10	20.3	17.5	10	19.5
6	8.5	9.0	19.8	17.0	10	20.1
7	8.5	9.5	19.5	16.8	11	20.2
8	9.2	9.0	19.4	16.9	10	19.4
9	7.3	9.0	19.1	16.8	9	19.6

Table B.2: Chlorine residual concentration (mg/L). Sampling points indicate different areas across London as shown in Figure 5.1

sampling points	Sampling time					
	Jan-15	Apr-15	Jul-15	Oct-15	Apr-16	Jul-16
1	0.2	0.3	0.1	0.4	0.25	0.2
2	0.2	0.25	0.2	0.5	0.4	0.3
3	0.3	0.3	0.2	0.3	0.4	0.2
4	0.3	0.2	0.3	0.3	0.5	0.3
5	0.2	0.2	0.2	0.2	0.3	0.3
6	0.1	0.3	0.2	0.4	0.4	0.2
7	0.3	0.3	0.2	0.3	0.5	0.1
8	0.2	0.1	0.3	0.4	0.2	0.2
9	0.4	0.4	0.3	0.5	0.5	0.4

Table B.3: Total organic carbon (TOC) concentration (mg/L). Sampling points indicate different areas across London as shown in Figure 5.1

Sampling points	Sampling times					
	Jan-15	Apr-15	Jul-15	Oct-15	Apr-16	Jul-16
1	2.1	3	2.6	1.9	1.7	0.9
2	1.7	1.8	1.8	2	1.8	2.1
3	2.3	2.1	1.9	3.2	2.4	2
4	2.3	2.1	2.6	1.9	2.1	2.5
5	2.4	2.1	2.6	2.9	2.5	2.2
6	2.8	2.7	2.6	3.2	2.7	2.5
7	2.5	1.4	2.7	3	2.3	2.7
8	2.3	2.9	2.3	2	2.5	2.1
9	1.9	2.2	1.8	1.9	1.8	1.8

Table B.4: pH. Sampling points indicate different areas across London as shown in Figure 5.1

sampling points	Sampling time					
	Jan-15	Apr-15	Jul-15	Oct-15	Apr-16	Jul-16
1	7.6	7.6	7.6	7.6	7.6	7.6
2	7.3	7.3	7.3	7.3	7.3	7.3
3	7.3	7.3	7.3	7.3	7.8	7.3
4	7.5	7.5	7.5	7.5	7.5	7.5
5	7.4	7.4	7.4	7.4	7.4	7.4
6	7.6	7.6	7.6	7.6	7.6	7.6
7	7.7	7.7	7.7	7.7	7.8	7.7
8	7.4	7.4	7.4	7.4	7.4	7.4
9	7.3	7.3	7.3	7.3	7.3	7.3

Appendix C. Raw data of total heterotrophic plate count bacteria, antibiotic resistant bacteria, antibiotic resistance genes and universal gene 16S rRNA

Table C.1: Raw data of heterotrophic plate count (HPC) bacteria in tap water samples. Sampling points indicate different areas across London as shown in Figure 5.1

Locations	HPC/ 100 ml					
	Jan-15	Apr-15	Jul-15	Oct-15	Apr-16	Jul-16
1	119	79	140	102	365	174
	95	91	81	90	325	144
	103	72	120	131	340	159
2	60	53	95	95	23	55
	68	56	79	102	25	72
	55	53	117	94	46	80
3	212	110	238	138	124	104
	193	123	200	88	137	118
	148	142	215	99	154	115
4	139	203	33	80	67	41
	123	196	20	73	36	40
	125	187	15	65	116	71
5	238	350	201	168	338	135
	275	580	169	185	294	146
	313	570	350	182	310	153
6	590	154	216	84	82	170
	620	109	239	195	108	165
	500	98	199	112	92	194
7	157	109	211	109	141	540
	136	106	186	106	121	510
	142	117	220	117	115	610
8	111	430	190	138	192	79
	102	138	104	88	123	67
	106	143	108	99	100	119
9	54	11	3	21	18	17
	51	18	5	24	29	18
	52	7	8	22	25	20

Table C.2: Raw data of antibiotic resistant bacteria from tap water samples. Sampling points indicate different areas across London as shown in Figure 5.1

Locations	Jan-15						Apr-15						Jul-15						Oct-15						Apr-16						Jul-16					
	Van	Ery	Am	Cip	Tet	Tri	Van	Ery	Am	Cip	Tet	Tri	Van	Ery	Am	Cip	Tet	Tri	Van	Ery	Am	Cip	Tet	Tri	Van	Ery	Am	Cip	Tet	Tri	Van	Ery	Am	Cip	Tet	Tri
1	1.7	9.2	9.2	0.0	0.0	20.2	10.1	11.4	20.3	0.0	0.0	34.2	16.4	30.0	2.1	0.0	0.0	35.0	8.8	3.9	15.7	0.0	0.0	34.1	0.0	23.6	21.1	0.0	0.0	18.6	10.3	12.1	10.3	0.0	0.0	14.4
	5.3	12.6	21.1	0.0	0.0	46.3	14.3	3.3	21.0	0.0	0.0	20.9	27.2	44.4	3.7	0.0	0.0	38.2	10.0	5.6	17.8	0.0	0.0	44.4	0.0	16.3	26.5	0.0	0.0	23.1	13.9	9.7	19.4	0.0	0.0	13.9
	2.9	1.9	18.9	0.0	0.0	32.0	12.5	5.6	9.7	0.0	0.0	26.4	24.2	15.0	4.2	0.0	0.0	13.3	10.7	4.6	14.5	0.0	0.0	30.5	0.0	17.9	23.5	0.0	0.0	23.5	8.2	10.7	28.3	0.0	0.0	15.7
2	4.0	15.0	0.0	0.0	0.0	50.0	13.2	17.0	1.9	0.0	0.0	39.6	5.3	75.8	22.1	3.2	0.0	78.9	11.6	18.9	1.1	3.2	0.0	37.9	8.7	21.7	21.7	0.0	0.0	60.9	12.3	21.2	14.5	0.0	0.0	10.9
	4.4	20.6	0.0	0.0	0.0	54.4	14.3	26.8	5.7	0.0	0.0	46.4	12.7	82.3	24.0	3.8	0.0	51.9	26.5	43.1	2.0	2.9	0.0	49.0	16.0	20.0	16.0	0.0	0.0	56.0	4.2	13.9	6.9	0.0	0.0	15.3
	1.8	12.7	0.0	0.0	0.0	54.6	22.6	26.4	3.8	0.0	0.0	41.5	15.4	58.1	20.5	4.3	0.0	51.3	20.2	37.2	2.1	5.3	0.0	43.6	8.7	15.2	8.7	0.0	0.0	32.6	6.3	15.0	7.5	0.0	0.0	18.8
3	3.3	17.0	58.9	0.0	0.0	35.4	55.5	19.1	36.4	0.0	0.0	57.3	16.0	60.0	60.0	0.0	0.0	23.1	24.1	2.9	23.2	2.9	2.9	21.7	48.4	58.1	36.0	0.0	0.0	69.4	21.2	21.2	27.9	1.9	0.0	26.0
	41.0	20.7	53.9	0.0	0.0	41.5	42.3	13.8	45.5	0.0	0.0	22.8	30.0	63.5	80.5	0.0	0.0	37.5	41.4	3.4	42.0	3.4	3.4	25.0	51.8	36.5	25.5	0.0	0.0	63.5	16.9	22.9	19.5	0.8	0.0	28.0
	38.9	18.9	85.8	0.0	0.0	30.4	45.8	11.3	23.9	0.0	0.0	38.7	30.2	62.3	76.3	0.0	0.0	37.7	50.8	14.1	44.4	4.0	4.0	30.3	47.4	44.8	26.0	0.0	0.0	46.1	21.7	20.0	21.7	0.0	0.0	37.4
4	47.3	5.0	25.9	20.9	0.0	10.1	35.5	33.5	24.1	5.9	0.0	67.0	21.1	33.3	21.1	21.1	0.0	72.7	25.0	62.5	1.3	0.0	0.0	70.0	19.4	37.3	32.8	0.0	0.0	38.8	12.2	17.1	0.0	0.0	0.0	34.1
	18.0	10.6	47.2	17.1	0.0	11.4	29.6	18.4	17.3	4.1	0.0	44.9	35.0	40.0	20.0	25.0	0.0	75.0	37.0	75.3	4.1	0.0	0.0	89.0	34.5	47.2	25.0	0.0	0.0	69.4	7.5	30.0	0.0	0.0	0.0	25.0
	27.6	8.0	16.8	14.4	0.0	22.4	25.1	24.6	18.7	2.7	0.0	52.4	40.0	46.7	26.7	26.7	0.0	86.7	32.3	46.2	3.1	0.0	0.0	66.2	20.7	29.2	1.7	0.0	0.0	22.4	11.3	23.9	0.0	0.0	0.0	14.1
5	25.6	19.1	20.5	4.0	0.0	63.0	14.6	13.1	24.6	1.7	0.6	75.4	49.8	28.4	35.8	0.0	2.0	75.6	61.9	30.4	17.9	0.0	0.0	77.3	10.4	17.8	14.5	18.6	0.0	35.5	13.3	5.9	9.6	0.0	0.0	51.1
	30.0	18.3	21.8	2.9	0.0	55.6	15.0	9.1	13.1	1.9	0.2	27.8	56.8	41.4	44.4	0.0	3.6	82.2	44.3	18.4	17.3	0.0	0.0	78.4	13.3	19.0	17.7	12.6	0.0	55.4	4.1	3.4	16.4	0.0	0.0	34.2
	26.7	14.2	13.5	3.3	0.0	45.9	5.3	8.8	12.1	0.4	0.2	38.1	34.3	21.7	20.0	0.0	0.6	43.7	46.7	19.2	8.8	0.0	0.0	80.7	24.2	19.4	14.8	16.1	0.0	47.4	5.9	2.0	11.8	0.0	0.0	19.6
6	23.3	76.7	12.5	0.0	0.0	72.9	7.1	3.2	5.2	1.8	0.0	22.7	26.3	42.1	18.1	3.1	0.0	52.1	20.2	81.0	59.5	11.9	2.4	72.6	58.5	36.6	9.8	0.0	0.0	58.5	32.9	12.9	3.5	3.5	0.0	33.5
	12.5	58.1	14.5	0.0	0.0	61.3	7.3	16.5	10.1	6.1	0.0	33.9	23.1	32.2	11.9	2.4	0.0	36.9	22.0	41.0	18.5	5.6	1.0	35.9	37.0	19.4	3.7	0.0	0.0	27.8	38.2	26.1	12.7	4.5	0.0	25.5
	10.8	64.0	12.2	0.0	0.0	69.6	9.2	15.3	15.3	12.5	0.0	24.5	22.4	38.7	13.8	4.1	0.0	45.3	10.0	67.0	25.0	13.4	0.9	68.9	48.2	38.0	8.7	0.0	0.0	38.0	35.6	9.3	7.7	1.0	0.0	25.5
7	9.2	41.4	47.8	0.0	2.6	38.2	29.4	9.2	41.3	11.0	11.0	50.5	18.8	27.1	31.9	0.0	4.0	43.6	16.5	62.4	6.4	0.0	6.4	50.5	3.5	19.9	5.0	0.0	0.0	27.0	20.3	2.8	59.3	0.0	0.0	51.9
	24.2	46.3	60.3	0.0	2.2	46.3	32.1	8.5	57.5	3.8	7.5	50.9	23.1	27.7	43.3	0.0	2.3	48.6	15.1	52.8	1.9	0.0	4.8	50.9	9.1	28.9	14.0	0.0	0.0	33.9	25.3	1.8	70.6	0.0	0.0	60.8
	33.8	33.8	54.2	0.0	0.7	38.0	33.3	7.7	29.1	2.4	5.1	35.9	25.0	25.0	29.1	0.0	3.3	39.5	39.3	60.7	4.3	0.0	4.3	35.9	5.2	21.7	8.7	0.0	0.0	30.4	18.0	1.3	49.1	0.0	0.0	57.4
8	28.9	45.1	17.1	24.3	8.1	60.8	11.6	0.7	44.9	1.2	4.0	53.6	19.7	16.4	18.9	5.4	2.7	51.5	20.3	7.2	3.6	0.0	0.0	18.8	26.6	13.8	18.8	0.0	0.0	57.3	20.3	15.2	10.1	1.3	1.3	46.8
	5.4	33.3	27.5	20.6	5.9	62.3	49.3	2.9	63.8	0.7	13.0	63.8	36.6	16.2	40.8	4.6	4.1	56.1	33.0	2.3	8.0	0.0	0.0	38.6	34.1	27.8	79.7	0.0	0.0	65.0	29.9	14.9	25.2	1.5	1.5	52.2
	4.9	32.1	17.9	8.5	4.7	64.0	33.6	2.6	39.9	6.0	7.0	51.0	43.5	17.7	28.6	3.6	7.5	46.7	61.6	6.1	7.1	0.0	0.0	35.4	35.4	35.4	68.3	0.0	0.0	63.0	14.3	12.6	10.1	3.4	0.8	21.8
9	3.8	20.4	3.7	0.0	0.0	48.2	0.0	9.1	9.1	0.0	0.0	45.5	0.0	0.0	0.0	0.0	0.0	0.0	13.3	15.0	9.2	0.0	0.0	34.6	33.3	27.8	16.7	0.0	0.0	50.0	29.4	17.6	16.7	0.0	0.0	29.4
	3.7	14.3	15.7	0.0	0.0	39.2	0.0	5.6	11.1	0.0	0.0	16.7	0.0	0.0	0.0	0.0	0.0	0.0	14.3	15.1	9.7	0.0	0.0	23.4	37.9	13.8	6.9	0.0	0.0	27.6	33.3	11.1	15.0	0.0	0.0	33.3
	7.7	11.5	7.7	0.0	0.0	32.7	0.0	14.3	28.6	0.0	0.0	42.9	0.0	0.0	0.0	0.0	0.0	0.0	11.3	12.2	17.0	0.0	0.0	23.3	12.0	20.0	32.0	0.0	0.0	16.0	15.0	15.0	16.7	0.0	0.0	25.0

Table C.3: Raw data of 16S rRNA gene from tap water samples. Sampling points indicate different areas across London as shown in Figure 5.1

locations	16s rRNA/100ml	
	Apr-16	Jul-16
1	9.16E+03	2.77E+02
	8.17E+04	2.36E+02
	4.70E+03	1.59E+02
2	5.52E+04	2.10E+03
	4.09E+04	3.65E+04
	1.87E+04	1.15E+04
3	2.15E+05	2.20E+03
	1.42E+05	9.82E+02
	1.32E+05	1.39E+03
4	5.40E+06	1.21E+05
	2.10E+06	6.49E+04
	3.18E+06	4.81E+04
5	4.81E+04	6.79E+02
	6.95E+04	3.03E+02
	6.95E+04	1.71E+02
6	3.82E+05	8.36E+04
	3.03E+04	3.57E+04
	2.10E+05	3.40E+04
7	2.77E+07	3.10E+05
	1.71E+07	5.65E+04
	1.59E+07	5.65E+04
8	5.15E+04	1.21E+04
	7.80E+04	9.16E+03
	1.26E+05	4.81E+03
9	3.65E+05	2.97E+02
	2.05E+05	3.33E+02
	1.26E+05	3.48E+02

Table C.4: Raw data of antibiotic resistance genes from tap water samples. Sampling points indicate different areas across London as shown in Figure 5.1

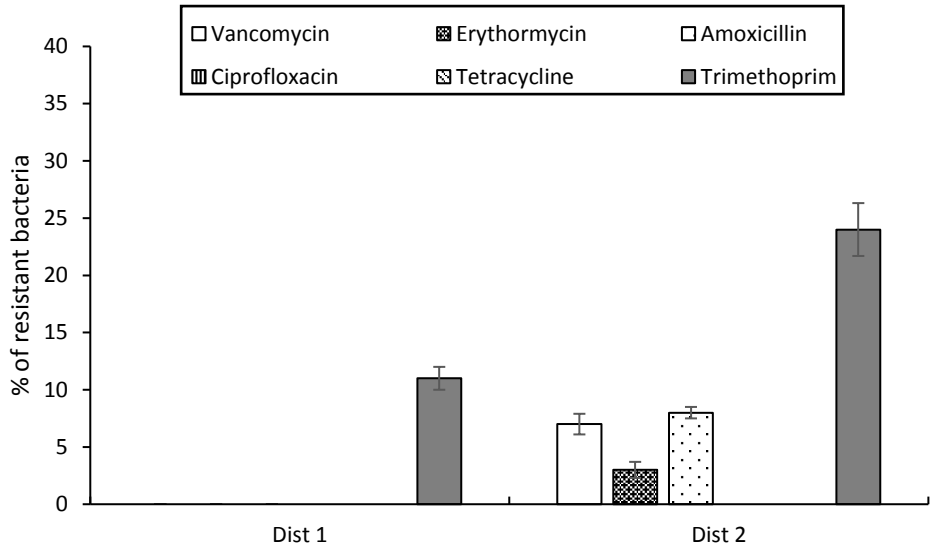
Sampling location	log resistant genes copies/100ml									
	Apr-16					Jul-16				
	tet-A	bla-tem	dfrA7	mph(A)	Sul1	tet-A	bla-tem	dfrA7	mph(A)	Sul1
	2.40	2.50	0.80	-0.31	1.76	-1.02	-1.36	-0.59	-0.30	1.09
	2.70	1.80	0.84	0.65	1.75	-1.15	-0.71	0.85	-0.88	0.16
1	2.50	1.91	0.59	0.88	1.71	-0.86	-0.84	0.85	-0.90	-1.21
	1.08	0.85	1.72	2.12	1.10	0.00	0.00	2.76	0.00	0.82
	1.09	0.78	3.31	2.79	1.13	0.00	0.00	2.77	0.00	0.76
2	1.28	0.80	2.89	3.05	1.45	0.00	0.00	2.66	0.00	0.69
	0.49	0.11	1.80	0.83	2.09	0.00	0.00	1.07	0.00	-1.30
	1.01	-0.45	2.37	0.49	2.04	0.00	0.00	0.98	0.00	-1.22
3	1.09	0.74	1.86	0.71	1.97	0.00	0.00	0.48	0.00	-1.19
	1.75	1.18	1.57	0.00	2.02	-1.26	0.00	-1.14	0.00	0.02
	1.57	1.52	1.58	0.00	1.93	-1.30	0.00	0.07	0.00	-0.42
4	1.84	1.32	1.57	0.00	1.97	-1.23	0.00	-1.34	0.00	0.02
	2.51	0.78	1.12	-0.03	3.25	-0.53	0.00	1.11	-0.03	1.45
	2.44	0.74	0.73	0.18	3.30	-1.12	0.00	0.73	0.13	0.91
5	2.46	0.81	0.42	0.13	3.48	0.00	0.00	0.42	-0.06	1.85
	0.87	-0.39	0.69	-0.58	2.93	-0.32	0.00	1.72	0.00	0.64
	0.94	-0.17	0.06	-0.50	3.01	-0.25	0.00	2.90	0.00	1.42
6	-0.11	-0.34	-0.37	-0.30	3.01	-0.01	0.00	1.90	0.00	1.19
	1.51	0.00	2.08	0.19	2.97	-0.58	0.00	-1.35	0.00	-0.51
	1.56	0.00	1.51	0.59	2.84	-1.35	0.00	-1.26	0.00	-0.56
7	1.64	0.00	1.65	-0.20		-1.15	0.00	-0.84	0.00	-0.09
	1.23	1.19	1.97	-0.38	3.00	-0.82	0.11	1.92	0.45	1.52
	1.92	1.04	2.30	-0.23	3.10	1.17	-0.37	1.61	0.35	2.11
8	1.66	0.89	2.10	-1.45	3.04	0.93	-0.24	1.80	-0.87	2.22
	0.49	0.11	1.80	0.83	0.67	0.79	-0.34	0.63	-0.42	0.73
	1.01	-0.45	2.37	0.49	0.62	-0.36	-1.05	0.47	-1.14	0.82
9	1.09	5.47	1.86	0.71	0.66	0.23	-0.64	0.55	-0.59	0.74

Appendix D. Antibiotic-resistant Bacteria and Antibiotic Resistance Genes in Chlorinated and Chloraminated Drinking Water Distribution Systems

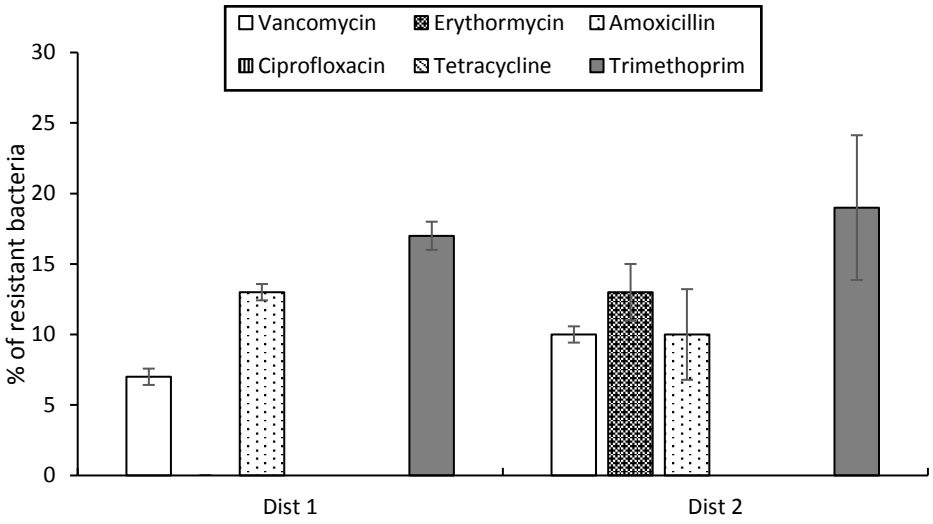
A small sub-study was conducted to compare ARBs and ARGs in chlorinated and chloraminated distribution systems with similar source waters. These water samples were only collected in October 2016 but not in the other sampling rounds of this research. Water samples were taken from two sampling points: (1) the finished water from the WTP, referred to as *Dist 1,* and (2) a sampling point farther from the WTP in the distribution network, referred to as *Dist 2*.

Figure D.1 shows the prevalence of ARBs in chlorinated and chloraminated water supplies. In the chlorinated water supply, only trimethoprim-resistant bacteria were detected at the *Dist 1* sampling point. On the other hand, three types of resistant bacteria were found in the chloraminated water supply, with trimethoprim resistance being the most frequent in both water supplies. More resistant bacteria were found along the distribution system, in both chlorinated and chloraminated systems. Tetracycline- and ciprofloxacin-resistant bacteria were not detected in any sampling points.

Figure D.2 summarises the quantity of ARGs found in chlorinated and chloraminated water systems. Antibiotic resistance genes *tet*-A, *bla*-$_{\text{TEM1}}$, *dfr*A7 and *sul*1 were found in the chlorinated water system. The quantities of the resistance genes were higher farther along the sampling points – for instance, the *sul*1 gene was 1.35-log higher at the sampling point farther from the WTP than at the first sampling point at the WTP. Interestingly, the quantities of *tet*-A and *bla*-$_{\text{TEM1}}$ genes at sampling point *Dist 2* decreased by 0.38-log, and 0.36-log, respectively.

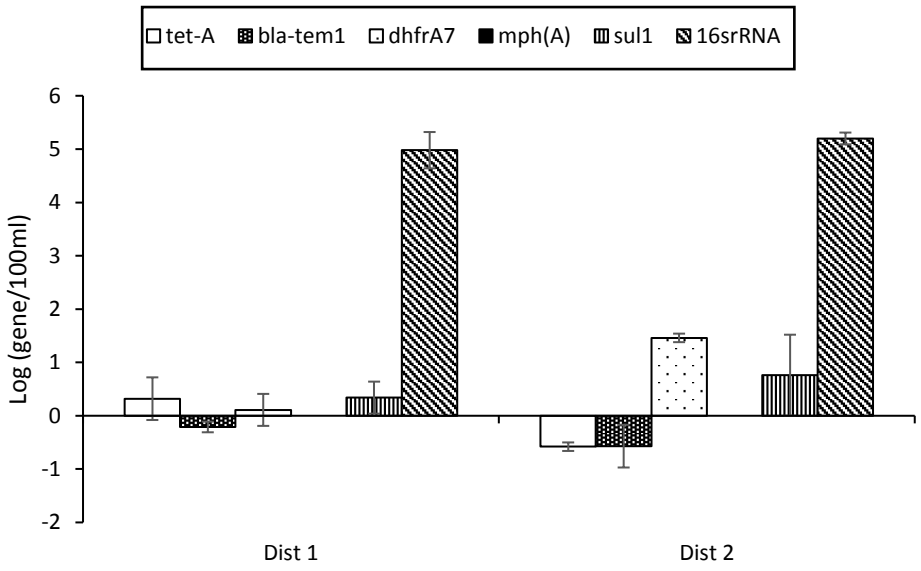


(a)

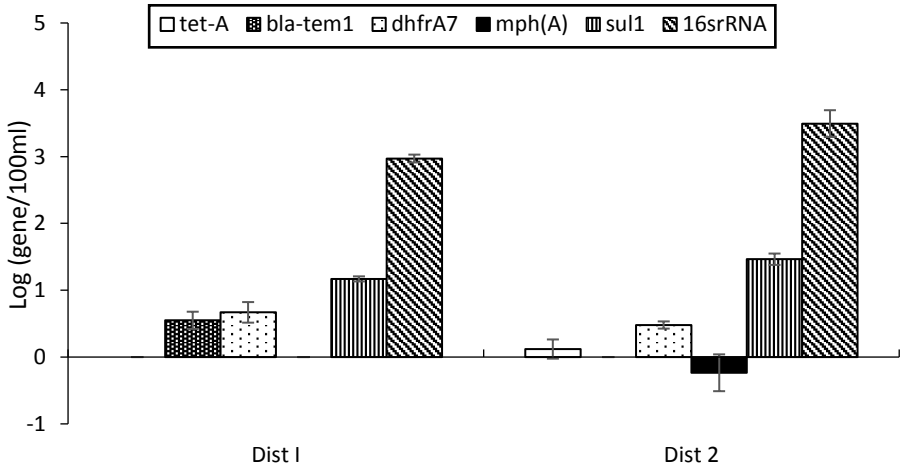


(b)

Figure D.1: Percentage of bacteria found in finished water from the WTP, referred to as *Dist 1*, and the sampling point farther from the WTP, referred to as *Dist 2*, that were antibiotic resistant in (a) chlorinated water system (b) chloraminated water system



(a)



(b)

Figure D.2: Quantity of antibiotic resistance genes found in finished water from the WTP, referred to as *Dist 1*, and the sampling point farther from the WTP, referred to as *Dist 2*, in (a) chlorinated water system (b) chloraminated water system

Appendix E. Quantitative PCR quality control

Appendix E.1: Melting curves

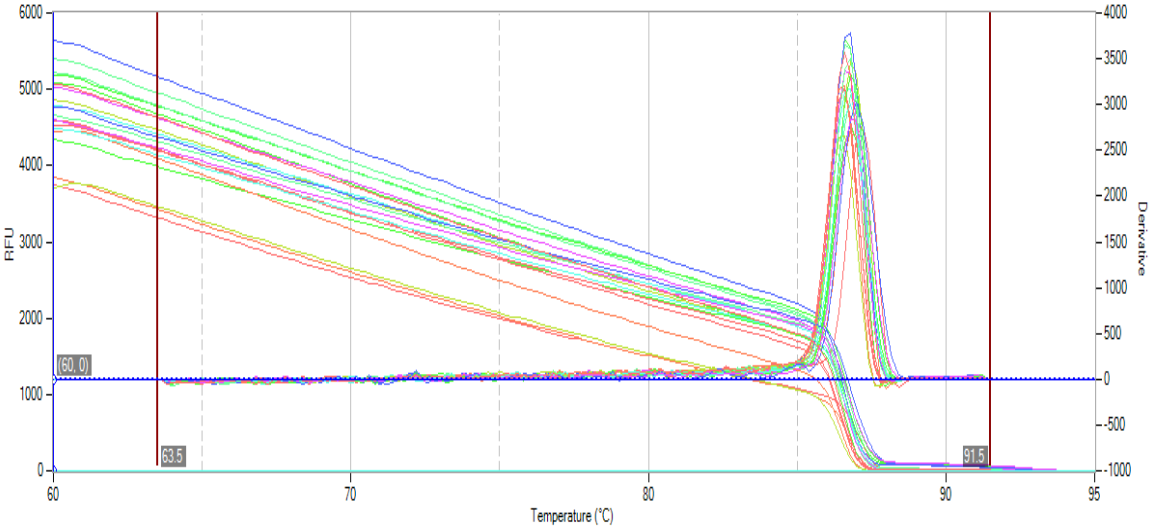


Figure E.1: *tet*(A) gene melting curve

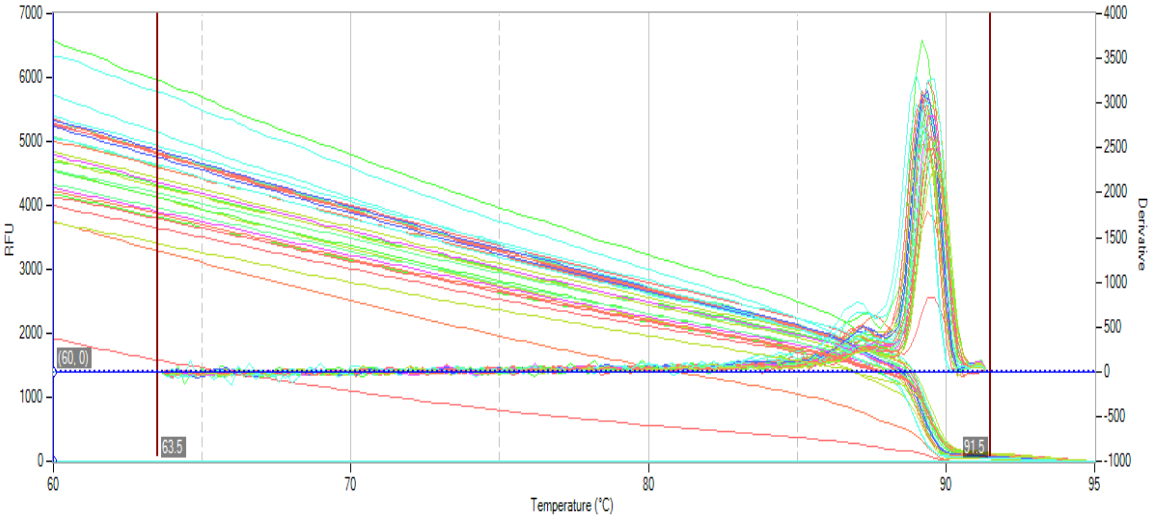


Figure E.2: *mph*(A) gene melting curve

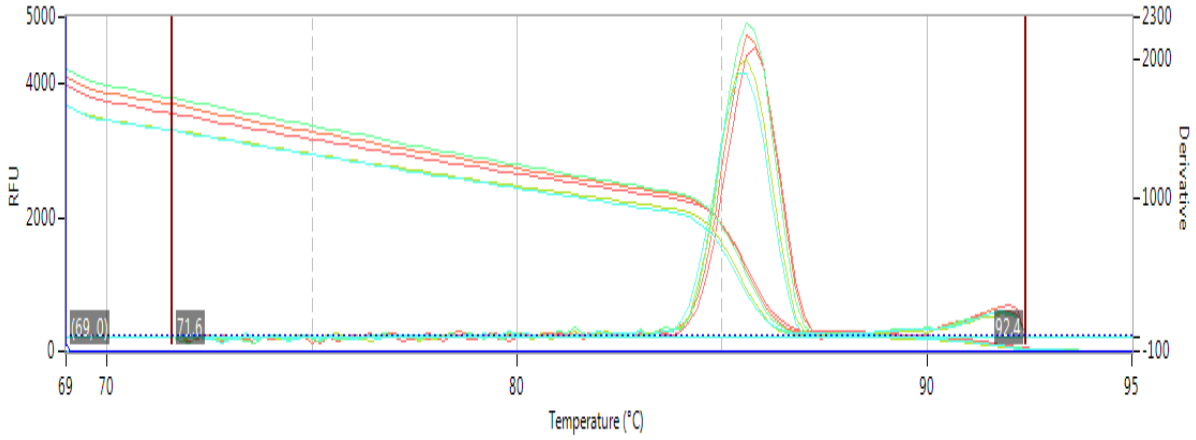


Figure E.3: *dhfr*A7 gene melting curve

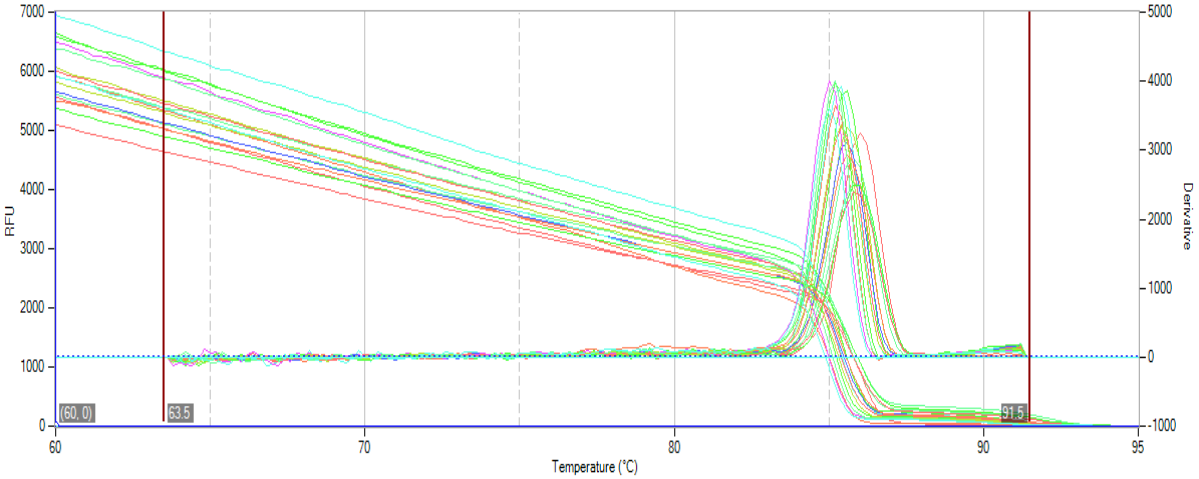


Figure E.4: *sul*1 gene melting curve

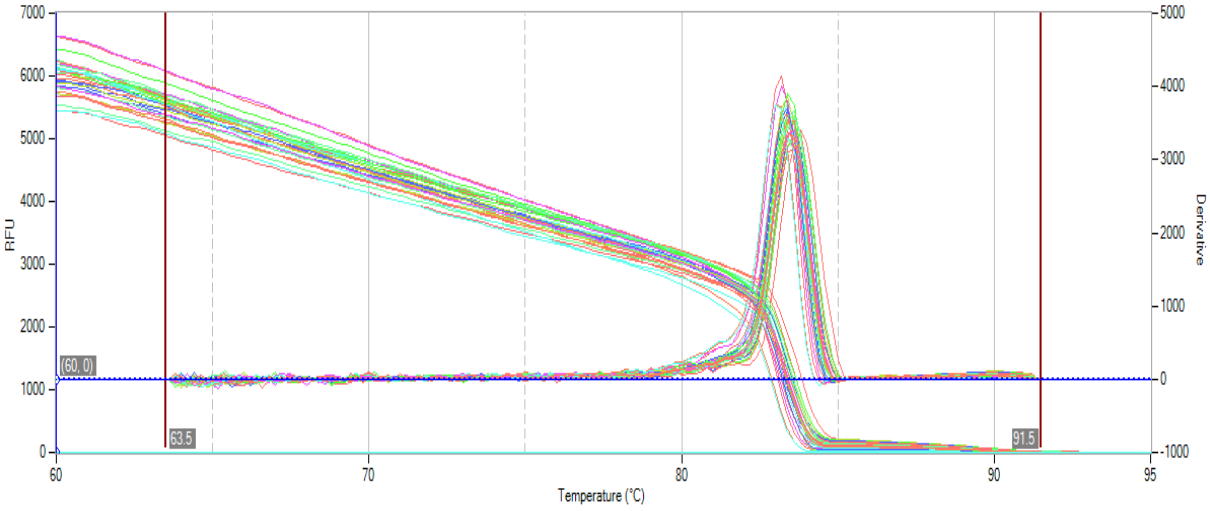


Figure E.5: *Bla*-$_{\text{TEM1}}$ gene melting curve

Appendix E.2: Electrophoresis results

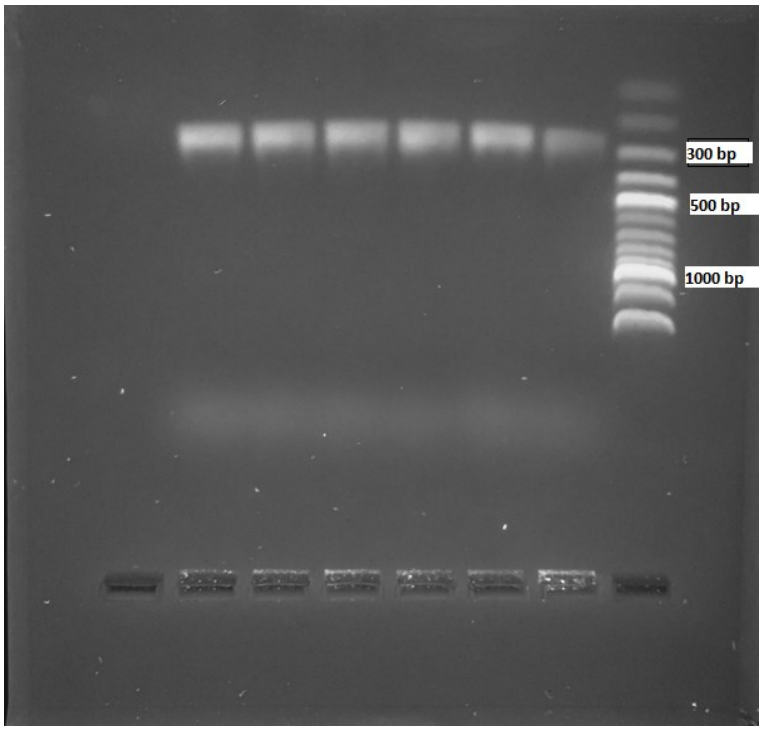


Figure E.6: *bla*-$_{TEM1}$ resistance gene, amplicon length 246 bp

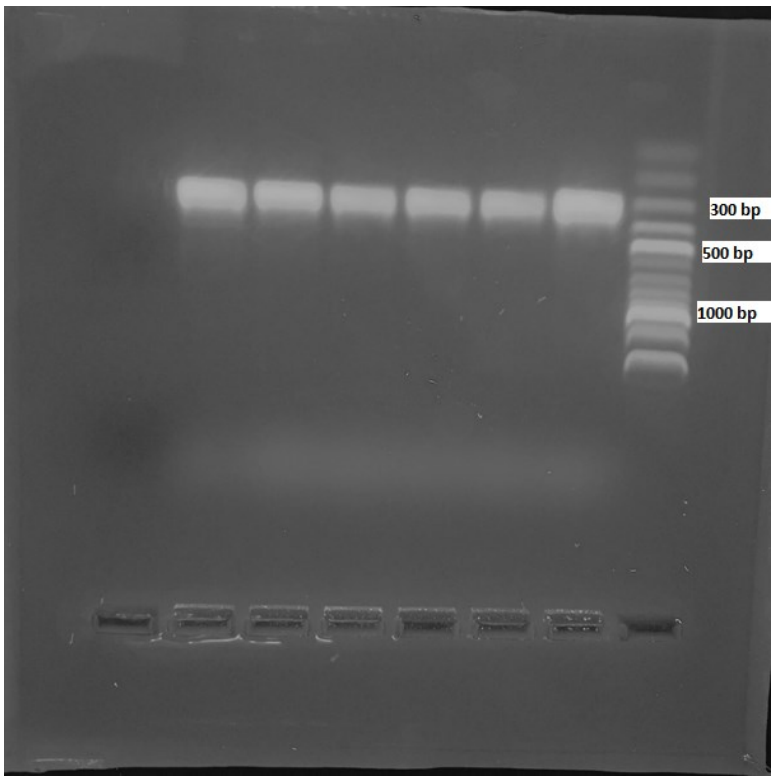


Figure E.7: *mph*(A) gene, amplicon length 293 bp

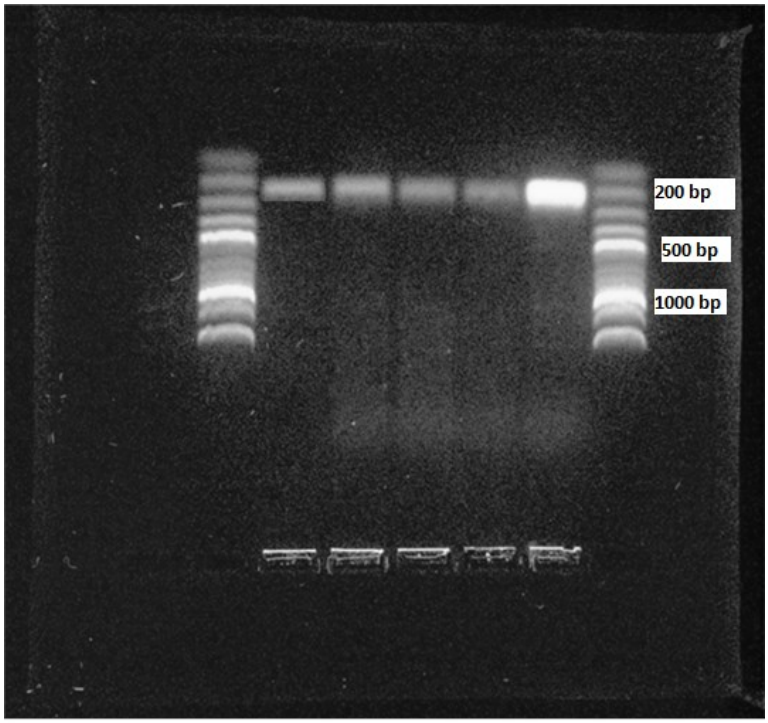


Figure E.8: *tet*(A) gene, amplicon length 190 bp

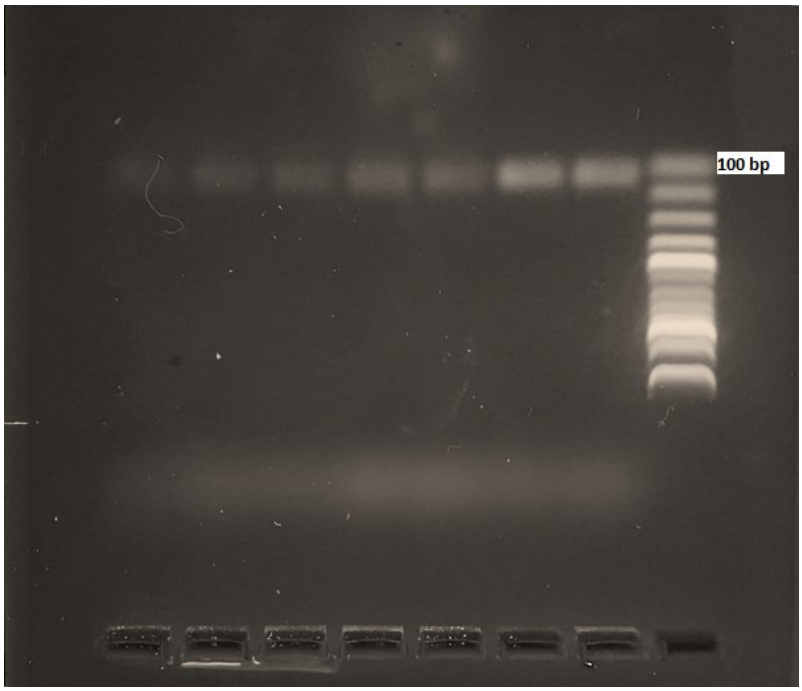


Figure E.9: *dfr*A7 gene, amplicon length 117 bp

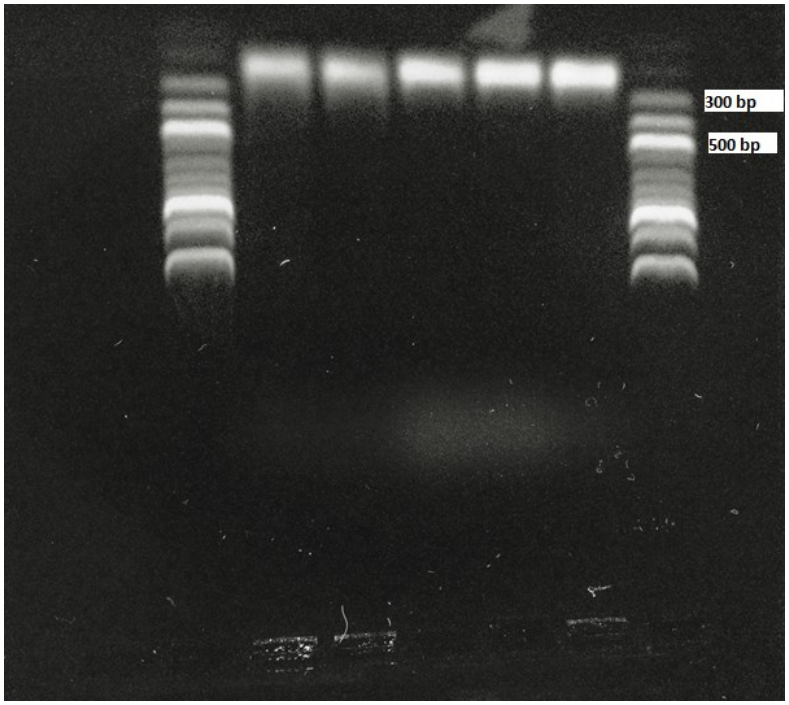


Figure E.10: *sul*1 gene, amplicon length 162 bp

Appendix F. Supplementary information for chlorination experiments

Appendix F.1: Raw data chlorination on antibiotic-resistant *E. coli*, antibiotic-sensitive *E. coli*, antibiotic-resistant *P. aeruginosa* and antibiotic-resistant heterotrophic bacteria (*P. luteola*)

Table F.1: Free chlorine control

Time (minutes)	Residual chlorine concentration (mg/L)
0	1
5	1
15	1
20	1
30	0.9
40	0.9

Table F.2: Enumeration control (in Phosphate buffer saline)

Time (minutes)	Colonies number (10^7 dilution)	CFU/ml	Free chlorine concentration (mg/L)
1	53	5.30E+07	0
	55	5.50E+07	
	52	5.20E+07	
5	56	5.60E+07	0
	49	4.90E+07	
	50	5.00E+07	
10	52	5.20E+07	0
	48	4.80E+07	
	55	5.50E+07	
15	56	5.60E+07	0
	50	5.00E+07	
	50	5.00E+07	
25	50	5.00E+07	0
	51	5.10E+07	
	50	5.00E+07	

Table F.3: Raw data 2 mg/L chlorination on antibiotic-resistant *E. coli* NCTC 13400

Strain	E. Coli 13400	Disinfectant	Cl_2	Concentration	2 mg/L			
t (min)	Plate 1	Plate 2	Plate 3	Mean Colonies	Cfu/ml	(N/N0)	Log_{10}(N/N0)	Ln (N/N0)
1st replicate								
	10^7 dilution							
0	35	40	35	36.67	3.67E+07	1	0	0
	10^2 and 10 dilution							
2	20	21	18	19.67	163.89	4.4697E-06	-5.35	-12.32
5	10	11	10	10.33	86.11	2.34848E-06	-5.63	-12.96
8	51	50	43	48.00	40.00	1.09091E-06	-5.96	-13.73
11	16	21	20	19.00	15.83	4.31818E-07	-6.36	-14.66
15	4	3	5	4.00	3.33	9.09091E-08	-7.04	-16.21
2nd replicate								
	10^7 dilution							
0	44	50	49	47.67	4.77E+07	1	0	0
	10^2 and 10 dilution							
2	28	21	30	26.33	219.44	4.60373E-06	-5.34	-12.29
5	9	11	11	10.33	86.11	1.80653E-06	-5.74	-13.22
8	82	75	71	76.00	63.33	1.32867E-06	-5.88	-13.53
11	18	17	13	16.00	13.33	2.7972E-07	-6.55	-15.09
15	5	5	7	5.67	4.72	9.90676E-08	-7.00	-16.13
3rd replicate								
	10^7 dilution							
0	52	50	48	50	5.00E+07	1	0	0
	10^2 and 10 dilution							
2	24	17	28	23.00	191.67	3.83333E-06	-5.42	-12.47
5	15	12	10	12.33	102.78	2.05556E-06	-5.69	-13.09
8	88	80	92	86.67	72.22	1.44444E-06	-5.84	-13.45
11	23	28	30	27.00	22.50	0.00000045	-6.35	-14.61
15	5	4	3	4.00	3.33	6.66667E-08	-7.18	-16.52

Table F.4: Raw data 1 mg/L chlorination on antibiotic-sensitive *E. coli* NCTC 12241

Strain	E. Coli 12241	Disinfectant	Cl2	Concentration	1 mg/L			
t (min)	Plate 1	Plate 2	Plate 3	Mean Colonie	Cfu/ml	(N/N0)	Log10(N/N0)	Ln (N/N0)
1st replicate								
	10^6 dilution							
0	135	123	117	125	1.25E+07	1	0	0
	10^2 dilution							
2	144	257	-	200.5	1670.83	1.34E-04	-3.87	-8.92
5	207	52	278	179	1491.67	1.19E-04	-3.92	-9.03
8	159	162	240	187	1558.33	1.25E-04	-3.90	-8.99
11	56	185	54	98.33	819.44	6.56E-05	-4.18	-9.63
15	15	13	8	12	144.00	1.15E-05	-4.94	-11.37
2nd replicate								
	10^6 dilution							
0	100	92	103	98.33	9.83E+06	1	0	0
	10^2 dilution							
2	27	125	30	60.67	505.56	5.14124E-05	-4.29	-9.88
5	19	5	13	12.33	102.78	1.0452E-05	-4.98	-11.47
8	13	16	3	9.5	79.17	8.05085E-06	-5.09	-11.73
11	9	6	10	7.5	62.5	6.35593E-06	-5.20	-11.97
15	8	2	7	4.5	16.67	1.69492E-06	-5.77	-13.29
3rd replicate								
	10^6 dilution							
0	99	135	82	105.33	1.05E+07	1	0	0
	10^2 dilution							
2	24	4	36	21.33	177.78	1.68776E-05	-4.77	-10.99
5	10	6	19	12.5	104.17	9.88924E-06	-5.00	-11.52
8	9	9	40	19.33	161.11	1.52954E-05	-4.82	-11.09
11	6	7	7	7	58.33	5.53797E-06	-5.26	-12.10
15	5	1	3	3	25	2.37342E-06	-5.62	-12.95

Table F.5: Raw data 2 mg/L chlorination on antibiotic-sensitive *E. coli* NCTC 12241

Strain	E. Coli 12241	Disinfectant	Cl_2	Concentration	2 mg/L			
t (min)	Plate 1	Plate 2	Plate 3	Mean Colonies	Cfu/ml	(N/N0)	Log_{10}(N/N0)	Ln (N/N0)
1st replicate								
	10^7 dilution							
0	19	23	20	20.67	2.07E+07	1	0	0
	10^2 and 10 dilution							
2	30	35	28	32.50	270.83	1.31048E-05	-4.88	-11.24
5	8	8	7	7.67	63.89	3.0914E-06	-5.51	-12.69
8	110	119	112	113.67	94.72	4.58333E-06	-5.34	-12.29
11	16	35	25	25.33	21.11	1.02151E-06	-5.99	-13.79
15	2	3	5	3.33	2.78	1.34409E-07	-6.87	-15.82
2nd replicate								
	10^7 dilution							
0	11	18	16	15	1.50E+07	1	0	0
	10^2 and 10 dilution							
2	28	21	30	26.33	219.44	1.46296E-05	-4.83	-11.13
5	9	11	11	10.33	86.11	5.74074E-06	-5.24	-12.07
8	101	108	98	102.33	85.28	5.68519E-06	-5.25	-12.08
11	18	17	13	16.00	13.33	8.88889E-07	-6.05	-13.93
15	5	5	7	5.67	4.72	3.14815E-07	-6.50	-14.97
3rd replicate								
	10^7 dilution							
0	19	25	28	24	2.40E+07	1	0	0
	10^2 and 10 dilution							
2	24	4	36	21.33	177.78	7.40741E-06	-5.13	-11.81
5	15	11	10	12.00	100.00	4.16667E-06	-5.38	-12.39
8	98	105	92	98.33	81.94	3.41435E-06	-5.47	-12.59
11	29	35	30	31.33	26.11	1.08796E-06	-5.96	-13.73
15	5	1	3	3.00	2.50	1.04167E-07	-6.98	-16.08

Table F.6: Raw data 1 mg/L chlorination on antibiotic-resistant *P. aeruginosa* NCTC 13437

Strain	P. aeruginosa 13437	Disinfectant	Cl_2	Concentration	1 mg/L			
t (min)	Plate 1	Plate 2	Plate 3	Mean Colonies	Cfu/ml	(N/N0)	Log_{10}(N/N0)	Ln (N/N0)
1st replicate								
	10^6 dilution							
0	150	155	150	151.67	1.52E+07	1	0	0
	10^2 and 10^3 dilution							
2	21	25	23	23.00	1916.67	0.00013	-3.90	-8.98
5	37	38	45	40.00	333.33	2.2E-05	-4.66	-10.73
8	38	39	35	37.33	311.11	2.1E-05	-4.69	-10.79
11	38	38	37	37.67	313.89	2.1E-05	-4.68	-10.79
15	30	25	26	27.00	225.00	1.5E-05	-4.83	-11.12
2nd replicate								
	10^6 dilution							
0	221	187	188	198.6666667	19866667	1	0	0
	10^2 and 10^3 dilution							
2	12	15	13	13.33	1111.11	5.6E-05	-4.25	-9.79
5	9	8	9	8.67	72.22	3.6E-06	-5.44	-12.52
8	6	8	6	6.67	55.56	2.8E-06	-5.55	-12.79
11	5	5	6	5.33	44.44	2.2E-06	-5.65	-13.01
15	3	2	3	2.67	22.22	1.1E-06	-5.95	-13.70
3rd replicate								
	10^6 dilution							
0	145	132	135	137.33	1.37E+07	1	0	0
	10^2 and 10^3 dilution							
2	128	132	122	127.33	10611.11	0.00077	-3.11	-7.17
5	177	139	140	152.00	1266.67	9.2E-05	-4.04	-9.29
8	135	122	130	129.00	1075.00	7.8E-05	-4.11	-9.46
11	85	60	76	73.67	613.89	4.5E-05	-4.35	-10.02
15	51	71	68	63.33	527.78	3.8E-05	-4.42	-10.17

Table F.7: Raw data 2 mg/L chlorination on antibiotic-resistant *P. aeruginosa* NCTC 13437

Strain	P. aeruginosa 13437	Disinfectant	Cl_2	Concentration	2 mg/L			
t (min)	Plate 1	Plate 2	Plate 3	Mean Colonies	Cfu/ml	(N/N0)	Log_{10}(N/N0)	Ln (N/N0)
1st replicate								
	10^6 dilution							
0	150	150	153	151.00	1.51E+07	1	0	0
	10^2 and 10^3 dilution							
2	81	70	70	73.67	613.89	4.1E-05	-4.39	-10.11
5	21	20	21	20.67	172.22	1.1E-05	-4.94	-11.38
8	68	65	60	64.33	53.61	3.6E-06	-5.45	-12.55
11	38	38	37	37.67	31.39	2.1E-06	-5.68	-13.08
15	21	25	26	24.00	20.00	1.3E-06	-5.88	-13.53
2nd replicate								
	10^6 dilution							
0	188	187	188	187.67	1.88E+07	1	0	0
	10^2 and 10^3 dilution							
2	35	41	43	39.67	330.56	1.8E-05	-4.75	-10.95
5	21	25	20	22.00	183.33	9.8E-06	-5.01	-11.54
8	55	58	65	59.33	49.44	2.6E-06	-5.58	-12.85
11	31	24	22	25.67	21.39	1.1E-06	-5.94	-13.68
15	17	11	20	16.00	13.33	7.1E-07	-6.15	-14.16
3rd replicate								
	10^6 dilution							
0	140	138	135	137.67	1.38E+07	1	0	0
	10^2 and 10 dilution							
2	75	70	88	77.67	647.22	4.7E-05	-4.33	-9.97
5	38	39	30	35.67	297.22	2.2E-05	-4.67	-10.74
8	28	24	30	27.33	22.78	1.7E-06	-5.78	-13.31
11	17	21	20	19.33	16.11	1.2E-06	-5.93	-13.66
15	13	17	16	15.33	12.78	9.3E-07	-6.03	-13.89

Table F.8: Raw data 2 mg/L chlorination on environmental strain antibiotic-resistant *P. luteola*

Strain	*P. luteola*	Disinfectant	Cl_2	Concentration	2 mg/L			
t (min)	Plate 1	Plate 2	Plate 3	Mean Colonies	Cfu/ml	(N/N0)	Log_{10}(N/N0)	Ln (N/N0)
1st replicate								
	10^5 dilution							
0	18	13	17	16.00	1.60E+05	1	0	0
	10, 10^2 and 10^3 dilution							
2	14	8	12	11.33	944.44	0.005902778	-2.23	-5.13
5	48	52	43	47.67	397.22	0.002482639	-2.61	-6.00
8	35	43	43	40.33	33.61	0.000210069	-3.68	-8.47
11	19	25	43	29.00	241.67	0.001510417	-2.82	-6.50
15	78	98	88	88.00	73.33	0.000458333	-3.34	-7.69
2nd replicate								
	10^5 dilution							
0	11	13	9	11.00	1.10E+05	1	0	0
	10, 10^2 and 10^3 dilution							
2	28	29	33	30.00	2500.00	0.022727273	-1.64	-3.78
5	165	158	150	157.67	1313.89	0.011944444	-1.92	-4.43
8	43	40	42	41.67	347.22	0.003156566	-2.50	-5.76
11	55	45	40	46.67	388.89	0.003535354	-2.45	-5.64
15	110	108	105	107.67	89.72	0.000815657	-3.09	-7.11
3rd replicate								
	10^5 dilution							
0	13	10	11	11.33	1.13E+05	1	0	0
	10, 10^2 and 10^3 dilution							
2	15	17	18	16.67	1388.89	0.012254902	-1.91	-4.40
5	83	78	80	80.33	669.44	0.005906863	-2.23	-5.13
8	28	29	27	28.00	233.33	0.002058824	-2.69	-6.19
11	23	26	21	23.33	194.44	0.001715686	-2.77	-6.37
15	98	94	97	96.33	80.28	0.000708333	-3.15	-7.25

Appendix F.2: Raw data chlorination on antibiotic resistance genes; chlorine dose 1, 2, and 5 mg/L; contact time 15 minutes

Table F.9: Target gene: *tet*(A)

Chlorine dose 1 mg/L						Chlorine dose 2 mg/L						Chlorine dose 5 mg/L					
target gene	Cq	Resistance gene number	Average	N/N0	Log N/N0	target gene	Cq	Resistance gene number	Average	N/N0	Log N/N0	target gene	Cq	Resistance gene number	Average	N/N0	Log N/N0
	1st replicate						1st replicate						1st replicate				
Control	15.67	6.72E+07	6.98E+07			Control	11.8	1.16E+08	9.18E+07			Control	12.77	3.66E+08	3.84E+08		
	15.77	6.29E+07					13.57	4.37E+07					12.59	4.11E+08			
	15.42	7.92E+07					13.65	4.18E+07					12.73	3.76E+08			
tet(A)	16.38	4.20E+07	5.03E+07	7.21E-01	-0.14	tet(A)	18.2	3.38E+06	3.41E+06	3.72E-02	-1.43	tet(A)	25.07	1.34E+05	1.88E+05	4.89E-04	-3.31
	15.94	5.62E+07					18.56	2.77E+06					24.18	2.37E+05			
	16.04	5.26E+07					17.86	4.08E+06					24.5	1.93E+05			
	2nd replicate						2nd replicate						2nd replicate				
control	15.67	6.72E+07	6.98E+07			control	11.8	1.16E+08	9.18E+07			control	12.77	3.66E+08	3.84E+08		
	15.77	6.29E+07					13.57	4.37E+07					12.59	4.11E+08			
	15.42	7.92E+07					13.65	4.18E+07					12.73	3.76E+08			
tet(A)	16.51	3.86E+07	4.55E+07	6.53E-01	-0.19	tet(A)	17.1	6.21E+06	5.11E+06	5.57E-02	-1.25	tet(A)	24.73	1.66E+05	2.34E+05	6.08E-04	-3.22
	16.23	4.64E+07					18.33	3.15E+06					23.89	2.86E+05			
	16.07	5.16E+07					17.17	5.98E+06					24.1	2.50E+05			
	3rd replicate						3rd replicate						3rd replicate				
control	15.67	6.72E+07	6.98E+07			control	11.8	1.16E+08	9.18E+07			control	12.77	3.66E+08	3.84E+08		
	15.77	6.29E+07					13.57	4.37E+07					12.59	4.11E+08			
	15.42	7.92E+07					13.65	4.18E+07					12.73	3.76E+08			
tet(A)	16.44	4.04E+07	4.14E+07	5.94E-01	-0.23	tet(A)	18.73	2.52E+06	6.44E+06	7.01E-02	-1.15	tet(A)	21.85	1.06E+06	1.09E+06	2.83E-03	-2.55
	16.43	4.07E+07					16.53	8.52E+06					21.35	1.46E+06			
	16.34	4.32E+07					16.58	8.28E+06					22.42	7.36E+05			

Table F.10: Target gene: *mph*(A)

Chlorine dose 1 mg/L						Chlorine dose 2 mg/L						Chlorine dose 5 mg/L					
target gene	Cq	Resistance gene number	Average	N/N0	Log N/N0	target gene	Cq	Resistance gene number	Average	N/N0	Log N/N0	target gene	Cq	Resistance gene number	Average	N/N0	Log N/N0
	1st replicate						1st replicate						1st replicate				
Control	14.28	1.51E+08	1.07E+08			Control	12.33	8.68E+07	9.18E+07			Control	12.28	4.44E+08	4.38E+08		
	15.52	8.51E+07					12.13	9.69E+07					12.11	4.87E+08			
	15.3	8.51E+07					12.13	9.69E+07					12.55	3.82E+08			
mph(A)	16.1	5.50E+07	6.15E+07	5.74E-01	-0.24	mph(A)	18.17	3.44E+06	3.72E+06	4.05E-02	-1.39	mph(A)	29.03	4.24E+04	5.89E+04	1.35E-04	-3.87
	15.96	5.89E+07					17.96	3.86E+06					27.98	7.58E+04			
	15.63	7.08E+07					17.96	3.86E+06					28.45	5.85E+04			
	2nd replicate						2nd replicate						2nd replicate				
control	14.28	1.51E+08	1.07E+08			control	12.33	8.68E+07	9.18E+07			control	12.28	4.44E+08	4.38E+08		
	15.52	8.51E+07					12.13	9.69E+07					12.11	4.87E+08			
	15.3	8.51E+07					12.13	9.69E+07					12.55	3.82E+08			
mph(A)	16.47	4.47E+07	4.47E+07	4.17E-01	-0.38	mph(A)	17.43	5.18E+06	6.31E+06	6.87E-02	-1.16	mph(A)	24.61	4.88E+05	5.12E+05	1.17E-03	-2.93
	16.46	4.47E+07					17.04	6.42E+06					23.69	8.11E+05			
	16.46	4.47E+07					16.8	7.34E+06					25.92	2.37E+05			
	3rd replicate						3rd replicate						3rd replicate				
control	14.28	1.51E+08	1.07E+08			control	12.33	8.68E+07	9.18E+07			control	12.28	4.44E+08	4.38E+08		
	15.52	8.51E+07					12.13	9.69E+07					12.11	4.87E+08			
	15.3	8.51E+07					12.13	9.69E+07					12.55	3.82E+08			
mph(A)	16.47	4.47E+07	5.34E+07	4.98E-01	-0.30	mph(A)	17.49	5.01E+06	1.54E+07	1.67E-01	-0.78	mph(A)	25.61	2.37E+05	3.20E+05	7.32E-04	-3.14
	15.66	7.08E+07					17.35	5.01E+06					24.69	4.88E+05			
	16.46	4.47E+07					18.19	8.60E+06					25.92	2.37E+05			

Table F.11: Target gene: *sul*1

Chlorine dose 1 mg/L						Chlorine dose 2 mg/L						Chlorine dose 5 mg/L					
target gene	Cq	Resistance gene number	Average	N/N0	Log N/N0	target gene	Cq	Resistance gene number	Average	N/N0	Log N/N0	target gene	Cq	Resistance gene number	Average	N/N0	Log N/N0
	1st replicate						1st replicate						1st replicate				
Control	15.59	4.34E+07	1.28E+08			Control	12.01	3.11E+08	2.66E+08			Control	12.06	6.40E+08	6.16E+08		
	15.56	4.42E+07					12.5	2.21E+08					11.66	8.42E+08			
	15.67	4.13E+07					13.18	1.38E+08					12.88	3.66E+08			
Sul1	15.65	4.18E+07	4.08E+07	3.19E-01	-0.50	Sul1	17.71	5.84E+06	6.13E+06	2.30E-02	-1.64	Sul1	24.44	1.37E+05	1.00E+05	1.63E-04	-3.79
	15.63	4.23E+07					17.81	5.44E+06					25.12	8.61E+04			
	15.79	3.83E+07					17.8	5.48E+06					25.26	7.82E+04			
	2nd replicate						2nd replicate						2nd replicate				
control	15.59	4.34E+07	1.28E+08			control	12.01	3.11E+08	2.66E+08			control	12.06	6.40E+08	6.16E+08		
	15.56	4.42E+07					12.5	2.21E+08					11.66	8.42E+08			
	15.67	4.13E+07					13.18	1.38E+08					12.88	3.66E+08			
Sul1	15.65	4.18E+07	4.08E+07	3.19E-01	-0.50	Sul1	17.6	6.30E+06	7.19E+06	2.70E-02	-1.57	Sul1	24.13	1.69E+05	1.50E+05	2.43E-04	-3.61
	15.63	4.23E+07					17.2	8.33E+06					24.22	1.59E+05			
	15.8	3.83E+07					17.46	6.95E+06					24.63	1.20E+05			
	3rd replicate						3rd replicate						3rd replicate				
control	15.59	4.34E+07	1.28E+08			control	12.01	3.11E+08	2.66E+08			control	12.06	6.40E+08	6.16E+08		
	15.56	4.42E+07					12.5	2.21E+08					11.66	8.42E+08			
	15.67	4.13E+07					13.18	1.38E+08					12.88	3.66E+08			
Sul1	15.97	3.42E+07	3.36E+07	2.62E-01	-0.58	Sul1	16.16	1.72E+07	1.43E+07	5.37E-02	-1.27	Sul1	23.13	3.35E+05	3.99E+05	6.48E-04	-3.19
	16.03	3.29E+07					16.55	1.31E+07					22.59	4.84E+05			
	16.49	2.47E+07					16.61	1.26E+07					22.95	3.79E+05			

Table F.12: Target gene: *bla*-$_{\text{TEM1}}$

Chlorine dose 1 mg/L						Chlorine dose 2 mg/L						Chlorine dose 5 mg/L					
target gene	Cq	Resistance gene number	Average	N/N0	Log N/N0	target gene	Cq	Resistance gene number	Average	N/N0	Log N/N0	target gene	Cq	Resistance gene number	Average	N/N0	Log N/N0
	1st replicate						1st replicate						1st replicate				
Control	10.83	1.95E+08	1.28E+08			Control	12.81	4.67E+08	3.90E+08			Control	10.54	2.28E+08	1.34E+08		
	12.32	8.70E+07					12.9	4.37E+08					11.94	1.07E+08			
	12.02	1.02E+08					13.57	2.66E+08					12.81	6.67E+07			
Bla-TEM1	12.51	7.72E+07	7.36E+07	5.75E-01	-0.24	Bla-TEM1	18.42	7.25E+06	6.13E+06	1.57E-02	-1.80	Bla-TEM1	24.95	9.35E+04	6.00E+04	4.48E-04	-3.35
	12.54	7.72E+07					19.06	4.50E+06					26.18	4.80E+04			
	12.82	6.64E+07					18.54	6.63E+06					26.59	3.85E+04			
	2nd replicate						2nd replicate						2nd replicate				
control	10.83	1.95E+08	1.28E+08			control	12.81	4.67E+08	3.90E+08			control	10.54	2.28E+08	1.34E+08		
	12.32	8.70E+07					12.9	4.37E+08					11.94	1.07E+08			
	12.02	1.02E+08					13.57	2.66E+08					12.81	6.67E+07			
Bla-TEM1	11.22	1.58E+08	9.49E+07	7.41E-01	-0.13	Bla-TEM1	19.39	3.53E+06	4.11E+06	1.05E-02	-1.98	Bla-TEM1	26.91	3.24E+04	3.36E+04	2.51E-04	-3.60
	12.65	7.28E+07					19.09	4.41E+06					28.23	1.58E+04			
	13.19	5.43E+07					19.09	4.41E+06					26.01	5.27E+04			
	3rd replicate						3rd replicate						3rd replicate				
control	10.83	1.95E+08	1.28E+08			control	12.81	4.67E+08	3.90E+08			control	10.54	2.28E+08	1.34E+08		
	12.32	8.70E+07					12.9	4.37E+08					11.94	1.07E+08			
	12.02	1.02E+08					13.57	2.66E+08					12.81	6.67E+07			
Bla-TEM1	12.25	9.03E+07	7.24E+07	5.66E-01	-0.25	Bla-TEM1	16.96	2.14E+07	1.54E+07	3.94E-02	-1.40	Bla-TEM1	23.15	2.48E+05	1.86E+05	1.39E-03	-2.86
	12.63	7.35E+07					17.35	1.60E+07					24.96	9.30E+04			
	13.22	5.34E+07					18.19	8.60E+06					23.4	2.16E+05			

Appendix F.3: Raw data chlorination on antibiotic resistance genes; chlorine dose 1, 2, and 5 mg/L; contact time 30 minutes

Table F.13: Target gene: *tet*(A)

Chlorine dose 1 mg/L						Chlorine dose 2 mg/L						Chlorine dose 5 mg/L					
target gene	Cq	Resistance gene number	Average	N/N0	Log N/N0	target gene	Cq	Resistance gene number	Average	N/N0	Log N/N0	target gene	Cq	Resistance gene number	Average	N/N0	Log N/N0
	1st replicate						1st replicate						1st replicate				
Control	14.63	1.33E+08	1.77E+08			Control	11.8	1.16E+08	6.73E+07			Control	12.77	3.66E+08	3.84E+08		
	14.03	1.98E+08					13.57	4.37E+07					12.59	4.11E+08			
	14.02	2.00E+08					13.65	4.18E+07					12.73	3.76E+08			
tet(A)	16.78	3.23E+07	3.08E+07	1.74E-01	-0.76	tet(A)	20.42	2.67E+06	2.68E+06	3.99E-02	-1.40	tet(A)	23.62	3.40E+05	3.58E+05	9.32E-04	-3.03
	16.89	3.00E+07					20.02	3.45E+06					23.74	3.15E+05			
	16.88	3.02E+07					20.92	1.93E+06					23.29	4.20E+05			
	2nd replicate						2nd replicate						2nd replicate				
control	14.63	1.33E+08	1.77E+08			control	11.8	1.16E+08	6.73E+07			control	12.77	3.66E+08	3.84E+08		
	14.03	1.98E+08					13.57	4.37E+07					12.59	4.11E+08			
	14.02	2.00E+08					13.65	4.18E+07					12.73	3.76E+08			
tet(A)	16.83	3.12E+07	3.29E+07	1.86E-01	-0.73	tet(A)	21.11	1.71E+06	2.06E+06	3.06E-02	-1.51	tet(A)	25.97	7.49E+04	8.40E+04	2.18E-04	-3.66
	16.46	3.99E+07					20.94	1.91E+06					25.14	1.28E+05			
	17.01	2.77E+07					20.48	2.56E+06					26.62	4.93E+04			
	3rd replicate						3rd replicate						3rd replicate				
control	14.63	1.33E+08	1.77E+08			control	11.8	1.16E+08	6.73E+07			control	12.77	3.66E+08	3.84E+08		
	14.03	1.98E+08					13.57	4.37E+07					12.59	4.11E+08			
	14.02	2.00E+08					13.65	4.18E+07					12.73	3.76E+08			
tet(A)	16.75	3.29E+07	3.56E+07	2.01E-01	-0.70	tet(A)	20.42	2.67E+06	2.17E+06	3.22E-02	-1.49	tet(A)	25.62	7.49E+04	2.36E+05	6.15E-04	-3.21
	16.48	3.93E+07					20.88	1.91E+06					24.52	2.14E+05			
	16.68	3.45E+07					20.92	1.93E+06					23.29	4.20E+05			

Table F.14: Target gene: *mph*(A)

Chlorine dose 1 mg/L						Chlorine dose 2 mg/L						Chlorine dose 5 mg/L					
target gene	Cq	Resistance gene number	Average	N/N0	Log N/N0	target gene	Cq	Resistance gene number	Average	N/N0	Log N/N0	target gene	Cq	Resistance gene number	Average	N/N0	Log N/N0
	1st replicate						1st replicate						1st replicate				
Control	12.14	4.79E+08	4.27E+08			Control	12.33	8.68E+07	9.18E+07			Control	12.28	4.44E+08	4.38E+08		
	14.53	1.28E+08					12.13	9.69E+07					12.11	4.87E+08			
	12.59	3.74E+08					12.13	9.69E+07					12.55	3.82E+08			
mph(A)	12.23	4.56E+08	2.21E+08	5.18E-01	-0.29	mph(A)	21.7	2.44E+06	1.72E+06	1.88E-02	-1.73	mph(A)	25.95	2.33E+05	1.76E+05	4.03E-04	-3.39
	12.11	4.87E+08					22.48	1.58E+06					26.64	1.59E+05			
	13.54	2.21E+08					23.05	1.16E+06					26.9	1.38E+05			
	2nd replicate						2nd replicate						2nd replicate				
control	12.14	4.79E+08	4.27E+08			control	12.33	8.68E+07	9.18E+07			control	12.28	4.44E+08	4.38E+08		
	14.53	1.28E+08					12.13	9.69E+07					12.11	4.87E+08			
	12.59	3.74E+08					12.13	9.69E+07					12.55	3.82E+08			
mph(A)	11.31	7.58E+08	1.90E+08	4.45E-01	-0.35	mph(A)	22.57	1.51E+06	1.46E+06	1.59E-02	-1.80	mph(A)	27.01	1.30E+05	1.13E+05	2.59E-04	-3.59
	14.02	1.70E+08					23.03	1.17E+06					26.49	1.73E+05			
	13.63	2.10E+08					22.35	1.70E+06					29.25	3.76E+04			
	3rd replicate						3rd replicate						3rd replicate				
control	12.14	4.79E+08	4.27E+08			control	12.33	8.68E+07	9.18E+07			control	12.28	4.44E+08	4.38E+08		
	14.53	1.28E+08					12.13	9.69E+07					12.11	4.87E+08			
	12.59	3.74E+08					12.13	9.69E+07					12.55	3.82E+08			
mph(A)	12.28	4.56E+08	3.89E+08	9.12E-01	-0.30	mph(A)	22.57	1.51E+06	1.53E+06	1.66E-02	-1.78	mph(A)	35.82	9.97E+02	4.18E+04	9.55E-05	-4.02
	12.31	4.89E+08					22.86	1.37E+06					28.61	5.35E+04			
	13.54	2.21E+08					22.35	1.70E+06					28.1	7.10E+04			

Table F.15: Target gene: *sul*1

Chlorine dose 1 mg/L						Chlorine dose 2 mg/L						Chlorine dose 5 mg/L					
target gene	Cq	Resistance gene number	Average	N/N0	Log N/N0	target gene	Cq	Resistance gene number	Average	N/N0	Log N/N0	target gene	Cq	Resistance gene number	Average	N/N0	Log N/N0
	1st replicate						1st replicate						1st replicate				
Control	12.71	4.11E+08	3.34E+08			Control	12.01	3.11E+08	2.66E+08			Control	12.06	6.40E+08	6.16E+08		
	13.1	3.15E+08					12.5	2.21E+08					11.66	8.42E+08			
	13.29	2.77E+08					13.18	1.38E+08					12.88	3.66E+08			
Sul1	13.4	2.57E+08	2.48E+08	7.42E-01	-0.13	Sul1	19.92	2.99E+06	3.32E+06	1.25E-02	-1.90	Sul1	23.61	2.41E+05	2.65E+05	4.29E-04	-3.37
	13.01	3.35E+08					19.59	3.75E+06					23.2	3.19E+05			
	14.16	1.53E+08					19.82	3.21E+06					23.66	2.33E+05			
	2nd replicate						2nd replicate						2nd replicate				
control	12.71	4.11E+08	3.34E+08			control	12.01	3.11E+08	2.66E+08			control	12.06	6.40E+08	6.16E+08		
	13.1	3.15E+08					12.5	2.21E+08					11.66	8.42E+08			
	13.29	2.77E+08					13.18	1.38E+08					12.88	3.66E+08			
Sul1	12.91	3.59E+08	2.01E+08	6.03E-01	-0.22	Sul1	19.54	3.75E+06	3.61E+06	1.35E-02	-1.87	Sul1	26.39	3.62E+04	8.66E+04	1.41E-04	-3.85
	13.53	2.35E+08					19.88	3.21E+06					25.28	7.72E+04			
	14.02	1.68E+08					19.34	3.86E+06					24.34	1.47E+05			
	3rd replicate						3rd replicate						3rd replicate				
control	12.71	4.11E+08	3.34E+08			control	12.01	3.11E+08	2.66E+08			control	12.06	6.40E+08	6.16E+08		
	13.1	3.15E+08					12.5	2.21E+08					11.66	8.42E+08			
	13.29	2.77E+08					13.18	1.38E+08					12.88	3.66E+08			
Sul1	13.36	2.55E+08	2.14E+08	6.41E-01	-0.19	Sul1	19.54	3.75E+06	3.79E+06	1.42E-02	-1.85	Sul1	25.39	4.02E+04	8.80E+04	1.43E-04	-3.85
	13.56	2.35E+08					19.55	3.75E+06					25.28	7.72E+04			
	14.16	1.53E+08					19.34	3.86E+06					24.34	1.47E+05			

Table F.16: Target gene: *Bla-*$_{TEM1}$

Chlorine dose 1 mg/L						Chlorine dose 2 mg/L						Chlorine dose 5 mg/L					
target gene	Cq	Resistance gene number	Average	N/N0	Log N/N0	target gene	Cq	Resistance gene number	Average	N/N0	Log N/N0	target gene	Cq	Resistance gene number	Average	N/N0	Log N/N0
	1st replicate						1st replicate						1st replicate				
Control	10.83	1.95E+08	1.28E+08			Control	12.81	4.67E+08	3.90E+08			Control	10.54	2.28E+08	1.34E+08		
	12.32	8.70E+07					12.9	4.37E+08					11.94	1.07E+08			
	12.02	1.02E+08					13.57	2.66E+08					12.81	6.67E+07			
Bla-TEM1	11.03	1.75E+08	1.06E+08	8.29E-01	-0.08	Bla-TEM1	21.01	7.89E+05	6.85E+05	1.76E-03	-2.76	Bla-TEM1	24.49	1.20E+05	9.23E+04	6.89E-04	-3.16
	12.54	7.72E+07					21.39	6.42E+05					25.02	9.00E+04			
	12.82	6.64E+07					21.44	6.25E+05					25.57	6.68E+04			
	2nd replicate						2nd replicate						2nd replicate				
control	10.83	1.95E+08	1.28E+08			control	12.81	4.67E+08	3.90E+08			control	10.54	2.28E+08	1.34E+08		
	12.32	8.70E+07					12.9	4.37E+08					11.94	1.07E+08			
	12.02	1.02E+08					13.57	2.66E+08					12.81	6.67E+07			
Bla-TEM1	11.22	1.58E+08	9.49E+07	7.41E-01	-0.13	Bla-TEM1	21.5	7.24E+05	6.87E+05	1.76E-03	-2.75	Bla-TEM1	27.18	2.80E+04	2.13E+04	1.59E-04	-3.80
	12.65	7.28E+07					21.35	6.92E+05					28.03	1.77E+04			
	13.19	5.43E+07					21.47	6.46E+05					27.96	1.83E+04			
	3rd replicate						3rd replicate						3rd replicate				
control	10.83	1.95E+08	1.28E+08			control	12.81	4.67E+08	3.90E+08			control	10.54	2.28E+08	1.34E+08		
	12.32	8.70E+07					12.9	4.37E+08					11.94	1.07E+08			
	12.02	1.02E+08					13.57	2.66E+08					12.81	6.67E+07			
Bla-TEM1	11.22	1.58E+08	1.03E+08	8.01E-01	-0.10	Bla-TEM1	20.98	7.44E+05	6.94E+05	1.78E-03	-2.75	Bla-TEM1	25.68	6.68E+04	7.29E+04	5.45E-04	-3.26
	12.65	7.28E+07					21.35	6.92E+05					25.02	9.00E+04			
	12.31	7.72E+07					21.35	6.46E+05					26.01	6.18E+04			

Appendix F.4: Raw data on antibiotic-resistant *E. coli* and *P. aeruginosa* regrowth following 1, 2 and 5 mg/L of chlorination

Table F.17: Raw data antibiotic-resistant *E. coli* regrowth following 1, 2, and 5 mg/L of chlorination

E. coli NCTC 13400																		
Chlorine dose 1 mg/L							Chlorine dose 2 mg/L							Chlorine dose 5 mg/L						
incubation time	plate 1	plate 2	plate 3	mean colonies	cfu/ml	log_{10} cfu/ml	incubation time	plate 1	plate 2	plate 3	mean colonies	cfu/ml	log_{10} cfu/ml	incubation time	plate 1	plate 2	plate 3	mean colonies	cfu/ml	log_{10} cfu/ml
1st replicate							1st replicate							1st replicate						
24 hr	175	154	154	161.00	1.97E+02	2.29	24 hr	12	9	12	11.00	3.00E+01	1.48	24 hr	90	60	72	74.00	3.00E+01	1.48
48 hr	188	183	185	185.33	2.26E+03	3.35	48 hr	4	5	4	4.33	3.30E+01	1.52	48 hr	4	8	8	6.67	3.60E+01	1.56
72 hr	59	58	50	55.67	6.68E+04	4.82	72 hr	4	8	6	6.00	5.40E+01	1.73	72 hr	8	11	11	10.00	2.80E+02	2.45
1 week	34	29	32	31.67	3.45E+05	5.54	1 week	15	31	20	22.00	2.48E+05	5.39	1 week	13	12	15	13.33	1.50E+04	4.18
2nd replicate							2nd replicate							2nd replicate						
24 hr	154	123	155	144	1.44E+02	2.16	24 hr	43	36	57	45.33333	4.70E+01	1.67	24 hr	43	42	55	46.67	5.10E+01	1.71
48 hr	41	43	39	41.00	4.98E+02	2.70	48 hr	17	15	19	17.00	2.04E+02	2.31	48 hr	11	12	15	12.67	1.38E+02	2.14
72 hr	50	37	47	44.67	6.00E+04	4.78	72 hr	18	36	31	28.33	3.40E+03	3.53	72 hr	29	22	25	25.33	2.52E+02	2.40
1 week	141	136	126	134.33	1.65E+05	5.22	1 week	48	39	39	42	5.04E+05	5.70	1 week	15	18	19	17.33	1.98E+04	4.30
3rd replicate							3rd replicate							3rd replicate						
24 hr	155	134	140	143.00	1.43E+02	2.16	24 hr	19	24	25	22.67	4.50E+01	1.65	24 hr	143	157	132	144.00	1.44E+02	2.16
48 hr	184	195	190	189.67	2.28E+03	3.36	48 hr	9	8	7	8.00	8.60E+01	1.93	48 hr	18	17	18	17.67	2.10E+02	2.32
72 hr	76	78	78	77.33	9.82E+04	4.99	72 hr	34	38	36	36.00	4.22E+03	3.63	72 hr	28	31	23	27.33	2.73E+02	2.44
1 week	17	19	24	20.00	2.52E+05	5.40	1 week	19	25	28	24.00	7.60E+04	4.88	1 week	13	15	18	15.33	1.68E+04	4.23
Control																				
Chlorine dose 1 mg/L							Chlorine dose 2 mg/L							Chlorine dose 5 mg/L						
incubation time	plate 1	plate 2	plate 3	mean colonies	cfu/ml	log_{10} cfu/ml	incubation time	plate 1	plate 2	plate 3	mean colonies	cfu/ml	log_{10} cfu/ml	incubation time	plate 1	plate 2	plate 3	mean colonies	cfu/ml	log_{10} cfu/ml
1st replicate							1st replicate							1st replicate						
24 hr	85	50	61	65.33	6.53E+07	7.82	24 hr	36	30	35	33.67	3.37E+07	7.53	24 hr	36	30	31	32.33	3.23E+07	7.51
48 hr	18	24	35	25.67	2.57E+07	7.41	48 hr	37	39	35	37.00	3.70E+07	7.57	48 hr	15	24	23	20.67	2.07E+07	7.32
72 hr	15	20	28	21.00	2.10E+07	7.32	72 hr	38	37	34	36.33	3.63E+07	7.56	72 hr	18	24	25	22.33	2.23E+07	7.35
1 week	15	25	26	22.00	2.20E+07	7.34	1 week	22	30	39	30.33	3.03E+07	7.48	1 week	20	28	23	23.67	2.37E+07	7.37

Table F.18: Raw data antibiotic-resistant *P. aeruginosa* regrowth following 1, 2, and 5 mg/L of chlorination

P. aeruginosa NCTC 13437

Chlorine dose 1 mg/L							Chlorine dose 2 mg/L							Chlorine dose 5 mg/L						
incubation time	plate 1	plate 2	plate 3	mean colonies	cfu/ml	$\log_{10}$ cfu/ml	incubation time	plate 1	plate 2	plate 3	mean colonies	cfu/ml	$\log_{10}$ cfu/ml	incubation time	plate 1	plate 2	plate 3	mean colonies	cfu/ml	$\log_{10}$ cfu/ml
1st replicate							1st replicate							1st replicate						
24 hr	62	62	70	64.67	6.47E+02	2.81	24 hr	220	210	202	210.67	2.00E+02	2.30	24 hr	13	5	6	8.00	8.00E+00	0.90
48 hr	18	41	30	29.67	2.70E+03	3.43	48 hr	83	89	89	87.00	8.70E+03	3.94	48 hr	71	87	74	77.33	7.73E+01	1.89
72 hr	42	24	19	28.33	2.83E+04	4.45	72 hr	73	87	89	83.00	8.30E+04	4.92	72 hr	84	104	85	91.00	9.10E+02	2.96
1 week	12	18	17	15.67	1.57E+06	6.19	1 week	31	41	11	27.67	2.77E+06	6.44	1 week	11	19	16	15.33	1.53E+04	4.19
2nd replicate							2nd replicate							2nd replicate						
24 hr	93	92	106	97	9.70E+02	2.99	24 hr	283	273	285	280.3333	2.80E+02	2.45	24 hr	6	4	3	4.33	4.00E+00	0.60
48 hr	116	113	121	116.67	1.17E+04	4.07	48 hr	123	117	130	123.33	1.23E+04	4.09	48 hr	14	22	13	16.33	1.63E+01	1.21
72 hr	205	165	135	168.33	1.68E+05	5.23	72 hr	82	89	46	72.33	6.23E+04	4.79	72 hr	102	98	104	101.33	1.01E+03	3.01
1 week	40	41	39	40	4.00E+06	6.60	1 week	19	14	21	18	1.80E+06	6.26	1 week	289	276	261	275.33	3.63E+05	5.56
3rd replicate							3rd replicate							3rd replicate						
24 hr	68	74	78	73.33	7.30E+02	2.86	24 hr	235	240	245	240.00	2.40E+02	2.38	24 hr	8	7	9	8.00	8.00E+00	0.90
48 hr	68	81	78	75.67	7.57E+03	3.88	48 hr	98	101	95	98.00	9.80E+03	3.99	48 hr	75	52	43	56.67	5.67E+01	1.75
72 hr	98	102	100	100.00	1.00E+05	5.00	72 hr	84	104	85	91.00	6.50E+04	4.81	72 hr	85	98	88	90.33	9.03E+02	2.96
1 week	12	18	28	19.33	1.93E+06	6.29	1 week	19	25	28	24.00	2.40E+06	6.38	1 week	96	105	110	103.67	1.04E+05	5.02

Control

Chlorine dose 1 mg/L							Chlorine dose 2 mg/L							Chlorine dose 5 mg/L						
incubation time	plate 1	plate 2	plate 3	mean colonies	cfu/ml	$\log_{10}$ cfu/ml	incubation time	plate 1	plate 2	plate 3	mean colonies	cfu/ml	$\log_{10}$ cfu/ml	incubation time	plate 1	plate 2	plate 3	mean colonies	cfu/ml	$\log_{10}$ cfu/ml
1st replicate							1st replicate							1st replicate						
24 hr	88	101	81	90.00	9.00E+07	7.95	24 hr	88	106	89	94.33	9.43E+07	7.97	24 hr	88	106	89	94.33	9.43E+07	7.97
48 hr	98	85	88	90.33	9.03E+07	7.96	48 hr	103	98	105	102.00	1.02E+08	8.01	48 hr	103	98	105	102.00	1.02E+08	8.01
72 hr	59	58	65	60.67	6.07E+07	7.78	72 hr	59	65	60	61.33	6.13E+07	7.79	72 hr	59	65	60	61.33	6.13E+07	7.79
1 week	80	71	71	74.00	7.40E+07	7.87	1 week	85	78	84	82.33	8.23E+07	7.92	1 week	85	78	84	82.33	8.23E+07	7.92

Appendix F.5: Antibiotic-resistant *Escherichia coli* and *Pseudomonas aeruginosa* regrowth with continuous chlorination

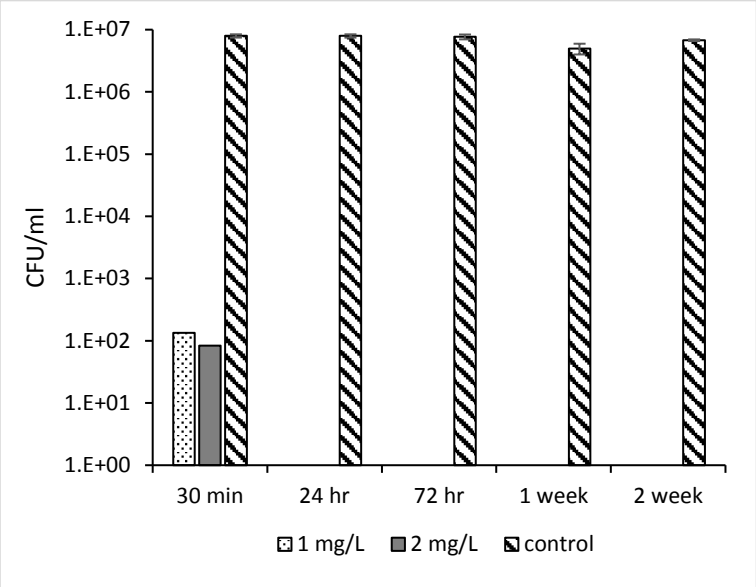


Figure F.1 *Escherichia coli* regrowth following continuous chlorination doses of 1 and 2 mg/L

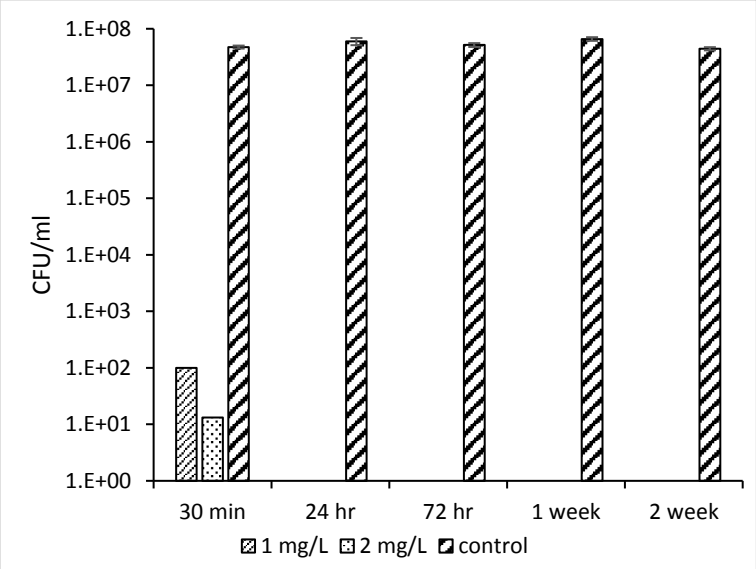


Figure F.2 *Pseudomonas aeruginosa* regrowth following continuous chlorination doses of 1 and 2 mg/L

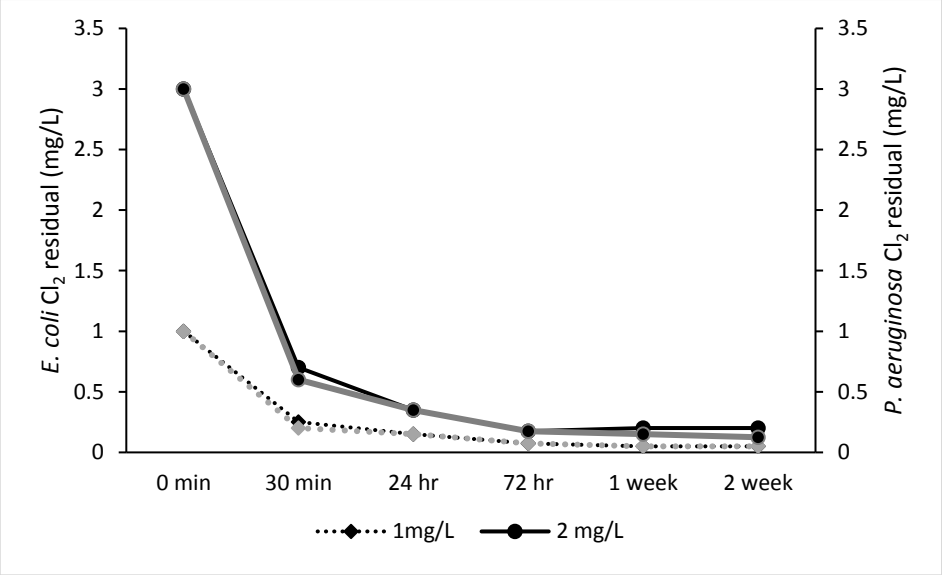


Figure F.3 Chlorine residual concentration (mg/L)

Appendix G: Raw data UV disinfection antibiotic-sensitive *E. coli*, antibiotic-resistant *E. coli*, antibiotic resistant *P. aeruginosa* and antibiotic-resistant heterotrophic bacteria (*P. luteola*)

Table G.1: Raw data UV disinfection antibiotic-resistant *E. coli* NCTC 13400

1 mJ/cm^2							3 mJ/cm^2						
Plate 1	Plate 2	Plate 3	Mean Colonies	Cfu/ml	(N/N0)	Log_{10}(N/N0)	Plate 1	Plate 2	Plate 3	Mean Colonies	Cfu/ml	(N/N0)	Log_{10}(N/N0)
1st replicate							1st replicate						
control 10^6 dilution							control 10^6 dilution						
37	22	17	25.33	2.53E+06	1	0	25	30	23	26.00	2.60E+06	1	0
10^3 dilution							10^2 dilution						
90	113	118	101.50	1.02E+04	0.004007	-2.40	30	25	31	27.50	27.50	1.058E-05	-4.98
2nd replicate							2nd replicate						
control 10^6 dilution							control 10^6 dilution						
38	50	42	43.3333	4.33E+06	1	0	25	30	35	30	3.00E+06	1	0
10^3 dilution							10^2 dilution						
60	63	62	61.67	6.17E+03	0.001423	-2.85	20	20	23	21.00	21.00	7.00E-06	-5.15
3rd replicate							3rd replicate						
control 10^6 dilution							control 10^6 dilution						
37	35	30	34	3.40E+06	1	0	45	51	60	52	5.20E+06	1	0
10^3 dilution							10^2 dilution						
76	82	80	79.33	7.93E+03	0.002333	-2.63	25	37	28	30.00	30.00	5.769E-06	-5.24
7mJ/cm^2							5 mJ/cm^2						
Plate 1	Plate 2	Plate 3	Mean Colonies	Cfu/ml	(N/N0)	Log_{10}(N/N0)	Plate 1	Plate 2	Plate 3	Mean Colonies	Cfu/ml	(N/N0)	Log_{10}(N/N0)
1st replicate							1st replicate						
Control 10^6 dilution							Control 10^6 dilution						
150	143	131	141.33	1.41E+07	1	0	123	118	115	118.67	1.19E+07	1	0
10 dilution							10^2 dilution						
8	10	11	9.00	9.00	6.37E-07	-6.20	21	25	21	23.00	23.00	1.938E-06	-5.71
2nd replicate							2nd replicate						
Control 10^6 dilution							Control 10^6 dilution						
130	130	135	131.667	1.32E+07	1	0	110	113	115	112.67	1.13E+07	1	0
10 dilution							10^2 dilution						
9	10	11	10.00	10.00	7.59E-07	-6.12	11	11	12	11.33	11.33	1.006E-06	-6.00
3rd replicate							3rd replicate						
Control 10^6 dilution							Control 10^6 dilution						
187	188	180	185	1.85E+07	1	0	187	188	180	185	1.85E+07	1	0
10 dilution							10^2 dilution						
8	8	8	8.00	8.00	4.32E-07	-6.36	11	12	11	11.33	11.33	6.126E-07	-6.21

Table G.2: Raw data UV disinfection antibiotic-sensitive *E. coli* NCTC 12241

1 mJ/cm²							3 mJ/cm²						
Plate 1	Plate 2	Plate 3	Mean Colonies	Cfu/ml	(N/N0)	Log_{10}(N/N0)	Plate 1	Plate 2	Plate 3	Mean Colonies	Cfu/ml	(N/N0)	Log_{10}(N/N0)
1st replicate							1st replicate						
control 10^6 dilution							control 10^5 dilution						
25	28	25	26.00	2.60E+06	1	0	64	66	60	63.33	6.33E+05	1	0
10^5 dilution							10^3 dilution						
20	21	24	20.50	2.05E+05	0.078846	-1.10	81	81	99	81.00	8.10E+03	0.0128	-1.89
2nd replicate							2nd replicate						
control 10^6 dilution							control 10^5 dilution						
16	18	20	18	1.80E+06	1	0	65	67	63	65	6.50E+05	1	0
10^5 dilution							10^3 dilution						
24	24	25	24.33	2.43E+05	1.35E-01	-0.87	94	88	101	94.33	9433.33	0.0145	-1.84
3rd replicate							3rd replicate						
control 10^6 dilution							control 10^5 dilution						
24	25	23	24	2.40E+06	1	0	37	35	38	36.67	3.67E+05	1	0
10^5 dilution							10^3 dilution						
27	24	25	25.33	2.53E+05	0.105556	-0.98	79	70	71	73.33	7333.33	0.02	-1.70
7mJ/cm²							5 mJ/cm²						
Plate 1	Plate 2	Plate 3	Mean Colonies	Cfu/ml	(N/N0)	Log_{10}(N/N0)	Plate 1	Plate 2	Plate 3	Mean Colonies	Cfu/ml	(N/N0)	Log_{10}(N/N0)
1st replicate							1st replicate						
Control 10^6 dilution							Control 10^5 dilution						
37	23	28	29.33	2.93E+06	1	0	67	60	65	64.00	6.40E+05	1	0
10 dilution							10 dilution						
12	8	9	10.00	10.00	3.41E-06	-5.47	12	19	16	15.50	15.50	2E-05	-4.62
2nd replicate							2nd replicate						
Control 10^6 dilution							Control 10^5 dilution						
17	26	31	24.67	2.47E+06	1	0	64	66	60	63.33333	6.33E+05	1	0
10 dilution							10 dilution						
15	17	12	14.67	14.67	5.95E-06	-5.23	18	20	17	18.33	18.33	3E-05	-4.54
3rd replicate							3rd replicate						
Control 10^6 dilution							Control 10^5 dilution						
31	28	27	28.67	2.87E+06	1	0	59	61	68	62.66667	6.27E+05	1	0
10 dilution							10 dilution						
13	12	10	11.67	11.67	4.07E-06	-5.39	33	35	31	33.00	33.00	5E-05	-4.28

Table G.3: Raw data UV disinfection *P. aeruginosa* NCTCT 13437

1 mJ/cm^2							3 mJ/cm^2						
Plate 1	Plate 2	Plate 3	Mean Colonies	Cfu/ml	(N/N0)	Log_{10}(N/N0)	Plate 1	Plate 2	Plate 3	Mean Colonies	Cfu/ml	(N/N0)	Log_{10}(N/N0)
1st replicate							1st replicate						
control 10^5 dilution							control 10^5 dilution						
138	108	110	118.67	1.19E+06	1	0	146	137	145	142.67	1.43E+06	1	0
10^4 dilution							10^2 dilution						
60	73	71	66.50	6.65E+04	0.056	-1.25	60	50	55	55.00	550.00	0.0004	-3.41
2nd replicate							2nd replicate						
control 10^5 dilution							control 10^5 dilution						
116	167	143	142	1.42E+06	1	0	130	135	124	129.667	1.30E+06	1	0
10^4 dilution							10^2 dilution						
31	67	55	51.00	5.10E+04	0.036	-1.44	30	15	21	22.00	220.00	0.0002	-3.77
3rd replicate							3rd replicate						
control 10^5 dilution							control 10^5 dilution						
130	137	146	137.6667	1.38E+06	1	0	186	167	175	176	1.76E+06	1	0
10^4 dilution							10^2 dilution						
23	51	36	36.67	3.66E+04	0.027	-1.58	80	70	41	63.67	636.67	0.0004	-3.44
7mJ/cm^2							5 mJ/cm^2						
Plate 1	Plate 2	Plate 3	Mean Colonies	Cfu/ml	(N/N0)	Log_{10}(N/N0)	Plate 1	Plate 2	Plate 3	Mean Colonies	Cfu/ml	(N/N0)	Log_{10}(N/N0)
1st replicate							1st replicate						
Control 10^5 dilution							Control 10^6 dilution						
138	145	138	140.33	1.40E+06	1	0	37	35	29	33.67	3.37E+06	1	0
10^2 dilution							10^2 dilution						
14	12	9	13.00	130.00	9E-05	-4.03	37	35	38	36.00	360.00	0.0001	-3.97
2nd replicate							2nd replicate						
Control 10^5 dilution							Control 10^6 dilution						
110	113	115	112.6667	1.13E+06	1	0	35	36	30	33.6667	3.37E+06	1	0
10^2 dilution							10^2 dilution						
15	17	12	14.67	146.67	1E-04	-3.89	69	59	55	61.00	610.00	0.0002	-3.74
3rd replicate							3rd replicate						
Control 10^5 dilution							Control 10^6 dilution						
187	188	180	185	1.85E+06	1	0	29	31	29	29.6667	2.97E+06	1	0
10^2 dilution							10^2 dilution						
19	17	16	17.33	173.33	9E-05	-4.03	33	35	31	33.00	330.00	0.0001	-3.95

Table G.4: Raw data UV disinfection on trimethoprim-resistant HPC bacteria (*P. luteola*)

1 mJ/cm²							3 mJ/cm²						
Plate 1	Plate 2	Plate 3	Mean Colonies	Cfu/ml	(N/N0)	Log_{10}(N/N0)	Plate 1	Plate 2	Plate 3	Mean Colonies	Cfu/ml	(N/N0)	Log_{10}(N/N0)
1st replicate							1st replicate						
control 10^5 dilution							control 10^5 dilution						
60	65	62	62.33	6.23E+05	1	0	60	65	61	62.00	6.20E+05	1	0
10^4 dilution							10^3 dilution						
66	45	32	55.50	5.55E+04	0.089	-1.05	32	31	35	31.50	3150.00	0.0051	-2.29
2nd replicate							2nd replicate						
control 10^5 dilution							control 10^5 dilution						
31	38	45	38	3.80E+05	1	0	31	39	40	36.66667	3.67E+05	1	0
10^4 dilution							10^3 dilution						
44	40	37	40.33	4.03E+04	0.1061	-0.97	18	30	35	27.67	2766.67	0.0075	-2.12
3rd replicate							3rd replicate						
control 10^5 dilution							control 10^5 dilution						
45	50	50	48.3333	4.83E+05	1	0	45	50	47	47.33333	4.73E+05	1	0
10^4 dilution							10^3 dilution						
58	45	43	48.67	4.87E+04	0.1007	-1.00	35	31	40	35.33	3533.33	0.0075	-2.13
7mJ/cm²							5 mJ/cm²						
Plate 1	Plate 2	Plate 3	Mean Colonies	Cfu/ml	(N/N0)	Log_{10}(N/N0)	Plate 1	Plate 2	Plate 3	Mean Colonies	Cfu/ml	(N/N0)	Log_{10}(N/N0)
1st replicate							1st replicate						
Control 10^5 dilution							Control 10^5 dilution						
60	65	61	62.00	6.20E+05	1	0	61	60	59	60.00	6.00E+05	1	0
10 dilution							10^2 dilution						
37	21	31	29.00	29.00	5E-05	-4.33	28	19	34	23.50	235.00	0.0004	-3.41
2nd replicate							2nd replicate						
Control 10^5 dilution							Control 10^5 dilution						
33	30	41	34.6667	3.47E+05	1	0	38	32	38	36	3.60E+05	1	0
10 dilution							10^2 dilution						
41	39	27	35.67	35.67	0.0001	-3.99	43	40	39	40.67	406.67	0.0011	-2.95
3rd replicate							3rd replicate						
Control 10^5 dilution							Control 10^5 dilution						
43	45	40	42.6667	4.27E+05	1	0	40	40	38	39.33333	3.93E+05	1	0
10 dilution							10^2 dilution						
55	52	40	49.00	49.00	0.0001	-3.94	38	37	40	38.33	383.33	0.001	-3.01

Appendix G.2: Raw data UV disinfection on antibiotic resistance genes; UV dose 20, 50, 100 and 200 mJ/cm^2

Table G.5: Target gene: *tet*(A)

UV dose 20 mJ/cm^2						UV dose 50 mJ/cm^2						UV dose 100 mJ/cm^2						UV dose 200 mJ/cm^2					
target gene	Cq	Resistance gene number	Average	N/N0	Log N/N0	target gene	Cq	Resistance gene number	Average	N/N0	Log N/N0	target gene	Cq	Resistance gene number	Average	N/N0	Log N/N0	target gene	Cq	Resistance gene number	average	N/N0	Log N/N0
	1st replicate						1st replicate						1st replicate						1st replicate				
Control	13.6	8.58E+07	1.34E+08			Control	12.99	1.70E+08	1.53E+08			Control	13.6	1.35E+08	1.34E+08			Control	13.6	8.58E+07	1.34E+08		
	13.6	1.35E+08					13.19	1.50E+08					13.6	1.35E+08					13.6	1.35E+08			
	13.6	1.32E+08					13.32	1.38E+08					13.6	1.32E+08					13.6	1.32E+08			
tet(A)	14.28	7.52E+07	1.19E+08	8.90E-01	-0.05	tet(A)	15.6	3.26E+07	3.94E+07	2.58E-01	-0.59	tet(A)	14.92	5.02E+07	5.93E+07	4.43E-01	-0.35	tet(A)	15.39	3.73E+07	3.48E+07	2.61E-01	-0.58
	13.31	1.39E+08					15.13	4.39E+07					14.54	6.38E+07					15.63	3.20E+07			
	13.27	1.43E+08					15.21	4.18E+07					14.65	6.38E+07					15.48	3.52E+07			
	2nd replicate						2nd replicate						2nd replicate						2nd replicate				
control	13.7	8.58E+07	1.34E+08			control	12.99	1.70E+08	1.53E+08			control	13.7	8.58E+07	1.18E+08			control	13.7	1.05E+08	9.14E+07		
	13.6	1.35E+08					13.19	1.50E+08					13.6	1.35E+08					13.6	9.91E+07			
	13.6	1.32E+08					13.32	1.38E+08					13.6	1.32E+08					13.6	8.38E+07			
tet(A)	13.6	1.16E+08	1.16E+08	8.72E-01	-0.06	tet(A)	13.84	9.94E+07	8.97E+07	5.87E-01	-0.23	tet(A)	14.68	5.84E+07	5.52E+07	4.70E-01	-0.33	tet(A)	16.1	1.61E+07	1.50E+07	1.64E-01	-0.79
	13.61	1.15E+08					14.04	8.76E+07					14.81	5.38E+07					15.98	1.44E+07			
	13.56	1.19E+08					14.14	8.22E+07					14.78	5.35E+07					16.21	1.44E+07			
	3rd replicate						3rd replicate						3rd replicate						3rd replicate				
control	13.7	8.58E+07	1.34E+08			control	12.99	1.70E+08	1.53E+08			control	13.7	8.58E+07	1.18E+08			control	13.7	8.32E+07	9.46E+07		
	13.6	1.35E+08					13.19	1.50E+08					13.6	1.35E+08					13.6	1.00E+08			
	13.6	1.32E+08					13.32	1.38E+08					13.6	1.32E+08					13.6	8.91E+07			
tet-A	13.6	1.16E+08	1.16E+08	8.71E-01	-0.06	tet-A	13.65	1.12E+08	1.03E+08	6.77E-01	-0.17	tet-A	15.25	4.07E+07	4.45E+07	3.78E-01	-0.42	tet-A	16.25	1.02E+07	9.16E+06	9.69E-02	-1.01
	13.52	1.15E+08					14.05	8.76E+07					15.05	4.62E+07					16.35	1.00E+07			
	13.56	1.19E+08					13.67	1.11E+08					14.9	4.65E+07					16.21	7.24E+06			

Table G.6: Target gene: *mph*(A)

UV dose 20 mJ/cm^2						UV dose 50 mJ/cm^2						UV dose 100 mJ/cm^2						UV dose 200 mJ/cm^2					
target gene	Cq	Resistance gene number	Average	N/N0	Log N/N0	target gene	Cq	Resistance gene number	Average	N/N0	Log N/N0	target gene	Cq	Resistance gene number	Average	N/N0	Log N/N0	target gene	Cq	Resistance gene number	average	N/N0	Log N/N0
	1st replicate						1st replicate						1st replicate						1st replicate				
Control	11.36	4.60E+07	3.27E+07			Control	13.43	3.55E+07	4.45E+07			Control	14.01	1.02E+08	1.07E+08			Control	14.01	1.02E+08	1.10E+08		
	12.04	3.34E+07					12.81	4.90E+07					13.83	1.15E+08					13.83	1.15E+08			
	13.28	1.86E+07					12.81	4.90E+07					13.99	1.05E+08					13.99	1.05E+08			
mph(A)	13.04	2.09E+07	2.12E+07	6.48E-01	-0.06	mph(A)	14.44	2.14E+07	2.24E+07	5.04E-01	-0.28	mph(A)	14.91	6.03E+07	5.21E+07	4.86E-01	-0.31	mph(A)	16.52	2.19E+07	2.25E+07	2.05E-01	-0.55
	12.91	2.19E+07					14.3	2.29E+07					14.8	6.46E+07					16.9	1.74E+07			
	13.05	2.08E+07					14.3	2.29E+07					15.95	3.16E+07					16.12	2.82E+07			
	2nd replicate						2nd replicate						2nd replicate						2nd replicate				
control	13.43	3.55E+07	4.39E+07			control	11.36	4.60E+07	3.27E+07			control	11.36	4.60E+07	3.27E+07			control	13.7	1.05E+08	9.14E+07		
	13.26	3.89E+07					12.04	3.34E+07					12.04	3.34E+07					13.6	9.91E+07			
	12.81	4.90E+07					13.28	1.86E+07					13.28	1.86E+07					13.6	8.38E+07			
mph(A)	13.18	3.98E+07	3.59E+07	8.16E-01	-0.09	mph(A)	12.89	2.24E+07	2.07E+07	6.34E-01	-0.20	mph(A)	13.17	1.96E+07	1.91E+07	5.83E-01	-0.23	mph(A)	16.27	2.51E+07	2.01E+07	2.19E-01	-0.66
	13.51	3.39E+07					13.16	1.97E+07					13.36	1.79E+07					16.9	1.70E+07			
	13.58	3.39E+07					13.12	2.01E+07					13.17	1.96E+07					16.8	1.81E+07			
	3rd replicate						3rd replicate						3rd replicate						3rd replicate				
control	14.01	1.02E+08	1.10E+08			control	13.43	3.55E+07	4.11E+07			control	13.84	1.16E+08	1.18E+08			control	11.36	4.60E+07	2.60E+07		
	13.83	1.15E+08					13.26	3.89E+07					14.02	1.04E+08					12.04	3.34E+07			
	13.99	1.05E+08					12.81	4.90E+07					13.61	1.34E+08					13.28	1.86E+07			
mph(A)	15.31	3.98E+07	9.45E+07	8.61E-01	-0.07	mph(A)	13.48	3.47E+07	2.87E+07	6.98E-01	-0.16	mph(A)	15.1	5.21E+07	5.24E+07	4.45E-01	-0.29	mph(A)	15.17	1.16E+07	1.27E+07	4.89E-01	-0.31
	14.47	7.76E+07					14.01	2.57E+07					15.5	5.26E+07					15.55	1.29E+07			
	13.24	1.66E+08					14	2.57E+07					15.5	5.26E+07					15.28	1.36E+07			

Table G.7: Target gene: *bla*-TEM1

UV dose 20 mJ/cm^2						UV dose 50 mJ/cm^2						UV dose 100 mJ/cm^2						UV dose 200 mJ/cm^2					
target gene	Cq	Resistance gene number	Average	N/N0	Log N/N0	target gene	Cq	Resistance gene number	Average	N/N0	Log N/N0	target gene	Cq	Resistance gene number	Average	N/N0	Log N/N0	target gene	Cq	Resistance gene number	average	N/N0	Log N/N0
	1st replicate						1st replicate						1st replicate						1st replicate				
Control	12.86	2.14E+08	1.91E+08			Control	12.25	3.24E+08	2.53E+08			Control	12.86	2.14E+08	1.91E+08			Control	12.86	2.14E+08	1.80E+08		
	13.13	1.78E+08					12.33	3.02E+08					13.13	1.78E+08					13.13	1.78E+08			
	13.1	1.82E+08					12.29	3.16E+08					13.1	1.82E+08					13.1	1.82E+08			
Bla-TEM1	13.04	1.91E+08	1.32E+08	6.88E-01	-0.16	Bla-TEM1	14.71	6.46E+07	6.93E+07	2.74E-01	-0.56	Bla-TEM1	16.32	1.91E+07	4.39E+07	2.29E-01	-0.64	Bla-TEM1	17.41	1.23E+07	1.31E+07	7.28E-02	-1.14
	15.05	5.37E+07					14.5	7.24E+07					15.01	5.50E+07					17.28	1.32E+07			
	13.89	1.51E+08					14.54	7.08E+07					14.94	5.75E+07					17.22	1.38E+07			
	2nd replicate						2nd replicate						2nd replicate						2nd replicate				
control	12.86	2.14E+08	1.91E+08			control	12.25	3.24E+08	2.53E+08			control	12.86	2.14E+08	1.91E+08			control	12.86	2.14E+08	1.80E+08		
	13.13	1.78E+08					12.33	3.02E+08					13.13	1.78E+08					13.13	1.78E+08			
	13.1	1.82E+08					12.29	3.16E+08					13.1	1.82E+08					13.1	1.82E+08			
Bla-TEM1	13.49	1.45E+08	1.47E+08	7.70E-01	-0.11	Bla-TEM1	14.51	7.76E+07	6.01E+07	2.38E-01	-0.54	Bla-TEM1	15.16	5.01E+07	4.76E+07	2.49E-01	-0.60	Bla-TEM1	17.84	9.33E+06	1.30E+07	7.21E-02	-1.14
	13.59	1.35E+08					15.46	4.27E+07					15.41	4.27E+07					17.09	1.48E+07			
	13.28	1.62E+08					15.46	4.27E+07					15.1	5.01E+07					17.02	1.48E+07			
	3rd replicate						3rd replicate						3rd replicate						3rd replicate				
control	14.3	5.75E+07	6.62E+07			control	11.41	1.48E+08	1.06E+08			control	11.41	1.48E+08	1.06E+08			control	11.41	1.48E+08	1.06E+08		
	14.2	6.17E+07					12.57	7.76E+07					12.57	7.76E+07					12.57	7.76E+07			
	14	7.08E+07					12.23	9.33E+07					12.23	9.33E+07					12.23	9.33E+07			
Bla-TEM1	14.75	4.27E+07	3.64E+07	5.50E-01	-0.26	Bla-TEM1	13.31	5.13E+07	4.89E+07	4.60E-01	-0.34	Bla-TEM1	15.12	1.86E+07	1.86E+07	1.75E-01	-0.76	Bla-TEM1	15.36	1.62E+07	1.60E+07	1.50E-01	-0.82
	14.3	2.95E+07					13.78	4.82E+07					17.92	3.80E+06					15.42	1.55E+07			
	14.98	3.72E+07					14.01	4.72E+07					18.06	3.55E+06					15.36	1.62E+07			

Table G.8: Target gene: *Sul*1

UV dose 20 mJ/cm²						UV dose 50 mJ/cm²						UV dose 100 mJ/cm²						UV dose 200 mJ/cm²					
target gene	Cq	Resistance gene number	Average	N/N0	Log N/N0	target gene	Cq	Resistance gene number	Average	N/N0	Log N/N0	target gene	Cq	Resistance gene number	Average	N/N0	Log N/N0	target gene	Cq	Resistance gene number	average	N/N0	Log N/N0
	1st replicate						1st replicate						1st replicate						1st replicate				
Control	13.84	1.16E+08	1.18E+08			Control	13.35	1.58E+08	1.42E+08			Control	13.84	1.16E+08	1.18E+08			Control	13.6	8.58E+07	1.34E+08		
	14.02	1.04E+08					13.47	1.46E+08					14.02	1.04E+08					13.6	1.35E+08			
	13.61	1.34E+08					13.76	1.22E+08					13.61	1.34E+08					13.6	1.32E+08			
Sul1	13.59	1.26E+08	1.13E+08	9.61E-01	-0.02	Sul1	16.4	2.32E+07	2.98E+07	2.10E-01	-0.68	Sul1	15.64	3.75E+07	3.71E+07	3.15E-01	-0.50	Sul1	16.57	2.09E+07	1.89E+07	1.41E-01	-0.85
	13.71	1.07E+08					15.52	4.04E+07					15.63	3.77E+07					16.84	1.76E+07			
	13.97	1.07E+08					16.24	2.57E+07					15.69	3.63E+07					16.8	1.81E+07			
	2nd replicate						2nd replicate						2nd replicate						2nd replicate				
control	13.84	1.16E+08	1.19E+08			control	13.35	1.58E+08	1.42E+08			control	13.84	1.16E+08	1.18E+08			control	13.7	1.05E+08	9.14E+07		
	14.02	1.04E+08					13.47	1.46E+08					14.02	1.04E+08					13.6	9.91E+07			
	13.61	1.34E+08					13.76	1.22E+08					13.61	1.34E+08					13.6	8.38E+07			
Sul1	14.17	9.42E+07	9.42E+07	7.94E-01	-0.1	Sul1	14.34	8.47E+07	7.20E+07	5.07E-01	-0.29	Sul1	15.64	3.75E+07	3.71E+07	3.15E-01	-0.50	Sul1	16.57	2.09E+07	1.89E+07	2.06E-01	-0.69
	14.05	9.42E+07					15.01	5.56E+07					15.63	3.77E+07					16.84	1.76E+07			
	14.05	9.42E+07					14.52	7.56E+07					15.69	3.63E+07					16.8	1.81E+07			
	3rd replicate						3rd replicate						3rd replicate						3rd replicate				
control	13.7	1.51E+08	1.40E+08			control	13.35	1.58E+08	1.42E+08			control	13.84	1.16E+08	1.18E+08			control	13.7	8.32E+07	9.46E+07		
	13.6	1.32E+08					13.47	1.46E+08					14.02	1.04E+08					13.6	1.00E+08			
	13.6	1.49E+08					13.76	1.22E+08					13.61	1.34E+08					13.6	8.91E+07			
Sul1	14.74	4.07E+07	5.74E+07	4.09E-01	-0.39	Sul1	15.2	8.17E+07	6.93E+07	4.88E-01	-0.31	Sul1	15.1	5.21E+07	5.24E+07	4.45E-01	-0.35	Sul1	15.31	1.66E+07	2.24E+07	2.37E-01	-0.62
	13.83	7.41E+07					15.01	5.56E+07					15.5	5.26E+07					15.9	2.88E+07			
	14.75	4.07E+07					15.82	7.06E+07					15.5	5.26E+07					16.3	2.18E+07			

Appendix G.3. Raw data on dark and light repair of antibiotic-resistant *E. coli* and *P. aeruginosa* following UV exposures of 3, 5 and 10 mJ/cm^2

Table G.9: Raw data *Escherichia coli* NCTC 13400 dark repair

UV dose 3 mJ/cm^2							UV dose 5 mJ/cm^2							UV dose 10 mJ/cm^2						
incubation time	plate 1	plate 2	plate 3	mean colonies	cfu/ml	$\log_{10}$ cfu/ml	incubation time	plate 1	plate 2	plate 3	mean colonies	cfu/ml	$\log_{10}$ cfu/ml	incubation time	plate 1	plate 2	plate 3	mean colonies	cfu/ml	$\log_{10}$ cfu/ml
1st replicate							1st replicate							1st replicate						
3 hr	74	68	65	69.00	6.90E+04	4.84	3 hr	27	26	26	26.33	2.63E+01	1.42	3 hr	80	81	80	80.33	8.03E+01	1.90
15 hr	114	118	115	115.67	1.15E+06	6.06	15 hr	60	61	63	61.33	6.10E+03	3.79	15 hr	12	15	13	13.33	1.30E+03	3.11
24 hr	41	38	40	39.67	3.95E+06	6.60	24 hr	30	30	31	30.33	3.00E+04	4.48	24 hr	70	71	75	72.00	7.20E+03	3.86
2nd replicate							2nd replicate							2nd replicate						
3 hr	62	63	65	63.33	6.30E+04	4.80	3 hr	12	13	18	14.33	1.43E+01	1.16	3 hr	70	75	79	74.67	7.46E+01	1.87
15 hr	108	109	110	109.00	1.09E+06	6.04	15 hr	70	70	75	71.67	7.16E+03	3.85	15 hr	12	9	13	11.33	1.10E+03	3.04
24 hr	34	38	40	37.3333	3.73E+06	6.57	24 hr	40	41	39	40.00	4.00E+04	4.60	24 hr	90	95	110	98.33	9.80E+03	3.99
3rd replicate							3rd replicate							3rd replicate						
3 hr	58	60	55	57.67	5.76E+04	4.76	3 hr	13	15	18	15.33	1.53E+01	1.19	3 hr	105	101	98	101.33	1.01E+02	2.00
15 hr	110	110	107	109.00	1.09E+06	6.04	15 hr	80	78	79	79.00	7.90E+03	3.90	15 hr	18	13	15	15.33	1.50E+03	3.18
24 hr	24	23	25	24.00	2.35E+06	6.37	24 hr	30	32	33	31.67	3.10E+04	4.49	24 hr	110	100	105	105.00	1.05E+04	4.02

control

UV dose 3 mJ/cm^2							UV dose 5 mJ/cm^2							UV dose 10 mJ/cm^2						
incubation time	plate 1	plate 2	plate 3	mean colonies	cfu/ml	$\log_{10}$ cfu/ml	incubation time	plate 1	plate 2	plate 3	mean colonies	cfu/ml	$\log_{10}$ cfu/ml	incubation time	plate 1	plate 2	plate 3	mean colonies	cfu/ml	$\log_{10}$ cfu/ml
0 hr	21	27	29	25.67	2.57E+06	6.41	0 hr	21	24	24	23.00	2.30E+06	6.36	0 hr	30	27	29	28.67	2.87E+06	6.46
3 hr	18	24	23	21.67	2.17E+06	6.34	3 hr	41	33	32	35.33	3.53E+06	6.55	3 hr	13	16	18	15.67	1.57E+06	6.19
15 hr	39	25	42	35.33	3.53E+06	6.55	15 hr	23	22	25	23.33	2.33E+06	6.37	15 hr	23	22	23	22.67	2.27E+06	6.36
24 hr	18	18	25	20.33	2.03E+06	6.31	24 hr	16	18	20	18.00	1.80E+06	6.26	24 hr	22	20	23	21.67	2.17E+06	6.34

Table G.10: Raw data *Escherichia coli* NCTC 13400 light repair

UV dose 3 mJ/cm^2							UV dose 5 mJ/cm^2							UV dose 10 mJ/cm^2						
incubation time	plate 1	plate 2	plate 3	mean colonies	cfu/ml	$\log_{10}$ cfu/ml	incubation time	plate 1	plate 2	plate 3	mean colonies	cfu/ml	$\log_{10}$ cfu/ml	incubation time	plate 1	plate 2	plate 3	mean colonies	cfu/ml	$\log_{10}$ cfu/ml
1st replicate							1st replicate							1st replicate						
3 hr	172	135	145	150.67	1.50E+03	3.18	3 hr	18	21	20	19.67	1.96E+01	1.29	3 hr	5	8	8	7.00	7.00E+00	0.85
15 hr	65	65	63	64.33	6.40E+04	4.81	15 hr	14	17	17	16.00	1.60E+02	2.20	15 hr	50	60	55	55.00	5.50E+01	1.74
24 hr	113	96	98	102.33	1.02E+05	5.01	24 hr	21	31	34	28.67	2.86E+02	2.46	24 hr	16	13	12	13.67	1.35E+02	2.13
2nd replicate							2nd replicate							2nd replicate						
3 hr	138	128	130	132.00	1.32E+03	3.12	3 hr	28	28	26	27.33	2.73E+01	1.44	3 hr	11	10	8	9.67	9.60E+00	0.98
15 hr	88	84	87	86.33	8.60E+04	4.93	15 hr	20	19	18	19.00	1.90E+02	2.28	15 hr	75	69	65	69.67	6.90E+01	1.84
24 hr	93	110	106	103	1.03E+05	5.01	24 hr	76	28	39	47.67	4.76E+02	2.68	24 hr	10	16	15	13.67	1.26E+02	2.10
3rd replicate							3rd replicate							3rd replicate						
3 hr	133	129	130	130.67	1.30E+03	3.11	3 hr	25	23	25	24.33	2.43E+01	1.39	3 hr	12	10	11	11.00	1.10E+01	1.04
15 hr	74	75	70	73.00	7.30E+04	4.86	15 hr	18	21	19	19.33	1.93E+02	2.29	15 hr	65	68	79	70.67	7.00E+01	1.85
24 hr	75	88	83	82.00	8.20E+04	4.91	24 hr	29	32	33	31.33	3.13E+02	2.50	24 hr	18	14	15	15.67	1.56E+02	2.19

Control

UV dose 3 mJ/cm^2							UV dose 5 mJ/cm^2							UV dose 10 mJ/cm^2						
incubation time	plate 1	plate 2	plate 3	mean colonies	cfu/ml	$\log_{10}$ cfu/ml	incubation time	plate 1	plate 2	plate 3	mean colonies	cfu/ml	$\log_{10}$ cfu/ml	incubation time	plate 1	plate 2	plate 3	mean colonies	cfu/ml	$\log_{10}$ cfu/ml
0 hr	18	18	19	18.33	1.83E+06	6.26	0 hr	18	19	21	19.33	1.93E+06	6.29	0 hr	21	18	20	19.67	1.97E+06	6.293730757
3 hr	12	15	15	14.00	1.40E+06	6.15	3 hr	21	20	20	20.33	2.03E+06	6.31	3 hr	19	19	20	19.33	1.93E+06	6.29
15 hr	13	15	18	15.33	1.53E+06	6.19	15 hr	19	22	21	20.67	2.07E+06	6.32	15 hr	21	18	18	19.00	1.90E+06	6.28
24 hr	18	18	21	19.00	1.90E+06	6.28	24 hr	17	19	20	18.67	1.87E+06	6.27	24 hr	18	19	21	19.33	1.93E+06	6.29

Table G. 11: Raw data *Pseudomonas aeruginosa* NCTC 13437 dark repair

UV dose 3 mJ/cm^2							UV dose 5 mJ/cm^2							UV dose 10 mJ/cm^2						
incubation time	plate 1	plate 2	plate 3	mean colonies	cfu/ml	$\log_{10}$ cfu/ml	incubation time	plate 1	plate 2	plate 3	mean colonies	cfu/ml	$\log_{10}$ cfu/	incubation time	plate 1	plate 2	plate 3	mean colonies	cfu/ml	$\log_{10}$ cfu/ml
1st replicate							1st replicate							1st replicate						
3 hr	314	297	314	308.33	3.07E+03	3.49	3 hr	136	115	131	127.33	1.27E+03	3.10	3 hr	21	33	24	26.00	2.70E+02	2.43
6 hr	117	110	124	117.00	1.17E+04	4.07	6 hr	63	64	48	58.33	5.83E+03	3.77	6 hr	35	36	43	38.00	3.55E+02	2.55
15 hr	32	32	33	32.33	3.23E+04	4.51	15 hr	18	15	15	16.00	1.60E+04	4.20	15 hr	130	129	125	128.00	1.30E+03	3.11
24 hr	24	23	26	24.33	2.43E+04	4.39	24 hr	28	10	17	18.33	1.83E+04	4.26	24 hr	267	258	240	255.00	2.63E+04	4.42
2nd replicate							2nd replicate							2nd replicate						
3 hr	295	285	285	288.33	2.92E+03	3.47	3 hr	255	280	256	263.67	2.64E+03	3.42	3 hr	35	61	44	46.67	4.80E+02	2.68
6 hr	129	106	121	118.67	1.12E+04	4.05	6 hr	92	99	90	93.67	9.37E+03	3.97	6 hr	43	55	58	52.00	7.50E+02	2.88
15 hr	10	29	12	17.00	1.70E+04	4.23	15 hr	15	21	23	19.67	1.97E+04	4.29	15 hr	11	23	29	21.00	1.26E+03	3.10
24 hr	75	109	89	91	9.10E+04	4.96	24 hr	18	19	19	18.67	1.63E+04	4.21	24 hr	245	240	250	245.00	2.45E+04	4.39
3rd replicate							3rd replicate							3rd replicate						
3 hr	312	293	328	311.00	3.09E+03	3.49	3 hr	290	309	263	287.33	2.89E+03	3.46	3 hr	43	33	38	38.00	3.80E+02	2.58
6 hr	99	107	70	92.00	1.75E+04	4.24	6 hr	110	124	123	119.00	1.19E+04	4.08	6 hr	107	92	88	95.67	9.00E+02	2.95
15 hr	28	27	29	28.00	2.80E+04	4.45	15 hr	13	18	12	14.33	1.43E+04	4.16	15 hr	20	17	28	21.67	1.85E+03	3.27
24 hr	46	31	51	42.67	4.40E+04	4.64	24 hr	21	18	19	19.33	1.93E+04	4.29	24 hr	267	265	280	270.67	2.72E+04	4.44

Control

UV dose 3 mJ/cm^2							UV dose 5 mJ/cm^2							UV dose 10 mJ/cm^2						
incubation time	plate 1	plate 2	plate 3	mean colonies	cfu/ml	$\log_{10}$ cfu/ml	incubation time	plate 1	plate 2	plate 3	mean colonies	cfu/ml	$\log_{10}$ cfu/	incubation time	plate 1	plate 2	plate 3	mean colonies	cfu/ml	$\log_{10}$ cfu/ml
0 hr	19	22	21	20.67	2.07E+06	6.32	0 hr	34	35	31	33.33	3.33E+06	6.52	0 hr	16	25	28	23	2.30E+06	6.36
3 hr	18	20	19	19.00	1.90E+06	6.28	3 hr	25	27	30	27.33	2.73E+06	6.44	3 hr	18	20	17	18.33	1.83E+06	6.26
6 hr	27	27	25	26.33	2.63E+06	6.42	6 hr	30	29	30	29.67	2.97E+06	6.47	6 hr	18	22	16	18.67	1.87E+06	6.27
15 hr	35	31	33	33.00	3.30E+06	6.52	15 hr	32	32	31	31.67	3.17E+06	6.50	15 hr	32	30	27	29.67	2.97E+06	6.47
24 hr	42	51	38	43.67	4.37E+06	6.64	24 hr	42	43	51	45.33	4.53E+06	6.66	24 hr	28	25	19	24.00	2.40E+06	6.38

Table G.12: Raw data *Pseudomonas aeruginosa* NCTC 13437 light repair

UV dose 3 mJ/cm^2							UV dose 5 mJ/cm^2							UV dose 10 mJ/cm^2						
incubation time	plate 1	plate 2	plate 3	mean colonies	cfu/ml	$\log_{10}$ cfu/ml	incubation time	plate 1	plate 2	plate 3	mean colonies	cfu/ml	$\log_{10}$ cfu/	incubation time	plate 1	plate 2	plate 3	mean colonies	cfu/ml	$\log_{10}$ cfu/ml
1st replicate							1st replicate							1st replicate						
3 hr	33	35	25	31.00	3.10E+03	3.49	3 hr	109	109	108	108.67	1.09E+04	4.04	3 hr	123	121	120	121.33	1.22E+03	3.09
6 hr	43	35	38	38.67	3.87E+04	4.59	6 hr	97	109	108	104.67	1.03E+04	4.01	6 hr	10	11	10	10.33	9.00E+02	2.95
15 hr	25	12	19	18.67	2.10E+04	4.32	15 hr	18	18	18	18.00	1.80E+04	4.26	15 hr	31	49	45	41.67	4.00E+03	3.60
24 hr	8	13	7	9.33	9.80E+04	4.99	24 hr	25	10	17	17.33	4.93E+04	4.69	24 hr	250	285	214	249.67	2.50E+04	4.40
2nd replicate							2nd replicate							2nd replicate						
3 hr	25	29	30	28.00	2.87E+03	3.46	3 hr	78	75	75	76.00	7.65E+03	3.88	3 hr	45	56	61	54.00	5.05E+02	2.70
6 hr	13	8	12	11.00	1.10E+04	4.04	6 hr	75	85	85	81.67	8.00E+03	3.90	6 hr	8	9	10	9.00	8.50E+02	2.93
15 hr	53	46	59	52.67	5.27E+04	4.72	15 hr	17	15	17	16.33	1.60E+04	4.20	15 hr	57	24	35	38.67	4.05E+03	3.61
24 hr	5	8	8	7	3.67E+04	4.56	24 hr	8	19	19	15.33	4.00E+04	4.60	24 hr	185	111	135	143.67	1.35E+04	4.13
3rd replicate							3rd replicate							3rd replicate						
3 hr	25	27	27	26.33	2.33E+03	3.37	3 hr	111	116	115	114.00	1.14E+04	4.05	3 hr	139	148	122	136.33	1.44E+03	3.16
6 hr	28	28	25	27.00	2.70E+04	4.43	6 hr	105	107	106	106.00	1.06E+04	4.03	6 hr	11	9	13	11.00	9.50E+02	2.98
15 hr	24	27	29	26.67	2.67E+04	4.43	15 hr	17	13	15	15.00	1.50E+04	4.18	15 hr	37	35	35	35.67	1.85E+03	3.27
24 hr	12	10	11	11.00	1.00E+05	5.00	24 hr	17	11	15	14.33	7.00E+04	4.85	24 hr	245	255	240	246.67	2.50E+04	4.40

Control

UV dose 3 mJ/cm^2							UV dose 5 mJ/cm^2							UV dose 10 mJ/cm^2						
incubation time	plate 1	plate 2	plate 3	mean colonies	cfu/ml	$\log_{10}$ cfu/ml	incubation time	plate 1	plate 2	plate 3	mean colonies	cfu/ml	$\log_{10}$ cfu/	incubation time	plate 1	plate 2	plate 3	mean colonies	cfu/ml	$\log_{10}$ cfu/ml
0 hr	30	27	29	28.67	2.87E+06	6.46	0 hr	35	37	31	34.33	3.43E+06	6.54	0 hr	30	27	29	28.67	2.87E+06	6.4573772
3 hr	31	33	29	31.00	3.10E+06	6.49	3 hr	32	28	29	29.67	2.97E+06	6.47	3 hr	31	33	29	31.00	3.10E+06	6.49
6 hr	18	25	31	24.67	2.47E+06	6.39	6 hr	30	23	27	26.67	2.67E+06	6.43	6 hr	18	25	31	24.67	2.47E+06	6.39
15 hr	39	25	42	35.33	3.53E+06	6.55	15 hr	25	39	30	31.33	3.13E+06	6.50	15 hr	39	25	42	35.33	3.53E+06	6.55
24 hr	18	18	25	20.33	2.03E+06	6.31	24 hr	29	33	31	31.00	3.10E+06	6.49	24 hr	18	19	24	20.33	2.03E+06	6.31

Appendix H. Raw data sequential UV/chlorine disinfection

Table H.1: Target gene: *bla*-$_{TEM1}$

UV dose 50 mJ/cm^2 + 2 mg/L of chlorination						UV dose 100 mJ/cm^2 + 2 mg/L of chlorination						UV dose 200 mJ/cm^2 + 2 mg/L of chlorination					
target gene	Cq	Resistance gene number	Average	N/N0	Log N/N0	target gene	Cq	Resistance gene number	Average	N/N0	Log N/N0	target gene	Cq	Resistance gene number	Average	N/N0	Log N/N0
1st replicate						1st replicate						1st replicate					
Control	14.26	1.18E+07	1.17E+07			Control	14.3	1.18E+07	1.17E+07			Control	14.26	1.18E+07	1.17E+07		
	14.4	1.10E+07					14.4	1.10E+07					14.4	1.10E+07			
	14.19	1.22E+07					14.2	1.22E+07					14.19	1.22E+07			
Bla-TEM1	22.29	2.95E+05	3.39E+05	2.91E-02	-1.54	Bla-TEM1	23.5	1.85E+05	1.65E+05	1.41E-02	-1.85	Bla-TEM1	29.15	9.74E+03	1.07E+04	9.17E-04	-3.04
	21.69	3.89E+05					24	1.46E+05					29.01	1.05E+04			
	22.02	3.34E+05					23.8	1.62E+05					28.77	1.19E+04			
2nd replicate						2nd replicate						2nd replicate					
control	14.26	1.18E+07	1.17E+07			control	14.3	1.18E+07	1.17E+07			control	14.26	1.18E+07	1.17E+07		
	14.4	1.10E+07					14.4	1.10E+07					14.4	1.10E+07			
	14.19	1.22E+07					14.2	1.22E+07					14.19	1.22E+07			
Bla-TEM1	22.51	2.67E+05	2.81E+05	2.41E-02	-1.62	Bla-TEM1	23.7	1.86E+05	1.65E+05	1.42E-02	-1.85	Bla-TEM1	29.15	9.74E+03	1.12E+04	9.60E-04	-3.02
	22.35	2.87E+05					24	1.46E+05					28.9	1.20E+04			
	22.34	2.88E+05					23.8	1.63E+05					28.77	1.19E+04			
3rd replicate						3rd replicate						3rd replicate					
control	14.3	1.18E+07	1.18E+07			control	14.3	1.18E+07	1.17E+07			control	14.26	1.18E+07	1.17E+07		
	14.2	1.13E+07					14.4	1.10E+07					14.4	1.10E+07			
	14	1.22E+07					14.2	1.22E+07					14.19	1.22E+07			
Bla-TEM1	22.41	2.68E+05	2.81E+05	2.39E-02	-1.62	Bla-TEM1	23.7	1.86E+05	1.71E+05	1.47E-02	-1.83	Bla-TEM1	29	9.79E+03	1.08E+04	9.22E-04	-3.04
	22.3	2.88E+05					23.8	1.63E+05					29.01	1.05E+04			
	22.34	2.88E+05					23.8	1.63E+05					28.8	1.20E+04			

Table H.2: Target gene: *Sul*1

UV dose 50 mJ/cm^2 + 2 mg/L of chlorination						UV dose 100 mJ/cm^2 + 2 mg/L of chlorination						UV dose 200 mJ/cm^2 + 2 mg/L of chlorination					
target gene	Cq	Resistance gene number	Average	N/N0	Log N/N0	target gene	Cq	Resistance gene number	Average	N/N0	Log N/N0	target gene	Cq	Resistance gene number	Average	N/N0	Log N/N0
1st replicate						1st replicate						1st replicate					
Control	13.38	1.76E+07	1.53E+07			Control	13.4	1.76E+07	1.53E+07			Control	13.38	1.76E+07	1.53E+07		
	13.87	1.41E+07					13.9	1.41E+07					13.87	1.41E+07			
	13.87	1.41E+07					13.9	1.41E+07					13.87	1.41E+07			
Sul1	20.56	6.53E+05	7.56E+05	4.95E-02	-1.31	Sul1	24.4	1.14E+05	3.36E+05	2.20E-02	-1.66	Sul1	24.05	1.32E+05	8.78E+04	5.75E-03	-2.24
	20.03	8.33E+05					20.1	8.03E+05					25.5	6.76E+04			
	20.17	7.81E+05					24.8	9.20E+04					25.61	6.43E+04			
2nd replicate						2nd replicate						2nd replicate					
control	13.38	1.76E+07	1.53E+07			control	13.4	1.76E+07	1.53E+07			control	13.38	1.76E+07	1.53E+07		
	13.87	1.41E+07					13.9	1.41E+07					13.87	1.41E+07			
	13.87	1.41E+07					13.9	1.41E+07					13.87	1.41E+07			
Sul1	20.61	6.38E+05	6.73E+05	4.40E-02	-1.36	Sul1	20.9	5.64E+05	5.44E+05	3.56E-02	-1.45	Sul1	24.15	1.32E+05	8.78E+04	5.75E-03	-2.24
	20.59	6.44E+05					21.1	5.05E+05					25.53	6.76E+04			
	20.3	7.36E+05					20.2	5.64E+05					25.61	6.43E+04			
3rd replicate						3rd replicate						3rd replicate					
control	13.38	1.76E+07	1.53E+07			control	13.4	1.76E+07	1.53E+07			control	13.38	1.76E+07	1.53E+07		
	13.87	1.41E+07					13.9	1.41E+07					13.87	1.41E+07			
	13.87	1.41E+07					13.9	1.41E+07					13.87	1.41E+07			
Sul1	20.1	8.33E+05	7.68E+05	5.03E-02	-1.30	Sul1	20.9	5.64E+05	5.44E+05	3.56E-02	-1.45	Sul1	24.75	1.02E+05	7.78E+04	5.09E-03	-2.29
	20.39	7.36E+05					21.1	5.05E+05					25.5	6.76E+04			
	20.3	7.36E+05					20.9	5.64E+05					25.61	6.43E+04			

Table H.3: Target gene: *tet*(A)

UV dose 50 mJ/cm^2 + 2 mg/L of chlorination						UV dose 100 mJ/cm^2 + 2 mg/L of chlorination						UV dose 200 mJ/cm^2 + 2 mg/L of chlorination					
target gene	Cq	Resistance gene number	Average	N/N0	Log N/N0	target gene	Cq	Resistance gene number	Average	N/N0	Log N/N0	target gene	Cq	Resistance gene number	Average	N/N0	Log N/N0
1st replicate						1st replicate						1st replicate					
Control	11.49	6.25E+07	6.52E+07			Control	11.5	6.25E+07	6.52E+07			Control	13.16	4.05E+07	3.01E+07		
	11.36	6.68E+07					11.4	6.68E+07					14.06	2.53E+07			
	11.37	6.64E+07					11.4	6.64E+07					14.12	2.46E+07			
tet(A)	20.33	9.65E+05	9.20E+05	1.41E-02	-1.55	tet(A)	21.5	3.69E+05	4.15E+05	6.36E-03	-2.20	tet(A)	25.99	5.06E+04	4.90E+04	1.63E-03	-2.79
	20.25	1.01E+06					21	4.91E+05					26.04	4.93E+04			
	20.72	7.88E+05					21.5	3.86E+05					26.12	4.73E+04			
2nd replicate						2nd replicate						2nd replicate					
control	11.49	6.25E+07	6.52E+07			control	13.2	4.05E+07	3.01E+07			control	13.16	4.05E+07	3.01E+07		
	11.36	6.68E+07					14.1	2.53E+07					14.06	2.53E+07			
	11.37	6.64E+07					14.1	2.46E+07					14.12	2.46E+07			
tet(A)	20.73	7.84E+05	8.51E+05	1.30E-02	-1.52	tet(A)	21.6	5.09E+05	5.42E+05	1.80E-02	-1.74	tet(A)	24.15	1.32E+05	8.78E+04	2.92E-03	-2.54
	20.42	9.21E+05					21.4	5.59E+05					25.53	6.76E+04			
	20.58	8.48E+05					21.4	5.59E+05					25.61	6.43E+04			
3rd replicate						3rd replicate						3rd replicate					
control	11.49	6.25E+07	6.52E+07			control	11.5	6.25E+07	6.52E+07			control	11.49	6.25E+07	6.52E+07		
	11.36	6.68E+07					11.4	6.68E+07					11.36	6.68E+07			
	11.37	6.64E+07					11.4	6.64E+07					11.37	6.64E+07			
tet(A)	20.53	7.86E+05	8.62E+05	1.32E-02	-1.51	tet(A)	21.6	5.09E+05	5.42E+05	8.31E-03	-2.08	tet(A)	21.18	4.43E+05	3.89E+05	5.96E-03	-2.22
	20.42	9.21E+05					21.4	5.59E+05					21.38	4.00E+05			
	20.38	8.78E+05					21.4	5.59E+05					21.8	3.23E+05			

Table H.4: Target gene: *mph*(A)

UV dose 50 mJ/cm^2 + 2 mg/L of chlorination						UV dose 100 mJ/cm^2 + 2 mg/L of chlorination						UV dose 200 mJ/cm^2 + 2 mg/L of chlorination					
target gene	Cq	Resistance gene number	Average	N/N0	Log N/N0	target gene	Cq	Resistance gene number	Average	N/N0	Log N/N0	target gene	Cq	Resistance gene number	Average	N/N0	Log N/N0
1st replicate						1st replicate						1st replicate					
Control	12.7	2.18E+07	2.03E+07			Control	12.7	2.18E+07	2.03E+07			Control	12.7	2.18E+07	2.03E+07		
	13.14	1.80E+07					13.1	1.80E+07					13.14	1.80E+07			
	12.78	2.10E+07					12.8	2.10E+07					12.78	2.10E+07			
mph(A)	20.9	5.82E+05	6.45E+05	3.18E-02	-1.50	mph(A)	22	3.52E+05	3.18E+05	1.57E-02	-1.80	mph(A)	29.28	1.43E+04	1.64E+04	8.08E-04	-3.09
	20.64	6.53E+05					22.8	2.55E+05					28.72	1.84E+04			
	20.48	7.01E+05					22.1	3.47E+05					28.97	1.64E+04			
2nd replicate						2nd replicate						2nd replicate					
control	12.7	2.18E+07	2.03E+07			control	12	4.63E+07	4.78E+07			control	12	4.63E+07	4.78E+07		
	13.14	1.80E+07					11.8	5.06E+07					11.82	5.06E+07			
	12.78	2.10E+07					12.1	4.63E+07					12.1	4.63E+07			
mph(A)	21.11	5.30E+05	5.29E+05	2.61E-02	-1.58	mph(A)	19.4	1.26E+06	1.06E+06	2.23E-02	-1.65	mph(A)	22.83	2.37E+05	1.79E+05	3.76E-03	-2.43
	21.02	5.52E+05					20.2	8.66E+05					23.6	1.63E+05			
	21.22	5.05E+05					22.5	2.78E+05					23.93	1.39E+05			
3rd replicate						3rd replicate						3rd replicate					
control	12.7	2.18E+07	2.03E+07			control	12.7	2.18E+07	2.16E+07			control	12.7	2.18E+07	2.03E+07		
	13.14	1.80E+07					12.7	2.18E+07					13.14	1.80E+07			
	12.78	2.10E+07					12.8	2.10E+07					12.78	2.10E+07			
mph(A)	21.01	5.31E+05	5.30E+05	2.61E-02	-1.58	mph(A)	20.7	5.84E+05	5.74E+05	2.66E-02	-1.57	mph(A)	29.28	1.43E+04	1.64E+04	8.08E-04	-3.09
	21.02	5.52E+05					20.8	5.75E+05					28.72	1.84E+04			
	21.2	5.06E+05					20.5	5.64E+05					28.97	1.64E+04			