THE ROLE OF WATER DISTRIBUTION SYSTEMS IN THE ENRICHMENT OF ANTIMICROBIAL RESISTANCE

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Thesis

Submitted for the fulfilment of the requirements for degree of

Doctor of Philosophy

Department of Civil and Environmental Engineering

University of Strathclyde, Glasgow, UK

2016

Declaration / Copyright

This thesis is the result of the author's original research. It has been composed by the author and has not been previously submitted for examination which has led to the award of a degree.

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Date: 25.8.2016

Acknowledgements

First of all, thanks to **Almighty Allah** for all His generous and plenitude of blessings throughout my life.

A warm gratitude to my mentor Dr. Charles W. Knapp, without his guidance and inspirations this research was not possible. His dynamic and compassionate supervision with myriad opportunities for my personal and professional developments made it possible for me to accomplish this difficult task on time. A great thank to Dr. Tara K. Beattie; my second supervisor, for her invaluable advices, discussions, reviews, and feed backs refining my research and the thesis. I pay my deepest gratitude to Dr. Christine Switzer as well for her affection, care, and encouragement during my stay in Strathclyde. I am also thankful to Dr. Ameet Pinto, University of Glasgow for his technical help in experiments.

I am appreciative to department's faculty and staff, especially Mrs. Mara Knapp for her countless support for providing all stuff as soon as possible. I am grateful to John Carlin, Darren and Gavin as well for their technical help in the construction of water distribution systems, and Athena SWAN committee members with whom I spend three years for the great purpose. I also appreciate the support provided by Disability Services and Equality and Diversity Centre of University of Strathclyde during my study in Glasgow.

Especial thanks to my whole family, my mother Ameena Khan and father Shafique Ahmed Khan for their unlimited prayers, unconditional love and care throughout my life. Thanks to my brothers Rizwan, Farhan, Irfan, and my younger sister Erum, I missed her most during my PhD.

Dear friends Alam, Catalina, Elly, Chris, Koi, Nui, Ann, Ronnie, Susmita, Sandra and Neil, thanks for your sincere advices, working with me till late hours, and chit chats. Thanks to Dr. Mahmood Ali, Dr. Zuhaib Siddique, and all colleagues in Pakistan who supported and prayed for my success during my long journey of PhD.

In the end, I am thankful to Schlumberger Foundation, its directors, staff, and members for their financial assistance and lavish scholarship from Faculty for the Future Scheme for four consecutive years, converting my dream into reality.

Sadia Khan
Dated: 25. 08. 2016

Abstract

The drinking-water environment is prone to chemical and biological pollution, and it remains a challenge to achieve contamination-free water for the consumer. Biological contaminants (microorganisms and their genetic traits) can become more difficult to treat, as compared to chemical, as they can escape treatment processes (including disinfection) and replicate. Their presence in the drinking water environment has become considered to be a hotspot for the dissemination of antimicrobial resistance genes (ARG). Moreover, the treatment process may create conditions that may exacerbate the development and dissemination of ARG.

The inter-relationship between disinfectant exposure, a common water-treatment process and the development of antibiotic resistance was investigated in this study. Potential human and animal pathogens from municipal drinking water, representing twenty-two genera, were characterized for antibiotic and disinfectant resistances. The co-existence of antibiotic resistant and transferable genes was found in bacteria, and significant but weak correlations were detected between disinfectant and antibiotic resistance (against sulfamethoxazole, tetracycline and amoxicillin).

The applicability of a minimum-selectable-concentration model (MSC) for the selection of resistant population was evaluated for the first time in this study. MSCs were found to be lower than minimum inhibitory concentrations (MIC) causing the enrichment of resistant populations at sub-inhibitory concentrations. Both MIC and MSC metrics should be considered when planning treatment against resistant organisms.

Finally, the potential roles of dispersal and gene enrichment in a model distribution system were explored. Different pipe surfaces, especially PVC, became colonized with bacteria and enriched the abundances of resistant populations in the presence of free chlorine. Additionally, resistant bacteria were enriched more in biofilm than water.

This thesis highlights how water-distribution ecosystems contribute to the emergence of resistance. The water-supply system must be considered for the control of resistant bacteria. Further, we must prevent the presence of microorganisms post-treatment as exposure to sub-inhibitory disinfectant levels causes the greatest risk.

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List of Publications

International, Peer-Reviewed Journal Papers

- **Sadia Khan**, Charles W. Knapp, and Tara K. Beattie (2016) Antibiotic resistant bacteria found in municipal drinking water. *Environmental Processes* 3(3): 541-552. Doi: 10.1007/s40710-016-0149-z.
- **Sadia Khan**, Tara K. Beattie, and Charles W. Knapp (2016) Relationship between antibiotic- and disinfectant-resistance profiles in bacteria harvested from tap water. *Chemosphere* 152:132-141. Doi:10.1016/j.chemosphere.2016.02.086.[1]
- **Sadia Khan**, Tara K. Beattie, and Charles W. Knapp (2016) Minimum selectable concentrations (MSCs): An approach for determining the selection of antimicrobial resistant bacteria. Manuscript submitted in *Ecotoxicology*.
- **Sadia Khan**, Tara K. Beattie, and Charles W. Knapp (2016) The ecotoxicological study in a simulated water distribution system. Manuscript under preparation.
- **Sadia Khan**, Tara K. Beattie, and Charles W. Knapp (2016) Disinfectant chemicals and ARB in drinking water: Impact on human health. Manuscript under preparation.
- Julie C. Anderson, Shira Joudan, Eira Shoichet, Leah D. Cuscito, Ayana E. C. Alipio, Craig S. Donaldson, **Sadia Khan**, Douglas M. Goltz, Charles W. Knapp, Mark L. Hanson, Charles S. Wong (2015). Reducing nutrients, organic micropollutants, antibiotic resistance, and toxicity in rural wastewater

[1] Supporting information of this chapter /paper is present in Appendix section.

effluent with subsurface filtration treatment technology. *Ecological Engineering*, 84: 375-385.

Conference Papers

- **Sadia Khan**, Charles W. Knapp, and Tara Beattie (2015) ARGs in water distribution system in Glasgow: Does the problem really exist? Oral presentation in International Water Resource Association, World Water Congress XV, (May 25-29, 2015, Edinburgh, Scotland, UK).
- **Sadia Khan**, Charles W. Knapp, and Tara Beattie (2015) Study of antibiotic resistance bacteria in water distribution system in Glasgow. Oral presentation in PhD Conference, Department of Civil and Environmental Engineering, University of Strathclyde, Glasgow, UK (November 5, 2014, Glasgow, UK).
- **Sadia Khan**, Charles W. Knapp, and Tara Beattie (2015) Problem of antibiotic resistance bacteria in tap-water in Glasgow, UK. Paper presentation in European Water Resource Association, 9th World Congress, Water Resources Management in a Changing World: Challenges and Opportunities (June 10-14, 2015, Istanbul, Turkey).
- **Sadia Khan**, Charles W. Knapp, and Tara Beattie (2015) Antibiotic resistance bacteria in a municipal water distribution system. Paper presentation in International Water Association, UK Young Water Professional Conference (April 15-17, 2015, Glasgow, UK).
- **Sadia Khan**, Charles W. Knapp, and Tara Beattie (2015) Diversity of microbial population in drinking water distribution system. Paper presentation in Faculty of Engineering Research Presentation Day, University of Strathclyde, Glasgow, UK (June 22, 2015).

- **Sadia Khan**, Charles W. Knapp, and Tara Beattie (2014) Superbugs: Do you know what is in your tap water? Paper presentation in Faculty of Engineering Research Presentation Day, University of Strathclyde, Glasgow, UK (June 25, 2014).

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Abbreviations

AMX	Amoxicillin
AOC	Assimilable Organic Carbon
APHA	American Public Health Association
ARB	Antibiotic Resistant Bacteria
ARG	Antibiotic Resistant Genes
ATCC	American Type Culture Collection
BAC	Biological Activated Carbon
BLAST	The Basic Local Alignment Search Tool
°C	Centigrade
CDC	Center for Disease and Control and Prevention
CFU	Colony Forming Unit
CIP	Ciprofloxacin
CLSI	Clinical and Laboratory Standard Institute
cm	Centimetre
cm^{-2}	Per Square Centimetre
cm^{3}	Cubic Centimetre
CPVC	Chlorinated Polyvinyl Chloride
DNA	Deoxyribonucleic Acid
DPD	N, N-Diethyl-ρ-Phenylenediamine
DRB	Disinfectant Resistant Bacteria
DW	Drinking Water
DWDS	Drinking Water Distribution System
DWTP	Drinking Water Treatment Plant

ECDC	European Center for Disease Prevention and Control
EDC	Endocrine Disrupting Chemicals
EDTA	Ethylene Diamine Tetra Acetic Acid
EPA	Environmental Protection Agency
EWRA	European Water Resource Association
F	Forward Primer
GI	Galvanized Iron
h	Hour
h^{-1}	Per Hour
HDPE	High Density Polyethylene
HGT	Horizontal Gene Transfer
HNO_3	Nitric acid
HPC	Heterotrophic Plate Count
IS	Insertion Sequences
L	Litre
L^{-1}	Per Litre
LB	Lysogeny Broth
LGT	Lateral Gene Transfer
ln	Natural Log
Log_{10}	Base 10 Logarithm Function
M	Molar
μ	Growth Rate Constant
MDR	Multi-Drug Resistant
μg	Microgram
mg	Milligram

MGE	Mobile Genetic Element
MIC	Minimum Inhibitory Concentration
MIC_{susc}	Minimum Inhibitory Concentration for Susceptible Strain
min	Minute
min^{-1}	Per Minute
mJ	Milli-Joule
µL	Microlitre
mL	Millilitre
mL^{-1}	Per Millilitre
mm	Millimetre
mM	Millimolar
MRSA	Methicillin Resistant *Staphylococcus aureus*
MSC	Minimum Selectable Concentration
mV	Milli-Volt
NA	Nutrient Agar
NaOCl	Sodium Hypochlorite
NCBI	The National Center for Biotechnology Information
NCTC	National Collection of Type Culture
NDM	New Delhi Metallo-β-lactamase
NH_4Cl	Ammonium Chloride
ηM	Nano-Molar
OD	Optical Density
PAI	Population Action International
PBS	Phosphate Buffer Saline
PCP	Personal Care Products

PE	Polyethylene
PP	Polypropylene
ppm	Part Per Million
PVC	Polyvinyl Chloride
QAC	Quaternary Ammonium Compound
qPCR	Quantitative Polymerase Chain Reaction
R	Reverse Primer
rpm	Revolutions Per Minute
rRNA	Ribosomal Ribonucleic Acid
RT-PCR	Real Time Polymerase Chain Reaction
s	Second
SCENIHR	Scientific Committee on Emerging & Newly Identified Health Risks
SD	Standard Deviation
SMX	Sulfamethoxazole
SPCA	Standard Plate Count Agar
sul	Sulfamethoxazole Resistant Gene
Table S	Table in Supplementary Document
TET	Tetracycline
TOC	Total Organic Carbon
TSB	Tyrptose Soya Broth
UNICEF	The United Nations Children Emergency Fund
UNU-INWEH	United Nation University-Institute of Water, Environment & Health
UV-VI	Ultraviolet-Visible
WHO	World Health Organization

Chapter 1

1.0 Introduction

The supply of safe and clean drinking water is fundamental to modern standards of living (Pruden 2014), but it is not easily available to all people. The extensive use of antimicrobial chemicals has created a global threat to public health in the form of resistance development in bacteria (CDC 2013). These chemical originate from the clinical environment (Berendonk et al. 2015; Li et al. 2016), industries and agriculture practices (Davies 2015; O'Neill 2015), ultimately dispersing into water bodies due to multiple anthropogenic activities. The problem of resistance has been increased to such an extent that the use of many antimicrobials has been banned in many parts of the world (Cogliani et al. 2011; McArthur et al. 2015). Although the release of these substances to the environment has been reduced through good management practices (O'Neill 2015), the emergence and dispersion of resistance continues and the key culprit has been previous overutilization (Llor and Bjerrum 2014).

Water resources (including: drinking water treatment plants, distribution systems, and points of use), polluted with antimicrobial resistant microorganisms and their resistance markers, have become a growing concern and represent a direct link between the environmental and human infections (Pruden et al. 2013). Outbreaks of typhoid due to multidrug resistant *Salmonella* in contaminated drinking water have been reported in Tajikistan (Mermin et al. 1999), and also in Africa (Walters et al. 2014). In Mexico, multidrug resistant bacteria have caused outbreaks of diarrhoea in children due to the consumption of contaminated water (Delgado-Gardea et al.

2016). In the USA alone, antibiotic resistant infections affect more than 2 million people, with no less than 23000 deaths annually due to the direct effect of these antibiotic resistant infections (CDC 2013). These infections cost $8 billion to the USA health system and other productivity losses (Ventola 2015).

The role of water supply infrastructure itself could be important as some studies have suggested that it can serve as a pathway in the spread of resistance (Szabo and Minamyer 2014). However the ecology of these systems, in terms of the enrichment and dispersion of antimicrobial resistance in the presence of disinfectant, remains relatively unknown, when compared to other environmental aspects of antimicrobial resistance. Disinfectants are used for the decontamination of biological agents from water systems (Lehtola et al. 2005), however many have limited effectiveness (Botsaris et al. 2015). Owing to the introduction of contaminants into the distribution systems from several sources, such as leakages in pipes, regrowth due to prolong storage of water, corroded and old infrastructure, inefficient treatment plants, contaminated pumps and plumbing systems in the buildings (Szabo and Minamyer 2014), it becomes challenging to get water free of biological agents at the point of use.

As mentioned previously, water supply infrastructure could be responsible for the propagation of resistance in these systems. This research aimed to find a linkage between the ecological factors in these systems and antibiotic and disinfectant resistances, an emerging issue of water distribution systems. This study focused on real life organisms, normally resident in municipal water distribution systems, rather than laboratory or clinical isolates. Evaluation of the role of low disinfectant levels in

resistance enrichment and the contribution of different physiological conditions to the final fate of resistant genes, were also considered as part of this study.

1.1 Structure of the Thesis

This work has been divided into seven chapters consisting of a brief literature review, research publications and overall conclusions. Detailed literature reviews and methodologies are incorporated into each of the publication chapters.

The literature review, in Chapter 2, provides background information about the research topic; including the major findings relevant to the ecotoxicology of water distribution systems with emphasis on the role of different factors that could contribute to the dispersion of bacteria and their genes in the water-supply system. The literature review is concise and focuses on key issues related to water-distribution systems, which are relevant to this study. Previous investigations are critically evaluated and research gaps (Section 2.7) discovered during the literature review are discussed. The major aims and objectives of this study (Section 2.9), and summary of the research process (Section 2.10) are also mentioned in this chapter.

The main body of the thesis comprises four chapters (Chapters 3-6), two of them (Chapter 3 and 4) have been published and one (Chapter 5) has been submitted as individual papers in international, peer-reviewed journals. The remaining one (Chapters 6) will be submitted for publication. Each chapter has its own introduction, methodology, results, discussion, conclusions and references sections and is presented in this dissertation in a similar form as it was published or will be submitted to journals. Each chapter has a preamble preceding the paper to provide additional background, disclosure of co-authors' contribution in the publication and journal information.

In relation to the individual result chapters (publications): Chapter 3 provides evidence of antibiotic- and disinfectant-resistant bacteria in drinking water and the confirmation of selected resistant genes and transferable elements. This chapter further discusses the risks of certain resistant genes in a water-distribution system, how these genes disperse and the factors promoting their existence.

Chapter 4 presents an evaluation of the susceptibilities of the bacteria isolated from municipal water-distribution systems (Chapter 3), to antibiotics and disinfectants. The link between antibiotic and disinfectant resistance is compared. Standard methods, such as the disk diffusion method (Clinical and Laboratory Standards Institute 2012b) and minimum inhibitory concentration (MIC) (Clinical and Laboratory Standards Institute 2012a), were used. However, the disk-diffusion method was adapted to screen bacteria against high concentrations of chlorine in this study; this method was further verified against a well-defined suspension test for studying the behaviour of six chlorine and monochloramine resistant bacteria.

Chapter 5 conceptualizes the idea of minimum selectable concentrations (MSCs) as an effective assay for comparing bacteria from a mixed population. Previously, this method has been used for antibiotic-resistance (Lundstrom et al. 2016) and has been adapted in this study against chlorine disinfectants. MSC is based on the theory that lower concentrations could cause the enrichment of resistant bacteria independent of MIC in the water environment; this is further discussed in this chapter.

In Chapter 6, the study analysed the potential of drinking water distribution systems (DWDS) to retain or disperse antibiotic-resistance genes. It ecotoxicologically verifies the MSCs model in a lab-scale microcosm of a water

supply system in the presence of different pipe materials, disinfectant concentrations and biofilms. This final study amalgamates the individual previous experiments into a pseudo-realistic scenario of what could happen in a water distribution system.

Chapter 7 is the final chapter of this dissertation and contains the overall conclusions from the study, summary of major findings, limitation of this research and the recommendations for further studies. Additional supplementary information from Chapter 4 and water quality parameters of Milngavie Water Treatment Works, Glasgow (which supplied water to the sampled area), are presented in the Appendix.

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Chapter 2

2.0 Literature Review

2.1 Background

Water is a basic necessity for life (Pruden 2014), is essential for the environment (Bogardi et al. 2012) and was recognized as a human right in 2010 by the United Nation General Assembly (WHO-UNICEF 2012). Despite the fact that 71% of planet Earth is covered with water, the shortage of clean drinking water is a worldwide issue due to limited freshwater resources. Between 1995 and 2025, the world population facing the problem of water scarcity will have increased from 485 million in 31 countries to 2.8 billion in 48 countries (PAI 1999). The main factors thought to be responsible for this issue are climate change, economic growth, increasing population, inefficient use of fresh water and pollution of natural water bodies (PAI 1997; Bogardi et al. 2012), which result in insufficient supply of water for a large part of the world's population (UNU-INWEH 2015).

A number of pollutants have been introduced into water bodies through natural cycles and anthropogenic activities (Wells et al. 2009). They include organic compounds, pharmaceuticals, pesticides, disinfectant by-products, personal care products (PPCPs), steroids, endocrine disrupting chemicals (EDCs) and heavy metals (Jelic´ et al. 2012; Boxall et al. 2012; Rivera-Utrilla et al. 2013; Chow et al. 2015; Li et al. 2016). Amongst the major causes of pollution are rapid pharmaceutical and industrial development and hospital wastes (Prado et al. 2008; McArthur et al. 2011), which result in increased generation of untreated contaminated wastewater with minimum recycling (Chow et al. 2015; Li et al. 2016). The release of these

wastewaters into the environment leads to pollution of natural water resources (Prado et al. 2008), endangered ecosystems (Cabello 2006) and human health issues (Bogardi et al. 2012; Berendonk et al. 2015). Ultimately, this creates water insecurity and contaminated drinking water (Bogardi et al. 2012).

Other important factors which could also contribute to the global problem of supply of safe drinking water are inefficient water treatment plants and contaminated distribution systems (Bergeron et al. 2015). These make it difficult for water-supply organizations to provide potable water at the consumer end. Since 1990, 2.6 billion people have gained access to improved drinking water sources. However, currently 663 million people do not have access to such water (WHO-UNICEF 2015). The use of piped water supplies on premises has increased worldwide since 1990 from 45% to 54% in 2010 (WHO-UNICEF 2012).

As previously mentioned, a number of pollutants have been reported. However, one of the emerging concerns in the environment is the presence of microorganisms originating from hospital environments which have resistant traits against antimicrobials (e.g., antibiotics and disinfectants). Water distribution systems (Samra et al. 2009), could become colonized by such pathogens and this could lead to their establishment in pipes and biofilms (Buse et al. 2012). Moreover, inappropriately maintained plumbing systems and storage of water in contaminated tanks and cisterns can also introduce pathogens into drinking water (Bagh et al. 2004; Falkinham 2015; Falkinham et al. 2015). This water could serve as a vehicle for the transfer of pathogens to healthy populations (Craun et al. 2006) and may cause outbreaks of water borne diseases in a susceptible population (Pedley et al. 2006).

2.2 Importance of Antimicrobials, Antimicrobial Resistance, and Related Genes in Water Distribution and Plumbing Systems

Antimicrobials are compounds used for the inhibition of the growth of microorganisms in industries and households and include antibiotics, antiseptics, and disinfectants. The effectiveness of each antimicrobial ranges from inhibition of reproduction (bacteriostatic) to killing of microorganisms (bactericidal). In drinking water, disinfectants are used to inhibit the growth of bacteria in treatment plants and distribution systems, while antibiotics are found in water due to their disposal from household, clinical, agricultural and multiple industrial sources. The importance of antibiotics and disinfectants in the environment is discussed below.

2.2.1 Antibiotics, Antibiotic Resistance and Genes

Antibiotics, being compounds that are either produced by microorganisms or synthesized commercially, are used to treat infections in hospitals and are commonly used as biocidal compounds in different industries, e.g. agriculture (Pruden et al. 2013), aquaculture (Cabello 2006), cosmetics production and animal rearing (Kummerer 2003; Phillips et al. 2004; Landers et al. 2012) to minimise undesired microorganisms (Kummerer 2009a). The inappropriate use of antibiotics has resulted in contamination of the natural environment (McArthur et al. 2015) and also the development and enrichment of resistant bacteria; including in drinking water (Dodd 2012). These organisms develop resistance to a wide variety of antimicrobials and biocides, even at much higher concentrations than their initial exposure and become

difficult to inhibit by conventional treatment methods. The presence of resistance gives these bacteria a selective advantage over susceptible populations. They tolerate the application of antimicrobials and biocides at concentrations used for their eradication and could transfer the resistance trait to other related bacteria—thus, potentially creating a large population of highly resistant organisms in the environment.

Antibiotic resistance could be defined as "*Ability of bacteria to survive, and even thrive, in the presence of antibiotics"* (Pruden 2014). The segment of DNA which encodes the antibiotic resistance trait is known as the antibiotic resistance gene (ARG) and bacteria containing these genes (or tolerating the specific concentration of antibiotic) are called antibiotic resistant bacteria (ARB). The presence of ARGs in bacteria is important in two ways. Firstly, the bacteria containing ARGs could have greater tendencies to survive than their wild type strains in the presence of antibiotics and other antimicrobials. Secondly, these ARBs have the capability to share these genes among other bacteria through the process of gene transfer (Schluter et al. 2007; Szczepanowski et al. 2009; Stalder et al. 2012).

A number of mobile genetic elements (MGEs) can be involved in the process of sharing and transferring resistance genes. Mobile genetic elements (MGEs) are "*Segments of DNA that encode enzymes and other proteins that mediate the movement of DNA within genomes (intracellular mobility) or between bacterial cells (intercellular mobility)*" (Frost et al. 2005). Examples of MGEs are given in Table 2.1.

Table 2.1 Examples of mobile genetic elements (MGE) in environment.

MGE	References
Integrons	(Jones et al. 1997; Rowe-Magnus et al. 2002; Roe et al. 2003; Xuejun and Weijin 2010; Hsu et al. 2014)
Plasmids	(Russell 1990; Lyon and Skurray 1987; Schmidt et al. 2001; Diab et al. 2002; Moura et al. 2012)
Genomic islands	(Stokes and Gillings 2011)
Gene cassettes	(Recchia and Hall 1995; Hall and Collis 1995; Hall et al. 1999; Koenig et al. 2009; Moura et al. 2010)
Transposons	(Devaud et al. 1982; Stokes et al. 2007; Knapp et al. 2008; Petrova et al. 2011; Cain and Hall 2012)

In the current era, antibiotic resistance is considered to be a critical challenge by several global agencies; including the World Health Organization (WHO) and the U.S. Centre for Disease Control and Prevention (CDC), because of the ever increasing rate of antibiotic resistance among disease-causing bacteria (Jones et al. 2008; Pruden 2014). For example, the recurrence of drug-resistant tuberculosis in many parts of the world (Zumla et al. 2012) and methicillin resistant *Staphylococcus aureus* (MRSA) in hospitals (Schito 2006; Klein et al. 2013), aquatic environments (Tolba et al. 2008; Levin-Edens et al. 2011; Levin-Edens et al. 2012; Porrero et al. 2014; Sood et al. 2014) and wastewater treatment plants (Borjesson et al. 2009; Borjesson et al. 2010; Goldstein et al. 2012).

A major concern is that ARB and ARGs are not limited to any specific environment. Any individual environment could serve as a source, sink, or reservoir of resistant bacteria and genes. A list is given below in Table 2.2. Besides the natural environment, there are some other major sinks of ARGs, e.g. human beings and

animals (Stokes and Gillings 2011), which are involved in the direct transfer of MGEs and linked resistance genes between sources and sinks.

Table 2.2 Sources, sinks and reservoirs of resistant bacteria in environment

Sink and sources of	**References**
1. Soil	(Riesenfeld et al. 2004; Knapp et al. 2010; Ji et al. 2012; Negreanu et al. 2012)
2. Water environments	(Baquero et al. 2008; Zhang et al. 2009; Finley et al. 2013; Coutinho et al. 2014; Vaz-Moreira et al. 2014)
2.1 Lakes	(Czekalski et al. 2012; Zhang et al. 2016)
2.2 Rivers	(Storteboom et al. 2010; McArthur et al. 2011; Jiang et al. 2013)
2.3 Sea water	(Alouache et al. 2012; Alves et al. 2014)
2.4 Surface water	(Figueira et al. 2012; Stoll et al. 2012)
2.5 Bottled water	(Falcone-Dias et al. 2012)
2.6 Potable tap-water	(Calomiris et al. 1984; Jazrawi et al. 1988; Diab et al. 2002; Faria et al. 2009; Samra et al. 2009; Vaz-Moreira et al. 2011; Coleman et al. 2012; Figueira et al. 2012)

The presence of ARB and ARGs in water engender a lot of concerns regarding the potential for transferring and exchanging resistance between biota of different aquatic compartments (Dodd 2012) and in human beings through drinking and recreational water sources (Buse et al. 2012; Machado and Bordalo 2014). The most common genes in drinking water treatment plants and finished water are *sul* and *tet* genes which show resistance against sulfonamide and tetracycline antibiotics, respectively (Pruden et al. 2006; Guo et al. 2014; Bergeron et al. 2015; Adesoji et al. 2016). Sources of *sul*1 and *sul*2 are mainly hospital waste streams (Czekalski et al. 2012), which could transfer these genes to water bodies (Prado et al. 2008). These

genes pass through water treatment plants, contaminating water resources and ultimately finding their way into drinking water distribution systems. Other ARGs, such as vancomycin and ampicillin resistant genes, *van* and *amp*, have been reported in drinking water biofilms (Schwartz et al. 2003).

2.2.2 Disinfectants, Disinfectant Resistance and Genes

The use of disinfectants or biocides is indispensable in many industries. A list of available disinfectants would be too long to produce and is beyond the scope of this study; however, in the drinking water industry, chlorine-based compounds such as chlorine and monochloramine are the first choice for the control of microorganisms (Rose et al. 2007). Interestingly, the most widely distributed disinfectant resistant genes in water, *qac* genes, are not associated with chlorine, but emerge due to the use of quaternary ammonium compounds (QACs). They are used as biocides in clinical settings, cosmetics production (Buffet-Bataillon et al. 2012) and the food processing industries (Holah et al. 2002). The intensive use of QACs in different industries has not only resulted in the development of disinfectant resistant bacteria (DRB), but also the selection of antibiotic resistant bacteria (Russell et al. 1998; McBain et al. 2004; Gaze et al. 2005) due to cross resistance of QACs and antibiotics as describe below (Morente et al. 2013). This intensive use has also resulted in the dispersal of resistance markers among the biota of natural (Gillings et al. 2009a) and anthropogenic environments, such as drinking water treatment plants (DWTP) (Farkas et al. 2013). Resistances to QACs and antibiotics often involve similar genetic elements and strains with plasmids (Bjorland et al. 2003), transposons

or integrons (Xuejun and Weijin 2010) and thus result in cross resistance for other antimicrobials (Morente et al. 2013).

Cross-resistance can be defined as "*the ability by which organisms become less susceptible to one drug in a class, causes them to also become less susceptible to others in the class*" (Sanders 2001). The presence of cross resistance can modify the minimum inhibitory concentration (MIC) value (*the minimum amount of antibiotic or disinfectant required to inhibit bacteria*) of antibiotics, e.g., chlorhexidine MIC is found to be higher in bacteria having *qac* genes (McGann et al. 2011). How quaternary ammonium compounds affect the MIC of other antimicrobial compounds depends upon the compound tested, resistance genes types, the methodology selected for study and the criteria used for interpretation (Buffet-Bataillon et al. 2012).

An important aspect of *qac* genes is that they do not only code for QACs resistance, but also code for resistance to a broad range of monovalent and divalent cationic compounds, which may belong to different chemical classes (Hassan et al. 2006). These include biguanides, diamidines and intercalating dyes (Jaglic and Cervinkova 2012). Many *qac* gene determinants have been reported, including *qac*A-H, *qac* J and *qac*Z. A high similarity in base pair is reported between *qac*A and *qac*B (McGann et al. 2011), between *qac*C and *qac* D (Mayer et al. 2001), and between *qac*E and *qac*F (Ploy et al. 1998), but this little difference in similarity creates strong impacts in substrate specificity for these genes (Mayer et al. 2001). *Qac* gene distribution is also related to the presence of certain environmental bacteria, e.g. Gram-positive groups (Kazama et al. 1998), *S. aureus* (Longtin et al. 2011; Ye et al. 2012; Zmantar et al. 2011), *Enterococcus* (Braga et al. 2011), *Aeromonas* (Chang et al. 2007), *Pseudomonas* (Kazama et al. 1998), *Acinetobacter* (Mak et al. 2009),

members of Enterobacteriaceae, Helicobacteriaceae and Xanthomonadaceae (Wang et al. 2008).

The importance of *qac* genes in the environment is due to their broad-spectrum resistance to related compounds (Jaglic and Cervinkova 2012), co-occurrence with antibiotic resistant genes (Farkas et al. 2013) and transferability, which make *qac* genes a potential target for study (See Sections 3.4.2 and 3.5.3).

2.2.3 Integrons and Associated Genes

Integrons are mobile genetic elements, usually located on chromosomes and plasmids. They are widely distributed in the environment and could acquire, exchange, express and maintain antibiotic resistance genes (Mazel 2006; Gillings et al. 2008; Cambray et al. 2010; Heuer and Smalla 2012; Jaglic and Cervinkova 2012; Stalder et al. 2012). As mentioned in Sections 2.2.1 and 2.2.2, ARGs, disinfectant resistance genes and integrons are often interlinked and both ARGs and disinfectant resistant *qac* genes are widely spread among integrons (Paulsen et al. 1993; Paulsen et al. 1996a; Jeong et al. 2009). Moreover, some integron classes such as type 1 (*intl*1) can carry both *qac* resistance genes and antibiotic resistance genes such as *sul*1 simultaneously (Adesoji et al. 2016); QACs resistance selection in any environment can also cause co-selection of antibiotic resistance (Gaze et al, 2005). For this reason, integron genes (i.e. *intl*1 and *intl*2) are studied with antibiotic and disinfectant resistant genes (i.e. *sul* and *qac*), as their presence shows that *sul* and *qac* may be present.

2.2.4 16S rRNA Genes

16S rRNA is a common housekeeping gene in qPCR (quantitative polymerase chain reactions for amplifying DNA and analysis) (Bergeron et al. 2015). It is present in almost all bacteria and its function has not changed with time (Drancourt et al. 2000). This gene can be used for phylogenetic determination of bacteria (Weisburg et al. 1991; Farkas et al. 2013), characterisation of genetic diversity in a complex bacterial community (Muyzer et al. 1993), determination of population activity in different environments; including drinking water (Keinanen-Toivola et al. 2006; Revetta et al. 2010) and identification of multiple resistant bacteria (Czekalski et al. 2012). Importantly, it can also be used to identify unculturable bacteria (Clarridge 2004), novel pathogens (Srinivasan et al. 2015) and aid the discovery of new bacteria (Woo et al. 2008).

2.3 Mode of Action of Antimicrobials

In the modern era, the use of biocides is unavoidable. They are not only used in clinical settings (Group 2008), but also in other sectors like the agriculture, food and water industries. They are used extensively in household items and cleaning products. Owing to their unlimited use, several problems have been encountered; including the emergence of resistance against them and knowledge of their mechanism of action becomes vital to overcome this issue. In the case of antibiotics, their mode of action is well documented in the literature; while for disinfectants, their mechanisms of action are not well understood. In a microbial cell, a disinfectant could have several target sites and collective damage at multiple points could be bactericidal (McDonnell and Russell 1999). Moreover, the disruption of electron

motive force and changes in the hydrophobicity of the cytoplasmic membrane by the interaction of biocide to outer cellular components create bactericidal effects. In addition, reversible activity of the cytoplasmic membrane and impairment of enzymatic activity cause bacteriostatic effects at lower biocide concentrations (Maillard 2002).

As mentioned in Section 2.2.2, chlorine and monochloramine are common disinfectants used for drinking water treatment. The mechanism of action of chlorine releasing agents involves increased permeability of outer membranes, adverse effects on cytoplasmic constituents (nucleic acids) and interaction with specific groups, e.g. thiol and sulfhydryl groups (Maillard 2002; Myers 2008), as well as metabolic inhibition (Denyer and Stewart 1998). Hypochlorite is more reactive than reactive oxygen species, e.g. oxide ion and hydrogen peroxide and also has better bactericidal activity, which gives it an advantage when used as a disinfectant (Miller and Britigan 1997). The main targets of hypochlorite are DNA, proteins and lipids. It can cause a mutation in the genome, disruption in membrane function and inactivation of proteins by oxidative unfolding (Davies 2005). Other reported modes of action for hypochlorite are induction of genes related to heat shock induction (Wang et al. 2009), thiol oxidation (Leichert et al. 2008), oxidative protein unfolding and irreversible aggregation of thermolabile proteins, which are essential for bacterial survival (Winter et al. 2008).

2.3.1 Factors Involved in the Efficacy of Disinfectants in Water Distribution Systems

Multiple factors can influence the efficacy of biocides in the water environment. Contact time is important, as most disinfectants do not work immediately and need longer exposure times, especially when lower concentrations are used. Biocide concentration also plays a major role in efficacy. Water conditions, such as the presence of organic matter, turbidity (LeChevallier et al. 1981), nutrient availability (Harakeh et al. 1985), pH (White 1999), temperature and the presence of biofilms (LeChevallier et al. 1988) can all lower the efficacy of antimicrobials in water systems.

Microbial physiology can also alter the efficacy of chemical disinfectants as the nature, state and composition of the outer membrane of microorganisms and sporulation could impact the effectiveness of biocides (Myers 2008). The lethal action of biocides is not the same for all organisms; it depends not only on the make-up of the organisms, but also on the chemical composition of the biocides (Myers 2008). For example, the presence of thymol (monotarpene phenol) (Jia et al. 2010; Nikolić et al. 2014), flavonoid (Uzel et al. 2005), hydroxyl group (Ben Arfa et al. 2006), halogen (Maris 1995; Denyer and Stewart 1998), alcohol and phenolic type compounds and silver containing compounds (McDonnell and Russell 1999). Hydrophobicity or lipophilicity of the compound or presence of free hydroxyl group cause alterations in the membrane permeability of bacteria through leakage of protons and are crucial for effectiveness (Ben Arfa et al. 2006).

2.4 Possible Mechanisms of Resistance Development against Antimicrobials

Antimicrobial resistance mechanisms found in the environment are much more diverse than their modes of action (Manaia et al. 2012). Biocide resistance has been classified into two types; intrinsic and acquired. Intrinsic resistance is natural resistance controlled by chromosomes, while acquired resistance occurs due to genetic manipulation, either by mutation or by gaining genetic elements like plasmids and transposons (Russell 1990). A specific concentration of disinfectant is required to effectively kill or inhibit bacteria. Owing to the presence of intrinsic resistance to biocides in bacteria, the target site of the bacteria is not exposed to the effective concentration and the concentration is unable to achieve the desired response. The presence of spores in *Bacillus* and *Clostridium* (Foegeding and Busta 1983), special cell wall composition in *Mycobacteria* (Russell 1996), peptidoglycan in Gram-positive bacteria (Poxton 1993), outer membrane of Gram-negative bacteria (Russell and Gould 1988) and physiological adaptation of bacteria in environment, are examples of intrinsic mechanisms of resistance in bacteria (McDonnell and Russell 1999).

Acquired resistance can be plasmid-mediated or non-plasmid-mediated. Plasmids are mobile genetic elements like integrons (discussed in Section 2.2.3). The resistance plasmids are R-plasmids, which contribute antimicrobial resistance to any microorganism, e.g., *Aeromonas salmonicida* (Schmidt et al. 2001). On the other hand, acquired non-plasmid encoded resistance can develop when bacteria are exposed to increasing concentrations of any disinfectant. An example of this is found in *Serratia marcescens*, which showed resistance to QACs (Prince et al. 1978).

Enzyme-catalysed antibiotic modification, such as resistance against heavy metals (Cloete 2003) and modification or bypass of drug target (Wright 2011) are also possible mechanisms of resistance development in bacteria.

Resistance to an antimicrobial of one class can also cause resistance to other antimicrobials of the same class (Canton and Ruiz-Garbajosa 2011), or resistance against biocide of a single class can cause resistance against another class of biocide having a similar mode of action. This process is known as "cross resistance" and is quite common in antibiotics and disinfectants (Chapman 2003) (see Section 2.2.2), for example, the cross resistance between triclosan and ciprofloxacin in *Pseudomonas aeruginosa* (Chuanchuen et al. 2001). This concurrent resistance to antibiotics and disinfectants could be due to gene encoding multidrug pumps (Lupo et al. 2012). These genes could be found on chromosomes in Gram-negative bacteria or on plasmids in Gram-positive bacteria (Webber 2002) and could develop both natural and acquired resistances. The presence of these genes on plasmids or transposons facilitates the transfer of efflux genes to other microorganisms. One efflux system could be responsible for the resistance to a variety of antimicrobials (Cloete 2003). Additionally, several efflux pumps could be expressed at the same time in a bacterium, causing multiple drug resistance (Myers 2008; Buffet-Bataillon et al. 2012). *P. aeruginosa* (Chuanchuen et al. 2001; Tabata et al. 2003), *Salmonella enterica serovar Typhimurium* (Karatzas et al. 2008) and *Escherichia coli* have this efflux system, which is involved in the extrusion of several biocides, e.g. QACs, benzalkonium chloride (Devaud et al.), triclosan and antibiotics, making the organisms resistant to these substances (Levy 2002; Bore et al. 2007).

Another mechanism of resistance found in bacteria is the generation of novel genetic response stimulated by the ecosystem. When cells are exposed to various antibiotics, a SOS response is induced, which affects the bacterial genome (Miller et al. 2004; Aertsen and Michiels 2006). The SOS response is a repairing mechanism that helps bacteria to adapt to different stresses by pausing cell division. It also increases its survival against antimicrobial exposure, which could otherwise be lethal (Miller et al. 2004). Horizontal transfer of antibiotic resistance genes and integron recombination (Guerin et al. 2009) events are examples of such induced responses.

Horizontal gene transfer (HGT); also known as lateral gene transfer (LGT), is a process of physical transfer of DNA among bacteria without any cell division, which incorporates itself into recipients in such a manner that it is stably inherited. ARGs can be transferred rapidly through this mechanism (Stokes and Gillings 2011). HGT is considered to be the major cause of widespread dispersion of ARGs on mobile genetic elements (MGEs), because these gene elements could enhance the transfer among diverse groups of bacteria present in different environments (Gaze et al. 2011). The MGEs that have ARGs are driven by three major processes, including conjugation (genes transfer through sex organelles), transduction (transfer of genes through phage or virus) and transformation (extracellular DNA uptake from dead cells) (Pruden 2014).

Another method, which transfers genetic material from ancestor to offspring, is known as vertical gene transfer or intrinsic gene transfer. Resistant genes remain in the same genera and do not transfer to other groups of bacteria, but the population having resistant genes increases.

Mutation is also a method of resistance acquisition, which causes changes in the genetic make-up of organisms and enables them to become resistant (Sanders 2001). The incorporation of resistant genes containing DNA into a recipient could also take place by homologous recombination, transposition or site specific recombination (Thomas and Nielsen 2005).

Beside these, new mechanisms of resistance are also arising. For example, the presence of the New Delhi metallo-β-lactamase 1 (NDM-1) gene has been reported in different countries in surface water and has become a worldwide public health concern in a short span of time (Kumarasamy et al. 2010; Walsh et al. 2011; McArthur et al. 2013; Ahammad et al. 2014). Traveling of people to developing countries such as India and Pakistan causes the transfer of this gene to United Kingdom (Kumarasamy et al. 2010).

2.5 Drinking Water Treatment Plants, Water Distribution Systems and Enrichment and Transfer of Resistance

As mentioned above, a variety of resistance mechanisms exist in nature, which can help bacteria to develop, transfer and enrich resistance traits where the surrounding environments favour these processes. Water habitats such as drinking water treatment plants (DWTP), drinking water distribution systems (DWDS) and plumbing and storage systems in buildings (see chapter 4 for detail) could provide a suitable environment for the enrichment and transfer of resistance traits and might serve as a source and reservoir of resistant bacteria and genes; similarly to the natural environment and animal and human populations, as discussed in Section 2.2.

The presence and propagation of a number of ARGs such as *erm*, *sul*, *tet*, *bla*, *cat*, *cmr*, and *amp* have been found in DWTP and DWDS following different treatments including ozonation, sedimentation, sand filtration, biological activated carbon (BAC) (Guo et al. 2014) and chlorination (Shi et al. 2013). These genes also code for resistance to a variety of antibiotics including aminoglycoside, β-lactam, chloramphenicol (Xi et al. 2009) sulfonamide, tetracycline (Guo et al. 2014), macrolide, loncosamide, and fluoroquinolone (Xu et al. 2016). Not only ARGs, but the MGE genes such as *Tnp* (transposon), *IS* (insertion sequences) and *intI* (integron) were also present with these ARGs, indicating the potential for transportation and dispersion of resistance through the drinking water system (Farkas et al. 2013; Xu et al. 2016). Further details can be found in Chapter 6.

ARB and ARGs can not be eliminated completely from the water by conventional and advanced treatments methods (Dodd 2012; McKinney and Pruden 2012). As mentioned above, drinking water harbours almost all types of ARGs and MGEs, and the incomplete removal of microorganisms and micro-contaminants from water supply systems has the potential to cause outbreaks of water-borne infections in human and animal consumers.

2.6 Health Care and Economic Burden due to Water Pollution

Water contamination is one of the major causes of water-borne diseases all over the world, imposing a burden on the population, especially for vulnerable populations such as children, the elderly and those with on-going illnesses. Outbreaks of water-borne disease have been reported in both developing regions (Ashbolt 2004) and developed countries due to the consumption of drinking water (Collier et al. 2012; ECDC 2014). This indicates that, wherever one may be, drinking

water may serve as a source of pathogens. Major water-borne diseases include diarrhoea, cholera, dysentery, gastroenteritis, hepatitis, typhoid and giardiasis (Ashbolt 2004; NYC 2016). Diarrhoea is one of the major causes of death among children under the age of five (UNICEF 2012) and globally water-borne disease causes 5 million deaths annually (Malik et al. 2012). According to the CDC, in the USA 65.5% of drinking water associated outbreaks during 2012 were due to the presence of *Legionella* in building plumbing systems (Beer et al. 2015). In India, where water pollution is very high, 37.7 million people suffer from water-borne illnesses annually, including 1.5 million children who die due to diarrhoea, resulting in a $600-million burden on the economy (Pathak 2015). This indicates that deficiencies in water systems (treatment and distribution), building premises and plumbing all contribute to the occurrence and transmission of pathogens at the point of use (Craun et al. 2001; Ashbolt 2004; Craun et al. 2006; Brunkard et al. 2011).

Water interlinks geophysical, socio-ecological and economical systems, creating a "global water system" (Bogardi et al. 2012). Water scarcity and pollution not only disturb all these systems (UN-WATER 2015), but also influence the production of food (PAI 1997), energy and industry sectors and growth and development processes of a country (UNU-INWEH 2015). Moreover, this reverses the economy, limits opportunities for economic growth (UN-WATER 2016), increases social inequality and impacts vulnerable populations; especially the poorest (UNU-INWEH 2015). The presence of ARBs and ARGs in water supply systems does not only pose a burden on treatment plants (as new and expensive technologies are required to cope with them), but also on the general population (it becomes

inevitable to purify water at the point of use, which is not economically feasible for all end users).

2.7. Gaps in the Research

Research into antibiotic resistance in the water environment is a very extensive field and it is not possible to cover all aspects in one thesis. Many studies will struggle to fully elucidate the diversity of this field. Many features need further and detailed investigation because of the link between DWDS and the general population and the potential for transferring water-borne diseases as discussed in Section 2.6. However, major research gaps highlighted in the production of this literature review include:

- The extent of microbial contamination of the system due to storage or outbreak of diseases due to disruption, repair and maintenance works on plumbing systems are not found in the literature. Decontamination data is also not available.
- The development of new treatment technologies for removing resistance genes during treatment and from distribution systems should be considered. Control strategies for antibiotic resistant bacteria and their genes are currently ignored and mostly focus on pathogenic bacteria.
- Water-system designs should minimize the risk of spread of antibiotic resistant bacteria and their genes; this requires further study.
- The impact of antibiotic resistance bacteria on human health is still unknown. A knowledge gap with respect to public health is present, as not much data is available about antibiotic resistance bacteria. Epidemiological studies are also limited and not carried out widely to include ARB as potential sources.

- Human health risk assessment data for exposure risk through distribution systems or through drinking water are not available. Studies for endemic risks or quantitative microbial risk assessment appear to be limited.
- Water quality data in distribution systems when outbreaks occur in certain areas is also not available and is not monitored.
- Statistics for water-borne illness due to water distribution system pollution appear to be incomplete and could better inform management practices.
- The rate of ARB and ARGs transfer within distribution systems and treatment plants and from these systems to plant or human population needs to be determined.
- There is no guideline level or any standard available for ARGs in drinking water. Coliform bacteria remain indicator organisms for drinking water quality determination, at 0 mL^{-1} of water. The safe level of ARGs and other bacteria need to be determined.

As mentioned earlier, it was not possible to cover all aspects in one research study, therefore the current work focused on first three of the major issues mentioned above;

1) plumbing systems and storage resulting in microbial contamination of drinking water at point of use;
2) use of minimum inhibitory concentration for the development of new treatment technologies; and
3) water system design or selection of pipes with minimum potential for resistance transfer. The exploration of enrichment of the resistant population within distribution systems in water and biofilms in pipes made of different materials.

2.8 Conclusion

Antibiotic resistance is not limited to the clinical environment and it is an emerging concern within water distribution systems. The presence of resistant organisms and genes in the DWDS, despite the use of treatment technologies and the potential of DWDS to transmit such pathogens to vulnerable populations creates an alarming situation. There is the need for appropriate planning and management to avoid the further introduction of resistant organisms into water bodies and the application of suitable treatment technologies for the complete elimination of these pollutants from water in drinking water treatment plants and distribution systems.

2.9 Aims and Objectives

The major aim of this research is to determine if DWDS ecology enhances the antibiotic and disinfectant resistances in drinking water. Information about several ecotoxicological factors is needed for investigation; microbial community structure in water distribution system, antibiotic and disinfectant resistant profiles of bacteria, confirmation of the presence of resistant genes (i.e., antibiotic resistant genes, disinfectant resistant genes, and transferable genes), information about water storage conditions and the impact of disinfectants and type of plumbing system.

To meet the above mentioned aim for this study, a set of hypotheses were used; detail of them is given below:

- drinking water contains antibiotic and disinfectant resistant bacteria;
- bacteria have resistant genes both for antibiotic and disinfectant;
- bacteria have potential to transfer resistance;
- storage conditions and plumbing can enrich ARB and DRB;

- resistant bacteria enrich at sub-inhibitory concentrations below MIC;
- ecological conditions in DWDS propagate the ARB; and
- presence of physical support such as a biofilm enriches the ARB.

To address the major aims and hypothesis of the study that the drinking water environment can enrich antimicrobial resistant bacteria and their genes, the research design involved the following objectives.

1. Collect drinking water samples from municipal water distribution systems from buildings with and without water storage facilities, e.g., cisterns (Chapter 3), to compare the impact of storage conditions and the populations that may exist.
2. Isolate bacteria from water samples using filtration techniques and identify bacterial pathogens by molecular techniques, targeting the 16S-rRNA genes (Chapter 3). This will be used to identify the bacteria communities in DWDS.
3. Confirm the presence of antibiotic (*sul*1 and *sul*2), disinfectant resistant genes (*qac*) and mobile genetic elements (*intI*1 and *intI*2) responsible for resistant gene transfer in the water environment using molecular techniques with specific primers for each gene (Chapter 3).
4. Antimicrobial susceptibility testing of bacterial isolates against four antibiotics (tetracycline, sulfamethoxazole, ciprofloxacin and ampicillin) and chlorine-based disinfectants and determine the linkage between antibiotic and disinfectant resistances (Chapter 4).
5. Comparison of the occurrence of resistant bacteria in samples collected from buildings with or without storage of water to evaluate the effect of water storage on bacterial resistance (Chapter 4).

6. Evaluation of the MSC model for the determination of risk of resistant populations at low biocidal concentration in water by comparing growth rates of susceptible and resistant species (Chapter 5).
7. Ecotoxicological study of the water distribution system using lab-scale microcosms to determine the effects of pipe materials, disinfectant and other chemical parameters on the existence, proliferation and dispersion of antimicrobial resistant bacteria and the fate of their genes in water distribution systems (Chapter 6).

2.10 Methods and Techniques Used for the Study

As discussed previously, water distribution systems can be a hot spot for biological agents such as antibiotic and disinfectant resistant bacteria and their genes, which survive disinfection processes and both conventional and advanced techniques (qualitative and quantitative) were needed to investigate them during this study.

For the isolation of bacteria from municipal drinking water, a traditional membrane filtration method was used. Antimicrobial susceptibilities of isolated bacteria against antibiotics were determined by the recommended agar dilution method (Clinical and Laboratory Standards Institute 2012a), while standard Kirby-Bauer disk diffusion method (Clinical and Laboratory Standards Institute 2012b) was adapted with some modifications against high concentration of disinfectants for preliminary evaluation of disinfectant resistance in bacteria. This was further confirmed by detailed suspension testing for antimicrobials. Bacterial cultures were stored in a bacterial bead preservation kit (Cryovials TS/71-MX, Technical Service Consultants LTD. UK) for experiments in this study.

Other techniques involved were bacterial DNA extraction by physical thermal treatment (Chapter 3 and 4) and ISOLATE ll Genomic DNA kit (BIOLINE, UK) (Chapter 6). The polymerase chain reaction (PCR) is a powerful amplification technique and can amplify small amounts of target DNA sequence (Lorenz 2012). General PCR with Bio-Rad iQ5 Real-Time PCR Detection System was used for the qualitative analysis of presence of bacteria (16S-rRNA), ARGs (*sul*1, *sul*2), MGE (*intI*1 and *intI*2) and disinfectant resistant genes (*qac*) by gene specific primers (Chapter 3). Confirmation of PCR products was carried out by Gel electrophoresis (Bio-Rad, UK). DNA was purified from PCR reaction products by QIAquick PCR Purification Kit (Qiagen, UK) (Chapter 6).

Quantitative analysis of the concentration of DNA in the purified PCR samples was carried out by using the EPOCH^{TM} Microplate spectrophotometric system (BioTek, UK) (Chapter 4 and 6). Quantitative real time PCR (qRT-PCR) data with standard curve were used for the calculation of gene copies of resistant genes and bacterial genes in test samples. This molecular technique has better sensitivity and reproducibility than general PCR for the quantification of microorganisms in the environment (Zhang and Fang 2006).

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Chapter 3

3.0 Antibiotic Resistant Bacteria Found in Municipal Drinking Water

3.1 Background

This chapter consists of a published paper and contains information about the occurrence of antibiotic and disinfectant resistant bacteria in a municipal water distribution system. This chapter demonstrates the use of molecular biology techniques for the identification of bacteria from water and confirmation of their resistant genes.

Sadia Khan designed and performed the experimental work. The paper was written by her and jointly reviewed and edited by all three authors. This paper has been published in *Environmental Processes* in February 2016 and is available online:[2] DOI: 10.1007/s40710-016-0149-z.

[2] An initial version of this paper has been presented at the 9th World Congress of the European Water Resources Association (EWRA) "*Water Resources Management in a Changing World: Challenges and Opportunities*", Istanbul, Turkey, June 10-13, 2015.

3.2 Abstract

Multidrug resistant bacteria in water supply systems have been emerging as a growing public health concern. Many factors affect the source and fate of these bacteria. However, conditions in plumbing systems may contribute to the dispersion of resistance genes among bacterial populations. Through the process of lateral gene transfer, resistance genetic material can be exchanged between species in the microbial population, intensifying the problem of resistance genes. The main aim of this study was to investigate the diversity of microorganisms in tap water in Glasgow, Scotland and the occurrence of certain antibiotic resistance genes and gene-transfer mechanisms. Results show that antibiotic resistant bacteria exist at the consumers' end of the distribution system, some of which also contain integrase genes, which can aid in the dispersion of resistance genes. The presence of such microorganisms indicates that further investigation should be made to assess the risks to public health.

3.2.1 Key words

Antimicrobial resistance, water distribution system, *intI*, *qac*, *sul*

3.3 Introduction

The supply of safe drinking water to the population of the world remains one of the major concerns for public health. Many factors impact this provision, including increasing world population, limited freshwater resources and pollution. Contemporary issues affecting supply include contamination with chemical compounds, e.g. pharmaceutical and personal care products and biological agents, which can contribute to increased antimicrobial resistance in bacteria (L. et al. 2006; Larsson et al. 2007; Pruden et al. 2013). The use of these chemicals has been increased to a point that the propagation of antimicrobial resistance has become unavoidable and it is now considered an emerging contaminant of concern in the environment (Pruden et al. 2006). In urban areas, water treatment plants utilise multiple technologies to remove many pollutants (Xu et al. 2007), while integrated constructed wetlands are used in rural areas for the removal of pollutants and resistance genes (Chen et al. 2015a). However, these systems are never entirely effective (Xu et al. 2007).

Various processes in treatment plants (Armstrong et al. 1981) and the physical and chemical nature of long distribution systems, facilitate the enrichment of bacteria and their genes. Attachment to particulates, capsule formation and increased tolerance of bacteria to chemical disinfectants help in the survival and spread of resistant organisms in water supply systems (Ridgway and Olson 1982; LeChevallier et al. 1984; LeChevallier et al. 1988; Bridier et al. 2011, Wingender and Flemming 2011).

The presence of antimicrobial resistant bacteria and their genes in water bodies is not limited to under-developed countries (Khan et al. 2013; Ahammad et al.

2014). They have been found in developed nations including Australia, Germany and the United Kingdom (Stoll et al. 2012; Khan et al. 2013; Ahammad et al. 2014). Many enteric bacteria with multiple drug resistance (MDR), e.g., *Escherichia, Enterobacter, Klebsiella, Salmonella* and *Shigella* species, have been found in both drinking and recreational water resources (Kumar et al. 2013). Previously, both Gram-positive and Gram-negative bacteria, including *Staphylococcus, Alcaligenes,* and *Acinetobacter* species, have been reported in drinking-water distribution systems (Armstrong et al. 1981). Antibiotic resistant *Pseudomonas* species have also been isolated from drinking water (Vaz-Moreira et al. 2012; Ribeiro et al. 2014). The presence of MDR in the clinical environment makes the treatment of diseases difficult (Ashbolt et al. 2013). Their presence in water bodies and therefore increased exposure risk to public, may necessitate enhanced water treatment and so increased expense.

Bacteria isolated from drinking water may not only contain antibiotic resistance, but they may also express resistance to commonly used disinfectants, including chlorine and monochloramine (Ridgway and Olson 1982; Chiao et al. 2014, Khan et al, 2016). These disinfectants are indispensable in water treatment plants to eradicate bacteria and minimise the growth of bacteria in the distribution system. Resistance develops upon exposure to sub-inhibitory concentrations over time and a number of bacterial species with monochloramine resistance have been reported in drinking water, including: *Coxiella, Desulfuromonas, Desulfomonile, Escherichia, Geobacter, Legionella, Mycobacterium* and *Sphingomonas* species (Chiao et al. 2014).

Beside physical and chemical processes, genetic factors contribute to the dispersion of resistance genes in drinking water distribution systems. Horizontal gene transfer (HGT) mechanisms move resistance genes from resistant to susceptible populations on mobile genetic elements like integrons (Mazel 2006; M. et al. 2008; Stalder et al. 2012; Jechalke et al. 2013). HGT spreads genetic elements and their genes among dissimilar groups of bacteria (Gaze et al. 2011), rapidly transferring antibiotic and disinfectant resistance genes (Boucher et al. 2007; Stokes and Gillings 2011; Mokracka et al. 2012). Integrons have a recombination system, which captures genes and serves as a reservoir of resistance genes (Stokes and Hall 1989; Demarre et al. 2007; Xu et al. 2011). The transfer of multiple genes on integrons via HGT intensifies the problem of resistance in bacterial communities, as it can link resistance traits between environmental bacteria and human pathogens in drinking water systems (Ribeiro et al. 2014).

Among multidrug resistance bacteria, class 1 integrons are most common (Shearer and Summers 2009) and they are related to the presence of resistance to quaternary ammonium compounds (QACs), *qac*EΔ1 genes and sulphonamide resistance *sul*1 determinants (Kucken et al. 2000). Almost half of the class 1 integrons contain a *qac* resistance genes cassette (Gillings et al. 2009b) and the prevalence of class 1 integrons in bacteria having previous exposure to QACs is greater than those without previous exposure (Gaze et al. 2005). Moreover, bacteria that possess or acquire plasmids, transposons or integrons, have greater QACs resistance than those which do not have any of these genetic elements (Bjorland et al. 2003). Bacteria often show cross resistance for QACs and antibiotics (Morente et al. 2013), including the use of efflux-pump systems to generate resistance to unrelated

broad-range antimicrobials (Buffet-Bataillon et al. 2012). Enzyme based mechanisms of antibiotic resistance are also found in bacteria. In the presence of antibiotics, bacteria may acquire gene encoding enzymes, which can destroy the antibiotic before reaching the target, resulting in the development of resistance in bacteria (Tenover 2006).

This study aimed to determine the prevalence of bacteria and resistance genes in drinking water. Assays included genes responsible for resistance to a wide variety of quaternary ammonium compounds (*qac* genes) and sulphonamide antibiotics (*sul*1 and *sul*2 genes), as these genes commonly co-occur. Mobile genetic integrons were detected via their integrase gene *intI*1 and *intI*2. The co-occurrence of *sul* and *qac* genes in bacteria from the water distribution system was also examined.

3.4 Materials and Methods

3.4.1 Sampling and Processing

Tap-water samples were collected from residences in Glasgow in sterile screw capped bottles and were processed within 2-4 hours of collection. For the uniformity in the samples, they were collected from the buildings with same distance from the treatment plants. These buildings have similar main structure and maintain similar public supply conditions except storage of water inside the buildings in cisterns (see Section 4.4.1). One hundred millilitres of each water sample were vacuum-filtered through 0.22 µm pore size cellulose nitrate gridded membranes (Millipore, UK), which were then aseptically placed on Standard Plate Count Agar APHA (Oxoid, UK). The plates were incubated for 48 hours at 37 ± 2°C. Selected bacterial isolates from the resultant growth were streaked on Nutrient Agar (Oxoid,

UK) plates to give isolated colonies; 4-5 colonies of each strain were preserved in glycerol using a Bacterial Beads Preservation Kit (Cryo vials TS/71-MX, Technical Service Consultants Ltd., UK) and stored at -80°C.

3.4.2 DNA Extraction and PCR Amplification

DNA of the bacterial isolates were thermally extracted by mixing strains with 100 µL of PBS (pH 7.4) and undergoing a series of freeze thaw cycles at -80°C and 70°C with continuous shaking between each cycle. The contents were centrifuged at 10,000 rpm for 5 minutes at the end of the fourth thermal cycle and DNA from the supernatant was stored at -80°C.

PCR reactions were performed with a Bio-Rad iQ5 Real-Time PCR Detection System for the presence of 16S-rRNA, *intI*1, *intI*2, *sul*1, *sul*2 and *qac* genes using previously described primers (Table 3.1; Pei et al. 2006; Luo et al. 2010; Caporaso et al. 2011; Jechalke et al. 2013). Twenty microliter PCR reactions consisted of 10 µL of MegaMix-Blue-PCR Mastermix with dye (Microzone Limited, UK), 1 µL of each primer (500 ηM final concentration; Sigma-Aldrich Life Science, UK), 6 µL of nuclease-free water, and 3 µL of DNA sample. Each PCR run consisted of initial denaturation at 95°C for 3 minutes; this was followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing for 30 seconds at annealing temperature (Table 3.1), extension at 72°C for 30 seconds and then a final extension at 72°C for 10 minutes. PCR products were further verified with 2 % agarose gel in 1x Tris Acetate-EDTA buffer; the size of amplified products was determined against a 50-bp incrementing DNA ladder (Fisher BioReagent, UK).

3.4.3 DNA Purification and Sequencing

PCR products from 16S-rRNA gene amplification were purified using the QIAquick PCR Purification Kit (Qiagen, UK) according to the manufacturer's instructions. The purified and cleaned amplicon concentration was determined by the EPOCH™ Microplate spectrophotometer system (BioTek, UK). Amplicon was mixed with 5 µM forward primer solution used in the PCR in a 1:1 ratio in a total volume of 10 µL and sent to LightRun Sequencing Service (GACT Biotech Ltd, London, UK) for sequencing. Bacteria were identified by comparing the sequences using the BLAST program through the National Centre for Biotechnology Information (NCBI) (http://blast.ncbi.nlm.nih.gov).

3.5 Results

3.5.1 Identification of Bacteria by16S-rRNA Sequencing: Bacterial Community Structure

Bacteria were isolated from tap water by a membrane filtration method (n=148) and 87 colonies were identified up to genus level by sequencing the V4 region of the 16S-ribosomal RNA gene. The water distribution system harboured three phyla of bacteria: Proteobacteria, Actinobacteria and Firmicutes. Among them, 54 (62.1 %) belonged to the phylum Proteobacteria. Sub grouping of this phylum indicated the presence of 10 alpha-proteobacteria (11.5 %), 38 beta-proteobacteria (43.7 %), 5 gamma-proteobacteria (5.7 %) and 1 epsilon-proteobacterium (1.2 %). Firmicutes were the second largest phyla found in drinking water and 18 (20.7 %)

bacteria belonged to this group, while 15 (17.2 %) bacteria were from Actinobacteria.

The presence of both Gram-negative and Gram-positive bacteria was confirmed in the tap water; among which some of the bacteria can be pathogenic. Species of *Bacillus*, *Paenibacillus*, *Brevibacillus*, *Kocuria*, *Staphylococcus*, *Arthrobacter*, *Comamonas*, *Acidovorax*, *Blastomonas*, *Variovorax*, *Escherichia* and pathogenic *Burkholderia* were found in the water distribution system.

3.5.2 Presence of Antibiotic Resistance Genes

Detection of the *sul*1 and *sul*2 genes specific for sulfonamide resistance was performed on 148 isolates and these genes were detected in 12 (8.1 %) isolates, thus confirming the presence of antibiotic resistant bacteria in the water distribution system; none contained both genes. *sul*1 genes were detected in 8 (5.4 %) bacteria, while *sul*2 genes were present in 4 (2.7 %) isolates (Table 3.2). In two (1.4 %) isolates, these genes were also positive for integrons, while in 10 (6.8%) isolates they were found singly without integrase genes, indicating that *sul* genes did not always correspond to *intI* genes as expected. This suggests that the *sul* genes in these bacteria might either be present on the chromosome, or are associated with other genetic elements (Gundogdu et al. 2011) than *intI* genes. The distribution of bacteria containing antibiotic resistance genes (ARGs) was widespread among organisms including *Bacillus*, *Cupriavidus*, *Variovorax*, *Kocuria*, *Ralstonia*, *Dermacoccus*, *Micrococcus* and *Staphylococcus* species from the samples.

3.5.3 Presence of Disinfectant Resistance Genes

Disinfectant resistance genes *qac* were not found in any of the isolates. *Qac* genes associated with class 1 integrons have a high occurrence rate in the environment, both in Gram-positive and Gram-negative bacteria (Jaglic and Cervinkova 2012); but in this study, the presence of *qac* genes was not detected.

3.5.4 Presence of Transferable Markers

PCR amplification analyses indicated that class-1 integrons existed in eight bacteria (5.4 %), while *intI*2 genes were not detected in any isolate. In this study, *Dermacoccus* sp. had both *sul*1 and *intI*1 genes, while *Micrococcus* sp. had *sul*2 and *intI*1 genes simultaneously. The presence of *intI*1 genes confirms the presence of transferable genetic element integrons in the bacteria of the water supply system, which could be involved in the dispersion of antibiotic and disinfectant resistance genes in the environment.

3.6 Discussion

The presence of antibiotic resistant bacteria and their genes in water is a major public health concern and a global challenge (Pruden et al. 2012; Pruden et al. 2013). They are a major contributory factor in water pollution, are found in natural water bodies (Ahammad et al. 2014), treated drinking water (Pruden et al. 2006) and drinking water distribution systems (Xi et al. 2009), can cause infectious diseases (Pruden et al. 2012) and are difficult to treat (Levy 2002). Currently, waterborne diseases are not only related to the presence of faecal bacteria, but also to the occurrence of opportunistic pathogens in water systems (Wang et al. 2013). Around

30 different bacterial genera have been isolated from drinking water in different studies including genera of *Pseudomonas*, *Klebsiella*, *Enterobacter*, *Escherichia* and *Proteus* (Norton and LeChevallier 2000; Allen et al. 2004; Gallego et al. 2005, Chiao et al, 2014). In developed countries, outbreaks of waterborne infections due to drinking water have been reported (Kilvington et al. 2004; Brunkard et al. 2011). Antibiotic resistant infections are also not uncommon in these countries. In the USA, two million people suffer from antibiotic resistant infections every year, resulting in 23,000 deaths (CDC 2013). Drinking water distribution systems host a variety of microorganisms (Simoes et al. 2010) and bacteria having antibiotic resistance can find their way into drinking water in these systems. From drinking water, they could colonize the gastrointestinal tract and serve as a potential health risk for the population; especially immune-compromised individuals (Lee et al. 2010).

Isolation of bacteria by membrane filtration demonstrated the presence of viable organisms, so it is a good indicator of the presence of living bacteria in the water environment, which can be actively involved in gene transfer among bacteria and spread of disease in humans. 16S-rRNA gene information is generally used for the identification of bacteria from different environments (Chakravorty et al. 2007). While a useful housekeeping genetic marker to classify bacteria, 1-14% of organisms remain unidentified as it has low phylogenetic power at species level and cannot discriminate some genera properly (Mignard and Flandrois 2006; Janda and Abbott 2007). In this study, 87% of bacteria were identified from the tap water, while 13% of bacteria were not characterized as no significant similarity was found. These bacteria belong to 22 genera (Table 3.2), and also included *Burkholderia*, some species of which are waterborne pathogens and may cause melioidosis in humans

(Howard and Inglis 2003). The isolation and identification of multiple types of bacteria at the point of use indicates that the distribution network or plumbing systems might be playing some role in the existence of these bacteria and the ecology of the system could be a contributing factor in their incidence (Hong et al. 2010).

A well-recognized factor which could contribute to the existence of antibiotic resistance bacteria at the consumer end is the presence of biofilms in the distribution system or plumbing system of the buildings from where water samples have been taken. The prevalence of antibiotic resistant bacteria in a water supply system, even in the presence of disinfectant, could be exacerbated by aging infrastructure, in which biofilms have formed on surfaces (Abe et al. 2012). The multiple layers of microorganisms in these biofilms decrease the residual disinfectant levels to the inner layers (see Sections 6.3 and 6.6 for details). Further, bacteria in these layers could develop and transfer resistance traits against antimicrobials to other susceptible populations present in these biofilms (Molin and Tolker-Nielsen 2003; Bridier et al. 2011). Water storage in tanks and cisterns also causes a decrease in the amount of residual disinfectant as water retention time increases, thus allowing bacteria to grow in the water. Disinfection itself could concentrate the antibiotic resistant bacteria and their genes in drinking water, as was found in the work of Shi et al. (2013), where chlorination caused the enrichment of *tet*, *amp* and *erm* genes.

The emergence of antibiotic resistance in the environment is not only due to physiological factors, but also depends upon genetic factors like the horizontal gene transfer (HGT) rate (Andersson and Hughes 2010). Co-selection of two different antibiotic resistance genes occurs through HGT due to the genetic linkage of these genes. For example, sulfonamide resistance genes are plasmid borne and are often

linked with other antibiotic resistance genes. It has also been found that *sul* and *intI* genes co-exist in water, which might be due to the presence of *sul*1 genes on the class 1 integrons (Chen et al., 2015b). This contributes to the reason why sulphonamide resistance has not declined even when the use of the antibiotics has been reduced (Enne et al. 2004).

Quaternary ammonium compound resistant genes are also present on mobile genetic elements, such as class 1 integrons (Chapman 2003). Bacterial strains, which acquire genetic units like plasmids, transposons or integrons, show a higher resistance to QACs (Bjorland et al. 2003). The selective pressure by quaternary ammonium compounds (Stalder et al. 2012) disperses *qac* genes and antibiotic resistance genes associated with the integrons (Paulsen et al. 1993; Paulsen et al. 1996b; Jeong et al. 2009). This indicates that cross resistance for QACs and antibiotics is possible (Morente et al. 2013). Other mechanisms like a multidrug efflux pump and modification of the cell wall also induce resistance in bacteria (Jaglic and Cervinkova, 2012), which allow them to survive in the presence of disinfectant. This could be a reason why in the current study, *qac* genes were not detected in any of the bacteria despite the fact that *intI*1 genes were present with antibiotic resistance genes. This suggests that in the absence of disinfectant resistant genes, other mechanisms of resistance might help bacteria persist in the water distribution system.

3.7 Conclusion

Isolation of antibiotic resistant bacteria from drinking water demonstrates a need for greater awareness of ecological interactions in drinking water and increased monitoring of distribution systems and plumbing. Presence of those genera, some

species of which could cause human diseases, indicates that water quality could not be guaranteed at the consumer end and future studies should focus on treatment considerations at point of use to guarantee safety.

Table 3-1 PCR Primers for targeting different genes.

Primer	Sequence (5′-3′)	PCR annealing temperature (°C)	Amplicon size (bp)	Reference
V4-16S-515F	TGTGCCAGCMGCCGCGGTAA	50	312	(Caporaso et al. 2011)
V4-16S-806R	GGCTACHVGGGTWTCTAAT			
qacEaIIF	CGCATTTTATTTTCTTTCTCTGGTT	60	Not detected	(Jechalke et al. 2013)
qacEaIIR	CCCGACCAGACTGCATAAGC			
int1-F	GGCTTCGTGATGCCTGCTT	57	148	(Luo et al. 2010; Chen et al. 2015b)
int1-R	CATTCCTGGCCGTGGTTCT			
int2-F	GTTATTTTATTGCTGGGATTAGGC	56	166	(Luo et al. 2010; Chen et al. 2015b)
int2-R	TTTTACGCTGCTGTATGGTGC			
sul1-F	CGCACCGGAAACATCGCTGCAC	56	163	(Pei et al. 2006; Chen et al. 2015b)
sul1-R	TGAAGTTCCGCCGCAAGGCTCG			
sul2-F	TCCGGTGGAGGCCGGTATCTGG	60.8	191	(Pei et al. 2006; Chen et al. 2015b)
sul2-R	CGGGAATGCCATCTGCCTTGAG			

Key: F = forward, R = reverse

Table 3-2 Detection of *intl*1, *intl*2, *sul*1, *sul*2 and *qac* genes in bacteria isolated from the drinking water distribution system.

N#	Phylum	Genus	Genes					Isolate identification (Isolate number assign in this study)
			***intI*1**	***intI*2**	***sul*1**	***sul*2**	***qac***	
1	Actinobacteria	*Arthrobacter*	-	-	-	-	-	DW(518)
1	Actinobacteria	*Arthrobacter*	+	-	-	-	-	DW(509)
2	Actinobacteria	*Dermacoccus*	-	-	-	-	-	DW(597, 603)
1	Actinobacteria	*Dermacoccus*	-	-	+	-	-	DW(608)
1	Actinobacteria	*Dermacoccus*	+	-	+	-	-	DW(607)
1	Actinobacteria	*Dietzia*	-	-	-	-	-	DW(625)
1	Actinobacteria	*Janibacter*	-	-	-	-	-	DW(644)
1	Actinobacteria	*Kocuria*	-	-	-	-	-	DW(565)
1	Actinobacteria	*Kocuria*	-	-	+	-	-	DW(620)
1	Actinobacteria	*Kocuria*	+	-	-	-	-	DW(513)
2	Actinobacteria	*Micrococcus*	-	-	-	-	-	DW(505, 637)
1	Actinobacteria	*Micrococcus*	+	-	-	-	-	DW(638)
1	Actinobacteria	*Micrococcus*	+	-	-	+	-	DW(512)
9	Alphaproteobacteria	*Blastomonas*	-	-	-	-	-	DW(525, 526, 547, 551, 553, 554, 556, 559, 599)
1	Alphaproteobacteria	*Sphingomonas*	-	-	-	-	-	DW(576)
8	Betaproteobacteria	*Acidovorax*	-	-	-	-	-	DW(516, 521, 537, 539, 540, 541, 544, 569)
5	Betaproteobacteria	*Burkholderia*	-	-	-	-	-	DW(530, 615, 617, 626, 643)
1	Betaproteobacteria	*Comamonas*	-	-	-	-	-	DW(503)
10	Betaproteobacteria	*Cupriavidus*	-	-	-	-	-	DW(501, 502, 504, 511, 522, 570, 578, 580, 587, 591)
1	Betaproteobacteria	*Cupriavidus*	-	-	-	+	-	DW(515)
2	Betaproteobacteria	*Cupriavidus*	-	-	+	-	-	DW(610, 622)
1	Betaproteobacteria	*Cupriavidus*	+	-	-	-	-	DW(604)

5	Betaproteobacteria	*Ralstonia*	-	-	-	-	-	DW(609, 613, 616, 618, 619)
1	Betaproteobacteria	*Ralstonia*	-	-	-	+	-	DW(614)
2	Betaproteobacteria	*Variovorax*	-	-	-	-	-	DW(546, 549)
2	Betaproteobacteria	*Variovorax*	+	-	-	-	-	DW(557, 600)
1	Epsilonproteobacteria	Not identified						DW(533)
5	Firmicutes	*Bacillus*	-	-	-	-	-	DW(514, 527, 529, 531, 640)
1	Firmicutes	*Bacillus*	-	-	-	+	-	DW(507)
1	Firmicutes	*Bacillus*	-	-	+	-	-	DW(532)
1	Firmicutes	*Brevibacillus*	-	-	-	-	-	DW(535)
5	Firmicutes	*Paenibacillus*	-	-	-	-	-	DW(552, 623, 634, 635, 641)
1	Firmicutes	*Paenibacillus*	-	-	+	-	-	DW(536)
3	Firmicutes	*Staphylococcus*	-	-	-	-	-	DW(538, 542, 632)
1	Firmicutes	*Staphylococcus*	-	-	+	-	-	DW(631)
2	Gammaproteobacteria	*Enhydrobacter*	-	-	-	-	-	DW(506, 508)
2	Gammaproteobacteria	*Escherichia*	-	-	-	-	-	DW(560, 611)
1	Gammaproteobacteria	*Pantoea*	-	-	-	-	-	DW(595)

Key: + = presence of gene, - = absence of gene

3.8 References

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Chapter 4

4.0 Relationship between Antibiotic- and Disinfectant-Resistance Profiles in Bacteria Harvested from Tap Water

4.1 Background

This paper describes the link between disinfectant and antibiotic resistances, in order to determine if disinfectant resistant bacteria also have greater antibiotic resistance, when compared to sensitive bacteria. The difference in the survival of antibiotic resistant and susceptible bacteria against disinfection was also highlighted in the paper. [3]

Sadia Khan designed and performed the research work and wrote the paper. Data analysis was carried out by Sadia Khan and Charles W. Knapp. All three authors edited and reviewed the paper.

This paper was published in *Chemosphere* in February 2016 and is available online. DOI:10.1016/j.chemosphere.2016.02.086. This paper was also highlighted as a press release by the University of Strathclyde, published as "Building owners 'face risks' from chlorine-resistant bacteria" http://www.strath.ac.uk/whystrathclyde/mediacentre/buildingownersfacerisksfromchlorine-resistantbacteria/ and was published in *ScienceDaily* on 18 March 2016. www.sciencedaily.com/releases/2016/03/160318131635.htm

[3] We are thankful to Dr. Ameet Pinto, University of Glasgow, Scotland, UK for his guidance in monochloramine experiments.

4.2 Abstract

Chlorination is commonly used to control levels of bacteria in drinking water. However, viable bacteria may remain due to chlorine resistance. What is concerning is that surviving bacteria, due to co-selection factors, may also have increased resistance to common antibiotics. This would pose a public health risk as it could link resistant bacteria in the natural environment to the human population. Here, the relationship between chlorine- and antibiotic-resistances was investigated by harvesting 148 bacteria surviving in chlorinated drinking-water systems and compared their susceptibilities to chlorine disinfectants and antibiotics. Twenty-two genera were isolated, including members of *Paenibacillus*, *Burkholderia*, *Escherichia*, *Sphingomonas* and *Dermacoccus* species. Weak, but significant, correlations were found between chlorine-tolerance and minimum inhibitory concentrations against the antibiotics tetracycline, sulfamethoxazole and amoxicillin, but not against ciprofloxacin. This suggests that chlorine-tolerant bacteria are more likely to also be antibiotic resistant. Further, antibiotic-resistant bacteria survived longer than antibiotic-sensitive organisms when exposed to free chlorine in a contact-time assay. However, there were few differences in susceptibility when exposed to monochloramine. Irrespective of antibiotic-resistance, spore-forming bacteria had a higher tolerance to disinfection compounds. The presence of chlorine-resistant bacteria surviving in drinking-water systems may also carry the additional risk of antibiotic resistance.

4.2.1 Key words

Susceptibility, antimicrobial-resistant bacteria, disinfectant-resistance, drinking-water

4.3 Introduction

Antibiotic-resistant bacteria (ARB) and their genes (ARG) are considered to be emerging environmental contaminants with a widespread distribution (Pruden et al. 2006; Diehl and Lapara 2010; Dodd 2012; Chen et al. 2015a). Both natural and anthropogenic activities are contributing to their development and dispersion in the environment (Allen et al. 2010; Gaze et al. 2011; Wellington et al. 2013) and water bodies (Pruden et al. 2012; Su et al. 2012). As the demand for safe drinking-water increases around the world (Brettar and Hofle 2008), these compromised natural-water resources could more increasingly become considered to be sources of either drinking-water or contamination to the distribution system.

Drinking-water treatment plants use a number of treatment methods to improve water quality, e.g., flocculation, sedimentation, filtration and disinfection. Among the processes, chemical disinfection contributes greatly to the control of microorganisms from treatment plant to point of use (Berry et al. 2006). However, it is known that chemical disinfection has limitations in its immediate and prolonged effectiveness and that multiple factors reduce the effectiveness of disinfectants against bacterial populations (Scully et al. 1999; Cherchi and Gu 2011; Jaglic et al. 2012; Bessa et al. 2014), including the presence of organic matter having amino nitrogen compounds (Scully and Hartman 1996), a bacterial growth phase (Cherchi and Gu 2011) and the presence of an extracellular polymeric matrix (Bridier et al. 2011; Wong et al. 2010).

It has increasingly been discovered that resistance traits horizontally transfer in microbial communities due to either cross-resistance (e.g., efflux mechanisms capable of detoxifying multiple stressors) or co-resistance (e.g., closely linked

genetic traits on a mobile genetic element) factors. For example, Templeton et al. (2009) found greater frequency of chlorine tolerance among antibiotic-resistant *E. coli* when compared to antibiotic-sensitive *E. coli* grown in the presence of chlorine (Templeton et al. 2009). Genetic factors, such as class 1 and class 2 integrons that transfer multiple resistance genes could be responsible for such traits (Gillings et al. 2009a; Ozgumus et al. 2009; Koczura et al. 2012; Mokracka et al. 2012; Su et al. 2012; Hsu et al. 2014; Chen et al. 2015a).

Wastewater treatment studies (Diehl and Lapara 2010; Burch et al. 2013) have reported decrease in total bacteria, but an increased ratio of resistant bacteria (Guo et al. 2014) following treatment; a similar trend may occur in drinking-water systems (Bergeron et al. 2015). There have been reports of drinking-water treatment plants (DWTP) (Armstrong et al. 1981; Armstrong et al. 1982; Xi et al. 2009; Farkas et al. 2013; Pruden et al. 2006) and water distribution systems (DWDS) (Laroche et al. 2010; Talukdar et al. 2013; Xi et al. 2009) influencing the emergence and spread of antibiotic-resistance. For example, the relative abundance of sulfonamide resistance genes increased from 3.5% to 33% in DWTP (Chao et al. 2013) and a broader range of ARGs was found (Fahrenfeld et al. 2013). Stressful environments such as extreme pH, high salinity, nutrient deprivation (Bessa et al. 2014), oxidation (Scully et al. 1999), or chlorine exposure (Ridgway and Olson 1982) promote populations with greater resistance. Sub-inhibitory concentrations not only select resistant populations, but could invoke a stress response, which may include genetic exchange.

Bacteria opportunistically colonise water distribution systems (Wang et al. 2013) and water meters (Hong et al. 2010). Additionally, localised disruptions in the

distribution mains (e.g., in building cisterns and plumbing) also introduce bacterial populations, which may include agents of waterborne disease, increasing health risks and maintenance costs to the system (Falkinham et al. 2015).

This study compares the susceptibilities of bacteria harvested from drinking-water taps to chlorine disinfectants and four antibiotics: tetracycline (TET), sulfamethoxazole (SMX), ciprofloxacin (CIP) and amoxicillin (AMX). It is hypothesized that bacteria isolated from water taps would have enhanced disinfectant- and antibiotic-resistance profiles. Further, it is determined whether disruptions to service lines provide a source of contamination and increase the risk of ARB and ARG.

4.4 Materials and Methods

4.4.1 Sampling and Bacteria Isolation

In the UK, most drinking-water is sourced from surface water (Scottish-Water 2012a, b) and does not deviate from many conventional water-treatment works: screening, coagulation, flocculation, sedimentation or clarification, filtration (rapid gravity, slow sand, or membrane) and pH adjustment. Both chlorination and chloramination are used for disinfection in Scotland, UK to provide good quality water for human use. Monochloramine is used in the distribution system as it has a longer residence time than chlorine and produces fewer by-products.

To compare tolerances between disinfectants and antibiotics, bacteria were harvested from 52 water samples, collected from flushed (5 min) taps in Glasgow, Scotland, UK (see Section 3.4.1). Samples were collected in sterile screw capped bottles and brought to the laboratory for processing within two hours to minimise

changes in the samples. Thirty-eight samples were collected from buildings that had tank cisterns for drinking-water storage, with tank capacities ranged from 16,000 to 27,000 L; the remaining 14 samples were from closed systems.

A vacuum-filtration method, with 0.22 µm pore-size cellulose-nitrate gridded membrane filters (Millipore, UK) was used to harvest cells from 100 mL of each water sample; the filter was placed on a Standard Plate Count Agar plate APHA (Oxoid, UK) and incubated for 48 h at 35 ± 2°C for the development of colonies. The plastic lid was retained to minimise aerosol contamination; sterilised distilled water was used as a control. Isolated bacterial strains were preserved by using a bacterial bead preservation kit (Cryo vials TS/71-MX, Technical Service Consultants Ltd. UK) and stored at -80°C throughout the study period. For each set of experiments, one bead was taken out from the cryovials, grown in LB broth overnight, and streaked on a Nutrient Agar (Oxoid, UK) plate to obtain isolated colonies.

4.4.2 Identification of Bacteria Isolates

Representative colonies were selected for phylogenetic characterisation by sequencing the V4 region of each 16S-rRNA gene. The DNA of bacterial isolates was extracted by a thermal freeze thaw method (Knapp et al. 2012), alternating between -80°C and 70°C in 100 µL PBS (phosphate buffer solution; pH 7.4). PCR reaction was performed with a Bio-Rad iQ5 Real-Time PCR Detection System. Forward and reverse primers (Sigma-Aldrich, Life Sciences, UK) were V4-16S-515F (5´-TGTGCCAGCMGCCGCGGTAA) and V4-16S-806R (5´-GGCTACHVGGGTWTCTAAT) (Caporaso et al. 2011). Each PCR reaction contained 10 µL of Universal Supermix (Bio-Rad, UK), 500 ηM of each primer, 0.1

µL SYBR green, 6 µL of nuclease free water and 3 µL of DNA template. A PCR run consisted of initial denaturation at 95°C for 3 min followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 50°C for 30 s, extension at 72°C for 30 s and then a 10 min final extension at 72°C. PCR product length was verified on 2% agarose gel (Bio-Rad, UK) with ethidium bromide (Sigma-Aldrich, UK) and a 50-bp DNA ladder.

A QIAquick PCR Purification Kit (Qiagen, UK) was used to purify PCR products. DNA concentrations were determined by the EPOCH^{TM} Microplate spectrophotometric system (BioTek, UK). Five µL of purified DNA was mixed with the same volume of 5 µM forward primer solution in total volume of 10 µL. Sequencing for the identification of bacteria was performed by LightRun Sequencing Service (GACT Biotech Ltd, London, UK). Bacteria were identified up to genus level by sequence comparisons using the BLAST program through the National Center for Biotechnology Information (NCBI) (http://blast.ncbi.nlm.nih.gov).

4.4.3 Disinfectant Susceptibility Testing

Testing was performed using the Kirby-Bauer disc diffusion method, as recommended by the Clinical and Laboratory Standards Institute (Clinical And Laboratory Standards Institute 2012b), against 127 bacterial isolates with disinfectant solutions of commercial bleach (4.5% sodium hypochlorite, Domestos™, UniLever, UK), 14.5% standard sodium hypochlorite (Alfa Aesar, UK), and a control (tap water) (Sassone et al. 2008; Poggio et al. 2010; Luddin and Ahmed 2013). Experiments were performed in duplicate and the mean zone of inhibition was determined for each isolate. It was arbitrarily considered bacteria having a zone $\leq$ 20

mm to be chlorine tolerant (or resistant), as high concentration of standard sodium chlorite (14.5%) was used.

4.4.4 Antibiotic Susceptibility Testing for MIC

Bacterial isolates were also tested for antibiotic susceptibility against tetracycline hydrochloride ('TET'; Sigma-Aldrich, UK), sulfamethoxazole ('SMX'; Molekula, UK), amoxicillin trihydrate ('AMX'; Alfa Aesar, UK) and ciprofloxacin ('CIP'; Fluka, UK) by Agar Dilution Method as recommended by the Clinical and Laboratory Standards Institute (Clinical and Laboratory Standards Institute 2012a). A master replica plate, containing 20-24 bacterial isolates, was freshly prepared for each experiment. The isolates were tested against a series of concentrations, 0.002–512 µg mL^{-1}, of each antibiotic in Mueller-Hinton Agar (Oxoid, UK) (Lederberg and Lederberg 1952; Lechevalier and Corke 1953; Harris 1963; Armstrong et al. 1981). All plates were incubated at 35 ± 2°C for 24 h. Minimum inhibitory concentrations (MIC) were calculated for each antibiotic (µg mL^{-1}) against all isolates. *E. coli* ATCC 25922 (NCTC 12241) was used as a control and the maximum MIC values of antibiotics against the organisms reported by CLSI were used as references for the interpretation (Clinical And Laboratory Standards Insitute 2011; Guo et al. 2013; Yuan et al. 2015). Any bacterium forming colonies above maximum MIC values mentioned by CLSI (TET $\geq$ 16 µg mL^{-1}, SMX $\geq$ 512 µg mL^{-1}, CIP $\geq$ 4 µg mL^{-1}, and AMX $\geq$ 32 µg mL^{-1}) was considered to be "resistant" to that antibiotic; those inhibited at lower concentrations were considered to be 'susceptible'.

4.4.5 Disinfectant Suspension Tests for Chlorine Resistance

Six isolates were selected for chlorine and monochloramine suspension tests to verify the Kirby-Bauer results at fixed concentrations and exposure time. Suspension tests were performed in 200 mL of 10 mM PBS at pH 7.0. All glassware was treated with 10% nitric acid overnight, soaked in bleach (5% sodium hypochlorite, Alfa Aesar), rinsed with nano-pure water, air-dried and autoclaved (Chiao et al. 2014). A stock solution of 14.5% sodium hypochlorite was used to prepare 0.5, 1.0, 2.0, 4.0 and 8.0 mg L^{-1} free chlorine solutions. Bacteria were grown overnight with continuous shaking in Tryptic Soya Broth (Fluka, UK), centrifuged at 3500 rpm for 15 min, washed 3 times with PBS, pH 7.0, and suspended in PBS to prepare the stock culture of $1x10^{8}$ cfu mL^{-1}. This stock culture was added to a free-chlorine solution to achieve a final bacterial count of $1x10^{5}$ cfu mL^{-1} and mixed well to ensure bacterial exposure to the disinfectant. At 0, 15 and 60 min contact times, 10 mL samples were taken out, dechlorinated with 100 µL of 1 M sodium thiosulfate (Fisher Scientific, UK) (Ridgway and Olson 1982) and 100 µL aliquots from disinfectant quenched samples were plated on Standard Plate Count Agar APHA (Oxoid, UK) plates after making dilutions in PBS, whenever required. Plates were incubated for 48 h at 35 ± 2 °C for heterotrophic plate count (HPC). Each experiment was reproduced three times and the mean was calculated from the three individual experiments.

Temperature and pH were recorded with a Multi 7 Mettler-Toledo meter (Mettler-Toledo International Inc., Columbus, OH, USA) at each time point of exposure. Free chlorine and total chlorine concentrations were determined using the N,N-diethyl-p-phenylenediamine (DPD) colorimetric method (APHA 1999) with

HACH DPD reagent and pocket colorimetric analysis system (HACH, USA) at 0, 15, and 60 min contact times. Two controls of PBS with bacteria without disinfectant and PBS with disinfectant and without bacteria were used for each set of experiments.

4.4.6 Disinfectant Suspension Test for Monochloramine Resistance

Monochloramine suspension tests were performed similarly to those described for the chlorine suspension test, except that PBS pH 8.0 was used for the experiments (Howard and Inglis 2005; Chiao et al. 2014). The monochloramine solution (10 mg L^{-1}) was prepared by mixing 68.9 µL of 14.5% NaOCl (Alfa Aesar, UK) and 2 mL of 1.91% NH_4Cl solutions (Sigma-Aldrich, UK) in a volumetric flask and making up the volume to 1 L with PBS, pH 8.0 (Driedger et al. 2001; Chiao et al. 2014). Five solutions of monochloramine were prepared similarly having concentrations of 0.5, 1.0, 2.0, 4.0 and 8.0 mg L^{-1}. Monochloramine concentration was determined using the Indophenol method with MonochlorF reagent (HACH, USA, Method 10172) and HACH Pocket colorimeter analysis system (Lee et al. 2007). The remaining protocol was the same as that used for the chlorine suspension test.

4.4.7 Data Collection and Statistical Analysis

Chlorine and monochloramine disinfectant suspension tests were performed against six identified bacterial isolates and mean cfu mL^{-1} ± SD were calculated for each contact time and concentration. Cell counts were $\log_{10}$ transformed before plotting. Statistical analysis was performed using Minitab version 17. MIC data was

compared against zones of inhibition of hypochlorite assays using the non-parametric Spearman correlation test.

4.5 Results

4.5.1 Water Conditions

Minimum free chlorine and total chlorine concentrations were found to be 0.01 mg L^{-1} and 0.1 mg L^{-1}, respectively at the time of sample collections. Thirty-eight samples were collected from buildings having a cold-water storage tank, or cistern, within the building, while 14 samples were collected from the buildings with completely closed supply lines (Table 4.1). Water storage tanks are inspected once every six months and disinfected generally on an annual basis in these buildings. All reported that drinking-water quality values were within permissible concentrations at the time of sampling; however, disinfection conditions declined at point of use.

4.5.2 Bacterial Communities in Drinking Water

Approximately 80% of water samples tested positively for at least one bacterium (per 100 mL water). The frequency of positive detections was similar between building types; however, cistern-related samples had greater abundances of bacteria; averaging 3.4 colony forming units (CFU) from cistern-systems, versus 1.4 CFU in buildings without cisterns.

Bacteria identified in this study included members from the phyla of Alphaproteobacteria (*Blastomonas* and *Sphingomonas*), Betaproteobacteria (*Acidovorax*, *Burkholderia*, *Comamonas*, *Cupriavidus*, *Ralstonia*, and *Variovorax*), Gammaproteobacteria (*Enhydrobacter*, *Escherichia*, and *Pantoea*), Actinobacteria

(*Arthrobacter*, *Dermacoccus*, *Dietzia*, *Janibacter*, *Kocuria*, and *Micrococcus*), and Firmicutes (*Bacillus*, *Paenibacillus*, *Brevibacillus*, and *Staphylococcus*) (Appendix, Table 8.2).

There were differences in bacterial communities found in drinking-water systems when the water had been stored before use. Twenty different genera were found in water samples collected from buildings having cisterns and eight genera were found in samples from buildings with closed systems (Table 4.1). *Bacillus*, *Burkholderia*, *Kocuria*, *Micrococcus*, *Paenibacillus*, and *Staphylococcus* were present in both types of buildings at relatively similar proportions. Fourteen groups were found only in the drinking-water samples taken from the buildings with a storage tank or cistern: *Cupriavidus*, *Blastomonas*, *Acidovorax*, *Variovorax*, *Arthrobacter*, *Escherichia*, *Enhydrobacter*, *Pantoea*, *Comamonas*, *Sphingomonas*, *Dietzia*, and an unrecognised Epsilonproteobacteria (Table 4.1), while *Janibacter* and *Brevibacillus* were present only in those samples taken from buildings without a drinking-water storage tank.

4.5.3 Disinfection Susceptibility Test by Disk Diffusion Method

This test assayed bacteria to determine their susceptibilities to sodium hypochlorite, either as 14.5% standard sodium hypochlorite solution or 4.5% commercial bleach on the same agar plate. Bacteria showed a broad range of susceptibility patterns producing zones of inhibition between 7 mm and 65 mm in diameter against the two disinfectants. Results were arbitrarily classified to facilitate analysis (there are no known standard metrics to define ‘resistance’ to high concentration of chlorine), and 13 (8.8%) bacteria showed zones of inhibition $\leq$ 20

mm in diameter; 96 (64.9%) isolates showed zones of inhibition between 21-40 mm, while 18 (12.2%) isolates produced zones of inhibition of > 41 mm against 14.5% sodium hypochlorite (Table 4.2). In the case of 4.5% commercial bleach, 98 (66.2%) isolates showed zone of inhibition $\leq$ 20 mm, 29 (19.6%) isolates showed between 21-40 mm, while no isolate showed any zone of inhibition $\geq$ 41 mm (Table 4.2).

Comparing the means of size of zone of inhibition with the two disinfectants indicated that (as expected) the standard sodium hypochlorite was more effective against the isolated bacteria (Appendix, Table 8.3), but interestingly 10 (6.8%) cultures (4 *Bacillus* spp., 2 *Acidovorax* spp., 1 *Burkholderia* sp., 1 *Paenibacillus* sp. and 2 unidentified bacteria) were more sensitive to commercial bleach (Appendix, Table 8.2). This may be due to the presence of other antimicrobial agents, e.g., non-ionic and cationic surfactants, or pH, of the commercial bleach solution. Twenty-one isolates were not tested as they did not form a proper lawn on the agar plate as required for the agar diffusion method; at least three attempts to create a lawn were made for each bacterium.

There were no differences in zones of inhibition to chlorine among bacteria collected from each building type (Mann Whitney, W = 7100, p = 0.75). There is no treatment-related bias to chlorine resistance based on the presence or absence of a cistern.

4.5.4 Antibiotic Susceptibility Test for MICs

To confirm the presence of ARB in tap water, antibiotic susceptibility testing was performed against four antibiotics to determine their MIC profiles: tetracycline (TET), sulfamethoxazole (SMX), ciprofloxacin (CIP) and amoxicillin (AMX). These

antibiotics belong to different antimicrobial classes and involve different mechanisms for resistance as they inhibit protein synthesis, folic-acid cycle, DNA gyrase (involved in DNA replication) and synthesis of cell walls, respectively (Kohanski et al. 2010).

Among the 148 isolates, 115 (77.7%) showed resistance against at least one antibiotic (Table 4.3), based on maximum values of MICs for organisms described by CLSI (Clinical And Laboratory Standards Insitute 2011; Guo et al. 2013; Yuan et al. 2015). Amoxicillin resistance was most prevalent, found in 96 (64.9%) isolates which were grown in AMX concentrations $\geq$ 32 µg mL^{-1} (Table 4.3), while sulfamethoxazole resistance was also widely distributed (45.9%, n = 68). Twenty bacteria (13.5%) were resistant to tetracycline, and thirteen (8.8%) possessed resistance against ciprofloxacin.

The presence of resistance traits against two or more antibiotics indicates that these organisms could have multidrug resistances. Multi-drug resistant bacteria were found in the drinking-water samples; six (4.1%) bacteria were resistant to all four antibiotics tested (TET, SMX, CIP, and AMX). Ten (6.8%) bacteria showed resistance against three antibiotics: 7 to TET, SMX, and AMX and 3 to SMX, CIP, and AMX. Out of 148 bacteria, 44 (29.7%) showed double resistance; further details can be found in Table 4.3.

Among building types, there were no differences between MIC for TET and SMX (Mann Whitney test: p = 0.42 and p = 0.30, respectively). Bacteria from cistern-systems had higher MIC for AMX (Mann Whitney test, $p < 0.001$) with median value of 64 µg mL^{-1} in cisterns, versus 0.125 µg mL^{-1} in closed systems. Conversely, bacteria in closed systems had higher MIC for CIP than those from

cisterns (Mann Whitney, $p < 0.01$): 0.063 µg mL^{-1} versus 0.016 µg mL^{-1}, respectively.

Bacteria showed similar resistance patterns against antibiotics and disinfectants (Appendix, Table 8.2). Spearman correlation tests ($p = 0.05$) indicate an inverse relationship between zones of inhibition against 14.5% standard sodium hypochlorite and antibiotic MICs. This suggests that bacteria with chlorine tolerance also tended to have greater tolerance to antibiotics. Correlations were weak but significant; AMX ($r = -0.30$; $p < 0.01$), SMX ($r = -0.28$; $p < 0.01$), and TET ($r = -0.22$; $p = 0.014$) (Table 4.5). There were no patterns between ciprofloxacin-resistance and chlorine tolerance ($r = -0.002$; $p = 0.98$).

4.5.5 Disinfection Suspension Test for Chlorine

Six bacteria were selected for the disinfectant suspension test on the basis of the number of antibiotics to which they were resistant: *Arthrobacter* (TET, SMX, CIP, and AMX), *Bacillus* (SMX and AMX), *Cupriavidus* (TET, SMX, CIP, and AMX), *Burkholderia* (type M: TET, SMX, and AMX), *Burkholderia* (type S: AMX) and *Paenibacillus* (No resistance) (Table 4.4). *Burkholderia* were represented with 'M' (multiple resistant) and 'S' (single resistant) to differentiate the two strains.

The chlorine suspension test was performed to evaluate contact time (0, 15 and 60 min) and disinfectant concentrations (0-8 mg L^{-1}) on inactivation of the bacteria at pH 7.0 and 20°C (Appendix, Table 8.4, 8.5). *Burkholderia* sp. (M) showed greater resistance to chlorine than other bacteria at 15 and 60 min contact times (Figure 4.1, a-f). A decrease of 2-3 log-units of cfu mL^{-1} was observed at concentrations 0.5-2 mg L^{-1} of free chlorine as compared to the control for all time

durations (versus log cfu = 5). However, to reduce viable counts further, required longer exposures and higher concentrations (4-8 mg L^{-1} free chlorine), while complete inhibition did not occur at any concentration or contact time against *Burkholderia* (M) (Figure 4.1, d). *Bacillus* sp. had the second highest survival rates at concentrations of 4.0 and 8.0 mg L^{-1}; however, viabilities were greater for *Bacillus* sp. than *Burkholderia* sp. (M) at quick exposures (0 min) at lower concentrations of 0.5-2 mg L^{-1} (Figure 4.1, c-d). These bacteria were resistant to three (TET, SMX and AMX) and two (SMX and AMX) antibiotics, respectively and had small zones of inhibition, 15 and 7 mm respectively, against standard sodium hypochlorite (Table 4.4).

Cupriavidus sp. and *Arthrobacter* sp. had resistances against all antibiotics (TET, SMX, CIP, and AMX); both had initial resistance to immediate exposure (0 min) to chlorine at 0.5 and 1.0 mg L^{-1}, but were inhibited with increased concentrations and contact times (Figure 4.1, a-b). They produced zones of inhibition of 35 and 40 mm in the disk diffusion method (Table 4.4).

Paenibacillus sp. and *Burkholderia* (S) sp. showed a decrease of 3-4 log-units at small doses of 0.5 and 1.0 mg L^{-1} at immediate contact (0 min) (Figure 4.1, e-f). *Paenibacillus* sp. was susceptible to all antibiotics tested in this study, while the *Burkholderia* sp. (S) had resistance against AMX only (Table 4.4) and they produced large zones of inhibition, 54 and 65 mm respectively, in the disinfectant susceptibility testing.

The results show that the six bacteria demonstrated similar patterns of resistances and susceptibilities in the agar diffusion test and the suspension test for disinfectants. Those that produced small zones of inhibition had greater survival in

the suspension tests. Additionally, all four bacteria having double, triple and quadruple antibiotic-resistances survived better than the single antibiotic-resistant and susceptible bacteria when exposed to free chlorine.

4.5.6 Disinfection Suspension Test for Monochloramine

The monochloramine suspension test was performed at pH 8.0 and 20°C (Appendix, Table 8.6, 8.7). The inhibitory effect of monochloramine was not as immediate as for free-chlorine exposure; rates of decrease in survival count were less than one-order of magnitude (Figure 4.2, a-f), as compared to free-chlorine where declines of 2-3 orders of magnitudes were observed. Among the six bacteria, *Burkholderia* sp. (M) showed the highest survival rates and was the only test microorganism that showed resistance to all concentrations even after 60 min contact time with both chlorine and monochloramine (Figure 4.1, d and 4.2, d). *Bacillus* sp. was inactivated at 4.0 mg L^{-1} at 15 min contact time, but showed growth at 8.0 mg L^{-1} at the same contact time (Figure 4.2, c). *Bacillus* sp. showed greater survival than the quadruple antibiotic-resistant species *Cupriavidus* and *Arthrobacter* at higher doses of 2-8 mg L^{-1} at 15 and 60 min contact time, but it showed less survival at immediate contact (0 min) (Figure 4.2, a-c). *Paenibacillus* sp., which was antibiotic sensitive showed greater survival rates than antibiotic-resistant *Cupriavidus* sp. *Arthrobacter* sp. and *Bacillus* sp. at brief (0 min) and 15-min exposures (Figure 4.2, e). The resistance of *Paenibacillus* sp. against monochloramine might also be due to the presence of spores, which allowed it to tolerate the high concentration of disinfectant. For all bacteria, declines in the viability count (cfu mL^{-1}) by monochloramine were less than those for the chlorine exposure, irrespective of their

antibiotic-resistances (Figure 4.2, a-f). Inhibition did not occur at low doses, as compared to chlorine where inhibition occurred even at 0.5 mg L^{-1} of free chlorine after 60 minutes, indicating that free chlorine has more inhibitory activity for bacteria of DWDS than does monochloramine.

4.6. Discussion

Drinking-water samples have diverse genera; some could be potentially pathogenic. For example, species of *Burkholderia* (Falkinham 2015), *Kocuria* (Purty et al. 2013), *Paenibacillus* (Ouyang et al. 2008), and *Dermacoccus* (Takahashi et al. 2015) can impact immune-compromised patients and have been transmitted via drinking water (Hunter 1997; Godoy et al. 2003). Many of these bacteria demonstrate antimicrobial-resistance, e.g., members of *Burkholderia cepacia* complex (Desai et al. 1998; Coenye et al. 2001) and *Cupriavidus*' resistance to metal (Vandamme and Coenye 2004). Moreover, the presence of *Pantoea* sp. (Pindi et al. 2013) and *Sphingomonas* sp. (Koskinen et al. 2000) are undesirable.

Different factors contribute to the introduction of bacteria into water distribution systems. In this study, most bacteria were from buildings with storage tanks, or cisterns, for drinking water. The building's plumbing represents an ideal place for opportunistic bacteria (Wang et al. 2012) by providing a low organic carbon level, a high surface to volume ratio and periods of stagnation (Falkinham 2015; Falkinham et al. 2015). During periods of stagnation or increased water-age, residual chlorine levels decline and the efficacy of bacterial growth inhibition becomes reduced (EPA 2002). The bacterial community structure in a distribution system becomes influenced (Wang et al. 2014), including those bacteria with antimicrobial resistance (Falkinham 2015; Falkinham et al. 2015).

The response of ARBs to chlorine varies widely (Shi et al. 2013) and it becomes very difficult to ascertain specific mechanisms from these observations. Disinfection efficiency does not remain the same throughout the supply system and gradients of exposure concentrations develop. Responses range from lethality/complete inhibition at high concentrations, selective survivability of resistant populations at sub-inhibiting concentrations, to triggering biochemical stress responses at much lower (sub-inhibitory) concentrations.

Surviving bacteria may innately have increased resistance. Spore-forming bacteria tend to be more resistant and Gram-negative bacteria are less susceptible than Gram-positive bacteria (Russell 1998). This might be a reason that in this study, the *Bacillus* species, having spores and antibiotic-resistance against two antibiotics, showed more tolerance to chlorine, as compared to multiple-antibiotic resistant *Cupriavidus* and *Arthrobacter* which do not form spores. Also, increases in the abundance of antibiotic-resistant *Pseudomonas*, *Acidovorax* and *Pleamonas* and ARGs have been observed after chlorine treatment (Jia et al. 2015).

One mechanism by which sub-inhibitory levels increase the risk of selection of ARB, is by chemical stress (Huang et al. 2013). Chlorine has been shown to increase the abundance of antibiotic-resistance bacteria and genes in opportunistic bacteria (Shrivastava et al. 2004; Shi et al. 2013). This is often attributed to the enrichment of bacteria with plasmids and integrons, which are involved in the transfer and enrichment of resistance markers among bacteria (Shi et al. 2013), as part of their stress-response mechanism. While not tested here, it remains a possibility in our systems; further investigation is required.

Inactivation of antibiotic-resistant and -sensitive bacteria diminishes when they have been previously exposed to chlorine disinfectant. Bacterial strains with antibiotic resistance have shown to be more tolerant to chlorination (Templeton et al., 2009; (Huang et al. 2013). Bacteria show a biphasic mode of inactivation during chlorine disinfection for drinking-water production. A sharp decline of 2-4 $\log_{10}$ in viable cells is not unusual and occurs within 15 min of exposure of 0.1-3 mg L^{-1} of free chlorine, indicating that chlorine does not require a long exposure time for effectiveness (Lee and Nam 2002). A 100-fold decrease in viability of bacteria after 60-minute exposure to 1 mg L^{-1} free chlorine, with bacteria viability decreasing quickly between 10-20 min of exposure to 1 mg L^{-1} of chlorine concentration (Howard and Inglis 2003). These authors also found that *E. coli* and *Ps. aeruginosa* growth decreased more than other bacteria, e.g. *Burkholderia* sp., during an initial five minutes contact with 1 mg L^{-1} chlorine. In this study, the same phenomenon was observed, with most bacterial inactivation occurring in the initial 15 min.

In many water distribution systems, residual disinfectant is present, which could select for disinfectant-resistant cells by allowing these bacteria to grow and decreasing the growth of other disinfectant-sensitive competitors (Falkinham et al. 2015). Populations might have had previous exposure to chlorine, which increased their resistance to it. This might be a reason that in our study, some isolated bacteria showed resistance against concentrated standard sodium hypochlorite and produce smaller zones of inhibition (< 20 mm).

4.7 Conclusion

In this study, greater numbers of bacteria were found in post-cistern systems - areas where chlorine efficacy could be reduced. These bacteria are likely to have, or

develop, disinfectant resistance, which could also carry higher risks of possessing resistance to antibiotics. More detailed investigation is required to properly conclude chlorination efficacy as part of drinking-water treatment protocols, including other possible disinfection methods which could remove bacteria from these systems. Also, the mechanisms for co-selection must be determined. Overall, the results provide additional evidence as to why care should be taken to minimise the introduction of bacteria into drinking-water distribution systems as these bacteria may cause public health risk with increased exposure and greater chances of antibiotic resistance.

Table 4-1 Bacteria found in buildings with cistern or storage tank and without cistern or storage tank.

Building type	Total samples collected	Positive	Bacteria selected	Bacteria submitted for identification	Not identified, no sequence found, no similarity found	Bacteria identified	Bacteria Identified in samples
Cistern	38	31	128	84	12	72	*Cupriavidus*=14, *Blastomonas*=9, *Acidovorax*=8, *Ralstonia*=6, *Burkholderia*=4, *Dermacoccus*=4, *Variovorax*=4, *Bacillus*=3, *Staphylococcus*=3, *Arthrobacter*=2, *Escherichia*=2, *Enhydrobacter*=2, *Kocuria*=2, *Micrococcus*=2, *Paenibacillus*=2, *Pantoea*=1, Epsilonproteobacteria=1, *Comamonas*=1, *Sphingomonas*=1, *Dietzia*=1
No Cistern	14	11	20	16	1	15	*Paenibacillus*=4, *Bacillus*=4, *Micrococcus*=2, *Burkholderia*=1, *Brevibacillus*=1, *Janibacter*=1, *Kocuria*=1, *Staphylococcus*=1
Total	52	42	148	100	13	87	

Table 4-2 Disinfectant susceptibility of isolates (zone of inhibition in mm) by Disk Diffusion Method.

Disinfectant	**Size of Zone of inhibition**	**No. of Organisms (%)**	**Organisms**
Standard Sodium hypochlorite (14.5%)	≤ 20 mm	13 (8.8)	5 *Bacillus* species, 1 *Burkholderia* specie, 1 *Paenibacillus* specie, 2 *Acidovorax* specie, 4 uncharacterised bacteria
	21-40 mm	96 (64.9)	14 *Cupriavidus* species, 6 *Blastomonas* species, 4 *Acidovorax* species, 4 *Staphylococcus* species, 4 *Variovorax* species, 2 *Paenibacillus* species, 2 *Arthrobacter* species, 2 *Bacillus* species, 2 *Dermacoccus* species, 2 *Enhydrobacter* species, 2 *Kocuria* species, 2 *Micrococcus* species, 2 *Ralstonia* species, 1 *Brevibacillus* specie, 1 *Comamonas* specie, 1 Epsilonproteobacteria, 1 *Pantoea* specie, 1 *Sphingomonas* specie, 43 uncharacterised bacteria
	≥ 41 mm	18 (12.2)	2 *Micrococcus* species, 2 *Paenibacillus* species, 1 *Acidovorax* specie, 1 *Blastomonas* specie, 1 *Escherichia* specie, 1 *Ralstonia* specie, 1 *Dietzia* specie, 1 *Burkholderia* specie, 8 uncharacterised bacteria
Commercial bleach (4.5% sodium hypochlorite)	≤ 20 mm	98 (66.2)	13 *Cupriavidus* species, 6 *Blastomonas* species, 4 *Acidovorax* species, 3 *Staphylococcus* species, 4 *Bacillus* species, 4 *Variovorax* species, 4 *Paenibacillus* species, 3 *Dermacoccus* species, 2 *Arthrobacter* species,, 2 *Enhydrobacter* species, 2 *Ralstonia* species, 1 *Kocuria* species, 1 *Micrococcus* species, 1 *Burkholderia* specie, 1 *Comamonas* specie, 1 Epsilonproteobacteria, 1 *Pantoea* specie, 1 *Sphingomonas* specie, 44 uncharacterised bacteria
	21-40 mm	29 (19.6)	3 *Acidovorax* species, 3 *Bacillus* species, 2 *Micrococcus* species, 1 *Cupriavidus* species, 1 *Blastomonas* specie, 1 *Staphylococcus* specie, 1 *Paenibacillus* specie, 1 *Brevibacillus* specie, 1*Dietzia* specie, 1 *Kocuria* specie, 1 *Ralstonia* specie,1 *Burkholderia* specie, 12 uncharacterised bacteria
	≥ 41 mm	0	No organism
	Not tested	21 (14.2)	3 *Ralstonia* species, 3 *Burkholderia* species, 2 *Dermacoccus* species, 1 *Kocuria* specie, 1 *Blastomonas* specie, 1 *Acidovorax* specie, 1 *Janibacter* specie, 1 *Paenibacillus* specie, 1 *Escherichia* specie, 7 uncharacterised bacteria

Table 4-3 Single and multiple antibiotic-resistances of bacteria isolated from drinking-water distribution system.

Resistant traits	Combinations	No. of Organisms (%)	Isolates
Quadruple	TET, SMX, CIP, and AMX	6 (4.1)	1 *Cupriavidus* specie, 1 *Arthrobacter* specie, 1 Epsilonproteobacteria, 1 *Kocuria* specie, 2 uncharacterised bacteria
Triple	TET, SMX, and AMX	7 (4.7)	1 *Cupriavidus* specie, 4 *Burkholderia* species, 2 uncharacterised bacteria
	SMX, CIP, and AMX	3 (2.0)	1 *Micrococcus* specie, 1 *Acidovorax* specie, 1 *Dermacoccus* specie
Double	SMX and AMX	34 (23.0)	9 *Cupriavidus* species, 1 *Comamonas* specie, 16 uncharacterised bacteria, 1 *Blastomonas* specie, 2 *Bacillus* specie, 1 *Acidovorax* specie, 2 *Staphylococcus* specie, 1 *Sphingomonas* specie, 1 *Kocuria* specie
	TET and AMX	5 (3.4)	1 *Cupriavidus* specie, 1*Dietzia* specie, 3 uncharacterised bacterium
	SMX and CIP	4 (2.7)	1 *Micrococcus* specie, 1*Kocuria* specie, 1 *Bacillus* specie, 1 *Dermacoccus* specie
	TET and SMX	1 (0.7)	1 *Staphylococcus* specie
Single	TET	1 (0.7)	1 Uncharacterised bacteria
	SMX	13 (8.8)	2 *Enhydrobacter* species, 1*Bacillus* specie, 1 *Arthrobacter* specie, 4 Uncharacterised specie, 1 *Brevibacillus* specie, 1*Dermacoccus* specie, 1 *Staphylococcus* specie, 2 *Micrococcus* species
	AMX	41 (27.7)	6 *Acidovorax* species, 18 uncharacterised bacteria, 1 *Bacillus* specie, 4 *Variovorax* species, 2 *Paenibacillus* species, 2 *Cupriavidus* species, 1 *Dermacoccus* specie, 5 *Ralstonia* species, 1 *Escherichia* specie, 1 *Burkholderia* specie
No Resistant	No Resistance	33 (22.3)	2 *Bacillus* species, 15 uncharacterised species, 4 *Paenibacillus* species, 8 *Blastomonas* species, 1 *Escherichia* specie, 1 *Pantoea* specie, 1 *Ralstonia* specie, 1 *Janibacter* specie

Resistance organisms: Tetracycline (TET) = 16 μg mL^{-1}, Sulfamethoxazole (SMX) = 512 μg mL^{-1}, Ciprofloxacin (CIP) = 4 μg mL^{-1} and Amoxicillin (AMX) = 32 μg mL^{-1}.

Table 4-4 Antibiotic and disinfectant resistance of six test bacteria.

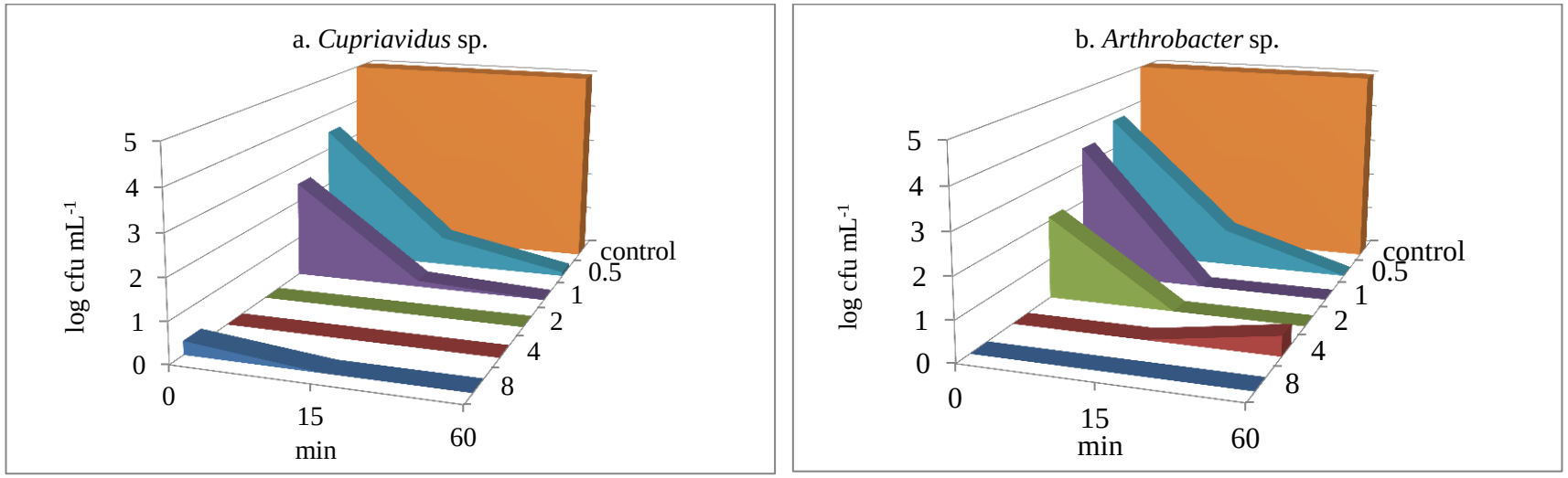
Code	Identification by 16S-rRNA	Antibiotic MICs (μg mL^{-1})				Resistant Traits for antibiotics	Size of zone of inhibition (mm ± SD) against NaOCl
		TET	SMX	CIP	AMX		
DW-515	*Cupriavidus sp.*	515	512	16	512	TET, SMX, CIP, and AMX	35 ± 2.8
DW-518	*Arthrobacter sp.*	512	512	512	512	TET, SMX,CIP, and AMX	40 ± 0.7
DW-527	*Bacillus sp.*	1	512	0.064	512	SMX and AMX	7 ± 0.0
DW-530	*Burkholderia sp.* (M)	64	512	0.064	512	TET, SMX, and AMX	15 ± 1.4
DW-641	*Paenibacillus sp.*	0.016	16	0.008	0.064	Susceptible	54 ± 2.1
DW-643	*Burkholderia sp.* (S)	8	8	0.032	512	AMX	65 ± 4.2

Resistant organisms: Tetracycline (TET) = 16 μg mL^{-1}, Sulfamethoxazole (SMX) = 512 μg mL^{-1}, Ciprofloxacin (CIP) = 4 μg mL^{-1} and Amoxicillin (AMX) = 32 μg mL^{-1}.

Table 4-5 Spearman correlation analysis for size of zone of inhibition by 14.5% standard NaOCl and minimum inhibitory concentrations (MIC) by four antibiotics (n=127). Significant level was $p<0.05$.

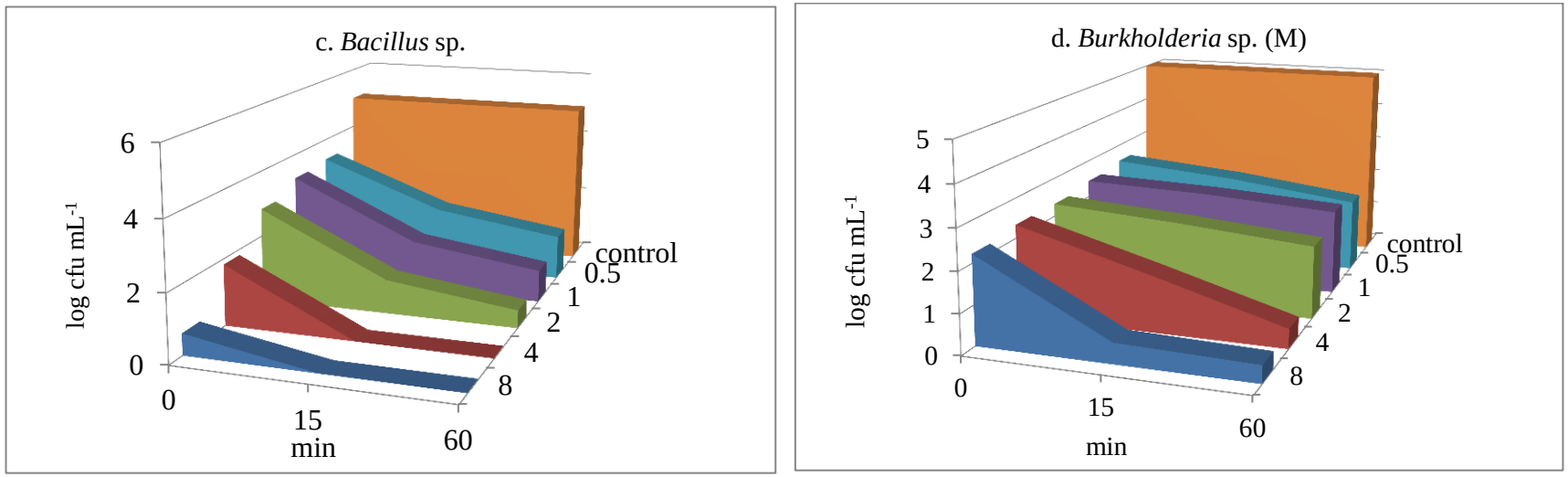
		TET	SMX	CIP	AMX
Standard NaOCl 14.5%	Spearman Correlation	-0.219	-0.278	-0.002	-0.303
	P value	0.014	0.002	0.981	0.001

Tetracycline (TET), Sulfamethoxazole (SMX), Ciprofloxacin (CIP), Amoxicillin (AMX).



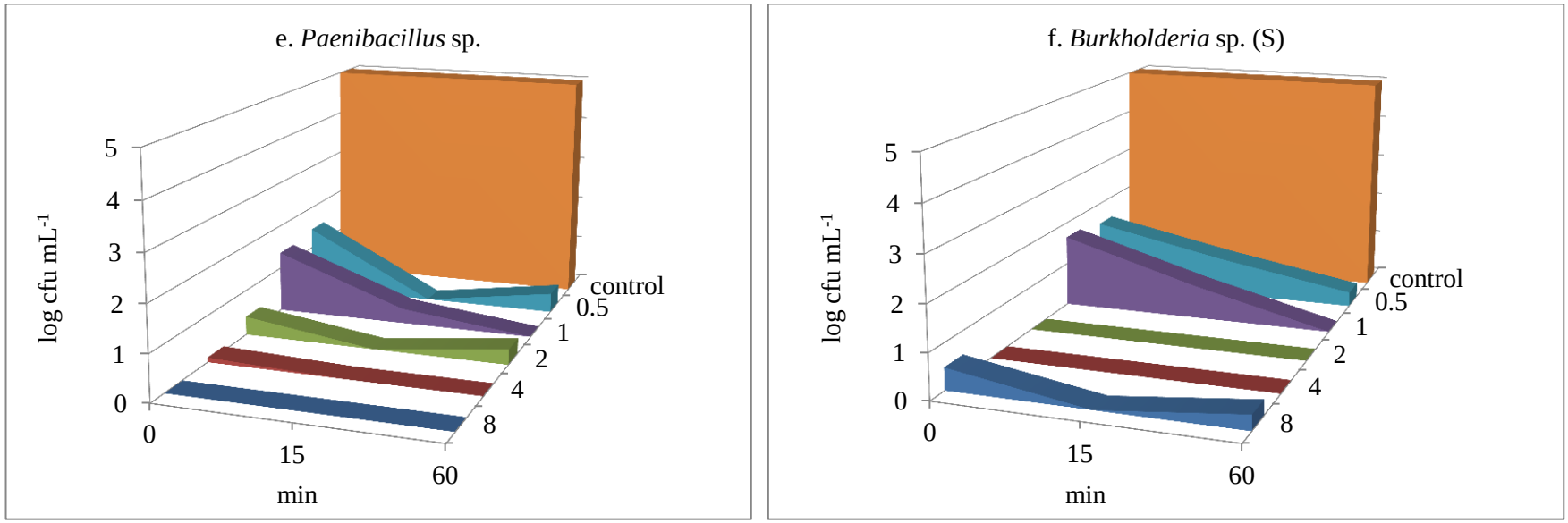
■ 0.5 mg L^{-1}, ■ 1.0 mg L^{-1}, ■ 2.0 mg L^{-1}, ■ 4.0 mg L^{-1}, ■ 8.0 mg L^{-1}, ■ C = control

Figure 4-1 (a-b) Effect of different concentrations of free chlorine on survival of bacteria (mean log cfu mL^{-1}) at different contact time (n=3).



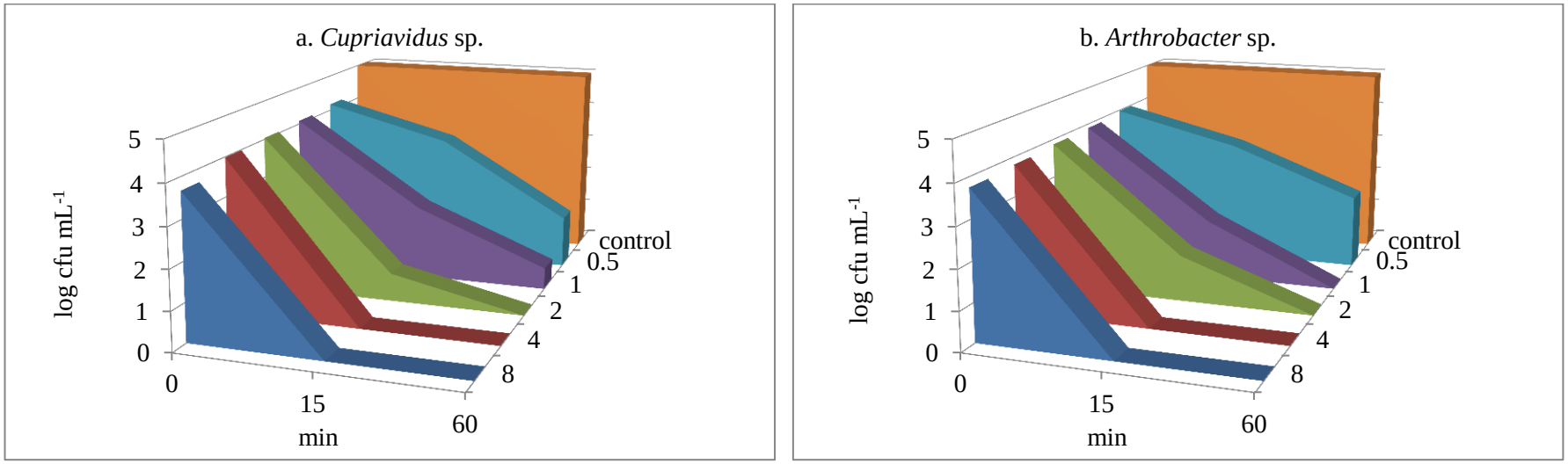
■ 0.5 mg L^{-1}, ■ 1.0 mg L^{-1}, ■ 2.0 mg L^{-1}, ■ 4.0 mg L^{-1}, ■ 8.0 mg L^{-1}, ■ C = control

Figure 4-1 (c-d) Effect of different concentrations of free chlorine on survival of bacteria (mean log cfu mL^{-1}) at different contact time (n=3).



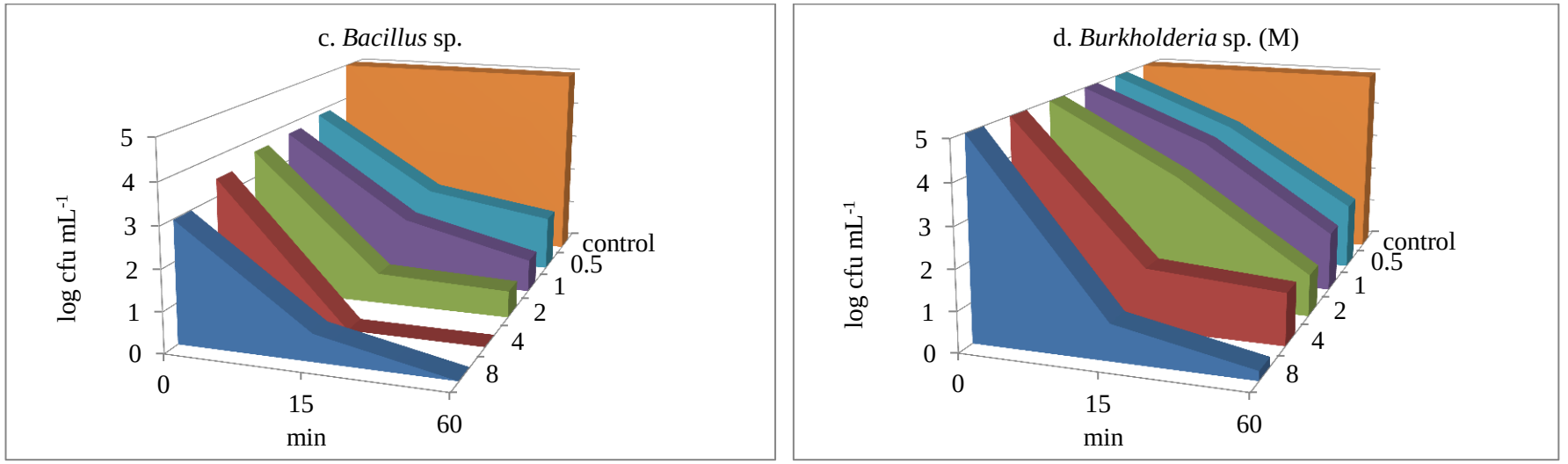
■ 0.5 mg L^{-1}, ■ 1.0 mg L^{-1}, ■ 2.0 mg L^{-1}, ■ 4.0 mg L^{-1}, ■ 8.0 mg L^{-1}, ■ C = control

Figure 4-1 (e-f) Effect of different concentrations of free chlorine on survival of bacteria (mean log cfu mL^{-1}) at different contact time (n=3).



■ 0.5 mg L^{-1}, ■ 1.0 mg L^{-1}, ■ 2.0 mg L^{-1}, ■ 4.0 mg L^{-1}, ■ 8.0 mg L^{-1}, ■ control

Figure 4-2 (a-b) Effect of different concentrations of monochloramine on survival of bacteria (mean log cfu mL^{-1}) at different contact time (n=3).



■ 0.5 mg L^{-1}, ■ 1.0 mg L^{-1}, ■ 2.0 mg L^{-1}, ■ 4.0 mg L^{-1}, ■ 8.0 mg L^{-1}, ■ control

Figure 4-2 (c-d) Effect of different concentrations of monochloramine on survival of bacteria (mean log cfu mL^{-1}) at different contact time (n=3).

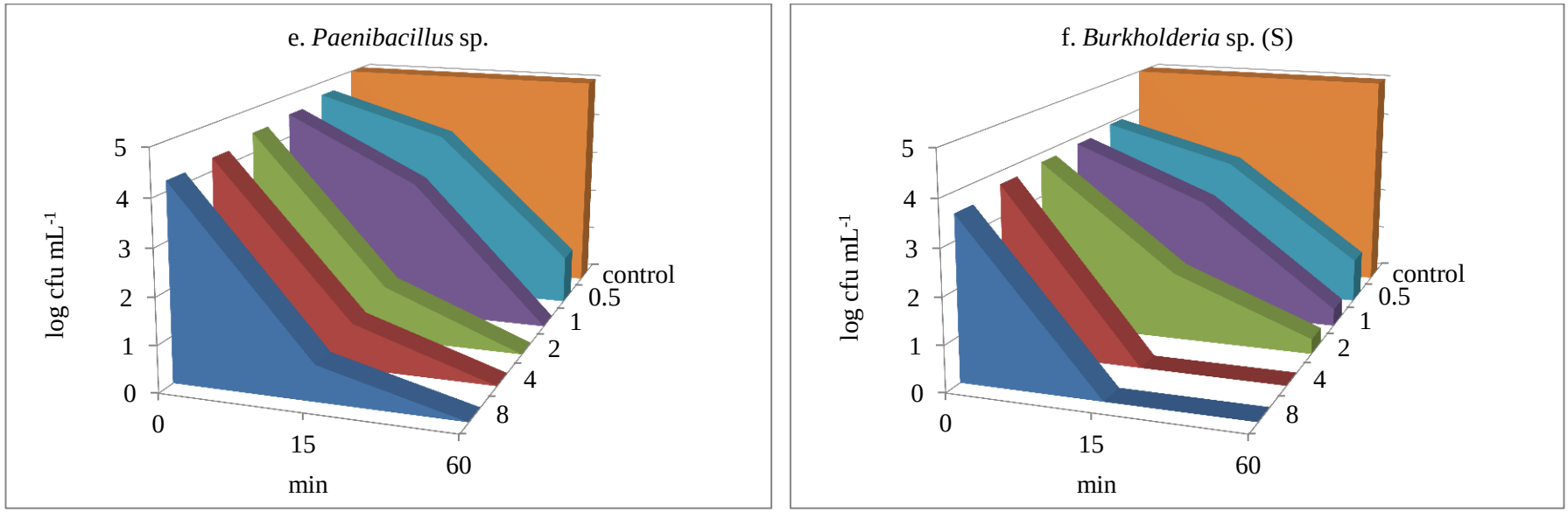


Figure 4-2 (e-f) Effect of different concentrations of monochloramine on survival of bacteria (mean log cfu mL^{-1}) at different contact time (n=3).

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Chapter 5

5.0 Minimum Selectable Concentrations (MSCs): An Approach for Determining the Selection of Antimicrobial Resistant Bacteria

5.1 Background

This paper has been submitted to the Journal *Ecotoxicology* in August 2016, and forms Chapter 5 of the thesis. This chapter investigates the minimum susceptibility concentrations (MSCs) of disinfectants for bacteria isolated from drinking water distribution systems. Eight different bacteria from four genera were used in the experiments. Free chlorine and monochloramine at different sub-lethal and lethal concentrations were applied and bacterial response in terms of growth rates was determined. This experiment was carried out to study the selection of a resistant bacterial population at very low concentrations; below the minimum inhibitory concentration of the disinfectants.

Sadia Khan designed and performed the experimental work, and wrote the paper. Sadia Khan and Charles W. Knapp did the data analysis, and Tara K. Beattie edited and reviewed the paper for publication with them.

5.2 Abstract

The use of antimicrobials is indispensable in many industries, especially drinking water production, to eradicate microorganisms. However, the growth of bacteria is not unusual in the presence of high concentrations of disinfectant, as these concentrations impose selective pressures on the bacterial population and can enrich resistant organism density. In addition, lower concentrations may also select for these bacteria due to the differences in bacterial growth rates at particular concentrations. This study was carried out to determine the minimal selective concentrations (MSCs) of free chlorine and monochloramine for the bacterial community residing in a water distribution system. Pairs of bacteria from the same genera were grown in the presence of sub-inhibitory to higher concentrations (0.01 – 10 mgL^{-1}) of disinfectants. The MSCs of free chlorine and monochloramine were found to range between 0.021 and 0.393 mg L^{-1}, which was 1/250 to 1/5.1 times lower than the MICs of susceptible bacteria (MIC_{susc}). This study indicates that sub-lethal concentrations of disinfectants in distribution systems could result in the selection of resistant bacterial populations in water distribution systems, which might help explain the failure of residual disinfectant within the water system and the occurrence of bacteria at point of use.

5.2.1 Key words

Minimal selective concentration (MSC), minimum inhibitory concentration (MIC), disinfectant, drinking water

5.3 Introduction

Overuse and misuse of antimicrobials during the last century have created issues related to the emergence and enrichment of resistant bacteria (Carlet et al. 2012), especially antibiotic resistant pathogens that could contaminate water supply systems and survive their disinfection (Khan et al. 2016b; Xu et al. 2016). Bacteria that have shown resistance to almost all antibiotics (Kummerer 2009b), have been found in the water, sanitation and agricultural industries (Kummerer 2009a; Li et al. 2016) because of selective pressures exerted by environmental concentrations of antimicrobials (Tello et al. 2012; Sandegren 2014). There are concerns that increased evolution and spread of antibiotic resistant pathogens will continue to occur due to these selective pressures (Baquero et al. 1998; Bengtsson-Palme and Larsson 2016) and that the driving forces behind the development and selection of resistance are not fully understood, due to the complexity of the interactions between bacteria, antimicrobials and environment.

Minimum Inhibitory Concentration (MIC) is used widely to understand the susceptibility and resistance of bacteria to antimicrobials, especially in the clinical setting; MIC represents population lethality. Resistant populations become selected at environmental concentrations higher than the MIC of susceptible bacteria (MIC_{susc}), while sub-MIC levels allow the continued growth of both susceptible and resistant genotypes (Andersson and Hughes 2014). High antimicrobial concentrations (>MIC) facilitate the population growth of resistant strains (Drlica 2003) and enrichment of the resistome (Kristiansson et al. 2011; Liu et al. 2012; Khan et al. 2013; Bengtsson-Palme et al. 2014a; Wang et al. 2015). However, concentrations below the MIC of disinfectants or their by-products could also favour highly resistant

bacteria (Li et al. 2016). As such, microbiologists have begun to define minimum selective concentrations (MSC), which represent the lowest concentration of antimicrobials that give the resistant strains a competitive advantage (Figure 5.1) (Andersson and Hughes 2014). This allows better understanding of the enrichment possibilities of resistant bacteria in those environments where low levels of antimicrobial are present, for example in soils and drinking water sources (Baquero et al. 1998; Baquero et al. 2008; Fram and Belitz 2011; Jiang et al. 2013; Khan et al. 2013).

Resistant strains may have low fitness cost at sub-lethal concentrations, which helps them to grow faster than a susceptible population (Gullberg et al. 2011), and this fitness cost, or competitive advantage, is the major factor which could affect the MSC (Sandegren 2014). The difference in fitness between susceptible and resistant organisms at sub-MIC values could result in much lower MSC values (than MIC) against some antimicrobials (Liu et al. 2011). Traditionally, it has been presumed that resistant bacteria have a competitive advantage at concentrations greater than the MIC (Sandegren 2014).

The resistant populations selected at sub-MIC could pose greater challenges to management than those selected at higher MIC (Andersson and Hughes 2012). They may increase complications in infection treatment (Andersson and Hughes 2010), and could become a public health concern (Capita et al. 2014). They do not lose their resistance traits in the absence of antimicrobials, are more stable and promote enrichment of resistance (Andersson and Hughes 2010, 2012). At low concentrations, not only could the risk of emergence of resistant populations in the environment increase (Knapp et al. 2008; Couce and Blazquez 2009), but the

problem of resistant bacteria could intensify by increased horizontal gene transfer (Couce and Blazquez 2009; Canton and Morosini 2011; Johnson et al. 2015), including induced transfer of plasmids and transposons (Barr et al. 1986; Doucet-Populaire et al. 1991) and enhanced recombination (Lopez et al. 2007; Lopez and Blazquez 2009). Increased rates of replication (Andersson and Hughes 2009, 2011) and mutation rates (Cortes et al. 2008; Morero et al. 2011; Thi et al. 2011; Gutierrez et al. 2013; Chow et al. 2015) have also been evidenced. Moreover, low concentrations contribute to signalling molecules for biofilm formation and gene expression (Andersson and Hughes 2014; Aka and Haji 2015; Ebrahimi et al. 2015). As such, sub-lethal concentrations could stimulate the spread of resistance in the environment and increase the likelihood of multi-resistant bacteria through genetic changes (Sandegren 2014).

There are multiple factors which can influence the MSC of disinfectants. In the presence of a complex microbial community, selective forces which can change the selectability of any population at sub-MIC levels include nutrient concentrations, pH and predation (Quinlan et al. 2011; Bengtsson-Palme et al. 2014b). A MSC model works best for planktonic bacteria growing in suspension form, rather than biofilm bacteria, as the presence of an extracellular matrix interferes with chemical concentrations in the biofilm (Canton and Morosini 2011). Furthermore, selection of resistance does not depend upon the initial number of resistant organisms in the system and any resistant organism could become enriched in a community (Gullberg et al. 2011).

While sub-lethal concentrations of antibiotic have been studied (e.g., Bengtsson-Palme and Larsson 2016), the impact of disinfectants and their residuals

have not been extensively investigated (Li et al. 2016). The purpose of this study is to examine the selection of resistant bacteria (Khan et al. 2016a) versus susceptible bacteria at specific sub-inhibitory concentrations of chlorine, either as free chlorine or monochloramine. Growth rates of susceptible and resistant bacteria were compared at different concentrations of disinfectants below the MICs of susceptible and resistant bacteria. The merit of using minimum selectability concentration (MSC) was examined as a novel toxicological approach to assess the emergence of antimicrobial resistant bacteria in the environment.

5.4 Materials and Methods

5.4.1 Bacterial Strains

Eight bacteria, belonging to four genera: *Bacillus*, *Paenibacillus*, *Acidovorax* and *Micrococcus*, which have previously been isolated (Khan et al. 2016b, Chapter 3), were chosen for this study. These bacteria were previously classified into three groups; resistant (R), intermediate (I) and susceptible (S), on the basis of size of zone of inhibition against the disinfectant 14.5% standard sodium hypochlorite by disk diffusion method (Khan et al. 2016a). Closely related bacteria were paired together and selected irrespective of their antibiotic resistance (Table 5.1).

Previously, bacteria were cryopreserved (Cryo vials TS/71-MX, Technical Service Consultants Ltd. UK) and stored at -80°C. For each experiment, a single bead was aseptically removed from the cryovials, grown in LB broth (Oxoid, UK) overnight and streaked on Nutrient Agar (Oxoid, UK) plates to give isolated colonies, which were used in the experiments. Bacterial strains have been identified by 16S-rRNA gene sequencing (Khan et al. 2016a), except *Bacillus subtilis* (R2),

which was acquired from a culture collection (National Collection of Type Cultures, UK; NCTC 10400).

5.4.2 Viable Cell Count by Turbidity (OD_{600}) Measurement (Standard Growth Curve)

To determine the relationship between OD and bacterial cell count, each bacterial isolate was grown overnight for maximum cell viability in 50 mL LB broth at 200 rpm on a shaker (Bench top Standard Analog, Orbital Shaker, VWR, UK) at 20°C. Next day, the culture was concentrated by centrifuging (Refrigerated Centrifuge, Eppendorf, UK) three times at 3500 rpm for 10 minutes and then suspended in 0.1% PBS in a total volume of 5 mL. This culture was used to make ten-fold serial dilutions from 1:10 to 1:10,000 and two-fold serial dilutions from 1:2 to 1:128. OD_{600} of each dilution was recorded with a UV-VIs spectrophotometer (Helios Zeta, Thermo Scientific, UK) by taking 4 mL from each dilution tube in a 1 cm wide cuvette. Sterile PBS (0.1%) was used as a blank. For the determination of number of bacteria (cfu mL^{-1}) at a specific OD, the dilution tubes were further diluted up to 1:10,000 in 10 mL PBS whenever required and 100 µL from the last dilution tube was transferred to Mueller Hinton Agar plates (Oxoid, UK) in duplicate, spread with a sterile spreader and incubated for 24 hours at 35 ± 2°C for the development of colonies. After 24 hours, colonies were counted on each plate and cfu mL^{-1} was calculated for each OD_{600} and dilution. *Ln*(OD_{600}) verses *ln*(cfu mL^{-1}) graph values were used for plotting and for the calculation of number of bacteria present at a specific OD in further experiments (Hall et al. 2014).

5.4.3 MIC for Chlorine

Experiments were performed in 50 mL screw capped glass vials in a total volume of 10 mL PBS, pH 7.0. Glass vials were pre-treated with 10% HNO_3 (prepared from 69%, AnalaR NORMAPUR, Prolabo VWR BDH) overnight, soaked in 1% NaOCl (Alfa Aesar, UK), rinsed with nano-pure water (18Ω) and sterilized before use. Bacterial strains were grown over night in LB broth with continuous shaking at 200 rpm at 20°C and washed three times with PBS pH 7.0 to remove organic material. The bacterial stock culture was suspended in the same buffer and diluted to a turbidity between 0.08-0.13 at OD_{600}, equivalent to a bacterial concentration of 1-1.5 x 10^8 cfu mL^{-1}. Chlorine solutions were prepared freshly at the time of each experiment, having concentrations of 0.001 mg L^{-1} to 10 mg L^{-1} from a standard stock solution of 14.5% sodium hypochlorite (Alfa Aesar, UK) in chlorine-demand free PBS. The bacterial stock culture was diluted, added at a concentration of 1x10^5 cfu mL^{-1} into vials, and the vials were incubated for 24 hours at 37°C. After incubation, 1 mL of the solution from each vial was spread with a sterile spreader onto Mueller Hinton Agar plates (Oxoid, UK) in duplicate and the plates were incubated for 24-48 hours at 37°C for the development of colonies. The lowest concentration of free chlorine without any sign of growth on representative plates after 48 hours was considered to be the MIC of free chlorine against that organism (Clinical and Laboratory Standards Institute 2012). The concentrations showing the appearance of colonies were considered to be non-inhibitory for the organism. The experiments were run in triplicate on three different days to determine the minimum inhibitory concentration (MIC) of disinfectant.

5.4.4 MIC for Monochloramine

For monochloramine experiments, PBS of pH 8.0 was used. Monochloramine solutions were prepared by mixing the appropriate volume of 1.9089% NH_4Cl (Sigma-Aldrich, UK) and 14.5% NaOCl (Alfa Aesar, UK) solutions. A series of monochloramine concentrations from 0.001 to 10 mg L^{-1} were prepared in PBS. The remaining protocol was the same as that used for chlorine (described above).

5.4.5 Selection of Medium for Growth Rate Experiment

For the determination of μ_{max} (ultimate population growth rate) and appropriate growing medium, experiments were carried out in different concentrations of LB broth, 0.1, 1.0, 5.0, 10, and 100%, and 10 mM PBS (pH: 7.0; representing 0% LB) in sealed serum vials. Hundred millilitre broths and PBS were inoculated with overnight grown cultures of *Bacillus* (R1 and R2) and *Paenibacillus* (R and S) species at a concentration of $1x10^6$ cfu mL^{-1} and allowed to grow with continuous shaking at 20°C. Optical densities (OD_{600}) were measured over 96 hours (6 hour intervals) with a UV-VIs spectrophotometer (Helios Zeta, Thermo Scientific, UK). Growth rate was calculated from the plots of $ln(OD_{600})$ verses time. The medium was selected on the basis of growth of bacteria and low chlorine demand. PBS had minimum chlorine demand but tested bacteria showed a negative growth rate so they were not used for further experiment. LB broth (0.1%) was selected as a medium for growth for further experiments of MSC of disinfectants, as it had low chlorine demand and bacteria grow well in the broth.

5.4.6 Preparation of Bacterial Inoculum for Growth Rate Experiments

A cryopreserved culture, previously stored at -80°C, was grown in LB broth overnight and streaked on Mueller Hinton Agar plates (Oxoid, UK) to verify culture purity. A single colony was transferred to 20 mL LB broth in a sealed glass bottle in an oxygen-limiting environment and grown overnight at 20°C to obtain a log phase culture with a high viability count. This culture was washed three times with chlorine demand free 0.1% LB and suspended in the same broth for growth rate experiments (Berney et al. 2006; Hall et al. 2014). The chlorine demand of the broth was calculated by the formula: chlorine demand = chlorine added concentration (mg L^{-1}) – chlorine residual concentration (mg L^{-1}) after 30 min contact time (HACH methods 10069 and 10223, DPD reagent, HACH, UK).

5.4.7 Growth Rate Experiments with Disinfectants for MSC

Experiments were performed in 0.1% LB broth in 100 mL sterile sealed serum vials to avoid the evaporation of chlorine during the experiments. Free chlorine solutions of 10 different concentrations from 0.01 to 10 mg L^{-1} were prepared in dilute LB. The overnight grown culture (as describe above) was diluted and added at a concentration of 1x 10^8 cfu mL^{-1} in the final volume of 100 mL and vials were sealed immediately and mixed well. The vials were incubated at 20°C with continuous shaking at 200 rpm for 24 h and OD_{600} were taken with a UV-VIs spectrophotometer (Helios Zeta, Thermo Scientific, UK) at 2 hour time intervals by removing 4 mL medium from each vial.

The growth rate constant (μ) was calculated for each bacterium from the previously determined growth curve (OD_{600} verses cfu mL^{-1}, Section 5.4.2) by converting the OD_{600} into cfu mL^{-1} and calculating the μ by the slope of the graph between *ln*(cfu mL^{-1}) versus time. The experiments were run in triplicate for each concentration and the mean growth rate constant was determined.

5.4.8 Data Analysis

Concentrations were log transformed before analysis. Statistical analysis was carried out using Minitab-17. Correlations were determined between concentrations of the two disinfectants and growth rates by Pearson's Correlation test (p = 0.05) (Table 5.2). Minimum selectable concentrations (MSCs) were determined from growth rates versus concentrations (log_{10} transformed) plots where the growth rate of resistant bacteria exceeded that of the susceptible population. Non-linear regression was performed using GraphPad Prism version 7.01 for Windows (GraphPad Software, La Jolla California USA) to calculate the MSC values from standard curves at 95% confidence interval.

5.5 Results

5.5.1 Zone of Inhibition by Selected Bacterial Strains

The eight bacteria (four different genera) were selected for this study. They were divided into three groups on the basis of size of zone of inhibition; Resistant (R) ≤ 20 mm, Intermediate (I) = 21 - 40 mm, and Susceptible (S) ≥ 41 mm, as described previously (Khan et al. 2016a). One member of each pair had a zone ≤ 20 mm, while the second member had a zone ≥ 41 mm, except for *Bacillus and*

Micrococcus spp.; both *Bacillus* produced < 20 mm zones and were differentiated by R1 and R2, while *Micrococcus* spp. produced 35 and 48 mm zones of inhibition and were differentiated by I and S, respectively. Other bacteria included *Paenibacillu*s spp. having 20 and 54 mm zones, and *Acidovorax* having 8 and 50 mm zones, respectively (Table 5.1).

5.5.2 MIC of the Bacterial Strains against Chlorine and Monochloramine

Bacteria were tested by the dilution method against a series of concentrations of free chlorine and monochloramine from 0.01 mg L^{-1} to 100 mg L^{-1} to determine the MICs of these disinfectants against the eight microorganisms. The MICs of free chlorine and monochloramine were in the ranges from 1-10.4 mg L^{-1}, and 2.1-10 mg L^{-1}, respectively (Table 5.1). *Bacillus* sp. (R1) showed the highest MICs for free chlorine and monochloramine, which were 10.4 ± 1.7 and 10.0 ± 3.8 mg L^{-1}, respectively. *Bacillus* sp. (R2) showed the lowest MIC 1.0 ± 0.6 mg L^{-1} for chlorine, while *Micrococcus* sp. (S) had the lowest MIC 2.1 ± 1.2 mg L^{-1} for monochloramine (Table 5.1).

5.5.3 Growth Rates of Bacteria in Different Media

The growth rates of *Bacillus* and *Paenibacillus* spp. were tested at six different concentrations of LB broth and were observed in the range of -0.076 to 0.462 h^{-1} in these media (Table 5.3). PBS (10 mM) showed the lowest growth rate and chlorine demand, but *Paenibacillus* sp. (S) did not grow well in PBS, so 0.1%

LB broth was selected for the further experiments; it had lowest chlorine demand, whilst supporting bacterial growth.

5.5.4 Minimum Selective Concentration (MSC) of Disinfectants

Bacteria, in their log phase of growth, were exposed to a series of concentrations (0.01-10 mg L^{-1}) of free chlorine and monochloramine and their growth rate constants (μ) were compared (Figure 5.2 and 5.3). Minimal selectable concentration (MSC) represented the sub-MIC concentration at which the more resistant organism's growth exceeded that of its competitor. Each bacterial pairing showed different behaviour with the different disinfectants—free chlorine and monochloramine. The *Micrococcus* assay showed the greatest difference between MIC (5.0 ± 1.7 mg L^{-1}) and MSC (0.046 mg L^{-1}), which was 110 fold lower than the MIC of the susceptible strain against chlorine (Table 5.4). While with monochloramine, *Acidovorax* assay MIC/MSC was more than that of any other bacteria. MSC was 0.021 mg L^{-1} which was $1/250^{th}$ the MIC value of the susceptible organism. Details of MSC and its ratio with MIC can be found in Table 5.4. The MSC for *Bacillus* against chlorine and monochloramine could not be calculated from the data, since the results suggested that the resistant strain had competitive advantage at much lower concentrations than used in this study, making determination difficult.

5.6 Discussion

Sub-lethal concentrations of antimicrobials can create conditions that selectively favour more resistant organisms (Chow et al. 2015). The enrichment of resistant bacteria can occur at concentrations many fold below the MIC_{susc} (Hughes

and Andersson 2012). In this study, the bacteria pairings had similar growth rates at very low chlorine and chloramine concentrations. Once chemical concentrations exceeded a particular threshold (the MSC), the growth rate of the more susceptible population declined when compared to the resistant population.

The relevance of the study suggests that we should also be concerned about the MSC with the MIC when examining antimicrobial resistance. Natural environments, which can be exposed to relatively low concentrations of antimicrobials, are also prone to the enrichment of resistance (Drlica 2003; Drlica and Zhao 2007), similarly to high concentration exposures (Myers 2008). This is also relevant along concentration gradients from high-exposure (e.g., over time for a degrading compound, or spatially when dispersed). For example, in drinking water treatment plants, a high concentration of residual disinfectant is applied to the system, but by the time the water reaches the point of use, the concentration may have reduced to sub-inhibitory levels because of the limited half-life of these disinfectants. This concentration gradient could increase the selection of resistant populations (Zhou et al. 2000) if bacterial contamination is allowed to enter the system. This could also become relevant to downstream areas where chlorinated water supplies discharge into the natural environment.

In this study, a series of concentrations of chlorine and monochloramine were used and enrichment of disinfectant resistant populations was observed in several cases (Figure 5.2 and 5.3), supporting the idea that certain low concentrations of chlorine and monochloramine could selectively enrich resistant bacteria. Similar results were obtained in a previous study where selection of multidrug resistant *Ps.*

aeruginosa was observed after treatment with a sub-optimal concentration of chlorine (Shrivastava et al. 2004).

Several mechanisms could be responsible for this behaviour of resistance development against chlorine based disinfectants at sub-MIC levels (Moen et al. 2012): increased surface hydrophobicity (Hostacka et al. 2003), changes in exopolymeric matrix (Dynes et al. 2009), detoxifying efflux genes (Mc Cay et al. 2010; Moen et al. 2012), differential expression of outer-membrane porin genes (Moen et al. 2012), morphological modifications, high enzyme activities (Gao and Liu 2014), transfer of conjugative plasmid carrying resistance traits (Johnson et al. 2015), and regeneration pathways (Drazic et al. 2015; Jozefczuk et al. 2010). A recent study showed that not only disinfectants, but also their by-products could enrich resistant bacteria at sub-lethal concentrations through chromosomal genetic mutation in water (Lv et al. 2014; Li et al. 2016). Environmental conditions could also have multiplicative effects in the enrichment process. For example, sub-inhibitory concentrations of benzalkonium chloride selects adaptive variants of *Ps. aeruginosa* in a magnesium limited medium, but not in organic-carbon rich conditions (Mc Cay et al. 2010).

Comparing bacterial growth rates is considered to be an important tool for understanding of microbial physiology (Hall et al. 2014). Bacterial growth rate data can be used in environmental studies for quantifying phenotypes (Warringer and Blomberg 2003) and their adaptation to environmental changes (Lindsey et al. 2013). In this study, growth rate data was applied to determine minimum selective concentrations of disinfectants which lead to increased resistance traits. It has been considered that the disinfectant resistance could enhance antibiotic resistance in the

environment and contribute to increased public health risk (Al-Jailawi et al. 2013; Capita et al. 2014; Seier-Petersen et al. 2014).

5.7 Conclusion

Seven drinking-water isolates and a single culture-collection strain were exposed to varying levels of chlorinated disinfectants. Results found that lower than expected concentrations (i.e., < MIC, a conventional metric for bacterial resistance) showed selective bias by providing resistance strains a competitive advantage in population growth. It is important to recognise sub-lethal effects of disinfectants on resistant strains because of their potential impact on drinking water contamination and human health. In the environment, sub-MIC levels of disinfectants are present as residuals which could select resistant bacteria and potentially facilitate the dissemination of resistant determinants among bacteria. There is a need for further investigation to understand the ecological responses of bacteria in the presence of sub-MIC level of disinfectants (and antibiotics) to overcome the problem of enriched antimicrobial-resistant (antibiotic resistant) populations that have become a concern on a global scale. Broadening ecotoxicological studies to strategically include selectivity metrics, e.g., MSC, would be an important step forward.

Table 5-1 Mean Minimum Inhibitory Concentrations of test organisms against free chlorine and monochloramine (n=3). R = resistant, I = intermediate, S = susceptible.

Organisms	**MIC (mg L^{-1} ± SD)^**		**Zone of inhibition (mm) against 14.5% standard NaOCl ***
	Free Chlorine	**Monochloramine**	
Bacillus sp. (R1)	10.4 ± 1.7	10.0 ± 3.8	8
Bacillus sp. (R2)	1.0 ± 0.6	5.0 ± 1.7	19^
Paenibacillus sp. (R)	10.0 ± 1.4	5.2 ± 1.6	20
Paenibacillus sp. (S)	5.2 ± 2.9	2.2 ± 1.1	54
Acidovorax sp. (R)	8.2 ± 2.0	8.2 ± 2.0	8
Acidovorax sp. (S)	2.0 ± 1.2	5.2 ± 1.6	50
Micrococcus sp. (I)	8.0 ± 3.1	4.8 ± 2.2	35
Micrococcus sp. (S)	5.0 ± 1.7	2.1 ± 1.2	48

* Unless otherwise stated, values were from Khan et al. 2016a.

^ Determined in this study.

Table 5-2 Correlation between growth rates and concentrations (*log* transformed) of free chlorine and monochloramine by Pearson correlation test ($\alpha = 0.05$).

Disinfectant	Organism	*R*-value	*P*-value
Chlorine	*Bacillus sp. R1*	-0.959	< 0.001
	Bacillus sp. R2	-0.893	0.001
	Paenibacillus sp. R	-0.977	< 0.001
	Paenibacillus sp. S	-0.954	< 0.001
	Acidovorax sp. R	-0.843	0.002
	Acidovorax sp. S	-0.760	0.011
	Micrococcus sp. I	-0.958	< 0.001
	Micrococcus sp. S	-0.976	< 0.001
Monochloramine	*Bacillus sp. R1*	-0.905	< 0.001
	Bacillus sp. R2	-0.941	< 0.001
	Paenibacillus sp. R	-0.962	< 0.001
	Paenibacillus sp. S	-0.961	< 0.001
	Acidovorax sp. R	-0.912	< 0.001
	Acidovorax sp. S	-0.943	< 0.001
	Micrococcus sp. I	-0.978	< 0.001
	Micrococcus sp. S	-0.926	< 0.001

Table 5-3 Growth rates of selected bacteria in different growth medium.

Organisms	Growth rates in Growth medium (h^{-1})					
	10 mM PBS	**0.1% LB**	**1% LB**	**5% LB**	**10%LB**	**100% LB**
Bacillus sp. (R1)	0.035 ± 0.01$^{\$}$	0.261^	0.224^	0.424^	0.181 ± 0.20*	0.304 ± 0.14*
Bacillus sp. (R2)	0.099 ± 0.10$^{\$}$	NT	NT	NT	0.462 ± 0.34$^{\$}$	0.237 ± 0.03$^{\$}$
Paenibacillus sp. (R)	0.028 ± 0.00$^{\$}$	0.197^	0.218^	0.283^	0.326 ± 0.16*	0.343 ± 0.28*
Paenibacillus sp. (S)	-0.076 ± 0.18$^{\$}$	NT	NT	NT	0.015 ± 0.21$^{\$}$	0.127 ± 0.13$^{\$}$

(NT = Not tested, *n=3, $^{\$}$n=2, ^n=1)

Table 5-4 Minimum selectable concentrations (MSC) of free chlorine and monochloramine for bacteria isolated from water distribution systems.

Organism	Free Chlorine			Monochloramine		
	MSC mg L^{-1}	**MIC_{susc} mg L^{-1}**	**MIC_{susc}/MSC**	**MSC mg L^{-1}**	**MIC_{susc} mg L^{-1}**	**MIC_{susc}/MSC**
Bacillus (R1)	NC	1.0	NC	NC	5.0	NC
Paenibacillus (R)	0.089	5.2	58.4	0.046	2.2	47.8
Acidovorax (R)	0.393	2.0	5.1	0.021	5.2	247.6
Micrococcus (I)	0.046	5.0	108.7	0.319	2.1	6.6

(NC = Not calculated)

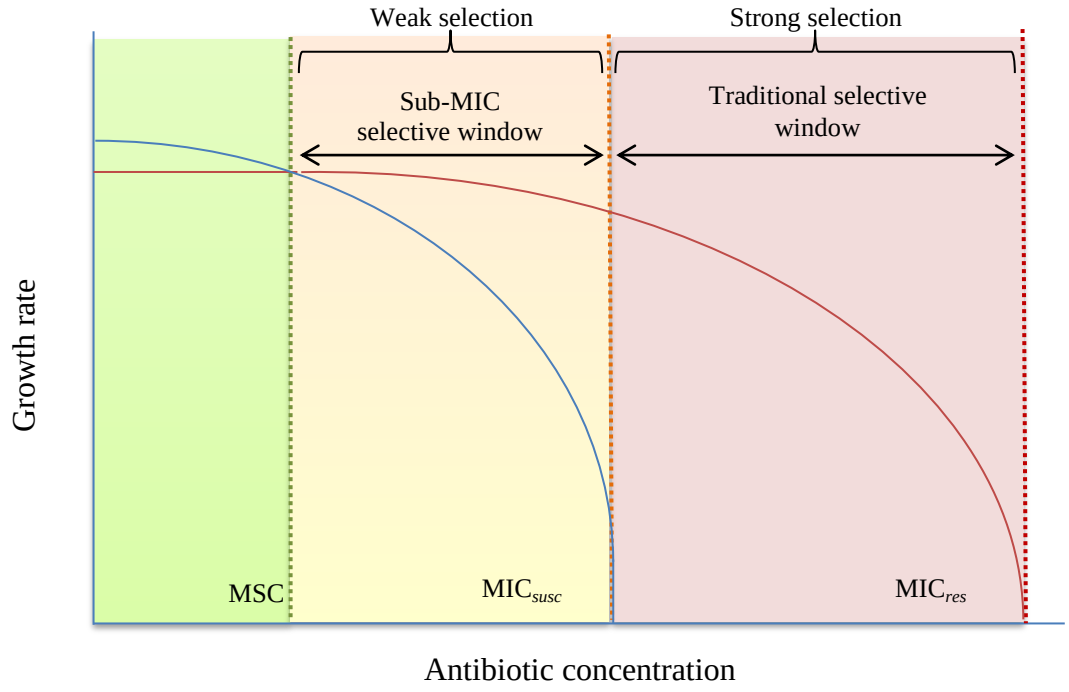

Figure 5-1 Schematic representation of growth rates as a function of antibiotic concentrations. MIC*susc* = Minimum inhibitory concentration for susceptible strain, MIC*res* = Minimum inhibitory concentration for resistant strain, MSC = Minimum selective concentration. Adapted from Gulberg et al. (2011) and Sandegren (2014).

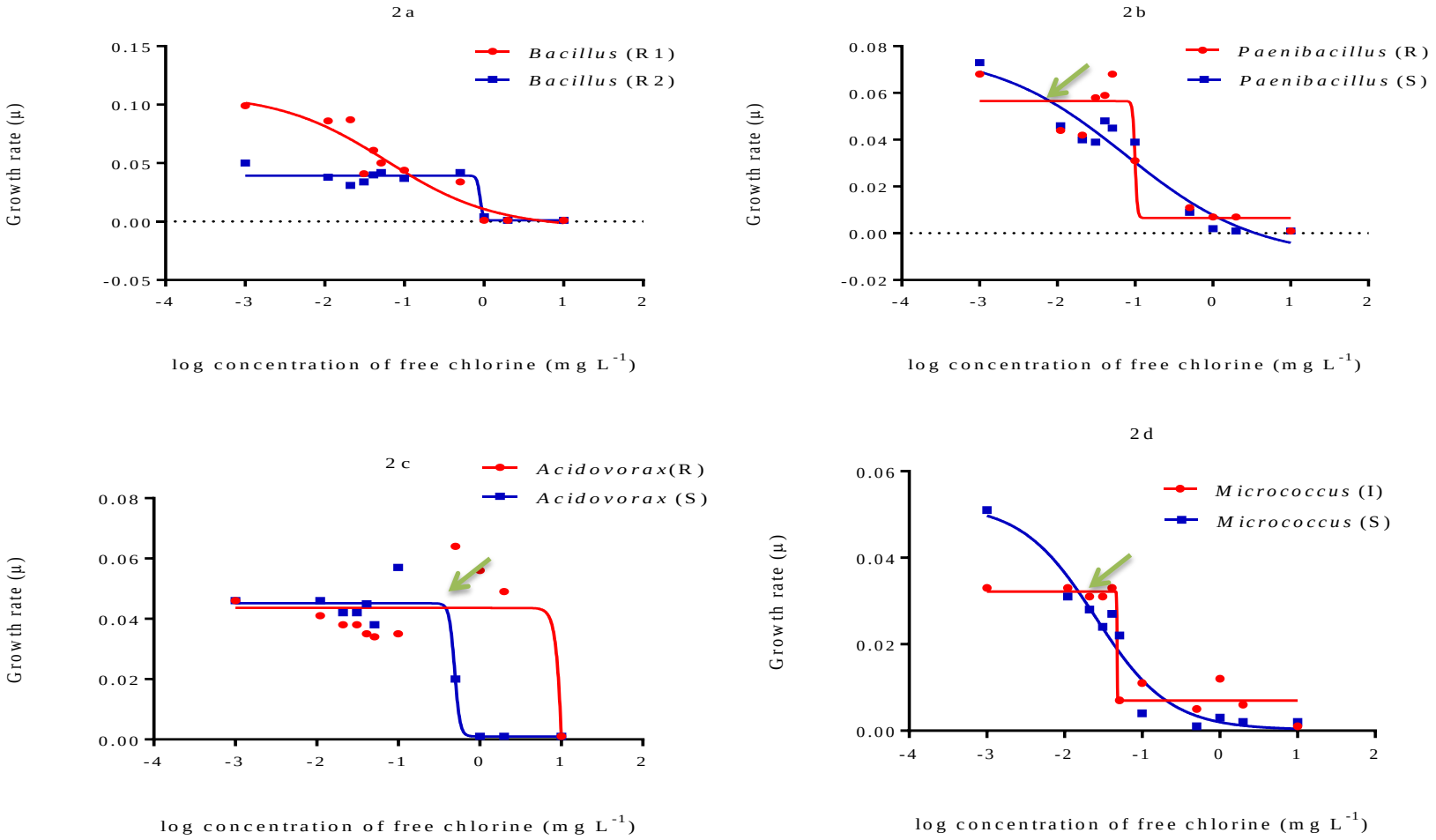

Figure 5-2 Minimum selectability concentrations (MSC) of *Bacillus*, *Paenibacillus*, *Acidovorax*, and *Micrococcus* species for free chlorine.

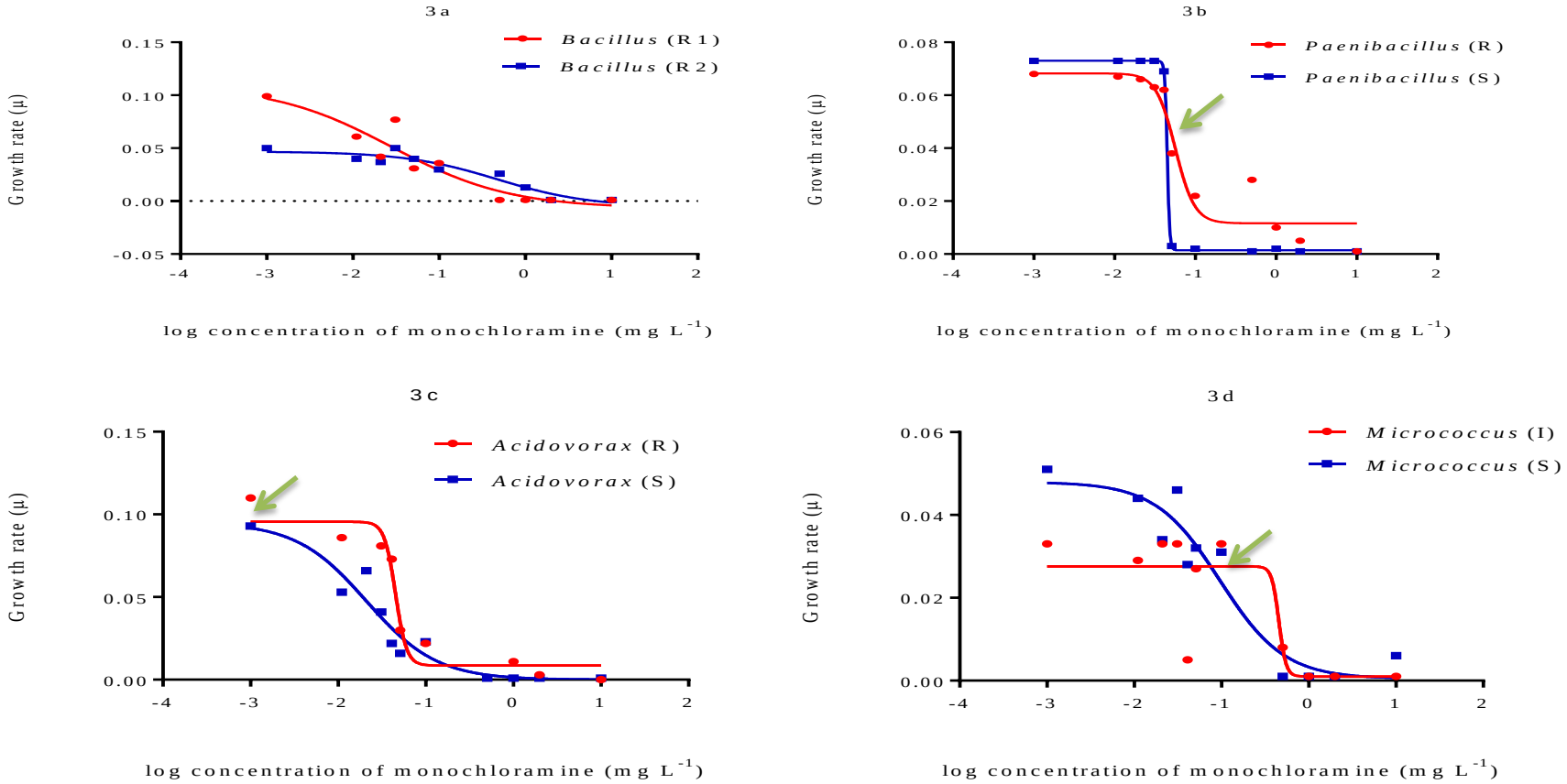

Figure 5-3 Minimum selectability concentrations (MSC) of *Bacillus*, *Paenibacillus*, *Acidovorax*, and *Micrococcus* species for monochloramine.

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Chapter 6

6.0 The Ecotoxicological Study in a Simulated Water Distribution System

6.1 Background

This manuscript will be submitted for publication in September 2016 and forms Chapter 6 of the thesis. This chapter continues from previous chapters to further confirm the hypothesis that ecotoxicological conditions enhance resistance in water distribution and plumbing systems by including water storage (used in Chapter 4), disinfectant concentration (Chapter 4), and their innate ability to survive and potentially be selected (Chapter 5). Here, elements of their interactions are combined into distribution-pipe microcosms, as a pseudo-realistic experimental examination of fate and effects. This chapter highlights that the different environmental conditions support the growth of resistant bacterial populations and thus could contribute to the dispersion of resistance through water distribution systems.

Sadia Khan designed and performed the experimental work for this study. She wrote the paper and did the data analysis with some guidance from Charles W. Knapp. All three authors jointly reviewed and edited the paper.

6.2 Abstract

To study the ecology of a water distribution system, it is necessary to understand its role in the fate of antibiotic-resistant bacteria and their genes and bacterial behaviour in plumbing systems. In this study, two microcosms each with different pipe materials including PVC, copper, cement and HDPE (used as base material for circulation lines and reservoir) were populated with disinfectant-resistant and susceptible natural bacterial populations (Chapter 4) in the absence and presence of 0.5 mg L^{-1} free chlorine.

It was hypothesised that pipe materials and chlorination differentially affect community composition and nature of bacterial presence (e.g., as planktonic or biofilm states) on different pipe surfaces. Results showed different enrichment of resistant bacteria. *X*-fold increase in resistant population was highest in cement pipes followed by copper and PVC. Growth potential in pipes were HDPE > PVC > copper > cement in suspension with PVC > cement > copper for biofilm development. In the mixed community assay, abundances of *Micrococcus* species became greater than *Paenibacillus* "R" and *Bacillus* "R1" in biofilm and water. This study indicates that antibiotic resistant bacteria are enriched differently in plumbing systems and the presence of residual chlorine fails to inhibit the proliferation of these bacteria.

6.2.1 Key words

Drinking water distribution system, resistance genes, pipe, chlorine, molecular, biofilm, antibiotic resistance genes (ARG)

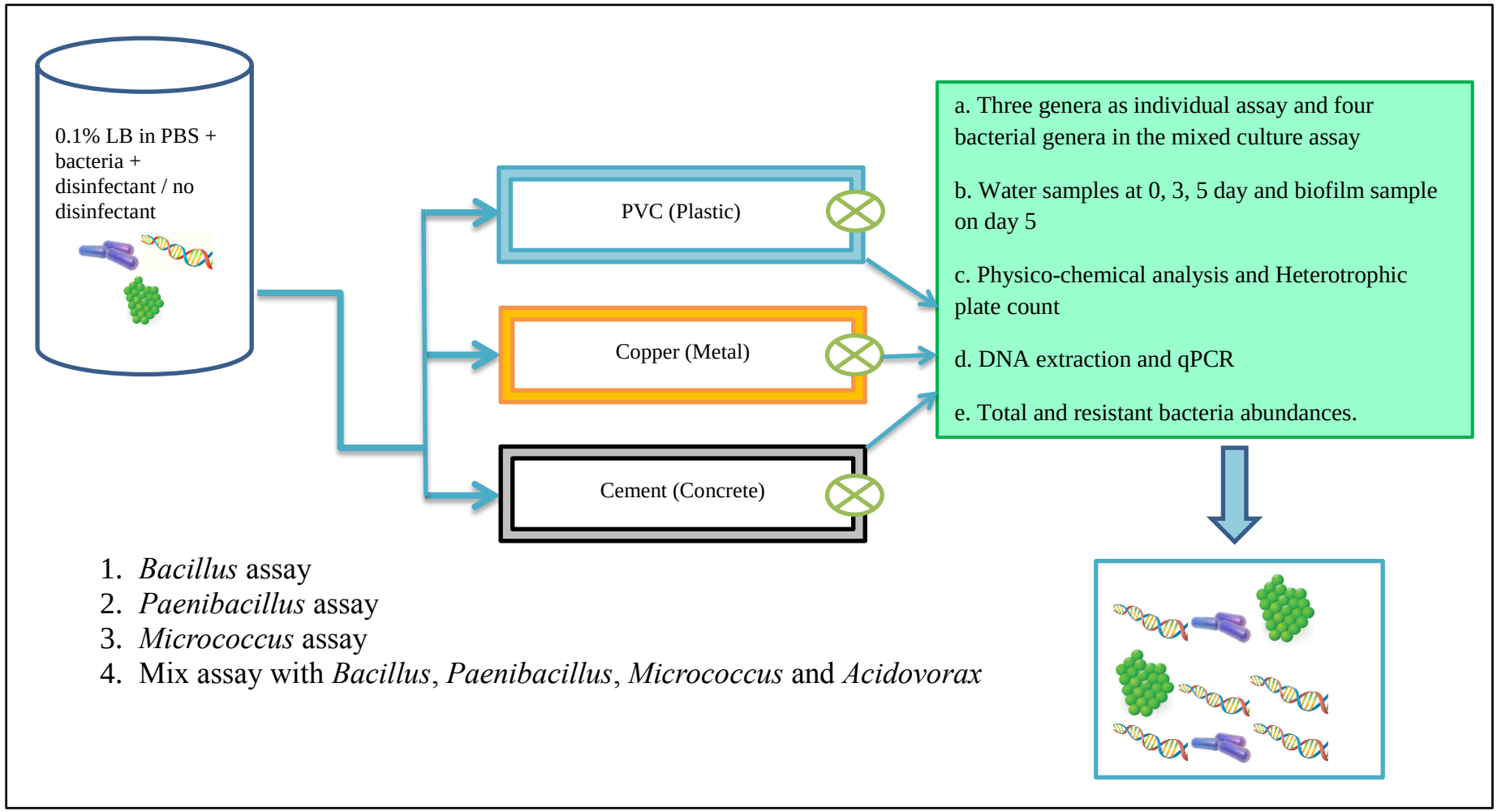

Figure 6-1 Graphical representation of simulated water distribution system studied (graphical abstract).

6.3 Introduction

Disinfectants are used to kill microorganisms in water treatment plants and distribution systems to maintain the potability of water (Dequeiroz and Day 2007; Morente et al. 2013), but they are unable to address the issue of pharmaceuticals; especially antibiotics and their resulting resistance genes (Jelic´ et al. 2012; Mathew and Unnikrishnan 2012; Bergeron et al. 2015; Xu et al. 2016). Disinfectants like UV and ozonation, which damage the DNA of bacterial cells and chlorination cannot fully eradicate antibiotic resistant bacteria (ARB) (Rizzo et al. 2013) at normal doses during the treatment process (MacLeod and Savin 2014; Yuan et al. 2015). Rather, much higher dosages are required: 400-12500 mJ cm^{-2} for UV, and > 100 mg L^{-1} for chlorine and ozone, respectively (McKinney and Pruden 2012; Zhuang et al. 2015). Unfortunately, water treatment plants do not use these high concentrations as they produce odour and taste problems and are difficult to achieve in water. Generally, the presence of disinfectants helps in the removal of bacteria from the system. Nonetheless, they can cause the proliferation of bacteria, e.g., the number of *Aeromonas* species increased after exposure to QAC-based disinfectants (McBain et al. 2004). Other disinfectants such as chlorine and monochloramine could enrich ARB in drinking water and distribution systems (Xi et al. 2009; Shi et al. 2013).

Not only disinfectants, but conditions in the water could also contribute to the emergence, enrichment and transfer of resistance traits among bacteria (Langsrud and Sundheim 1998; Gao and Liu 2014). For example, conditions can impact the microorganisms' growth in suspension and biofilm (Boe-Hansen et al. 2002) and contribute to biofilm formation, composition, and its extent in the pipes (Douterelo et al. 2016). Moreover, nutrient limitation and the innate reduced growth rate of biofilm

bacteria could also facilitate resistance against biocides (Boe-Hansen et al. 2002; Boe-Hansen et al. 2003; Ito et al. 2009).

Biofilms provide physical support for the attachment of bacteria in the distribution system and often occur during periods of nutrient limitation. Bacterial physiology becomes different throughout the depth of a biofilm and reflects the gradient of nutrients—there are more nutrients on surface of biofilm and fewer with depth ultimately influencing growth and metabolic rates of bacteria. Further, the physical biofilm structure, especially cell density, facilitates horizontal gene transfer (Molin and Tolker-Nielsen 2003; Merod and Wuertz 2014), which helps to propagate resistance traits and improve the survival of bacterial members.

Besides nutrient availability (Park and Hu 2010), other factors contribute to biofilm formation in distribution systems, including: operational conditions (Douterelo et al. 2013), source water (Zeng et al. 2013), excretion of extracellular polymeric substances by bacteria (Fish et al. 2015) and physicochemical properties of their environment (e.g., disinfectants and pipe material) (Roeder et al. 2010; Allion et al. 2011; Xue and Seo 2013; Wang et al. 2014). Operational parameters such as hydraulic regime, or flow rate of the water supply system, influence the biofilm formation, shaping physical structure and microbial communities (Douterelo et al. 2013; Holinger et al. 2014; Douterelo et al. 2016).

Bacteria as biofilms tend to have increased survival in disinfectant presence (Brown and Gilbert 1993; Lund and Ormerod 1995) and better tolerance of metal salts (especially those bacteria found along the inner surfaces of biofilms and exposed to metal pipe materials); biofilms absorb metal ions and work as selective barriers against toxic chemicals (Grumbein et al. 2014). As such, simultaneous

selection of metal-tolerant and antimicrobial-resistant bacteria could occur in distribution systems (Calomiris et al. 1984; Gullberg et al. 2014). Additionally, the sloughing of resistant bacteria from the biofilm causes a continual flux of bacteria in the distribution system, and results in increased variations in types and incidences of resistant organisms from site to site (Armstrong et al. 1981; Armstrong et al. 1982).

In water distribution systems and water fittings, a variety of metal pipes are installed, e.g., galvanized iron (GI), lead, copper, cast iron and steel (Sarin et al. 2004; Xie and Giammar 2011; Nguyen et al. 2012; Yang et al. 2014; Zhu et al. 2014). Distribution systems also have plastic pipes, for example chlorinated polyvinyl chloride (CPVC), polypropylene (PP), polyethylene (PE), polyvinylchloride (PVC) (Lasheen et al. 2008; Kelley et al. 2014; Liu et al. 2014); and other materials, e.g. cement pipes, such as asbestos and concrete (Niquette et al. 2000a; Wang et al. 2011) and rubber materials (Colbourne et al. 1984). Different studies indicate that these pipe materials could have different potentials for biofilm formation (Colbourne et al. 1984; Rogers et al. 1994; Lehtola et al. 2004a; Douterelo et al. 2014). They could also serve as venues for biocide-, metal- and antibiotic-resistance development (Pal et al. 2014). Moreover, bacterial exposure to multiple stressors, such as metals and disinfectants (SCENIHR 2009, 2010), could enhance the spread of antibiotic resistance.

The biofilms along the surfaces of pipes have greater variety in terms of bacterial species than finished water because biofilm provides suitable environment for the survival of different microorganisms (Chu et al. 2003) and contribute to the retention of pathogens in treated water systems (Colbourne et al. 1984; Rogers et al. 1994). The presence of multiple bacterial populations in biofilms ecologically assists

in the exchange and metabolism of substrates (Wolfaardt et al. 1994) and gene transfer (Blaustein et al. 2016) and, as such, depending upon the presence of certain bacterial species in water, could also serve as a sink of resistant genes. For example, *E. coli* significantly contributes to the gene-transfer process (Bailey et al. 2010), because of its ability to transfer genes from donor to recipient cells through conjugation. While the presence of slime-forming bacteria and heterotrophic aerobic bacteria facilitate metal tolerance and decomposition of asbestos cement pipes in water distribution system (Wang and Cullimore 2010). These bacteria produce fatty acids which cause the leaching and corrosion of metals and asbestos cement.

Microcosm experimental systems help understanding of natural and engineered ecosystems, as they represent an analogue to the actual large system. In this study, microcosms were used to examine environmental factors that may influence population selection in the municipal water supply system. It was hypothesized that conditions within distribution systems, e.g., the presence of different types of pipes, water age or stagnation and disinfectant residuals shape the microbial community and help to promote the dissemination of antimicrobial-resistant bacteria. Further, the system examined the synergistic effect of disinfectant and pipe material in the enrichment of resistant populations and the formation of biofilms in the distribution system.

6.4 Materials and Methods

6.4.1 Bacterial Strains

Eight bacterial species included in this study were the members of genera *Bacillus*, *Acidovorax*, *Paenibacillus* and *Micrococcus*—two populations from each

genus. All were previously isolated and selected from drinking-water distribution systems and identified by molecular techniques (Khan et al. 2016a; Chapter 4), except *Bacillus* specie (R2), which was purchased from NCTC (*Bacillus subtilis* NCTC 10400). Some of these bacteria have resistance and transferable genes, which further aid in their identification in a mixed community (Table 6.1).

6.4.2 Microcosm Construction

Two simulated water-distribution systems were prepared (Figure 6.1—graphical abstract), consisting of pipes connected to a water reservoir (HDPE hub) directly at one end (Figure 6.1) that fed the water into the system (total internal volume of each pipe was 102.09 cm^3 and surface area was 240 cm^2) and four channel peristaltic pumps (MP model M312, Miniplus 3, Gilson, Inc., France) were used for continuous flow of water (flow rate = 2 mL min^{-1}) in the pipes. Test-treatment piping materials were attached to this pumping mechanism and tested in parallel as individual systems. Pipe materials tested consisted of PVC, copper and cement. All pipes had equal length (45 cm) and diameter (1.7 cm).

The water systems were twice disinfected by applying 500 mg L^{-1} of sodium hypochlorite (Wang et al. 2012) for 2 hours over two consecutive days, while pipes and tubing were disinfected overnight to reduce the chlorine demand of the system and avoid microbial contamination.

6.4.3 Experimental Set-up

Bacteria from the tap water were isolated and stored as mentioned previously (Khan et al. 2016b; Chapter 3). Each population was grown separately in 50-mL LB broth at room temperature (20ºC) in a shaker at 200 rpm (Bench top Standard

Analog, Orbital Shaker, VWR, UK), centrifuged at 3000 rpm for 10 min (Eppendorf, UK), washed three times with 10 mM PBS (phosphate buffered saline, pH 7.0) and re-suspended in the same buffer. A diluted suspension of bacterial cells was added at a concentration of 1 x 10^5 cfu mL^{-1} and a single dose of free chlorine at a concentration of 0.5 mg L^{-1} was used in the water reservoir. Free-chlorine and bacteria-containing water from the same reservoir was circulated in PVC, copper and cement pipes for uniformity of the ecotoxicological conditions in the pipes. The control system had the same setup as the experimental reservoir but contained bacteria without the disinfectant.

Experimental runs were created to challenge populations within the same genus, but with different disinfectant susceptibilities, in each chlorine treatment. It was hypothesised whether conditions within the microcosms would selectively favour one type of population over the other. Additionally, a mixed community, comprising both species from *Bacillus*, *Paenibacillus*, *Micrococcus* and *Acidovorax,* were run to further examine community dynamics.

The retention time of water in the distribution system was five days and 1-mL sample was taken from each pipe on days 0, 3 and 5 for heterotrophic plate count. Biofilm samples were taken on day 5 with the help of sterile cotton swabs from all the pipes at a specific sampling place that was exposed to circulating water from an area of 4 cm^2 of each pipe and stored at -20°C for molecular analysis. 50 mL water sample from each pipe was filtered through a 0.22 µm pore-size cellulose nitrate gridded membrane filter on day 0 and 5 (Millipore, UK) and the filter paper was stored at -20°C for further analysis.

6.4.4 Sample Collection and Water-Quality Analyses

Bulk water samples were collected on days 0, 3 and 5 to analyse for temperature, pH, dissolved oxygen (DO), total organic carbon (Blaustein et al.) and free chlorine. Temperature and DO were determined by pocket DO200 dissolved oxygen meter (VWR, International); pH was monitored by High Performance pH/mV/°C meter (Model S40 SevenMulti™, Mettler Toledo™); TOC concentration was measured by using Apollo 9000 Combustion TOC Analyser (Teledyne Tekmar, Ohio, USA); and free chlorine was determined by the N, N-diethyl-p-phenylenediamine (DPD) colorimetric method (Hach DPD reagent and colorimetric analysis system, Hach, USA).

6.4.5 DNA Extraction and Primers

DNA was extracted from cotton swabs and filters samples by an ISOLATE II Genomic DNA kit (Bioline, UK) as per manufacturer's instructions and eluted DNA was stored at -80°C for qPCR analysis. Four sets of forward and reverse primers *Bacillus*-F and *Bacillus*-R, PAEN-843-F and PAEN-1484-R, WFB1 and WFB2 and ML-recA-F and ML-recA-R (Sigma-Aldrich, Life Science, UK) were used for the quantification of *Bacillus*, *Paenibacillus*, *Acidovorax* and *Micrococcus* species, respectively, in these samples (Table 6.2). Details of all primers including those used for the determination of the abundance of antibiotic resistance genes (ARGs; *sul*1, *sul*2) and the mobile genetic element (MGE; *intl*1), which were used to identify individual types of bacteria within each genera and 16S-rRNA genes are provided in Table 6.2.

6.4.6 PCR Amplification and Quantification

Real-time PCR assays were performed in duplicate for the quantification of each set of organisms (*Bacillus*, *Paenibacillus*, *Acidovorax*, *Micrococcus* spp.), 16S-rRNA gene concentration (representing a surrogate measure of 'total bacteria'), ARGs (*sul*1, *sul*2), and *intI*1 by using Sso Fast^{TM} EvaGreen Supermix (Bio-Rad, UK) in a Bio-Rad iCycler/iQ6 instrument system (Bio-Rad, UK). qPCR reactions were performed in a total volume of 20 µL and consisted of 10 µL of Supermix, 1-µL specific primer (500 nM), 6-µL nuclease-free water and 3 µL of sample DNA. The PCR cycle began with initial denaturation at 95°C for 90 sec, followed by 40 cycles of denaturation at 95°C for 10 sec, then annealing for 30 sec at primer specific temperature, and a final elongation at 55ºC for 6 sec. Details of annealing temperatures for all primers sets are given in Table 6.2. Serially diluted standards of known concentration of DNA and a control without any DNA were run in duplicate with each primer in all qPCR reactions. Standards were used ranging from $1x10^8$ to $1x10^3$ genes uL^{-1}. For the determination of relative abundances of resistant populations and bacterial genera to total bacteria (16S-rRNA) in each exposure experiment, the 16S-rRNA gene abundances were also calculated.

6.4.7. Data Analyses

All data values were log transformed before analysis. All absolute gene abundances were normalised to 16S-rRNA abundances in order to calculate the relative abundances (as per "total bacteria"). Bacterial abundances over the total surface area of pipes and total volume of water were calculated and percentages of bacteria in the biofilms were determined with respect to total bacterial count in each

pipe system. Fold increases in total bacterial abundances and resistant species abundances were also calculated from bacterial abundances on day 0 and day 5. Statistical analysis was carried out by t-test. Data was analysed by Excel 2010 (Microsoft Office 2010, Microsoft Corporation, USA). The Pearson correlation was calculated to determine the relationship between physicochemical properties and different resistant population abundances by using MiniTab version 17.

6.5 Results

6.5.1 Water Chemistry

Water pH were different in cement and copper pipes. Increases were observed on day 3 and 5, in the range of 7.6 - 11.1 and 7.6 - 12.1 for copper and cement pipes, respectively, in both chlorination treatments (Figure 6.2, a-d). Copper pipes showed the highest value (pH = 11.1) on day 3 when *Bacillus* species were present in the system (Figure 6.2, a), while the cement pipe's maximum pH (12.0 – 12.1) was on day 5 when *Micrococcus* species were in the system (Figure 6.2, c). The pH values were 12.0 and 12.1, respectively. pH in the HDPE hub and PVC pipe were lower and ranged from 7.5-7.6 and 7.6 to 9.7, respectively (Figure 6.2, a-d), indicating very slight pH change in the HDPE and PVC, while the system's pH remained more in the unchlorinated control.

Dissolved oxygen concentrations ranged from 7.5 - 11.1 ppm. The highest DO was in the copper pipe on day 3 when *Bacillus* species had grown without chlorine (Figure 6.3, a); in the presence of free chlorine, the maximum DO was 10.9 ppm, when a mixed culture was present in the system (Figure 6.3, d). Changes in DO concentrations were also observed in PVC and cement pipes in the chlorine-free

water system, where DO ranges were 7.6 - 9.7 ppm and 7.6 - 9.6 ppm, respectively. DO concentrations in PVC and cement pipes in the chlorinated water system were 7.6 - 7.9 and 7.6 - 8.2 ppm. Overall, copper pipes showed greater changes in DO concentration than other pipes, both in chlorinated (Up to 3.5 ppm) and unchlorinated water systems (up to 3.2 ppm) from day 0 to day 3 and 5, in all bacteria and mixed culture experiments (Figure 6.3, a-d). In the presence of free chlorine, changes in DO were less than in unchlorinated systems in all pipe materials except cement.

Total organic carbon (TOC) concentrations were in the range of 3.09 - 734 ppm-C. TOC of the feed water was 3.09 – 10.8 ppm-C in non-chlorinated systems and 11.7 - 20.4 ppm-C in chlorinated systems. The maximum concentration was observed in unchlorinated PVC pipes, followed by cement pipes (540 ppm-C) in the *Bacillus* sp. experiment (Figure 6.4, a), while in *Paenibacillus*, *Micrococcus* and mixed-culture experiments, copper pipes had a higher TOC after 5 days of incubation (Figure 6.4, b-d). In the chlorinated water system, the PVC pipes showed a higher TOC for *Bacillus* sp. after 5 days; similar to the unchlorinated system. In the *Paenibacillus* and *Micrococcus* spp. experiments, the copper pipes had the maximum TOC (Figure 6.4, b-c), while the HDPE hub had more TOC with the mixed culture than the test pipe materials (Figure 6.4, d). Results indicate that different types of pipe materiel impact the TOC concentration differently. In 62.5% of the treatments, the TOC concentration was higher in copper pipes. In contrast to this, in only 25% and 12.5% (data not shown) treatments the TOC concentration was higher in the PVC pipes and HDPE hub (stagnant water).

To measure the chlorine decay in the water system, water with 0.5 mg L^{-1} free chlorine was allowed to circulate in the pipes, while it remained stagnant in the HDPE hub over 5 days. On day 3, the chlorine concentrations were in the range of 0.03-0.28, which were further reduced to 0.02-0.25 by day 5 (Figure 6.5, a-d). The copper pipes showed the highest residual free-chlorine concentrations on day 3 and 5, which were 0.28 and 0.25 mg L^{-1}, respectively (Figure 6.5). The lowest residual chlorine concentration was 0.02 mg L^{-1} and depletion of no residual chlorine was observed at the end of any experiment.

6.5.2 Heterotrophic Plate Count

Cultures were each added at a concentration of $1x10^5$ cfu mL^{-1} into the water systems and the bacterial plate count of *Paenibacillus* and *Micrococcus* species decreased upon initial contact with 0.5 mg L^{-1} free-chlorine on day 0, but the HPC did not decrease in the *Bacillus* or mixed experiments (Figure 6.6, a-d). In the chlorinated *Bacillus* system, growth inhibition occurred by day 3 in the PVC and cement pipes, while there was a 4-5 log decrease in HPC in the copper pipe and HDPE hub (Figure 6.6, a). In the *Paenibacillus* assay, there was a 1-2 log decrease by 3 day and a further decrease of the same magnitude by day 5, except in the chlorinated copper pipe where the HPC was higher on day 5 than day 3 and the chlorinated HPDE hub where growth inhibition occurred by day 5 (Figure 6.6, b).

The *Micrococcus* species showed the greatest reduction in growth (up to log 2) by day 0 and continued until day 3; no live bacteria were isolated from water taken from the test pipes in the chlorinated system (Figure 6.6, c). There was regrowth of bacteria (up to log 2) in the chlorinated PVC pipes in the *Micrococcus* assay by day 5, while no regrowth was observed in the copper and cement pipes with

HDPE hub (Figure 6.6, c). In the non-chlorinated system, there was more growth on day 5 when compared to day 3 in the PVC and copper pipes. This was not the case in the cement pipe and HDPE hub. In the mixed species assay, there was higher cfu mL^{-1} in the copper pipes by day 5 than day 3, both in the chlorinated and non-chlorinated systems (Figure 6.6, d). This indicates that the presence of certain pipe materials, especially PVC and copper, support the growth of bacteria in the distribution system.

6.5.3 Abundances of Resistant and Total Populations in Water in Single Genus Assays

In the water samples, increases in the abundance of total and resistant bacterial populations for each genus were calculated by normalizing the day 5 (linearized) bacterial abundances to initial day 0 bacterial abundances. Bacteria communities were compared in the static HDPE reservoir as a "control". Results indicated that there were mostly declines in the concentration of both total and resistant bacterial populations in the pipes (Table 6.3).

In the *Bacillus* assay, there were obvious declines in total populations among all treatments (1-3 log). However, the resistant bacterial population (*Bacillus* R1) did not decline to the same extent (often only 1 log) and in a single case (copper pipe) the population number actually increased.

The *Paenibacillus* species showed the highest increase (up to 2 log) in the concentrations of both total and resistant populations (Table 6.3). The total *Paenibacillus* population increased in the chlorinated PVC pipes (up to 2 log), while increases in the resistant population in this pipe were less than those for the total population (up to 1 log).

The *Micrococcus* species showed a relative increase by day 5 in resistant population concentrations in respect of day 0 in all of the pipes without chlorine, but not in the chlorinated pipes (Table 6.3). An increase in total *Micrococcus* species population was not observed in any case. There was increase in the *Micrococcus* S population (with *sul*2 and *intl*1) in the chlorinated copper pipes only, while there was an increase in the abundances of *Micrococcus* S species in all non-chlorinated pipes (Table 6.3). The cement pipes had a greater increase (up to 2 log) than the PVC and copper pipes.

The effects of storage conditions were also observed in some cases, both with and without chlorine. The *Paenibacillus* and more resistant *Bacillus* were able to grow during storage; chlorination only had a slight impact on the *Bacillus*. Increases in the resistant population of above two genera in the presence of free chlorine (Table 6.3) showed that storage conditions might promote the enrichment of both total and resistant populations of some bacteria.

6.5.4 Abundances of Resistant and Total Populations in Water in Mixed Genera Water Distribution System

In the mixed genera assay, increases in the abundance of total and resistant bacterial populations were not observed in any of the pipes except in the chlorinated PVC pipes (Table 6.4). Resistant-gene containing *Micrococcus* (*Micrococcus* S) and total resistant bacteria increased in abundances in these pipes.

The effects of storage in the presence of chlorine were prominent in the mixed genera assay. Increases in the resistant population of *Bacillus, Paenibacillus, Micrococcus* and total resistant bacterial species were observed in the chlorinated HDPE hub after 5 days (Table 6.4). The maximum increase was observed in the

resistant *Micrococcus* species population (up to 2 log). Increases in the total bacterial species abundances were also observed among *Paenibacillus*, *Micrococcus* and *Acidovorax* species, but not *Bacillus*, during storage without chlorine.

6.5.5 Abundances of Bacteria in Biofilm in Single Genus Assays

The biofilm-forming potential of each bacterial genus was determined by the presence of bacterial genes (with genera specific primers) by swabbing pipe surfaces on day 5. For the evaluation of the fate of bacteria in the water distribution system, the percentage of biofilm bacteria (total and resistant) were calculated in relation to total bacteria in the whole system (total surface area and total volume of pipe).

Results indicate that bacteria tend to remain in the biofilm as compared to water (Figure 6.7, a-c). For *Bacillus*, the more resistant population ended up in the biofilm than did the total population on PVC materials, regardless of chlorine content (Figure 6.7, a). Moreover, higher percentages of resistant bacteria were found in biofilms in the chlorinated copper and cement pipes, while total bacteria were greater in the biofilms of the non-chlorinated pipes.

The percentage of total bacteria ending up in the biofilm in the *Paenibacillus* assay was higher in the chlorinated copper and cement pipes when compared to non-chlorinated pipes, while the PVC, non-chlorinated pipes contained a higher percentage of total bacteria (Figure 6.7, b). The percentage of resistant *Paenibacillus* (*Paenibacillus* R) was greater in the chlorinated copper and non-chlorinated PVC and cement pipes.

In the *Micrococcus* species assay, the percentage of resistant *Micrococcus* in the biofilm by day 5 was similar (100%) in the chlorinated and non-chlorinated PVC pipes (Figure 6.7, c); chlorination had no effect on the fate of the resistant population

in PVC pipes. However, the percentage of total bacteria was slightly higher (99.6%) in the chlorinated PVC biofilms than the non-chlorinated (98.6%). A large difference was observed in the copper and cement pipes. In the non-chlorinated copper pipes, 95% of the resistant *Micrococcus* was in the biofilm by day 5, while only 1.02% of the total *Micrococcus* was settled down in the biofilm. In the cement pipes, 99.8% of the total bacteria were in the biofilm, while only 40.2% of resistant bacteria were in the biofilm (Figure 6.8, c). The percentage of total and resistant populations ending up in the biofilm in the chlorinated copper and cement pipes were also similar.

Among all bacteria tested in the single genus assay, the percentages of resistant populations were higher in chlorinated pipes than non-chlorinated; except for *Micrococcus* in the PVC pipes (Figure 6.7, a-c). The resistant genes containing *Micrococcus* S (with *sul*2 and *intl*1 genes) were more prevalent in the biofilm than *Bacillus* R1 (*sul*1) and *Paenibacillus* R (*sul*1) in the chlorinated PVC, copper and cement pipes and non-chlorinated PVC pipes. However, the *Paenibacillus* R1 percentages were higher in the biofilms of non-chlorinated copper and cement pipes. The presence of two different genetic traits in *Micrococcus* might be a reason for the increased abundances of this species in the biofilm, providing them selective advantage over other bacteria.

6.5.6 Abundances of Bacteria in Biofilm in Mix Genus Assay

In the mixed genera assay, the greatest percentage of resistant *Bacillus* and *Paenibacillus* were present in the biofilms of the chlorinated cement pipe while the lowest percentage was found in the chlorinated copper pipes. Resistant bacteria (*Bacillus* R1 and *Paenibacillus* R) were higher in the chlorinated pipes than non-chlorinated in all cases (Figure 6.8, a-b).

In the case of *Micrococcus* species in the mixed assay, > 99% resistant bacteria formed a biofilm by day 5. Among three resistant bacteria, the percentages of bacteria of *Micrococcus* S (with *sul*2 and *intl*1) were higher in biofilms than other bacteria. No resistant bacteria were determined for *Acidovorax* because of the absence of a resistant trait.

6.5.7 Relationship between Physicochemical Properties and Abundances of ARGs, MGEs and Bacteria

A Pearson correlation analysis ($\alpha = 0.05$) was carried out between the physico-chemical properties DO, TOC, Cl_2, pH and HPC and different gene abundances. A significant negative correlation was found between the DO and the absolute abundance of 16S-rRNA ($r = -0.880$, $p = 0.021$) and the relative abundance of the resistant population (*Paenibacillus* R) ($r = -0.887$, $p = 0.018$) in the *Paenibacillus* assay in water (Table 6.5) and the resistant population of *Micrococcus* genus (*Micrococcus* S) ($r = -0.914$, $p = 0.011$) in the biofilm in the mixed genera assay. In the *Micrococcus* assay, a significant negative correlation occurred between the TOC and the absolute 16S-rRNA abundances ($r = -0.824$, $p = 0.044$) in biofilm.

pH appears to be the most effective driver for the system, there were significant correlations between pH and the relative resistant populations and absolute 16S-rRNA gene abundances (Table 6.5). Correlations were $r = 0.948$, $p = 0.004$ in biofilm for *Bacillus* R1, $r = 0.819$, $p = 0.046$ for resistant *Paenibacillus* populations and $r = 0.849$, $p = 0.016$ for *Micrococcus* S in circulating water, and $r = -0.875$, $p = 0.01$ for total bacteria in water in the *Micrococcus* assays. In the mixed genera assay, a significant negative correlation ($r = -0.935$, $p = 0.006$) was found between pH and total 16S-rRNA gene abundances in biofilm.

6.6 Discussion

Supply of potable drinking water is the fundamental prerequisite for the general population, but it is quite difficult to achieve because of the unsterility of the water distribution system (Wang et al. 2014). The drinking water environment could provide several ecological advantages to certain microbial populations, which both individually and collectively influence the drinking water microbiome. This study demonstrated that a combination of engineering and ecological factors could influence the distribution of microbes to promote resistant bacteria with resistant genes in both circulating water and biofilms. Physico-chemical parameters such as DO, TOC and pH contribute to the propagation of the microbial population in the DWDS besides the major impacting factors, which include pipe material, residual disinfectant, attachment with surfaces and water age.

The standard for DO in drinking water is 4-6 ppm while in current experiments the minimum DO concentration was 7.52 ppm on day 0 (Figure 6.3, a-d). High DO speeds up corrosion in water pipes such as those made of iron (Sarin et al. 2004), steel (Jung et al. 2013) and copper (Vargas et al. 2010). The standard for TOC in drinking water is 2-4 ppm-C and the WHO limit is 3 ppm-C (Khademikia et al. 2013). In this study, the initial concentration was > 3.09 ppm-C (Figure 6.4, a-d). The presence of high TOC in water increases the chlorine demand and the formation of disinfectant by-products (Ramavandi et al. 2015). The loss of chlorine dioxide has been reported due to the presence of TOC (Zhang et al. 2008). In the current study, the decline in the free chlorine concentration during the initial 3 days in closed systems might be due to the high chlorine demand as more TOC was present in the

systems, and the release of substances from pipe materials or corrosion could increase both the TOC and chlorine demand.

Regrowth or recolonization of bacteria could occur in the pipe material (Manaia et al. 2012). This was also observed in this study for the *Micrococcus* and mixed species assays (Figure 6.6, c-d). Many factors contribute to the regrowth of bacteria in the water distribution systems, for example the presence of assimilable organic carbon (AOC) (Liu et al. 2002) and inorganic nutrients such as nitrate and phosphorus (Chu et al. 2005). The storage of water in containers of PE and galvanized steel (Momba and Kaleni 2002) and for longer periods of 7 days (Mellor et al. 2013) also have proliferation impacts. Furthermore, the concentration of disinfectant residual or loss of disinfectant (Zhang and DiGiano 2002) and the presence of new pipes (Nguyen et al. 2012) allow bacteria to regrow in the distribution systems. Not only bacteria, but a vast difference in ARGs has been observed at the point of use when the reclaimed water has passed through the distribution system (Fahrenfeld et al. 2013). This indicates that some bacteria regrowth occurs during the passage of water through the pipe system. Experiments showed that the colonization of the pipes could occur within 5 days and the number of bacteria increased up to 10^5 cfu cm^{-2}(Frias et al. 2001). In the current study, the bacterial abundances reached up to log $10^{6.5}$ cfu cm^{-2} in the biofilm when the culture was used at a concentration of $1x10^5$ in water.

During this study, only *Micrococcus* showed significant correlation with the concentration of TOC in biofilm and 16S-rRNA gene abundances (but only in the single genus experiment), while in the water and mixed genera assays, no such relationship was found. It has been shown in studies that nutrients like phosphorous,

nitrogen and sulphur have no contribution to the bacterial growth in distribution systems (Frias et al. 2001). Organic carbon, especially the biodegradable organic carbon, is the only nutrient that increases bacteria in circulation; but it has little effect on the enrichment of bacteria in biofilms (Ellis et al. 1999; Frias et al. 2001). Contrary to this, other studies have shown that the number of bacteria in a biofilm could correlate with the presence of biodegradable organic carbon (BOC) (Volk and LeChevallier 1999), nitrogen and phosphorus (Chu et al. 2005) and the bacterial growth in biofilm and circulation water could be controlled by minimizing the amount of BOC and nutrients.

Results in this study indicate that bacteria have different potentials to form biofilms (Figure 6.7 and 6.8). Other studies also indicate that biofilm formation is not the same for all types of pipes used in water distribution and plumbing systems; plastic pipes; especially those made of polyethylene (PE) are more prone to biofilm formation when compared with copper pipes (Lehtola et al. 2004b). Iron, steel and cement pipes support the growth of bacteria but may have different potentials depending upon the bacterial community. In one study, *Actinobacteria* and *Clostridia* were dominant in plastic pipes, *Gammaproteobacteria* and *Bacilli* were dominant in cast iron pipes, while *Alphaproteobacteria* had a tendency to colonise both types equally (Douterelo et al. 2014).

In the other study, stainless steel supported less biofilm formation than elastomeric surfaces including latex and ethylene propylene, while PVC was between the two (Rogers et al. 1994). In yet another study, iron pipes supported more heterotrophic bacteria when compared to PVC, while PVC pipes promoted the attachment of pathogens like *Shigella* and *Vibrio* spp. biofilms (Ren et al. 2015).

Biofilm formation was faster on plastic pipes when compared to copper pipes (Lehtola et al. 2004b), and fewer cfu mL^{-1} were found on copper pipes in drinking water distribution systems (Schwartz et al. 1998). This might be due to copper toxicity (Yu et al. 2010). In contrast to this, another study showed that the lowest fixation of bacterial biomass was on plastic surfaces like PE and PVC and the greatest on grey iron (Niquette et al. 2000b). Rubber material also supports the growth of pathogens, while plastic material does not (Colbourne et al. 1984).

For plumbing in the drinking water distribution system, chlorinated polyvinyl chloride has been suggested because of its very smooth surfaces and low biofilm formation potential (Yu et al. 2010). New pipe materials have numerous surface structures, which could support the attachment of bacteria. Copper pipes have relatively smooth surfaces when compared to steel pipes and more bacteria attach to steel pipes (Yu et al. 2010), yet, copper pipes facilitate attachment of bacteria more than chlorinated PVC pipes (Yu et al. 2010). New metallic piping, such as iron and copper, can accelerate chlorine (Zhang and Edwards 2009) and chloramine decay (Nguyen et al. 2012). Additionally, not all organisms in a distribution system develop biofilms.

Biofilm bacteria demonstrate different behaviour than planktonic cells, and due to their physiological adaptation, they exhibit greater resistance to disinfectants (Costerton et al. 1987; Russell 1998; Brown and Gilbert 1993). One study found that the biofilm bacteria were > 3000 times more resistant to hypochlorous acid when compared to free-living bacteria (LeChevallier et al. 1988). This allows not only the susceptible bacteria to survive, but the pathogenic bacteria could also escape from the disinfection process.

As the presence of antibiotics exerts a selective pressure on ARGs even at sub-inhibitory concentrations, metals (especially the heavy metals) could serve as selective agents for the enrichment of antibiotic resistance genes (Graham et al. 2011; Knapp et al. 2011). Like antibiotics and metals, the presence of disinfectants can also imposed selective pressure in the distribution system, supporting the survival of organisms having resistant traits (Xi et al. 2009). Under selective pressures, organisms in the distribution system can acquire new resistant traits (Figueira et al. 2012).

A low concentration of disinfectant exerts selection pressure on the resistant populations, i.e. both disinfectant and antibiotic resistant. As found previously in Chapter 5, a low concentration of chlorine and monochloramine selected the disinfectant-resistant population and the population with antibiotic resistant genes. This was further confirmed during this ecotoxicological study, where increases in the abundance of disinfectant-resistant populations with antibiotic resistant genes were observed in *Bacillus* and *Paenibacillus* species. This is not universal, as *Micrococcus* species showed unique behaviour; in the MSC study the disinfectant resistant population (*Micrococcus* I) was selected, while in ecotoxicological study the organism with antibiotic resistant genes (*Micrococcus* S) was in greater abundance. The ecotoxicological conditions, such as pipe material, might be the reason for their differential behaviour, which indicates that different environmental conditions could select different resistant populations in DWDS environments and there are likely to be undetected (or unknown) traits that may confer selective advantage.

A hindrance in DWDS disinfection is that chlorine's ability to destroy the antibiotics (Li and Zhang 2013) and ARGs (Dodd 2012) is different. Chlorine

efficacy depends upon how much penetrates the biofilms on pipes, the thickness of biofilm and the dominant species of biofilm, all of which determine the final inhibitory effects of the disinfectant. This phenomenon also depends upon the water chemistry, pipe material and corrosion conditions (Norton et al. 2004) of the system. An important factor for consideration during the disinfection process of water is the possibility of viable but non-cultivable bacteria, which survive the disinfection shock and could be involved in the transfer of genetic material after disinfection.

In the current study, it was found that the pipe materials had an impact on the growth of bacteria in water distribution system and PVC pipes had a greater abundance of bacteria and resistance genes than other pipes. The highest resistant populations were in biofilm both in single and mixed assays, which indicates that resistant populations become enriched in the biofilm (Figure 6.7 and 6.8), while some proliferated more in circulating water e.g., *Micrococcus* and *Paenibacillus* in single genera assays and *Bacillus* and *Paenibacillus* in mixed assays, respectively. Stagnant water had an impact on the concentration of bacteria (Lautenschlager et al. 2010); bacterial abundances were highest in HDPE hub, in which water was allowed to stagnate for 5 days.

6.7 Conclusion

This study revealed the impact of DWDS ecology on the profiles of resistant bacteria with different factors contributing to the enrichment of resistant bacteria. Also the presence of residual chlorine had little effect on the control of bacterial growth in circulating water and biofilms. The absence of significant correlation between HPC (viable count) and genes (non-viable) indicate that the quality testing at the point of use is not enough for the guaranteed potability of water as many more

bacterial components (e.g. resistant genes) could be present and not be eliminated by disinfectant. To avoid the outbreak of infections in sensitive populations due to the use of ARB and ARG containing water, there is a need for further research to determine the best disinfection and management strategies, which could inactivate or minimise resistant bacteria in the DWDS.

Table 6-1 Bacteria and their resistance and transferable genes.

Name of Organisms	**Genes**	**Chlorine Susceptibility**	**MIC mg L^{-1}**	**MSC mg L^{-1}**	**Antibiotic Susceptibility**
Bacillus R1	*sul*1	Resistant	10.4 ± 1.7	NC	Susceptible
Bacillus R2	-	Resistant	1.0 ± 0.6		Susceptible
Paenibacillus R	*sul*1	Resistant	10.0 ±1.4	0.089	Susceptible
Paenibacillus S	-	Susceptible	5.2 ± 2.9		Susceptible
Micrococcus I	-	Intermediate	8.0 ± 3.1	0.046	Resistant (SMX, CIP)
Micrococcus S	*sul*2, *intI*1	Susceptible	5.0 ± 1.7		Resistant (SMX, CIP, AMX)
Acidovorax R	-	Resistant	8.2 ± 2.0	0.393	Resistant (AMX)
Acidovorax S	-	Susceptible	2.0 ± 1.2		Resistant (AMX)

Key: SMX = Sulfamethoxazole, CIP = Ciprofloxacin, AMX = Amoxicillin, NC = not calculated

Table 6-2 PCR Primers used for the detection of *Bacillus*, *Paenibacillus, Acidovorax* and *Micrococcus* species, total bacteria, MGE, and ARGs in simulated water distribution system.

Primer	Sequence (5′-3′)	PCR annealing temperature (°C)	Amplicon size	Reference
B. subtilis-F	GCGGCGTGCCTAATACATGC	60	267	(De Clerck et al. 2004)
B. subtilis-R	CTCAGGTCGGCTACGCATCG			
PEAN 843-F / 862 F	TCGATACCCTTGGTGCCGAAGT	58	664	(Pettersson et al. 1999)
RNA 1484-R	TACCTTGTTACGACTTCACCCCA			
WFB1-F	GACCAGCCACACTGGGAC	65	360	(Walcott and Gitaitis 2000)
WFB2-R	CTGCCGTACTCCAGCGAT			
ML-recA-F	GCCCTGGACCCGGTCTACGCCCG	60	310	(Hilbert and Busse 2010)
ML-recA-R	CGCCGATCTTCTCGCGCAGCTGG			
V4-16S-515F	TGTGCCAGCMGCCGCGGTAA	50	312	(Caporaso et al. 2011)
V4-16S-806R	GGCTACHVGGGTWTCTAAT			
int1-F	GGCTTCGTGATGCCTGCTT	57	148	(Luo et al. 2010; Chen et al. 2015)
int1-R	CATTCCTGGCCGTGGTTCT			
sul1-F	CGCACCGGAAACATCGCTGCAC	56	163	(Pei et al. 2006; Chen et al. 2015)
sul1-R	TGAAGTTCCGCCGCAAGGCTCG			
sul2-F	TCCGGTGGAGGCCGGTATCTGG	60.8	191	(Pei et al. 2006; Chen et al. 2015)
sul2-R	CGGGAATGCCATCTGCCTTGAG			

F = forward, R = reverse.

Table 6-3 Changes in total and resistant bacterial abundances after 5 days in water in different pipe materials in single genus assay.

Assay	Organism Type	PVC	PVC+Cl	Copper	Copper+Cl	Cement	Cement+Cl	HDPE	HDPE+Cl
Bacillus	Total *Bacillus*	--	-	---	---	---	--	-	-
	Resistant *Bacillus*	-	-	+	-	-	-	++	+
Paenibacillus	Total *Paenibacillus*	ND	++	--	---	--	ND	++	++
	Resistant *Paenibacillus*	+	+	0	-	-	0	+	+
Micrococcus	Total *Micrococcus*	--	-	--	--	ND	ND	-	-
	Resistant *Micrococcus*	+	-	+	+	++	-	-	0

Key: 0 = no change, ND = non-detected, - = < 1 log decline, + = < 1 log increase, -- = < 2 log decline, ++ = < 2 log increase, --- = 2+ log decline, +++ = 2+ log increase

Table 6-4 Changes in total and resistant bacterial abundances after 5 days in water in different pipe materials in mix genera assay.

Genus	Organism type	PVC	PVC+Cl	Copper	Copper+Cl	Cement	Cement+Cl	HDPE	HDPE+Cl
Bacillus	Total *Bacillus*	---	--	---	---	---	---	-	-
	Resistant *Bacillus*	-	-	---	-	--	--	-	+
Paenibacillus	Total *Paenibacillus*	---	--	---	---	---	---	+	+
	Resistant *Paenibacillus*	-	-	---	-	--	--	-	+
Micrococcus	Total *Micrococcus*	--	-	---	-	-	-	+	-
	Resistant *Micrococcus*	-	+	-	-	--	--	0	++
Acidovorax	Total *Acidovorax*	--	-	---	--	--	--	+	+
	Resistant *Acidovorax*	ND	ND	ND	ND	ND	ND	ND	ND
16S-rRNA	Total bacteria	--	--	--	--	---	--	-	-
	Resistant bacteria	0	+	-	-	-	--	+	++

Key: 0 = no change, ND = non-detected, - = < 1 log decline, + = < 1 log increase, -- = < 2 log decline, ++ = < 2 log increase, --- = 2+ log decline, +++ = 2+ log increase

Table 6-5 Correlation analysis between physicochemical properties and bacterial abundances. Significant limit was $p < 0.05$.

Assay	Genes	Pearson correlation	Water					Biofilm				
			DO	**TOC**	**Cl**	**pH**	**HPC**	**DO**	**TOC**	**Cl**	**pH**	**HPC**
Bacillus	*Bacillus* R1/16S-rRNA relative	*r*= value	-0.167	0.028	-0.596	0.535	0.276	0.433	-0.504	-0.59	0.948	-0.233
		p value	0.692	0.947	0.119	0.172	0.509	0.391	0.308	0.912	**0.004**	0.657
	16S-rRNA absolute	*r*= value	0.628	-0.226	0.32	-0.554	0.203	-0.39	-0.004	0.287	-0.671	0.382
		p value	0.096	0.591	0.439	0.154	0.63	0.445	0.994	0.582	0.144	0.455
Paenibacillus	*Paenibacillus* R/16S-rRNA relative	*r*= value	-0.887	0.782	0.036	0.819	-0.097	-0.103	0.152	0.342	0.434	0.325
		p value	**0.018**	0.066	0.946	**0.046**	0.325	0.868	0.808	0.573	0.465	0.593
	16S-rRNA absolute	*r*= value	0.88	-0.778	-0.043	-0.806	0.055	-0.085	-0.124	-0.509	-0.347	-0.189
		p value	**0.021**	0.068	0.936	0.053	0.918	0.892	0.843	0.381	0.567	0.76
Micrococcus	*Micrococcus* S/16S-rRNA relative	*r*= value	-0.307	0.329	-0.171	0.849	-0.232	0.633	0.308	0.005	-0.316	-0.422
		p value	0.504	0.427	0.715	**0.016**	0.616	0.177	0.552	0.992	0.542	0.705
	16S-rRNA absolute	*r*= value	0.464	-0.302	0.196	-0.875	0.119	0.047	-0.824	0.72	-0.468	-0.533
		p value	0.294	0.51	0.674	**0.01**	0.799	0.93	**0.044**	0.106	0.349	0.276
MIX	*Bacillus* R1 + *Paenibacillus* R/16S-rRNA relative	*r*= value	0.47	-0.093	0.198	-0.596	-0.041	-0.284	-0.113	0.403	0.181	0.478
		p value	0.239	0.826	0.639	0.119	0.924	0.585	0.831	0.428	0.731	0.338
	Micrococcus S/16S-rRNA relative	*r*= value	0.552	0.413	0.12	-0.058	-0.013	-0.914	0.283	0.193	0.22	0.209
		p value	0.156	0.309	0.778	0.891	0.976	**0.011**	0.587	0.715	0.675	0.692
	16S-rRNA absolute	*r*= value	0.235	0.056	-0.364	-0.362	-0.25	0.64	-0.567	-0.015	-0.935	-0.503
		p value	0.576	0.895	0.376	0.378	0.55	0.171	0.24	0.978	**0.006**	0.309

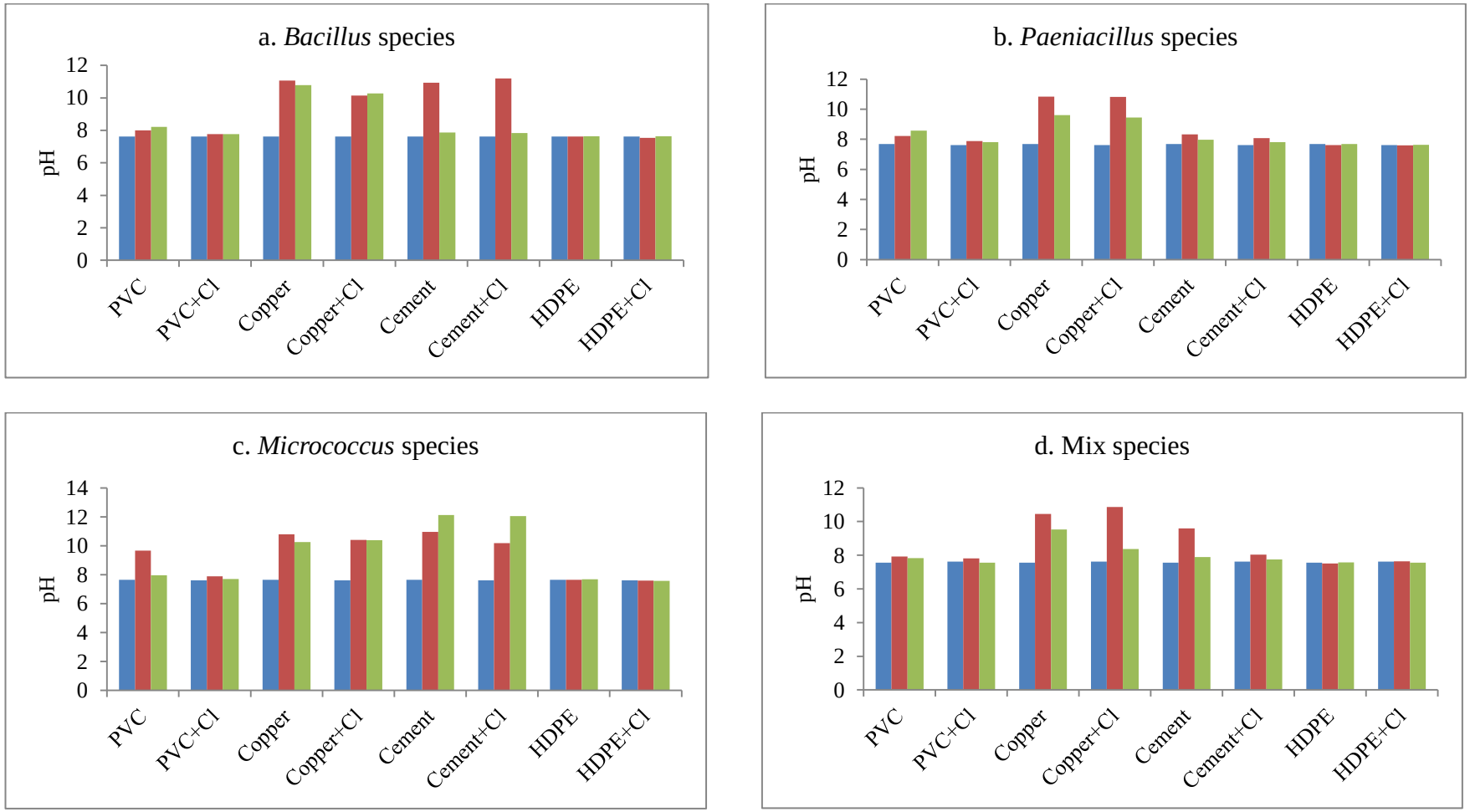

Figure 6-2 pH of water in different pipes in the absence and presence of 0.5 mg L^{-1} free chlorine.

Key: ■ Day 0 ■ Day 3 ■ Day 5.

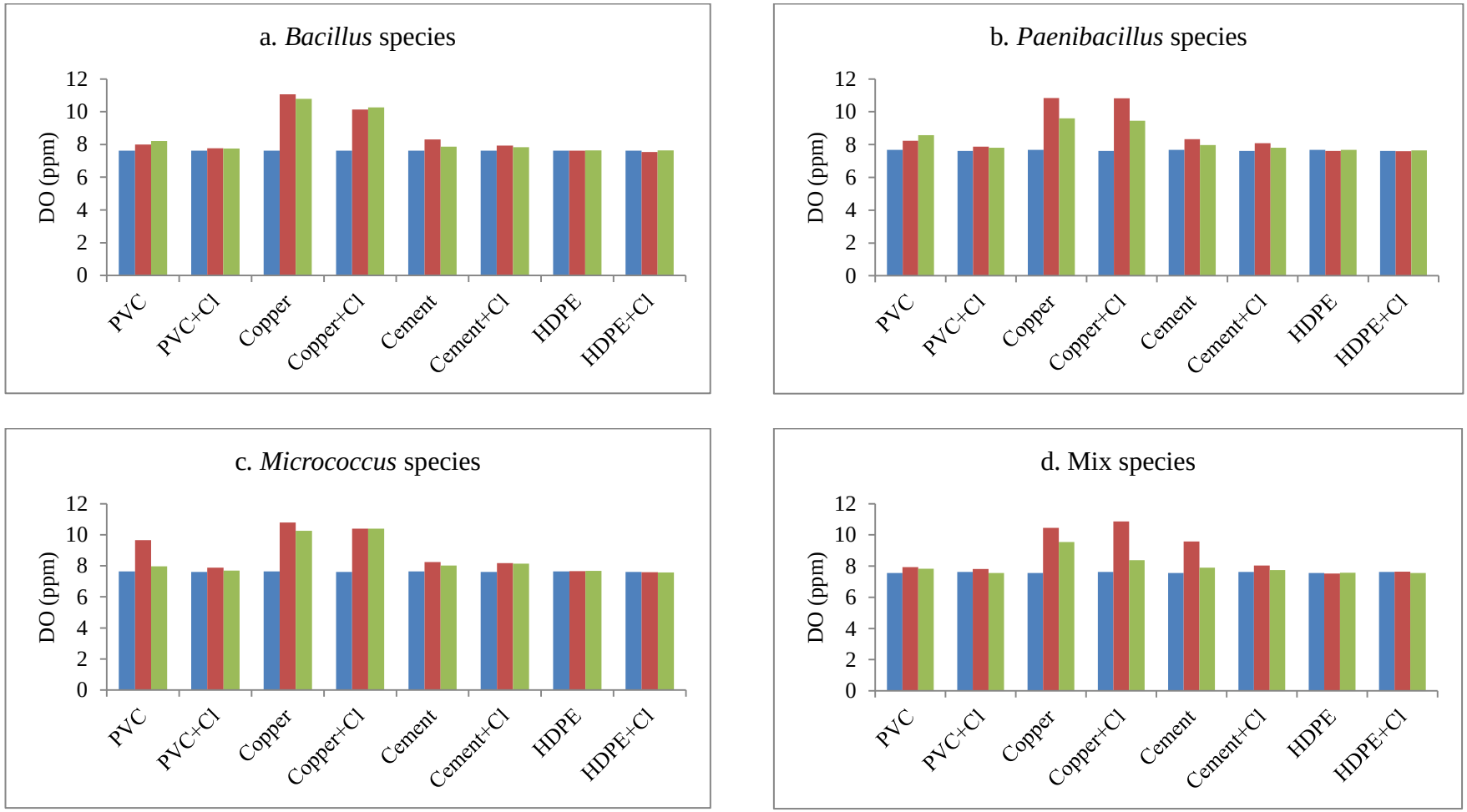

Figure 6-3 Dissolve oxygen concentrations (ppm) in water in different pipes in the absence and presence of 0.5 mg L^{-1}free chlorine.
Key: ■ Day 0 ■ Day 3 ■ Day 5.

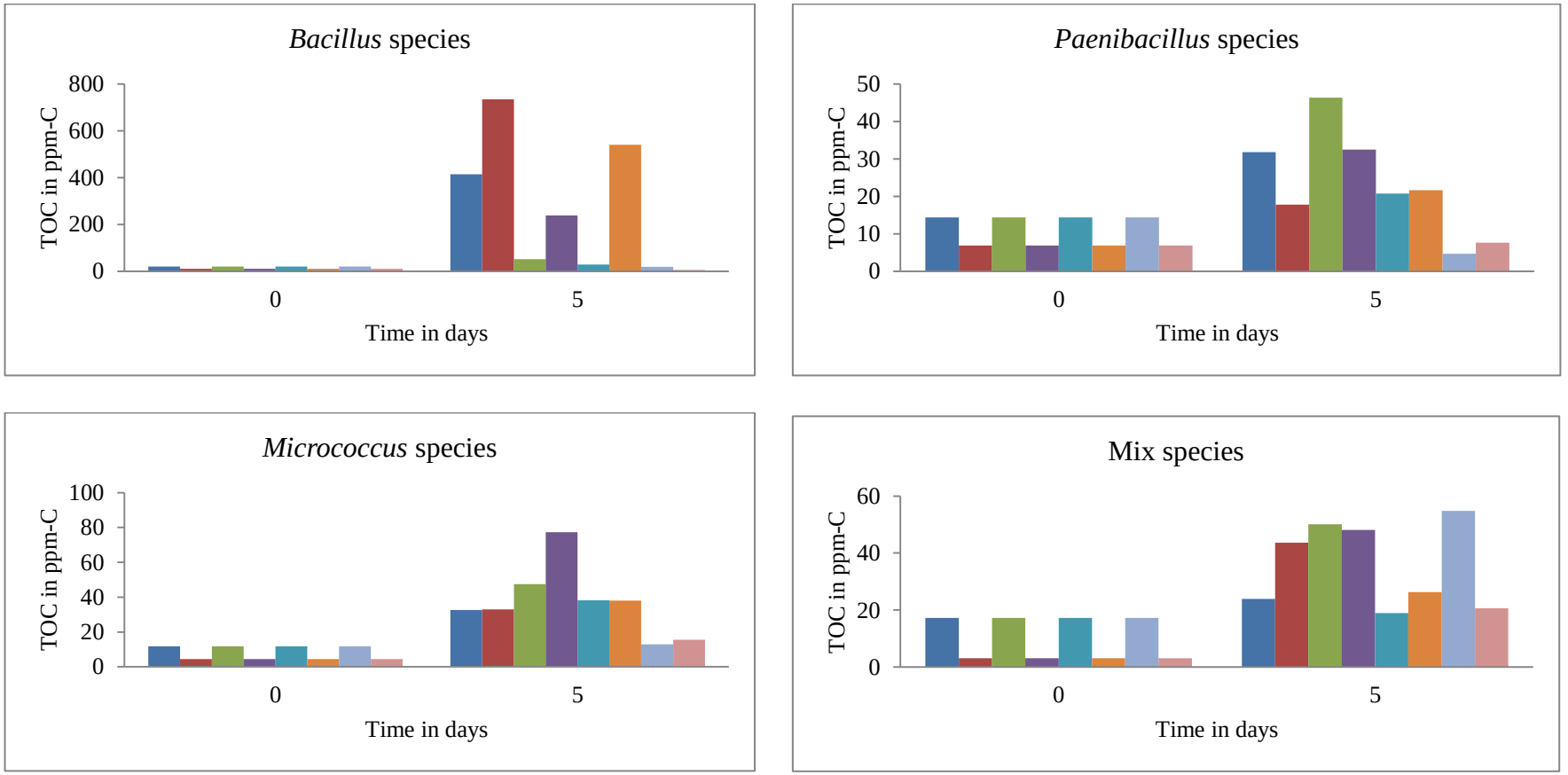

Figure 6-4 Concentrations of total organic carbon (ppm-C) in water in different pipes in the absence and presence of 0.5 mg L^{-1} free chlorine.

Key: PVC+Cl_2 PVC Copper+Cl_2 Copper Cement+Cl_2 Cement HDPE+Cl_2 HDPE.

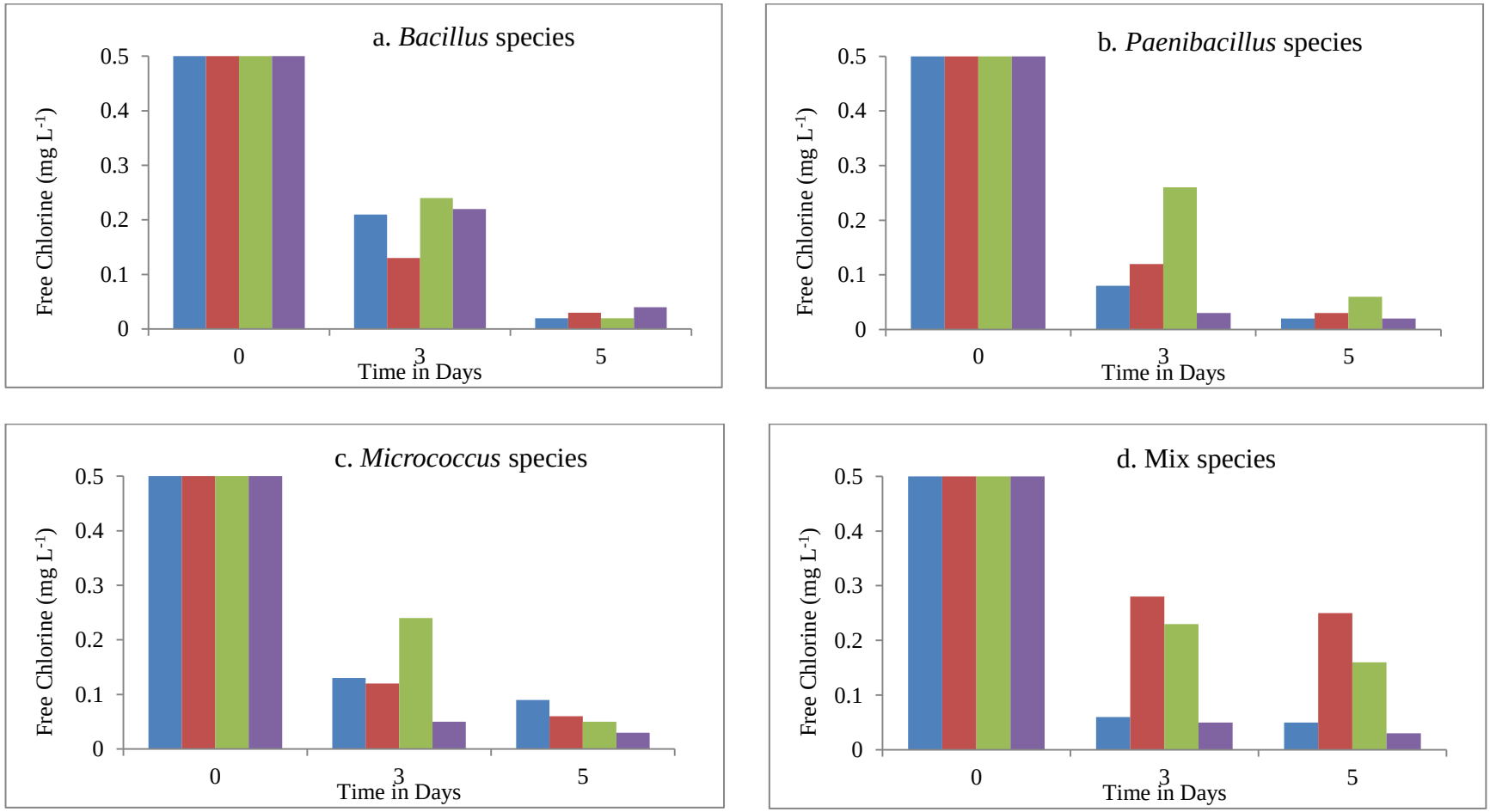

Figure 6-5 Concentrations of free chlorine (mg L^{-1}) in different pipes over time.

Key: PVC Copper Cement HDPE.

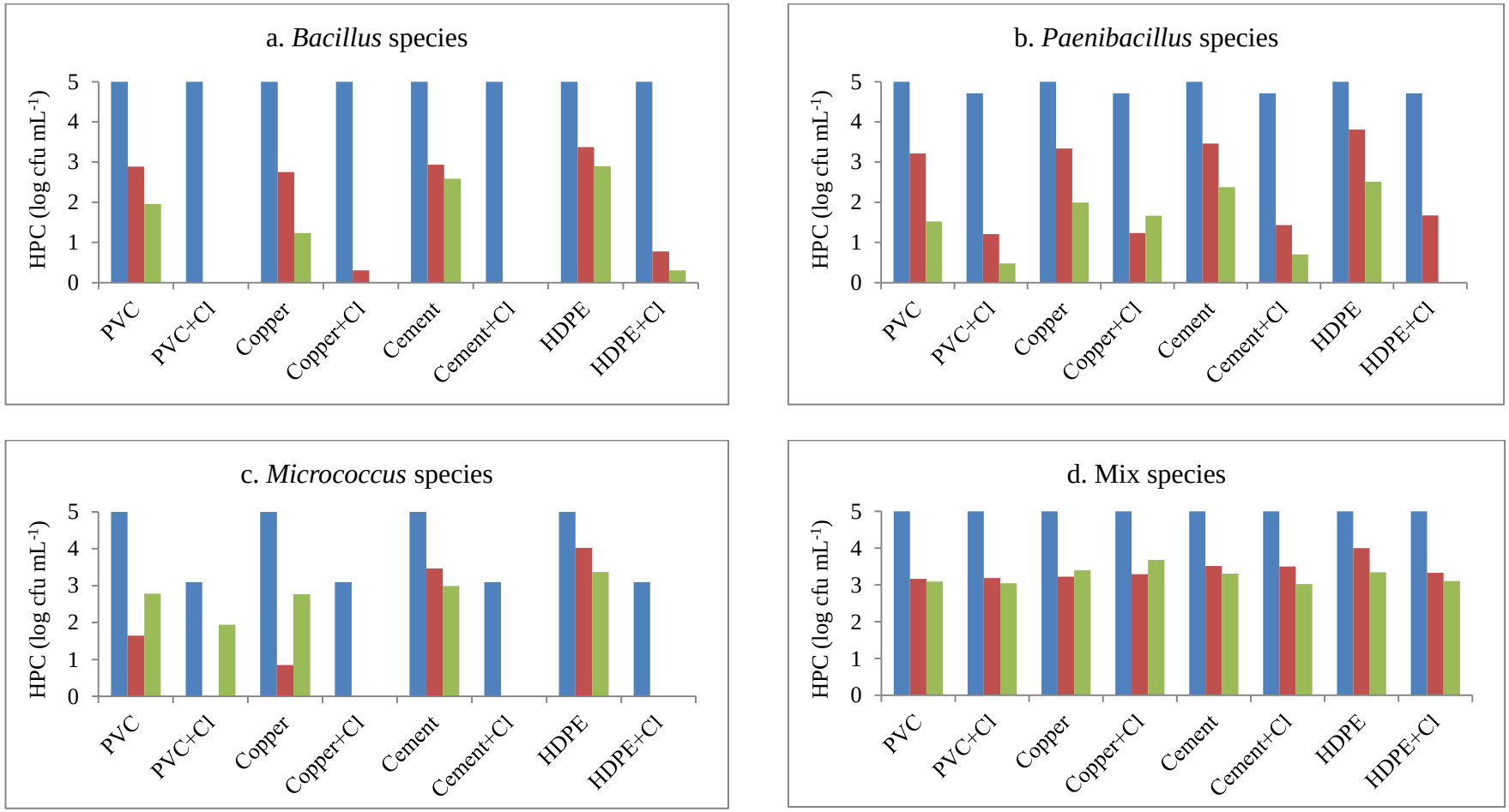

Figure 6-6 Heterotrophic Plate Count (cfu mL^{-1}) in different pipes in the absence and presence of 0.5 mg L^{-1} free chlorine.

Key: Day 0 Day 3 Day 5.

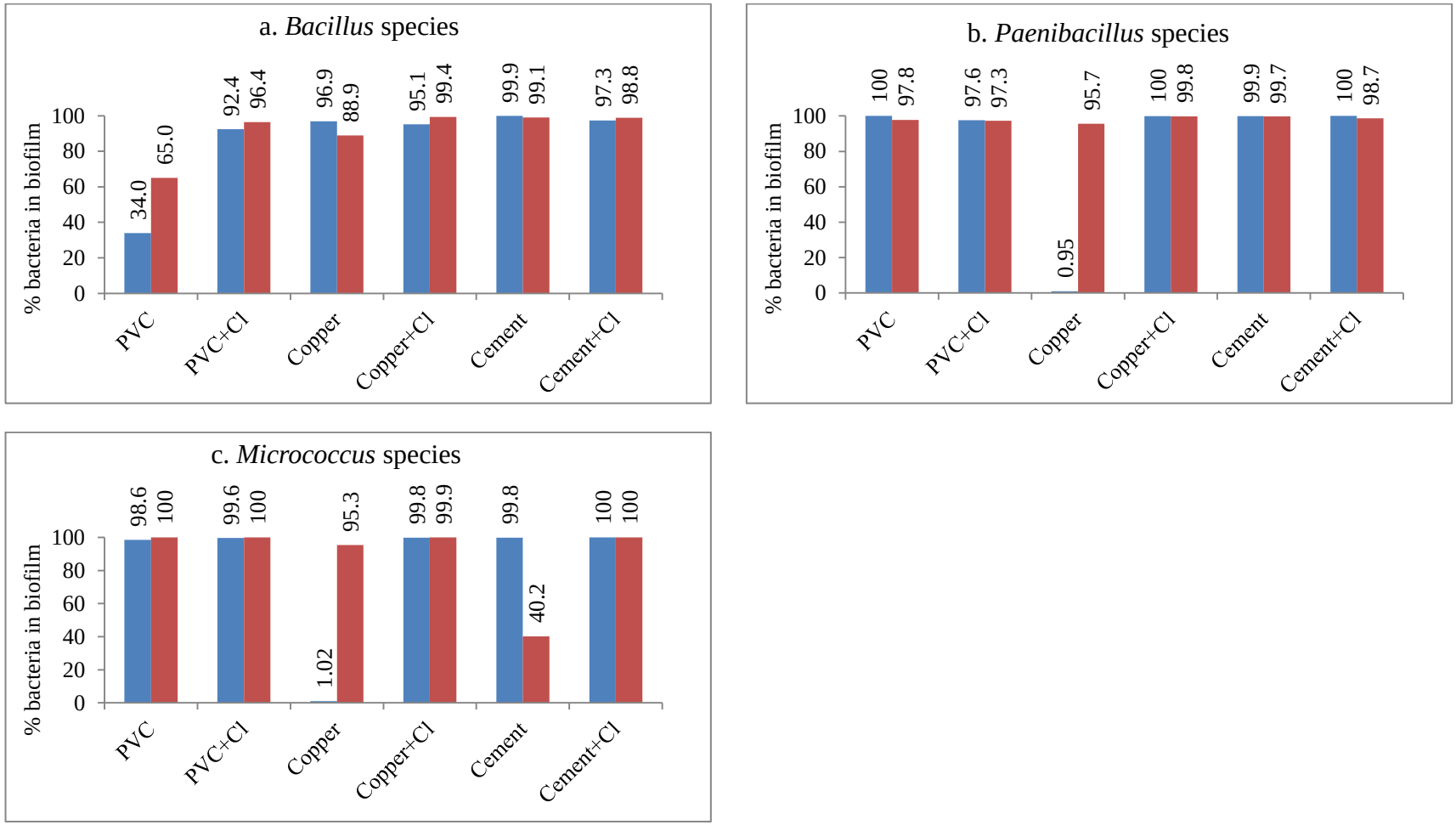

Figure 6-7 Percent bacterial abundances in biofilm after 5 days in different pipes in single genus assays.

Key: ■ Total bacteria ■ Resistant bacteria.

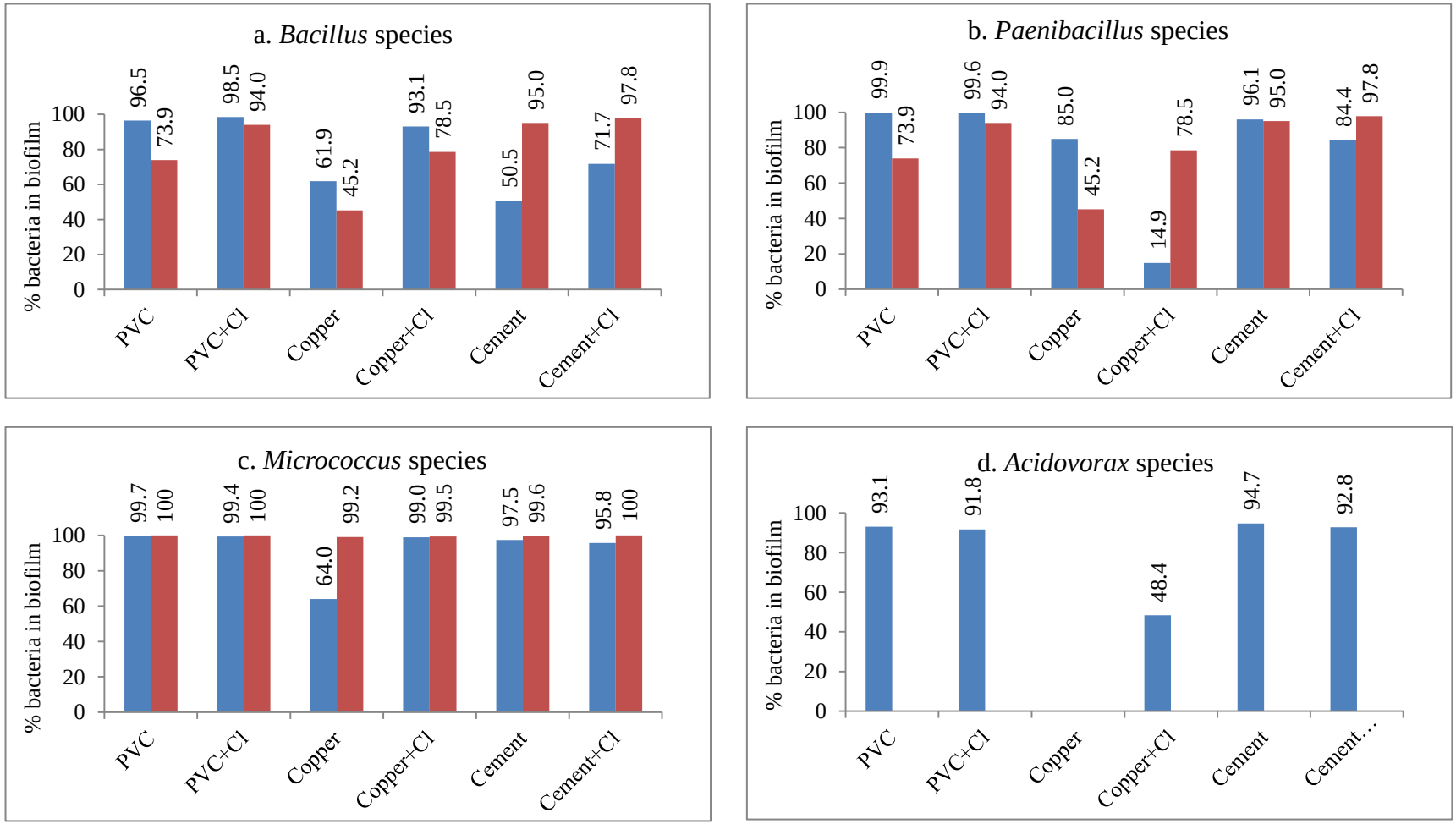

Figure 6-8 Percent bacterial abundances in biofilm after 5 days in different pipes in mix genera assay.

Key: ■ Total bacteria ■ Resistant bacteria.

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Chapter 7

7.0. Conclusion

This study was carried out to address the role DWDS have in the development of resistance among bacteria inhabiting such systems. The major aim was to determine whether DWDS ecology boosts the antibiotic- and disinfectant resistances of bacteria in drinking water. DWDS are not simple ecosystems; to understand them requires the consideration of a series of variables including residual disinfectants, distribution pipes, storage facilities and plumbing systems within buildings that deliver drinking water. Many factors were studied here and the results have increased the understanding of DWDS and which factors contribute to the propagation of resistant organisms and traits in these systems.

DWDS can disseminate potentially pathogenic bacteria. The recovery of *Burkholderia* from tap-water samples was surprising as some species can cause fatal human diseases and demonstrated that pathogens can be dispersed to the human population through DWDS. The key finding of *Burkholderia* species and other potential emerging waterborne pathogenic bacteria in water samples (see Sections 3.6 and 4.6) indicates a need for vigilance for further epidemiological evidence. *Burkholderia* can be transferred from person to person and from the environment but also remains as transient infections among people. Disruption and repair of plumbing system could introduce them in the drinking water from environment (WHO, 1996). *Burkholderia* species are environmental organisms, which can grow in drinking water, muddy water, soil and dilute disinfectant (Agency, 2002). *Burkholderia* is common in Australia and South East Asia (WHO, 1996) and could be transferred

into United Kingdom through the patients traveling to these countries. The same was found with *bla*$_{\text{NDM-1}}$ resistant gene (Kumarasamy et al. 2010) (see Section 2.4).

The MDR bacteria isolated in this study, with quadruplet resistance, are other examples of the potential role of DWDS in the transfer of resistant organisms to water consumers, which clearly represents a concern with respect to human health risk. Of similar concern was the isolation of bacteria with a higher MIC for the AMX antibiotic from buildings with water storage cisterns, indicating that storage conditions might enhance antibiotic resistance as well. This study highlighted that storage conditions in buildings and cistern construction could serve as potential sources of water contamination. In the same manner, building-plumbing, leakages, repairs and maintenance work should also be considered as potential sources of resistant organisms in DWDS.

Water treatment companies supply highly regulated water in UK, but they do not have control over maintenance work and plumbing system in buildings. There is a need to educate building owners regarding repair work and storage conditions in building as it could increase the risk of resistance development.

The testing of bacterial isolates from DWDS for the presence of *qac* genes indicated that these genes were not present in these bacteria. The distribution of *qac* genes is likened with the presence of certain bacterial species, which serve as source and sink of *qac* genes (Section 2.2.2). The absence of *qac* genes might also be owing to the treatment plant efficiency; multiple treatment processes remove the bacteria harbouring *qac* genes.

While the presence of bacteria is informative, knowing the propensity of them to become a larger problem (as microorganisms replicate) would be good to

know. As such, minimum selectable concentration (MSC) would be innovative and useful technique for determining the selection of potential resistant population in DWDS in the presence of disinfectant. Although MSC has been applied successfully to antibiotics; this study took this novel approach with its application to disinfectants. The results suggested that potentially resistant organisms could surpass the growth of susceptible populations at low concentrations of disinfectants; the knowledge of MIC and MSC is equally important and could be helpful in predicting populations. The MSC model should be studied in detail with other disinfectants for the selection of resistant populations in DWDS.

It was surprising to observe that the selection of resistant organisms is not solely based on the presence of either disinfectant or antibiotic resistant genes, or both. The ecology of the system could select any resistant organisms. Low residual disinfectant concentrations, due to the half-life of disinfectants and the storage of water, which results in the gradual depletion of disinfectant from the system, could (in part) be responsible for the dissemination of resistant organisms in DWDS and should be studied to find their impact on the resistance acquisition in the DWDS environment.

Difference in water-storage conditions resulted in the isolation of wider community of bacteria, while all other parameters were similar (see Sections 3.4.1 and 4.4.1). As samples were taken after tap flushing for 5 minutes to avoid the contamination of bacteria in samples, presence of bacteria in these samples indicated that isolated bacteria were from distribution or plumbing systems.

Environmental conditions, e.g. the presence of chlorine or the nature of plumbing systems in DWDS, can enrich the resistant microbial populations. This was

confirmed by ecotoxicological study in simulated water distribution system. Previously, it was thought that the presence of disinfectants such as chlorine could transform the antibiotic resistome. Unexpectedly, in the ecotoxicological study, no significant correlations were found in the bacterial and gene abundances vs. chlorine presence, but total bacterial and resistant population abundances were higher in chlorinated pipes and biofilms. In the initial suspension test described before (Section 4.5.5), increased survival of multiple antibiotic resistant bacteria in the presence of chlorine indicated that there was some link that needs to be further investigated.

Physical support, e.g. the presence of a biofilm could enrich resistant populations in DWDS. Growth patterns were similar on agar and in suspension. Similarly, no significant difference in mean relative abundances were found in biofilm or water in most of the bacteria in mixed species assay, except in *Micrococcus* species where the relative bacterial abundances in the biofilm were significantly higher than those suspended in the water. However, mean abundances were higher in the biofilm in all cases. In contrast to total bacterial abundances, resistant population abundances were significantly higher in biofilm than in circulating water, which demonstrated that the biofilm environment supports the enrichment and transfer of resistant genes in DWDS (the close contact of bacteria could enhance the horizontal gene transfer as compared to circulating water).

The mechanism of biofilm resistance to antimicrobial is not fully understood, and several mechanisms could be involved in decreased efficacy of antimicrobials (see Section 6.3 and 6.6). For example, nutrient limitation in biofilm results in slower growth of bacteria causing a dormant phase of growth (phenotypic changes), which

decreases susceptibility to antimicrobials (Cerca et al. 2014). The dormant phase is a state in which bacteria are metabolically inactive and do not undergo genetic changes. This condition occurs in deeper layers of mature biofilms found on aging infrastructure, where there is low nutrients and restricted penetration of antimicrobials (Ito et al. 2009).

Overall, this study supports the perceptions that certain environmental factors of DWDS, such as the presence of low concentration of disinfectants, storage conditions, plumbing system maintenance, pipe materials and biofilms are possible causes of enhancement of antimicrobial resistant populations in these systems. There is a link between disinfectant and antibiotic resistances in the water environment and disinfectant resistance promotes antibiotic resistance. This research provides meaningful insight into the factors which could directly or indirectly link with DWDS pollution and result in public health concerns. This study is useful for management and planning personnel involved in the operation and maintenance of DWTP and DWDS for future development and improvement in these systems.

7.1 Key Findings

The current study resulted in a number of findings. The key findings are discussed below.

1. Bacteria isolated from the DWDS have resistance to common antibiotics and disinfectants; some of them were highly resistant when tested with disk diffusion method with high concentration of disinfectant. Classification of bacteria, for the first time, on the basis of zone of inhibition against disinfectant is very useful technique for water treatment plants, water

suppliers, and bottled water industries as a quick and cheap method of detection of disinfectant-resistant bacteria in drinking water.

2. The isolation of *Burkholderia* species from buildings is an important finding in this study. This should become concerning for treatment plants, water supply companies and plumbing maintenance as they could be introducing this potential pathogenic bacterium into the DWDS. Those who have recently renovated or maintained their plumbing systems should test their drinking water for possible contamination. They are potential risks for serious infection outbreaks from this pathogenic bacterium.
3. The minimum selectable concentrations (MSC) of chlorine and monochloramine can promote resistance acquisition in water distribution system bacterial populations. This information is useful for water treatment plants and water supply companies who deal with the resistant organisms. Enrichment of resistance at low concentration indicates that the residual concentration of disinfectant applied in DWDS is not as beneficial as it appears and could detrimentally promote resistant populations. There is a need for careful use of disinfectants in DWDS by treatment plants and supply companies.

Some of the most impactful findings have presented, other important findings from this study are further given below.

1. The drinking water microbiome has a diverse microbial population, as 22 different genera were found in the distribution system including potential human, plant and animal pathogens. The most significant were *Burkholderia* species because of their pathogenicity and antibiotic resistance (see above).

2. Microbial populations in the distribution system have ARGs and MGEs, demonstrating the potential for dissemination and enrichment of resistance traits.
3. Bacteria isolated from the tap-water did not have broad spectrum disinfectant resistant *qac* genes; however they showed high resistance to sodium hypochlorite.
4. There was significant correlation between disinfectant and antibiotic resistances; disinfectant resistant bacteria were more likely to be antibiotic resistant.
5. Antibiotic resistant bacteria tolerated chlorine longer than sensitive bacteria.
6. Pipe materials within the distribution infrastructure have the potential to influence bacterial growth; PVC pipes enriched bacterial species more than copper or cement.
7. Water storage conditions and containers increased antibiotic resistance and supported the growth of resistant bacterial populations. Bacteria from systems with water storage cisterns had higher MIC against some antibiotics, e.g. AMX. Bacterial abundance of resistant populations was greater in HDPE containers than in pipes.
8. Biofilms can enrich the resistant population; populations with resistant traits *Bacillus* R1, *Paenibacillus* R and *Micrococcus* S were found to be enriched more within biofilms than in the free water, in both single and mixed populations.
9. Enrichment of resistant populations was different in single species populations when compared with mixed bacterial populations; *Paenibacillus*

R enriched more in single than in mixed, *Micrococcus* S enriched more in mixed than in single.

7.2 Limitations of Research

It is essential here to know and identify the study's limitations for future research. During this study, all samples were collected from taps; nothing can be inferred about the municipal treatment process. Milngavie Water Treatment Works, Glasgow, where the water was sourced, follows the standards for water treatment, and all parameters tested were within the permissible range (Appendix, Table 8.1); however, the possibility of leakage or interruption in the distribution system cannot be ignored. The bacteria could have easily been obtained from taps, or could become present in the pipework following past renovations or breaks in the systems, including improperly maintained building cisterns.

A number of resistance genes have been reported in literature to be in the natural environment and most of them end up in water ecosystems. Although technology has been developed to study multiple genes simultaneously, it is not possible to study all resistance genes within the bacteria isolated from the water distribution system, so the most prevalent genes (based on literature) were selected for this study. Additionally, it was not the objective of this study to detect all types of genes. However, future research could focus on these.

The selection of the disk diffusion method for the disinfectant resistance investigation and direct use of high concentrations of disinfectants are some of the shortcomings of this research. The disk diffusion method is basically a standard protocol for antibiotic susceptibility testing, which was modified in this study for chlorine based compounds. Sodium hypochlorite was arbitrarily classified in this

study; its applicability for other disinfectants needs further investigation and standardization. A standard range of zone of inhibition for classification of disinfectant resistant and susceptible bacteria is also necessary for future work.

7.3 Recommended Future Research

From the conclusions drawn through this study, the author recommends the following research.

- Evaluation of the contribution of storage conditions in buildings in the enrichment of resistant bacteria and genes in water distribution systems.
- Development of a standard method of classification of bacteria into resistant and susceptible to disinfectants on the basis of size of zone of inhibition by disk diffusion method similar to antibiotics.
- Investigation of plumbing system maintenance work and storage tanks conditions within DWDS when water is suspected as the source of water-borne disease.
- Assessment of the MSC model for other disinfectants with sub-inhibitory concentrations to confirm its applicability in DWDS.
- Developing or testing of new materials of plumbing pipes for DWDS which can minimize the enrichment of ARGs in water in the presence of disinfectants.
- Investigation of the impact of resistant of these organisms on public health.

7.4 References

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Appendix

A. Water Quality in Glasgow

In Glasgow, most water is supplied through Milngavie Water Treatment Works situated north west of the city. The treatment works uses lime water for water conditioning, coagulation, flocculation, filtration, acid dosing and sodium hypochlorite for disinfection of water (Scottich-Katrine 2008)[4]. Permissible concentrations for Milngavie Water Treatment Works, Glasgow is given below.

Table 8-1 Permissible concentrations for Milngavie Water Treatment Works, Glasgow[5].

Parameter	Concentration with unit
pH	6.5-9.5
Turbidity	< 4.0 NTU
Hardness	< 2500 µS cm^{-1} at 20 °C
Nitrite	< 0.5 mg L^{-1}
Nitrate	< 50 mg L^{-1}
Chloride	< 250 mg L^{-1}
Fluoride	< 250 mg L^{-1}
Sulphate	< 250 mg L^{-1}
Copper	< 2 mg L^{-1}
Iron	< 200 µg L^{-1}
Manganese	< 50 µg L^{-1}
Aluminium	< 200 µg L^{-1}
Sodium	< 200 mg L^{-1}
Lead	< 25 µg L^{-1}

[4] SCOTTICH-KATRINE 2008. Water treatment and supply, Katrine water project.

[5] The Public Water Supplies (Scotland) Regulations 2014.

Trihalomethane	< 100 µg L^{-1}.

B. Supplementary Information of Chapter 4

Table 8-2 MIC values of all antibiotics against isolates and size of zone of inhibition against Standard NaOCl and commercial bleach.

Code	Identification by 16S-rRNA*	Antibiotic MICs (μg mL^{-1})				Resistant Traits for antibiotics	Zone of inhibition (mm ± SD) (n=2)	
		TET	SMX	CIP	AMX		Pure NaOCl (14.5%)	Commercial Bleach (4.5%)
501	*Cupriavidus*	4	512	0.064	512	SA	32 ± 0.7	17 ± 0.7
502	*Cupriavidus*	1	512	0.032	512	SA	30 ± 1.4	16 ± 1.4
503	*Comamonas*	1	512	0.032	512	SA	28 ± 0.7	16 ± 1.4
504	*Cupriavidus*	2	512	0.032	512	SA	35 ± 7.1	18 ± 1.4
505	*Micrococcus*	2	512	16	0.25	SC	35 ± 0.7	20 ± 3.5
506	*Enhydrobacter*	0.5	512	0.032	0.25	S	40 ± 1.4	19 ± 4.2
507	*Bacillus*	1	512	2	0.25	S	36 ± 1.4	24 ± 5.0
508	*Enhydrobacter*	0.5	512	0.032	0.25	S	40 ± 1.4	18 ± 0.7
509	*Arthrobacter*	0.5	512	0.5	0.125	S	25 ± 0.0	20 ± 2.8
510	No significant similarity found	1	512	2	512	SA	36 ± 0.7	16 ± 1.4
511	*Cupriavidus*	0.5	512	0.016	512	SA	30 ± 1.4	18 ± 0.7
512	*Micrococcus*	1	512	16	32	SCA	48 ± 0.7	22 ± 2.8
513	*Kocuria*	2	512	16	1	SC	NT	NT
514	*Bacillus*	1	32	0.032	1	No Resistance	30 ± 5.7	24 ± 0.7
515	*Cupriavidus*	512	512	16	512	TSCA	35 ± 2.8	20 ± 1.4
516	*Acidovorax*	0.125	128	0.064	512	A	34 ± 1.4	15 ± 0.7

517	Not identified	512	512	512	512	TSCA	35 ± 2.8	17 ± 2.1
518	*Arthrobacter*	512	512	512	512	TSCA	40 ± 0.7	20 ± 0.7
519	Not identified	1	128	0.25	32	A	40 ± 0.7	20 ± 0.7
520	Not identified	0.25	128	0.064	2	No Resistance	35 ± 1.4	22 ± 0.7
521	*Acidovorax*	1	128	0.064	64	A	26 ± 1.4	15 ± 1.4
522	*Cupriavidus*	128	512	2	512	TSA	26 ± 0.7	15 ± 0.7
523	No sequence data	8	32	0.016	16	No Resistance	40 ± 1.4	17 ± 0.7
524	Not identified	0.25	32	0.125	0.125	No Resistance	34 ± 2.8	22 ± 0.0
525	Not identified	0.125	64	0.032	0.064	No Resistance	34 ± 1.4	20 ± 2.8
526	*Blastomonas*	0.5	512	0.064	64	SA	NT	NT
527	*Bacillus*	1	512	0.064	512	SA	7 ± 0.0	15 ± 0.0
528	Not identified	1	512	0.064	0.125	S	10 ± 0.0	20 ± 1.4
529	*Bacillus*	1	512	16	0.25	SC	15 ± 1.4	20 ± 1.4
530	*Burkholderia*	64	512	0.06	512	TSA	15 ± 1.4	20 ± 0.7
531	*Bacillus cereus*	1	128	0.125	512	A	10 ± 0.7	22 ± 0.0
532	*Bacillus subtilis*	4	128	0.016	0.064	No Resistance	8 ± 0.7	15 ± 0.0
533	Epsilonproteobacteria	128	512	32	512	TSCA	32 ± 1.4	20 ± 0.0
534	No sequence data	2	128	0.008	0.25	No Resistance	15 ± 1.4	22 ± 2.1
535	*Brevibacillus*	2	512	0.032	2	S	26 ± 0.7	27 ± 2.1
536	*Paenibacillus*	8	256	0.008	0.25	No Resistance	20 ± 0.7	18 ± 3.5
537	*Acidovorax*	0.5	512	0.016	64	SA	34 ± 1.4	24 ± 0.0

538	*Staphylococcus*	2	512	0.25	32	SA	30 ± 0.7	15 ± 1.4
539	*Acidovorax*	2	512	32	32	SCA	25 ± 0.7	20 ± 0.0
540	*Acidovorax*	0.125	8	0.032	32	A	8 ± 0.0	21 ± 1.4
541	*Acidovorax*	1	32	0.032	64	A	10 ± 1.4	18 ± 2.8
542	*Staphylococcus*	1	512	0.25	64	SA	28 ±1.4	24 ± 1.4
543	no sequence data	0.125	8	0.032	64	A	30 ± 2.12	14 ± 0.7
544	*Acidovorax*	1	8	S	64	A	50 ± 2.8	21 ± 0.7
545	No significant similarity found	0.251	4	S	64	A	NT	NT
546	*Variovorax*	2	4	1	128	A	35 ± 7.1	20 ± 1.4
547	*Blastomonas*	0.125	128	0.016	0.25	No Resistance	60 ± 2.1	20 ± 0.7
548	Not identified	0.125	64	0.064	1	No Resistance	NT	NT
549	*Variovorax*	0.5	32	0.064	32	A	32 ± 0.7	20 ± 0.7
550	Not identified	2	128	1	32	A	40 ± 1.4	30 ± 2.8
551	*Blastomonas*	0.125	32	0.032	0.064	No Resistance	30 ± 1.4	20 ± 0.0
552	*Paenibacillus*	S	128	0.032	32	A	42 ± 1.4	16 ± 1.4
553	*Blastomonas*	0.125	128	0.032	0.064	No Resistance	40 ± 0.7	20 ± 3.5
554	*Blastomonas*	S	128	0.032	0.25	No Resistance	35 ± 1.4	18 ± 2.1
555	Not identified	0.125	4	0.032	1	No Resistance	36 ± 1.4	17 ± 2.8
556	*Blastomonas*	0.125	32	0.032	S	No Resistance	40 ± 1.4	24 ± 1.4
557	*Variovorax*	0.5	4	0.064	32	A	32 ± 1.4	17 ± 0.0
558	Not identified	0.5	4	0.064	32	A	37 ± 0.7	14 ± 1.4

559	*Blastomonas*	0.125	32	0.016	0.25	No Resistance	32 ± 7.1	20 ± 2.8
560	*Escherichia*	0.5	4	0.064	1	No Resistance	NT	NT
561	Not identified	0.5	128	0.032	0.25	No Resistance	36 ± 1.4	18 ± 2.8
562	Not identified	0.5	8	0.064	1	No Resistance	47 ± 4.2	18 ± 3.5
563	Not identified	0.064	4	0.016	S	No Resistance	NT	NT
564	No sequence data	0.5	512	0.016	0.125	S	34 ± 0.0	22 ± 0.0
565	*Kocuria*	512	512	256	64	TSCA	33 ± 1.4	22 ± 0.7
566	Not identified	0.5	32	0.064	0.06	No Resistance	40 ± 2.1	30 ± 1.4
567	Not identified	0.5	512	0.125	1	S	47 ± 5.0	15 ± 0.0
568	Not identified	0.25	128	0.064	0.25	No Resistance	40 ± 1.4	21 ± 5.0
569	*Acidovorax*	0.5	8	0.064	128	A	NT	NT
570	*Cupriavidus*	0.5	512	0.064	512	SA	30 ± 1.4	14 ± 0.0
571	Not identified	4	128	0.032	512	A	25 ± 2.8	9 ± 0.0
572	Not identified	4	512	0.032	512	SA	30 ± 4.2	9 ± 0.0
573	Not identified	4	512	0.032	512	SA	28 ± 0.7	20 ± 0.7
574	Not identified	4	512	0.032	512	SA	27 ± 0.7	19 ± 0.0
575	Not identified	2	512	0.032	512	SA	34 ± 1.4	16 ± 1.4
576	*Sphingomonas*	2	512	0.032	512	SA	35 ± 0.7	17 ± 2.1
577	*Physcomitrella*	4	256	0.032	512	A	20 ± 0.7	15 ± 2.8
578	*Cupriavidus*	2	256	0.032	512	A	27 ± 2.8	9 ± 0.0
579	No sequence data	1	512	0.032	512	SA	22 ± 4.2	16 ± 1.4

580	*Cupriavidus*	1	512	0.032	512	SA	24 ± 2.8	10 ± 0.0
581	Not identified	2	512	0.064	512	SA	33 ± 2.8	16 ± 1.4
582	Not identified	2	512	0.064	512	SA	24 ± 0.7	16 ± 0.0
583	Not identified	4	256	0.064	512	A	30 ± 2.8	18 ± 1.4
584	Not identified	2	256	0.064	512	A	30 ± 2.1	17 ± 4.2
585	Not identified	2	512	0.064	512	SA	32 ± 2.8	18 ± 0.7
586	No sequence data	2	512	0.064	512	SA	32 ± 0.7	16 ± 2.1
587	*Cupriavidus*	2	512	0.064	512	SA	30 ± 0.0	19 ± 0.7
588	Not identified	2	512	0.064	512	SA	35 ± 1.4	20 ± 1.4
589	Not identified	2	512	0.064	512	SA	29 ± 1.4	19 ± 0.0
590	Not identified	2	512	0.064	512	SA	29 ± 1.4	20 ± 1.4
591	*Cupriavidus*	1	512	0.064	512	SA	28 ± 1.4	21 ± 0.0
592	Not identified	1	512	0.032	512	SA	26 ± 4.2	19 ± 1.4
593	Not identified	1	512	0.064	512	SA	30 ± 5.7	18 ± 0.7
594	Not identified	8	512	0.064	512	SA	32 ± 2.8	18 ± 0.7
595	*Pantoea*	0.5	128	0.016	1	No Resistance	35 ± 4.2	20 ± 0.0
596	No sequence data	0.5	64	0.064	512	A	30 ± 2.1	15 ± 0.0
597	*Dermacoccus*	4	256	0.064	64	A	30 ± 0.7	20 ± 0.0
598	Not identified	0.5	64	0.064	64	A	40 ± 2.8	24 ± 1.4
599	*Blastomonas*	0.125	8	0.064	1	No Resistance	30 ± 2.8	13 ± 0.0
600	*Variovorax*	0.5	32	0.064	64	A	37 ± 4.2	15 ± 0.0

601	Not identified	0.5	32	0.064	64	A	36 ± 5.7	16 ± 0.7
602	Not identified	0.5	128	0.064	64	A	36 ± 2.1	15 ± 0.7
603	*Dermacoccus*	4	512	32	1	SC	NT	NT
604	*Cupriavidus*	0.5	32	0.064	512	A	32 ± 0.7	18 ± 0.0
605	Not identified	0.125	128	0.064	1	No Resistance	42 ± 4.2	18 ± 0.7
606	No sequence data	0.5	32	0.064	64	A	37 ± 2.8	15 ± 1.4
607	*Dermacoccus*	0.5	512	0.125	1	S	NT	NT
608	*Dermacoccus*	4	512	32	64	SCA	40 ± 6.4	14 ± 2.8
609	*Ralstonia*	8	64	0.016	64	A	NT	NT
610	*Cupriavidus*	8	512	0.016	512	SA	30 ± 8.5	13 ± 4.2
611	*Escherichia*	8	32	0.016	128	A	45 ± 3.5	17 ± 0.7
612	No sequence data	8	64	0.016	64	A	NT	NT
613	*Ralstonia*	8	64	0.016	64	A	35 ± 3.5	17 ± 0.0
614	*Ralstonia*	8	64	0.016	64	A	38 ± 5.7	16 ± 1.4
615	*Burkholderia*	16	512	0.064	512	TSA	NT	NT
616	*Ralstonia*	8	32	0.016	0.5	No Resistance	NT	NT
617	*Burkholderia*	16	512	0.06	512	TSA	NT	NT
618	*Ralstonia*	8	8	0.016	64	A	44 ± 1.4	22 ± 1.4
619	*Ralstonia*	8	8	0.016	64	A	NT	NT
620	*Kocuria*	2	512	2	64	SA	30 ± 2.8	15 ± 0.7
621	Not identified	16	512	0.125	512	TSA	NT	NT

622	*Cupriavidus*	16	8	0.016	512	TA	21 ± 0.7	16 ± 0.0
623	*Paenibacillus*	8	8	0.064	32	A	35 ± 1.4	20 ± 0.0
624	Not identified	8	16	0.016	64	A	NT	NT
625	*Dietzia*	16	32	0.016	64	TA	44 ± 1.4	30 ± 0.0
626	*Burkholderia*	16	512	0.125	512	TSA	NT	NT
627	Not identified	8	4	0.032	64	A	NT	NT
628	No sequence data	8	8	0.016	64	TA	35 ± 5.0	15 ± 0.0
629	Not identified	16	32	0.016	64	TA	46 ± 2.8	30 ± 1.4
630	Not identified	16	32	0.016	32	A	50 ± 5.7	28 ± 1.4
631	*Staphylococcus*	16	512	0.016	4	TS	25 ± 3.5	16 ± 0.0
632	*Staphylococcus*	0.25	512	1	0.032	S	32 ± 2.8	19 ± 0.0
633	Not identified	S	32	0.008	0.125	No Resistance	41 ± 0.7	16 ± 0.0
634	*Paenibacillus*	S	8	0.004	0.125	No Resistance	33 ± 1.4	20 ± 2.8
635	*Paenibacillus*	S	32	0.004	0.064	No Resistance	NT	NT
636	Not identified	S	16	0.032	0.064	No Resistance	40 ± 1.4	25 ± 0.0
637	*Micrococcus*	S	512	0.016	0.125	S	45 ± 2.1	25 ± 0.0
638	*Micrococcus*	1	512	1	0.125	S	40 ± 1.4	20 ± 0.0
639	Not identified	0.5	512	0.5	0.125	S	47 ± 2.1	28 ± 0.0
640	*Bacillus*	8	512	0.016	512	SA	16 ± 2.8	13 ± 1.4
641	*Paenibacillus*	S	16	0.008	0.064	No Resistance	54 ± 2.1	36 ± 1.4
642	Not identified	32	16	0.008	0.5	T	20 ± 2.8	19 ± 0.0

643	*Burkholderia*	8	8	0.032	512	A	65 ± 4.2	25 ± 1.4
644	*Janibacter*	S	256	S	0.064	No Resistance	NT	NT
645	Not identified	8	16	0.032	0.064	No Resistance	24 ± 0.7	20 ± 0.0
646	Not identified	256	16	0.008	32	TA	40 ± 1.4	20 ± 0.0
647	Not identified	64	512	0.008	512	TSA	27 ± 1.4	16 ± 0.0
648	Not identified	256	512	512	512	TSCA	42 ± 2.1	18 ± 0.0
S. aureus		0.125	512	0.5	0.125	S	47 ± 4.2	40 ± 2.8
E. coli MC1000		128	32	S	64	TA	25±1.41	35 ± 0.0
E. coli NCTC 12241, ATCC25922		2	64	0.008	4	Sensitive, Control	13 ± 0.0	13 ± 2.8
Ps. aeruginosa PAO1		16	512	0.125	128	TSA	12 ± 0.7	14 ± 0.0

Resistance organisms: TET: $\geq$ 16 µg mL^{-1}, SMX: $\geq$ 512 µg mL^{-1}, CIP: $\geq$ 4 µg mL^{-1} and AMX: $\geq$ 32 µg mL^{-1}

*= submitted for publication elsewhere

Table 8-3 Statistical analysis for disk diffusion using the Tukey Method (Tukey pairwise comparison) and 95% Confidence.

Disinfectant treatment	N	Mean	Grouping	
Commercial Bleach 4.5%	127	18.72	A	
Standard NaOCl 14.5%	127	32.55		B

Table 8-4 Mean pH ± SD (n=3) for disinfectant suspension test for Chlorine.

		pH ± SD (n=3)					
Conc.	**Time**	***Cupriavidus***	***Arthrobacter***	***Bacillus***	***Burkholderia* (M)**	***Paenibacillus***	***Burkholderia* (S)**
C-1 (Bacteria)	0 min	7.0 ± 0.0	6.9 ± 0.2	6.9 ± 0.2	6.9 ± 0.1	7.0 ± 0.0	7.0 ± 0.0
	15 min	7.0 ± 0.0	6.9 ± 0.1	6.8 ± 0.2	7.0 ± 0.1	7.0 ± 0.0	7.0 ± 0.0
	60 min	7.0 ± 0.0	6.9 ± 0.1	6.8 ± 0.2	7.0 ± 0.0	7.0 ± 0.0	7.0 ± 0.0
0.5 mg L^{-1}	0 min	7.1 ± 0.0	7.0 ± 0.2	6.8 ± 0.2	6.9 ± 0.1	7.1 ± 0.0	7.0 ± 0.0
	15 min	7.0 ± 0.1	7.0 ± 0.2	6.8 ± 0.2	6.9 ± 0.1	7.0 ± 0.0	6.9 ± 0.1
	60 min	7.0 ± 0.1	7.0 ± 0.2	6.8 ± 0.2	6.9 ± 0.1	7.0 ± 0.0	6.9 ± 0.1
1.0 mg L^{-1}	0 min	7.1 ± 0.1	7.1 ± 0.3	6.8 ± 0.2	7.0 ± 0.1	7.0 ± 0.0	6.9 ± 0.1
	15 min	7.1 ± 0.1	7.1 ± 0.3	6.8 ± 0.2	6.9 ± 0.1	7.0 ± 0.0	7.0 ± 0.0
	60 min	7.0 ± 0.1	7.1 ± 0.3	6.8 ± 0.2	6.9 ± 0.1	7.0 ± 0.0	7.0 ± 0.0
2.0 mg L^{-1}	0 min	7.1 ± 0.1	7.1 ± 0.3	6.8 ± 0.2	6.9 ± 0.1	7.0 ± 0.0	6.9 ± 0.1
	15 min	7.1 ± 0.1	7.1 ± 0.3	6.8 ± 0.2	6.9 ± 0.1	7.0 ± 0.0	6.9 ± 0.1
	60 min	7.0 ± 0.1	7.2 ± 0.3	6.8 ± 0.1	6.9 ± 0.1	7.0 ± 0.0	6.9 ± 0.1
4.0 mg L^{-1}	0 min	7.1 ± 0.2	7.1 ± 0.3	6.8 ± 0.2	6.9 ± 0.1	7.0 ± 0.0	6.9 ± 0.2
	15 min	7.0 ± 0.1	7.1 ± 0.3	6.8 ± 0.2	6.9 ± 0.1	7.1 ± 0.0	6.9 ± 0.1
	60 min	7.0 ± 0.2	7.1 ± 0.3	6.8 ± 0.2	6.9 ± 0.1	7.1 ± 0.0	6.9 ± 0.1
8.0 mg L^{-1}	0 min	7.1 ± 0.1	7.1 ± 0.3	6.9 ± 0.3	6.9 ± 0.1	7.0 ± 0.0	7.0 ± 0.0
	15 min	7.1 ± 0.1	7.1 ± 0.3	6.8 ± 0.2	6.9 ± 0.1	7.0 ± 0.0	7.0 ± 0.0
	60 min	7.0 ± 0.1	7.1 ± 0.3	6.8 ± 0.2	6.9 ± 0.1	7.0 ± 0.0	7.0 ± 0.0
C-2(Cl_2)	0 min	7.0 ± 0.1	7.0 ± 0.2	6.9 ± 0.2	7.0 ± 0.0	7.0 ± 0.1	6.9 ± 0.1
	15 min	7.0 ± 0.1	6.9 ± 0.1	6.8 ± 0.2	7.0 ± 0.0	7.0 ± 0.1	6.9 ± 0.1
	60 min	7.0 ± 0.1	6.9 ± 0.1	6.8 ± 0.2	6.9 ± 0.1	7.0 ± 0.0	6.9 ± 0.1

C=control

Table 8-5 Mean Temperature ± SD (n=3) for disinfectant suspension test for Chlorine.

		Temperature °C ± SD (n=3)					
Conc.	**Time**	***Cupriavidus***	***Arthrobacter***	***Bacillus***	***Burkholderia* (M)**	***Paenibacillus***	***Burkholderia* (S)**
C-1 (Bacteria)	0 min	22.3 ± 1.0	22.3 ± 1.0	22.8 ± 1.7	22.3 ± 0.8	22.9 ± 0.2	21.7 ± 1.0
	15 min	22.3 ± 1.0	22.5 ± 0.5	22.9 ± 1.5	22.2 ± 0.9	22.8 ± 0.2	22.0 ± 1.0
	60 min	22.2 ± 0.7	22.3 ± 0.7	22.8 ± 1.3	22.1 ± 0.7	22.6 ± 0.3	21.8 ± 1.4
0.5 mg L^{-1}	0 min	22.5 ± 1.3	22.4 ± 0.6	22.6 ± 0.8	23.1 ± 0.7	23.1 ± 0.1	22.3 ± 1.1
	15 min	22.6 ± 1.2	22.5 ± 0.5	22.7 ± 0.8	23.1 ± 0.8	23.2 ± 0.4	22.0 ± 1.0
	60 min	22.3 ± 0.9	23.0 ± 1.3	23.0 ± 1.1	23.0 ± 1.0	22.9 ± 0.8	25.6 ± 5.0
1.0 mg L^{-1}	0 min	22.8 ± 1.4	22.5 ± 0.8	22.8 ± 0.9	22.7 ± 0.9	23.2 ± 0.4	22.0 ± 1.0
	15 min	22.8 ± 0.7	22.5 ± 0.8	22.5 ± 0.8	22.4 ± 0.8	23.2 ± 0.4	22.0 ± 1.0
	60 min	22.8 ± 1.0	23.1 ± 2.1	22.6 ± 1.4	22.4 ± 0.8	23.0 ± 0.6	22.0 ± 1.0
2.0 mg L^{-1}	0 min	22.7 ± 1.3	22.5 ± 0.6	22.2 ± 1.4	23.0 ± 0.8	22.9 ± 0.4	21.9 ± 1.4
	15 min	23.2 ± 1.3	22.6 ± 0.6	22.3 ± 1.4	22.7 ± 0.9	22.9 ± 0.4	21.9 ± 1.2
	60 min	22.7 ± 0.7	23.0 ± 1.3	22.4 ± 1.6	22.7 ± 0.7	23.2 ± 0.6	21.9 ± 1.4
4.0 mg L^{-1}	0 min	22.4 ± 1.2	22.7 ± 0.6	22.7 ± 1.4	22.7 ± 0.7	22.8 ± 0.3	22.0 ± 1.0
	15 min	22.5 ± 1.1	22.6 ± 0.4	22.8 ± 1.5	22.5 ± 0.7	23.1 ± 0.2	21.9 ± 1.2
	60 min	22.4 ± 0.7	22.8 ± 0.8	23.2 ± 1.1	22.4 ± 0.6	23.2 ± 0.7	21.9 ± 1.2
8.0 mg L^{-1}	0 min	22.8 ± 1.5	22.8 ± 0.7	23.1 ± 1.1	22.8 ± 0.7	22.6 ± 0.6	22.0 ± 1.0
	15 min	22.7 ± 1.1	22.7 ± 0.6	23.3 ± 1.1	22.0 ± 1.0	22.9 ± 0.5	22.0 ± 1.0
	60 min	22.5 ± 0.7	22.4 ± 0.3	23.0 ± 1.3	22.0 ± 1.1	22.7 ± 0.5	22.0 ± 1.0
C-2(Cl_2)	0 min	22.1 ± 1.2	22.5 ± 1.1	22.5 ± 1.6	22.0 ± 1.2	23.2 ± 0.5	22.0 ± 1.0
	15 min	22.4 ± 0.8	22.3 ± 1.0	22.5 ± 1.6	22.0 ± 1.3	22.9 ± 0.1	22.0 ± 1.0
	60 min	22.3 ± 0.8	22.0 ± 0.8	22.8 ± 1.1	22.0 ± 1.4	22.9 ± 0.3	21.9 ± 1.0

C=control

Table 8-6 Mean pH ± SD (n=3) for disinfectant suspension test for Monochloramine.

		pH ± SD (n=3)					
Conc.	**Time**	***Cupriavidus***	***Arthrobacter***	***Bacillus***	***Burkholderia* (M)**	***Paenibacillus***	***Burkholderia* (S)**
C-1 (Bacteria)	0 min	8.1 ± 0.2	7.9 ± 0.1	8.0 ± 0.1	8.0 ± 0.0	8.0 ± 0.1	8.0 ± 0.2
	15 min	8.1 ± 0.1	7.9 ± 0.1	8.0 ± 0.1	7.9 ± 0.0	8.0 ± 0.1	8.0 ± 0.1
	60 min	8.0 ± 0.0	7.9 ± 0.1	8.3 ± 0.7	7.9 ± 0.0	8.0 ± 0.2	8.0 ± 0.2
0.5 mg L^{-1}	0 min	8.1 ± 0.2	7.9 ± 0.2	8.0 ± 0.1	7.9 ± 0.0	8.0 ± 0.0	8.0 ± 0.1
	15 min	8.1 ± 0.1	7.9 ± 0.2	8.0 ± 0.1	7.9 ± 0.0	8.0 ± 0.1	8.0 ± 0.1
	60 min	8.0 ± 0.1	7.9 ± 0.2	8.0 ± 0.2	7.9 ± 0.0	8.0 ± 0.1	8.0 ± 0.1
1.0 mg L^{-1}	0 min	8.1 ± 0.2	7.9 ± 0.2	8.0 ± 0.2	7.9 ± 0.0	8.0 ± 0.1	8.0 ± 0.1
	15 min	8.1 ± 0.1	7.9 ± 0.1	8.0 ± 0.1	7.9 ± 0.0	8.0 ± 0.1	8.0 ± 0.1
	60 min	8.0 ± 0.1	7.9 ± 0.1	8.0 ± 0.1	7.9 ± 0.0	8.0 ± 0.1	8.0 ± 0.1
2.0 mg L^{-1}	0 min	8.1 ± 0.2	7.8 ± 0.2	8.0 ± 0.1	7.9 ± 0.0	8.0 ± 0.0	8.0 ± 0.1
	15 min	8.1 ± 0.1	7.8 ± 0.2	8.0 ± 0.2	7.9 ± 0.0	8.0 ± 0.0	8.0 ± 0.2
	60 min	8.0 ± 0.1	7.8 ± 0.2	8.0 ± 0.1	7.9 ± 0.0	8.0 ± 0.1	8.0 ± 0.1
4.0 mg L^{-1}	0 min	8.1 ± 0.2	7.8 ± 0.3	8.0 ± 0.1	7.9 ± 0.0	8.0 ± 0.0	8.0 ± 0.1
	15 min	8.0 ± 0.1	7.8 ± 0.3	8.0 ± 0.1	7.9 ± 0.0	8.0 ± 0.0	8.0 ± 0.1
	60 min	8.0 ± 0.1	7.8 ± 0.3	8.0 ± 0.1	7.9 ± 0.0	8.0 ± 0.0	8.0 ± 0.1
8.0 mg L^{-1}	0 min	8.1 ± 0.2	7.7 ± 0.3	8.0 ± 0.1	7.9 ± 0.1	8.0 ± 0.1	8.0 ± 0.1
	15 min	8.0 ± 0.1	7.7 ± 0.4	8.0 ± 0.1	7.9 ± 0.1	8.0 ± 0.1	8.0 ± 0.1
	60 min	8.0 ± 0.1	7.7 ± 0.4	8.0 ± 0.1	7.9 ± 0.1	8.0 ± 0.1	8.0 ± 0.1
C-2 (NH_2Cl)	0 min	8.1 ± 0.1	7.9 ± 0.1	7.9 ± 0.0	7.9 ± 0.0	7.9 ± 0.2	8.0 ± 0.2
	15 min	8.0 ± 0.1	7.9 ± 0.1	7.9 ± 0.0	7.9 ± 0.0	7.9 ± 0.1	8.0 ± 0.1
	60 min	8.0 ± 0.1	7.9 ± 0.1	7.9 ± 0.1	7.9 ± 0.0	8.0 ± 0.1	8.0 ± 0.1

C=control

Table 8-7 Mean Temperature ± SD (n=3) for disinfectant suspension test for Monochloramine.

		Temperature °C ± SD (n=3)					
Conc.	**Time**	***Cupriavidus***	***Arthrobacter***	***Bacillus***	***Burkholderia* (M)**	***Paenibacillus***	***Burkholderia* (S)**
C-1 (Bacteria)	0 min	21.7 ± 1.0	23.9 ± 0.6	22.5 ± 1.0	23.0 ± 1.4	22.8 ± 0.3	22.9 ± 0.6
	15 min	21.8 ± 0.6	23.6 ± 0.7	22.1 ± 0.7	22.5 ± 1.0	22.6 ± 0.6	22.8 ± 0.1
	60 min	21.7 ± 0.6	22.5 ± 1.4	22.2 ± 0.7	22.4 ± 0.9	23.0 ± 0.2	22.6 ± 0.3
0.5 mg L^{-1}	0 min	22.0 ± 1.1	23.4 ± 0.6	22.8 ± 0.5	22.2 ± 1.4	22.9 ± 0.4	23.2 ± 0.6
	15 min	22.0 ± 0.8	23.2 ± 0.7	22.8 ± 0.5	22.3 ± 1.3	23.1 ± 0.6	23.5 ± 0.5
	60 min	22.1 ± 1.1	23.3 ± 0.6	22.9 ± 0.5	22.3 ± 1.2	23.0 ± 0.7	23.3 ± 0.7
1.0 mg L^{-1}	0 min	22.2 ± 1.0	23.8 ± 0.4	22.8 ± 0.6	22.7 ± 1.4	22.6 ± 0.3	23.0 ± 0.1
	15 min	22.2 ± 0.9	23.7 ± 0.1	23.0 ± 0.6	22.2 ± 1.2	22.8 ± 0.3	23.0 ± 0.1
	60 min	22.3 ± 1.0	23.7 ± 0.6	23.0 ± 0.8	22.5 ± 0.9	22.9 ± 0.5	23.5 ± 0.5
2.0 mg L^{-1}	0 min	22.1 ± 1.2	23.4 ± 0.6	22.5 ± 0.7	22.5 ± 1.1	22.8 ± 0.3	22.8 ± 0.2
	15 min	22.2 ± 0.9	23.4 ± 0.5	22.5 ± 0.6	22.2 ± 0.9	22.9 ± 0.3	22.8 ± 0.3
	60 min	22.0 ± 1.1	23.3 ± 0.6	22.6 ± 0.4	22.4 ± 0.8	23.0 ± 0.4	23.0 ± 0.6
4.0 mg L^{-1}	0 min	22.2 ± 1.0	23.1 ± 0.8	22.5 ± 0.5	22.7 ± 1.1	22.8 ± 0.2	22.9 ± 0.3
	15 min	21.9 ± 0.8	23.1 ± 0.6	22.5 ± 0.3	22.4 ± 0.8	23.2 ± 0.2	23.0 ± 0.4
	60 min	22.1 ± 1.3	22.6 ± 1.2	22.9 ± 0.8	22.7 ± 0.9	23.2 ± 0.5	23.2 ± 0.2
8.0 mg L^{-1}	0 min	22.0 ± 1.0	23.0 ± 1.0	22.5 ± 0.8	22.5 ± 1.0	23.2 ± 0.2	23.2 ± 0.4
	15 min	21.8 ± 0.8	22.7 ± 1.0	22.4 ± 0.4	22.9 ± 1.3	23.2 ± 0.5	23.0 ± 0.3
	60 min	22.1 ± 1.4	22.3 ± 1.2	22.8 ± 0.8	22.8 ± 1.3	23.1 ± 0.5	23.3 ± 0.2
C-2 (NH_2Cl)	0 min	22.0 ± 0.9	23.5 ± 0.3	22.8 ± 0.9	22.8 ± 1.0	22.8 ± 0.2	23.2 ± 1.2
	15 min	21.8 ± 0.7	23.3 ± 0.9	22.1 ± 0.5	22.5 ± 1.0	22.7 ± 0.2	23.0 ± 0.2
	60 min	21.7 ± 0.6	22.7 ± 1.5	21.9 ± 0.7	22.1 ± 1.4	22.6 ± 0.4	22.9 ± 0.5

C=control