Controlling waterborne antimicrobial resistant healthcare associated infections

By

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LIST OF ABBREVIATIONS

AGRTP	Australian government research training program
ATCC	American Type Culture Collection
B	Biofilm forming
BAC	Benzalkonium chloride
BCYE	Buffered charcoal yeast extract
BSE	Back scattered electron
C	Corrosive
CAP	Canonical analysis of principal coordinates
CAZ	Ceftazidime
CDC	Centers for Disease Control and Prevention
CFU	Colony forming units
CFX	Cefoxitin
CIP	Ciprofloxacin
CLSI	Clinical and laboratory standards institute
CN	Gentamicin
CRAB	Carbapenem resistant *Acinetobacter baumannii*
CRE	Carbapenem resistant Enterobacterales
CRPA	Carbapenem resistant *Pseudomonas aeruginosa*
DOR	Doripenem
DWDS	Drinking water distribution system
DWG	Drinking water guideline
DWPI	Drinking water associated pathogens that can cause infections in immunocompromised or otherwise susceptible individuals
EDAX	Energy dispersive X-ray analysis
eDNA	Environmental DNA
EDS	Energy dispersive spectroscopy
EPS	Extracellular polymeric substances
ESBL	Extended-spectrum beta-lactamases
EU	European Union
EUCAST	The European committee on antimicrobial susceptibility testing
FAM	Fluorescein amidites
FEP	Cefepime
FISH	Fluorescence in situ hybridization
FLA	Free living amoeba

FQ	Fluorescent quencher
FSC	Forward scatter
g	Gram
GU	Genomic unit
GVPC	Glycine vancomycin polymyxin cycloheximide
HAIs	Healthcare associated infections
HD	Hospital drain
HF	Hospital faucet
HGT	Horizontal gene transfer
HI-FBS	Heat-inactivated fetal bovine serum
HPC	Heterotrophic plate count
Hr	Hour
I	Intermediate
IBC	Institutional biosafety committee
IMI	Imipenem
ISO	International organization for standardization
KPC	Klebsiella pneumoniae carbapenemase-producing bacteria
L	Litre
mL	Millilitre
LEV	Levofloxacin
M	Mole
MAC	Mycobacterium avium complex
MDR	Multi-drug resistant
MER	Meropenem
MIC	Microbially influenced corrosion
Min	Minute
mm	Millimetre
MRSA	Methicillin resistant *Staphylococcus aureus*
NSW	New South Wales
NTM	Non tuberculous mycobacteria
OD	Optical density
OPPPs	Opportunistic premise plumbing pathogens
OTU	Operational taxonomic unit
P	Pathogenic genera
PBS	Phosphate buffered saline
PC2	Physical containment facility level 2

PCoA	Principal coordinates analysis
PCR	Polymerase chain reaction
PEN	Penicillin
PERMANOVA	Permutational analysis of variance
pg	Picogram
PI	Propidium iodide
PIP	Piperacillin
PMA	Propidium monoazide
POU	Point of use
ppb	Parts per billion
PYG	Peptone yeast glucose broth
Q	Quencher
QAC	Quaternary ammonium compound
qPCR	Quantitative polymerase chain reaction
R	Resistant
RD	Residential drain
RF	Residential faucet
S	Sensitive
s	Second
SBREC	Social and behavioural research ethics committee
SD	Standard deviation
SE	Standard error
SE	Secondary electron
sg	Serogroup
SIMPER	Similarity percentages
SSC	Side scatter
SXT	Trimethoprim sulfamethoxazole
TO	Thiazole orange
TOB	Tobramycin
TZP	Piperacillin tazobactam
UK	United Kingdom
µM	Micrometre
US	United States
USD	United States dollar
US EPA	United States Environmental Protection Authority
VBNC	Viable but non culturable

VFC+qPCR	Viability based flow cytometry + quantitative polymerase chain reaction
VRE	Vancomycin-resistant Enterococci
WHO	World Health Organisation

DECLARATION

I certify that this thesis:

1. does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university

2. and the research within will not be submitted for any other future degree or diploma without the permission of Flinders University; and

3. to the best of my knowledge and belief, does not contain any material previously published or written by another person except where due reference is made in the text.

Signed.....Claire Hayward.....

Date.....12/12/2024.....

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STATEMENT OF CO-AUTHORSHIP

The following people contributed to the publication of the work undertaken as part of this thesis, The co-authors are list in the order that the co-authored publications appear in the thesis:

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PUBLICATIONS

Published manuscripts

Hayward, C., Ross, K. E., Brown, M. H., Bentham, R., & Whiley, H. (2022). The presence of opportunistic premise plumbing pathogens in residential buildings: A literature review. Water, 14(7). https://doi.org/10.3390/w14071129

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Hayward, C., Ross, K. E., Brown, M. H., Nisar, M. A., Hinds, J., Jamieson, T., Leterme, S. C., & Whiley, H. (2024). Handwashing basins and healthcare associated infections: Bacterial diversity in biofilms on faucets and drains. *Science of The Total Environment*, *949*, 175194. https://doi.org/https://doi.org/10.1016/j.scitotenv.2024.175194

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Unpublished manuscripts currently under review

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This thesis is based on published and un-published manuscripts, therefore some unavoidable repetition between chapters occurs.

The thesis was written in Australian English; however, words in American English will appear in some chapters that include published and unpublished manuscripts due to the language style used by the journal.

ABSTRACT

Opportunistic premise plumbing pathogens (OPPPs) are an emerging public health threat. These are waterborne pathogens share characteristics such as biofilm formation, survival in low nutrient environments, disinfectant resistance and growth in amoeba hosts that enable them to persist in drinking water plumbing systems. Globally, the incidence of OPPP healthcare associated infections (HAI) is increasing, and the significance and severity of these infections is also increasing due to the rise in antimicrobial resistance.

The aim of this thesis was to explore factors influencing OPPPs in Australian hospital and residential drinking water plumbing systems. This included investigating prevalence, antimicrobial resistance, interactions with protozoan hosts and the effects of factors such as building type, product design, and plumbing materials on the growth and proliferation of these pathogens. The data presented in this thesis can be used to inform future guidelines to mitigate infection risks in healthcare and residential environments.

A systematic literature review found that residential buildings are often overlooked as a source of waterborne infections. However, they are significant reservoirs for OPPPs such as Legionella pneumophila, Pseudomonas aeruginosa, Acinetobacter baumannii and Mycobacterium avium complex (MAC). These environments were also found to harbor non-waterborne pathogens such Staphylococcus aureus and Enterobacteriaceae, challenging the traditional focus of guidelines that prioritize healthcare facilities and traditionally waterborne bacteria.

Molecular surveillance of Australian hospitals and residential drinking water plumbing systems revealed widespread colonisation by P. aeruginosa (41%), S. aureus (26%), Legionella spp. (26%), L. pneumophila (24%), and A. baumannii (14%). Residential properties had a statistically higher prevalence of OPPPs, particularly associated with biofilm colonization. Additionally, drain biofilms were identified as the primary reservoirs for key antimicrobial resistant (AMR) threats identified by the World Health Organization (WHO) and Centers for Disease Control and Prevention including carbapenem resistant A. baumannii and P. aeruginosa, and methicillin resistant S. aureus. This study is the first to explore these risks within Australian healthcare and residential settings, providing insights into the role of drain biofilms as hidden contributors to the global AMR crisis. However, the influence of building characteristics, including stagnation, hot water system type, and building age, on pathogen prevalence was inconsistent, underscoring the complexity of these environments. A complementary 16S rRNA analysis of biofilms from hospital and residential handwashing

basins revealed diverse prokaryotic communities, with high abundances of potentially corrosive, biofilm-forming, and pathogenic genera, including those not typically waterborne. β-diversity analysis showed a statistically significant difference in bacterial communities between building types (hospital vs. residential, $p = 0.0415$). However, in hospitals, there was no significant difference between drain and faucet biofilms, suggesting that microbial communities are highly similar between these two sites. This study highlights the potential mechanisms of microbial transfer between these sites and discusses how interventions aimed at one site could influence the microbial dynamics of the other.

Handwashing basins were identified as a niche for diverse OPPP communities in the earlier stages of this thesis. These findings informed the development of a model system designed to investigate transmission pathways, including aerosolization and retrograde contamination, under controlled conditions. This model demonstrated that lower flow rates led to increased aerosol production from contaminated drains ($p=0.021$) and greater retrograde contamination from the drain up to the faucet compared to higher flow rates. This suggests that water-saving recommendations, which often promote lower flow rates, may inadvertently increase the risk of microbial transmission. This is particularly concerning in healthcare settings, where infection control must take precedence over water conservation goals. Future guidelines must consider the potential conflict between infection control measures and water-saving initiatives, particularly for vulnerable populations in hospital or home healthcare settings.

To understand the potential public health implications of a legislative change promoting a shift away from brass to "lead-free" alternative plumbing materials such as stainless steel, a laboratory model study investigated the effects of plumbing materials on biofilm formation, OPPP growth, and lead leaching under stagnant conditions. Bioreactors inoculated with *A. baumannii*, *P. aeruginosa*, MAC, and *Acanthamoeba polyphaga* were analysed using selective culture and viability-based flow cytometry. Each OPPP responded differently to brass and stainless steel, with brass exhibiting greater antimicrobial activity. However, both materials leached lead above WHO guidelines after 10 weeks of stagnation, with stainless steel showing lead deposits likely from post manufacturing contamination. These results emphasize the importance of evidence-based plumbing material selection that considers both microbial and chemical risks.

This research advocates for a dynamic multi-barrier approach to OPPP surveillance and drinking water treatment, emphasizing the need for evidence-based, multidisciplinary regulatory decisions to effectively control AMR waterborne HAIs. Collaboration between

microbiologists, engineers, and public health professionals with inform future guidelines to better protect vulnerable individuals and improve water quality.

1. INTRODUCTION

This chapter provides background information on opportunistic premise plumbing pathogens (OPPPs) and their importance from a healthcare infection control perspective. It also describes factors associated with the growth and proliferation of these pathogens in both residential and hospital drinking water systems, and current approaches used to detect and characterise the risk in both drinking water and biofilm samples. This chapter concludes by detailing the aims and objectives for this thesis.

1.1. Healthcare associated infections

Healthcare-associated infections (HAIs) occur in patients receiving medical treatment or those who are in long term care (Centers for Disease Control and Prevention, 2017). This may take place in hospitals, nursing homes, aged care facilities and office-based practices such as dental clinics and general practices as well as those receiving healthcare at home (Haque et al., 2018). Common types of HAIs include surgical site infections, bloodstream infections, urinary tract infections, and pneumonia, which can complicate treatment and prolong hospital stays (Centers for Disease Control and Prevention, 2014). The United States (US) Centers for Disease Control and Prevention (CDC) estimates that over 1.7 million HAIs occur annually in the US alone, resulting in approximately 99,000 associated deaths (Centers for Disease Control and Prevention, 2019c). These infections are often caused by opportunistic pathogens that exploit niches in the healthcare environment (Spelman, 2002).

1.2. Opportunistic premise plumbing pathogens

OPPPs are a group of microorganisms that share characteristics such as resistance to disinfection, survival in free living amoeba hosts and biofilm formation, that increase their ability to persist in drinking water plumbing systems (Falkinham, 2015). In recent literature, these pathogens are also referred to as “drinking water associated pathogens that

predominantly cause infections in immunocompromised individuals" or DWPI (Proctor et al., 2022). This definition has been proposed as a broader umbrella term that encompasses diverse genera that can grow in drinking water systems, particularly in biofilms formed on water fixtures. However, throughout this thesis, they are referred to as OPPPs for consistency. These pathogens, including *Legionella pneumophila*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Mycobacterium avium* complex, can thrive in drinking water plumbing systems, and cause severe infections in immunocompromised individuals, the elderly, and newborns (Falkinham et al., 2015). Unlike gastrointestinal pathogens that are often transmitted through drinking the water, OPPPs infections can also be from inhaling, aspirating or coming into contact with contaminated water sources through wounds or medical devices (Collier et al., 2021). As a result, drinking water plumbing systems in hospitals, residential properties, and other buildings are increasingly recognized as reservoirs of these pathogens. Efforts to combat waterborne antimicrobial resistance must include targeted surveillance and monitoring of hospital water systems, implementation of effective water management plans, and adoption of a collaborative evidence-based approach to infection control and drinking water treatment.

1.2.1. Drinking water

Drinking water, also commonly called potable water, refers to water that is intended for human consumption, whether it is from a municipal treated drinking water distribution system, from rainwater harvesting, groundwater or surface water sources (National Health and Medical Research Council, 2011). However, even within thoroughly treated drinking water systems, OPPPs can persist. The Australian Drinking Water Guidelines outline methods to manage drinking water quality that balance the risk of pathogen persistence with the aesthetic quality of water (National Health and Medical Research Council, 2011). However, conditions where water stagnates, residual disinfection diminishes, or temperatures rise to favour microbial growth may contribute to deteriorated microbial water quality (Falkinham, 2015; Nisar et al., 2023b). This thesis explores the potential health risks

associated with the presence of OPPPs in drinking water, and the efficacy of current treatment approaches.

1.2.2. Biofilms

Biofilms in drinking water plumbing systems are a key contributor to microbial growth and proliferation (Batté et al., 2003). Biofilms are communities of microorganisms, including bacteria, yeast, fungi, protozoa, and viruses, that are embedded within an extracellular polymeric substance (EPS) matrix (Mann et al., 2012; Yu et al., 2010). The diverse microorganisms within these biofilms exhibit different phenotypes compared with planktonic cells, particularly when they interact with other organisms that are in close proximity within the biofilm (Liu et al., 2016; Mann et al., 2012; Mazzotta et al., 2020). Biofilms can also provide physical protection from routine disinfection methods and sheer stress (LeChevallier et al., 2024; Nisar et al., 2023b). Bacteria in biofilms can also have significantly higher resistance to antibiotics and disinfectants compared with planktonic cells, which are influenced by both genetic and environmental factors (Shree et al., 2023). The biofilm EPS matrix provides a physical and chemical barrier that protects the bacteria and creates a microenvironment with altered pH, ionic gradients, and diffusion of antimicrobial agents (Muhammad et al., 2020). These barriers, combined with the production of EPS, create a physical resistance by trapping and neutralizing disinfectants (Muhammad et al., 2020; Shree et al., 2023). Biofilms also facilitate genetic resistance through horizontal gene transfer within the EPS matrix, enabling the dissemination of antimicrobial resistance genes between the bacterial population (Abe et al., 2020). For example, bacteria in biofilms have been found to be up to 3000 times more resistant to free chlorine than planktonic cells. This poses a significant challenge in maintaining safe drinking water and controlling OPPP proliferation (LeChevallier et al., 2024; Soto-Giron et al., 2016). This combination of genetic resistance mechanisms and the protective biofilm environment underscores the complexity of addressing OPPP growth in drinking water plumbing systems and highlights the need of tailored control strategies. This thesis explores the dynamics of OPPPs in drinking water plumbing biofilms, and how abiotic factors influence prevalence and community composition.

1.2.3. Antimicrobial resistance

Antimicrobial resistance represents a significant and growing threat to global public health. It has been estimated that in 2019, antimicrobial resistance contributed to more than 4.95 million deaths globally, and is predicted to overtake cancer as the leading cause of death by 2050 (World Health Organization, 2020a). Antimicrobial resistant (AMR) pathogens are of particular concern in healthcare settings, where they complicate HAIs and result in increased morbidity and mortality rates. The World Health Organisation (WHO) has highlighted AMR *A. baumannii, P. aeruginosa* and *S. aureus* as critical and high priority pathogens of concern (World Health Organization, 2015, 2020a). In water related devices such as showers and handbasins, these pathogens can persist in biofilms that are difficult to eliminate but are in direct contact with vulnerable people (LeChevallier et al., 2024). The protective environment of biofilms not only enhances the survival of these pathogens against disinfectants but also facilitates the transfer of resistance genes among microbial communities (Abe et al., 2020). As rates of antimicrobial resistance increases, there is a need for comprehensive strategies to monitor, control, and reduce its impact on drinking water management and infection control practices.

CITATION: The presence of opportunistic premise plumbing pathogens in residential buildings: A systematic review

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1.2.4. Abstract

Opportunistic premise plumbing pathogens (OPPP) are microorganisms native to the plumbing environment and present an emerging infectious disease problem. They share characteristics such as disinfectant resistance, thermal tolerance, and biofilm formation. Colonisation of domestic water systems presents an elevated health risk for immune compromised individuals receiving healthcare at home. Literature that identified the previously described OPPPs *Aeromonas* spp., *Acinetobacter* spp., *Helicobacter* spp., *Legionella* spp., *Methylobacterium* spp., *Mycobacteria* spp., *Pseudomonas* spp. and *Stenotrophomonas* spp. in residential drinking water systems were systematically reviewed. By applying the PRISMA guidelines, 214 studies were identified from Scopus and Web of Science databases, including 30 clinical case investigations. Tap components and showerheads were the most frequently identified source of OPPPs. Sixty four of these studies detected additional clinically relevant pathogens that are not classified as OPPPs in these reservoirs. There was considerable variation in detection methods including traditional culturing and molecular approaches. These identified studies demonstrate that current drinking water treatment methods are ineffective against many waterborne pathogens. It is critical that as healthcare at home services continue to be promoted, we must understand the emergent risks posed by OPPPs in residential drinking water. Future research is needed to provide consistent data on the prevalence of OPPPs in residential water and the incidence of waterborne home care associated infections. This will enable the contributing risk factors to be identified and effective controls developed.

1.2.5. Introduction

Access to safe drinking water and sanitation has been recognized by the United Nations General Assembly as a human right (World Health Organization, 2019). Diseases such as cholera and typhoid have decreased in developed countries due to effective drinking water disinfection and distribution (World Health Organization, 2019). Recognition of additional waterborne illnesses such as pneumonia, bloodstream infections and skin diseases has increased in recent decades (Centers for Disease Control and Prevention, 2020b; Collier et

al., 2021). The World Health Organization has estimated that in 2016, 1.9 million deaths could have been prevented with access to safe water, sanitation, and hygiene (World Health Organization, 2019). Despite approximately 94% of the United States (US) population having access to public water systems, there are an estimated 7.2 million waterborne infections each year (Centers for Disease Control and Prevention, 2020b; U.S. Environmental Protection Agency, 2020a). Of these, the CDC has estimated that over 2.3 million waterborne enteric illnesses and 96,000 waterborne respiratory illnesses were acquired domestically (Collier et al., 2021). This disease transmission is commonly attributed to aging infrastructure, private unregulated systems, and inconsistent disinfection protocols (Dieter et al., 2018). Once municipal water reaches residential properties, microbial water quality can be difficult to maintain due to warm and cold water outlets, showers and home appliances creating unique environmental niches (Neu et al., 2020).

Opportunistic premise plumbing pathogens (OPPPs) are waterborne microorganisms that inhabit water distribution systems and premises plumbing (Falkinham, 2015). OPPPs have been distinguished from other drinking water contaminants as they are adapted to growth and proliferation in drinking water systems (Falkinham et al., 2015). This growth can be promoted and influenced by water stagnation, increased water residence times, application of subinhibitory disinfectant concentrations and fluctuating water temperatures (Mogoa et al., 2011; Moore et al., 2006). Due to the complex design and age of residential plumbing infrastructure, maintaining parameters such as these is an ongoing challenge (Wang et al., 2012). OPPPs share characteristics such as disinfectant resistance, biofilm formation, amoeba digestion resistance, and growth under oligotrophic conditions. Although *Legionella pneumophila*, *Pseudomonas aeruginosa* and *Mycobacterium avium* have been considered model OPPP, the definition has expanded to include species such as *Acinetobacter baumannii*, *Stenotrophomonas maltophila*, *Helicobacter pylori*, *Aeromonas hydrophila* and *Methylobacterium* spp. (Falkinham, 2015). Of all waterborne disease transmission in the US in 2014, Legionnaires' disease, pneumonia caused by *Pseudomonas* spp. and non-tuberculous Mycobacteria infection have been attributed with the largest number of deaths

(Collier et al., 2021). *Legionella* spp. infection almost exclusively presents as a non-transmissible respiratory infection such as Legionnaires' disease or Pontiac fever (Nisar et al., 2020a). Whereas other OPPPs such as *P. aeruginosa, Mycobacteria* spp. and *A. baumannii* each cause a range of potentially transmissible and antimicrobial resistant infections including pneumonia, septicaemia and dermal infection, further complicating their management (Falkinham, 2015).

Legionnaires' disease is the only OPPP caused infection that is a nationally notifiable disease in the US. The CDC reported that in 2018 there were approximately 10,000 cases of Legionnaires' disease (Collier et al., 2021). However, it has been suggested that the incidence of Legionnaires' disease was underestimated, and the true number of cases may be 1.8 to 2.7 times higher than what is reported. Additionally, the origins of these infections are rarely identified as environmental sampling is typically only conducted in response to extended outbreaks. Outbreaks in domestic settings are less easily detected due to the inherent low numbers of exposed occupants at individual premises; though the sum total of exposed individuals is likely to exceed those in large buildings. As such, it is difficult to quantify the total public health risk associated with various environmental reservoirs The elderly, newborns, and those with compromised immune systems are especially vulnerable to waterborne infections. The number of individuals with conditions that may put them at risk of OPPP infection such as advanced age, cancer, and immunodeficiency are increasing (Collier et al., 2021). Life expectancy has increased by more than six years since 2000 and the number of cancer diagnoses worldwide is set to increase by 47% in 2040 (World Health Organization, 2020b). Healthcare at home has been emerged as an alternative to extensive inpatient hospital stays (de Sousa Vale et al., 2019; Di Mascolo et al., 2017). Services such as chemotherapy, tracheotomy care, and ventilator support are being facilitated by government healthcare and disability support schemes in countries such as the United Kingdom, US, and Australia (Houston et al., 2020; Landers et al., 2016; Montalto et al., 2020). These 'at home' alternatives are receiving further attention in the wake of the COVID-19 pandemic to reduce the burden on the healthcare system and to support those with

potential long term respiratory side effects (Brocard et al., 2021). When in a hospital or healthcare facility, a patient's risk of healthcare-associated infection (HAI) and exposure to environmental risks has been minimised by implementing infection control and prevention guidelines with varying success (Australian Commission on Safety and Quality in Healthcare, 2019; Centers for Disease Control and Prevention, 2017). Despite such initiatives, the US CDC reported significant increases in four of the six monitored HAIs from 2019-20 even with decreased surveillance activities due to shortages in personnel and equipment (Weiner-Lastinger et al., 2021). Conversely, patients receiving healthcare in residential properties may have poor access to plumbing, sanitation, and ventilation that are overlooked by these guidelines (Di Mascolo et al., 2017). Major outbreaks of OPPP infection are typically associated with larger buildings, such as hospitals, resulting in drinking water guidelines focusing on the unique risks posed by this infrastructure. Without consistent environmental surveillance of residential properties, which can vary significantly in size, age, occupancy, and infrastructure quality, it is difficult to identify and quantify the unique risks posed by OPPPs in in these settings. If healthcare services continue to move patient care away from the hospital environment, further research is required to identify and quantify potential risks and tailor infection surveillance and prevention guidelines to the patient and their property.

Previous literature reviews have identified and characterized emerging OPPPs to increase awareness and encourage novel control procedures in healthcare settings (Cervia et al., 2008; Collier et al., 2021; Deshmukh et al., 2016; Falkinham et al., 2015; Hayward et al., 2020; Kanamori et al., 2016; Soto-Giron et al., 2016). It is evident that OPPPs and other clinically relevant bacterial species are present in drinking water and pose a significant public health threat to many demographics. However, this risk is not reflected appropriately in current infection control and surveillance guidelines as water related devices are consistently underestimated as sources of infection in outbreak response investigation protocols (Anaissie et al., 2002). This systematic review uniquely focuses on the role of residential drinking water in the transmission of OPPP infection. Common water related devices,

surveillance protocols and detection methods are discussed. It is essential that water industry, homeowners and healthcare providers understand the scope and risks posed by OPPPs in residential buildings. This will facilitate the implementation of effective control protocols that can be implemented as health care at homes services are progressed as an alternative to hospital admission for at risk individuals.

1.2.6. Materials and methods

This systematic review was based on an adapted version of the PRISMA statement presented in Figure 1.1 (Page et al., 2021). Relevant studies were identified using two different databases, Web of Science and Scopus, using the search terms presented in Table 1.1. A detailed search strategy was established to ensure a thorough review of all identified OPPP in residential drinking water.

Table 1.1 Complete search strategy and all keywords used to identify relevant literature.

Search terms employed to identify relevant literature
Stenotrophomonas OR *Aeromonas* OR *Acinetobacter* OR *Legionella* OR *Mycobacterium** OR "nontuberculous mycobacteria*" OR *Pseudomonas* OR *Methylobacterium* or *Helicobacter* or "opportunistic premise plumbing pathogen*" or "opportunistic waterborne pathogen*" or "legionnaires disease" or legionellosis or "pontiac fever" or pneumonia
AND
Home or house or residence* or domestic or household or private
AND
Water or potable or shower or tap* or drain or bath or sink or bathroom or plumbing or faucet or biofilm or aerosol or "drinking water"

'' indicates wildcard symbol used when variations of the search term may be possible*

All titles and abstracts of published literature were manually reviewed to ensure that they reported the presence of *Aeromonas* spp., *Acinetobacter* spp., *Helicobacter* spp., *Legionella* spp., *Methylobacterium* spp., *Mycobacterium* spp., *Legionella* spp. or *Stenotrophomonas*

spp. to the genus level. The study must also have reported this presence in a residential drinking water source or water-related device. Studies were excluded if they were not written in English, if they were reviews, if they reported on clinical infection but did not identify a contributing residential water source or studies investigating wastewater. Study site, reservoir, pathogen, country and year of the study, bacterial isolation methods and antimicrobial characteristics were collected from each article as appropriate.

1.2.7. Results

Three thousand, four hundred and two papers were retrieved from SCOPUS and Web of Science using the search terms identified (Table 1.1). After applying inclusion and exclusion criteria (Figure 1.1), a total of 214 papers were included for review and are presented in Table 11.1.

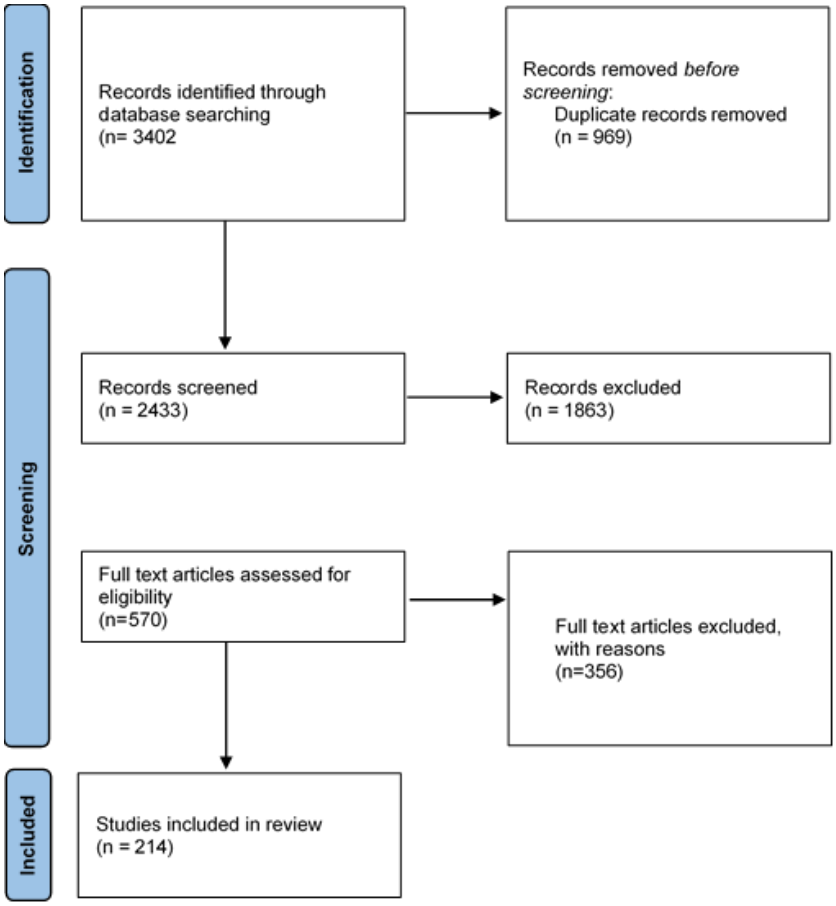

Figure 1.1 Flow diagram presenting the search strategies used, based on the PRISMA statement reporting guidelines for systematic literature reviews (Page et al., 2021)

1.2.7.1. Study sites

Of the 214 papers included for review, 82 studies were from Europe, 66 from North America, 43 from Asia, 11 from Africa, seven from Oceania and two from South America (Figure 1.2, Table 11.1). Three studies investigated residential drinking water from two or more countries from different continents (Gattlen et al., 2010; Oxford et al., 2013; Ristola et al., 2015). One hundred and ninety-one studies sampled water from private houses, seven from residential drinking water distribution systems (DWDS), one from an accommodation site, one from an apartment building and one from a dormitory (Table 11.1). Twelve studies sampled from two or more sites that included both private houses and public or healthcare facilities such as retirement homes, hotels, universities, commercial buildings, and schools (Abubakar et al., 2013; Ahmadrajabi et al., 2016; Barna et al., 2016b; Briancesco et al., 2014; Donohue et al., 2019; Kobayashi et al., 2014; Mathias et al., 2007; Moore et al., 2006; Prevost et al., 1997; Rakić et al., 2011; Sanden et al., 1992; Watson et al., 2004). OPPP were found in taps and tap components such as handles and aerators (103 studies), shower and shower components such as shower heads and hoses (62 papers), potable water samples (51 studies), hot water systems (16 studies), drain holes (16 studies), baths (15 studies), water storage (six studies), ice and ice machines (three studies), rainwater (three studies), sink surfaces and U bends (three studies), cooling towers (two studies), private wells (two studies), biofilm (one study), building inlet (one study), garden hose (one study), garden sprinkler (one study), washing machine (one study), water meter (one study) and water purifier (one study) (Table 11.1).

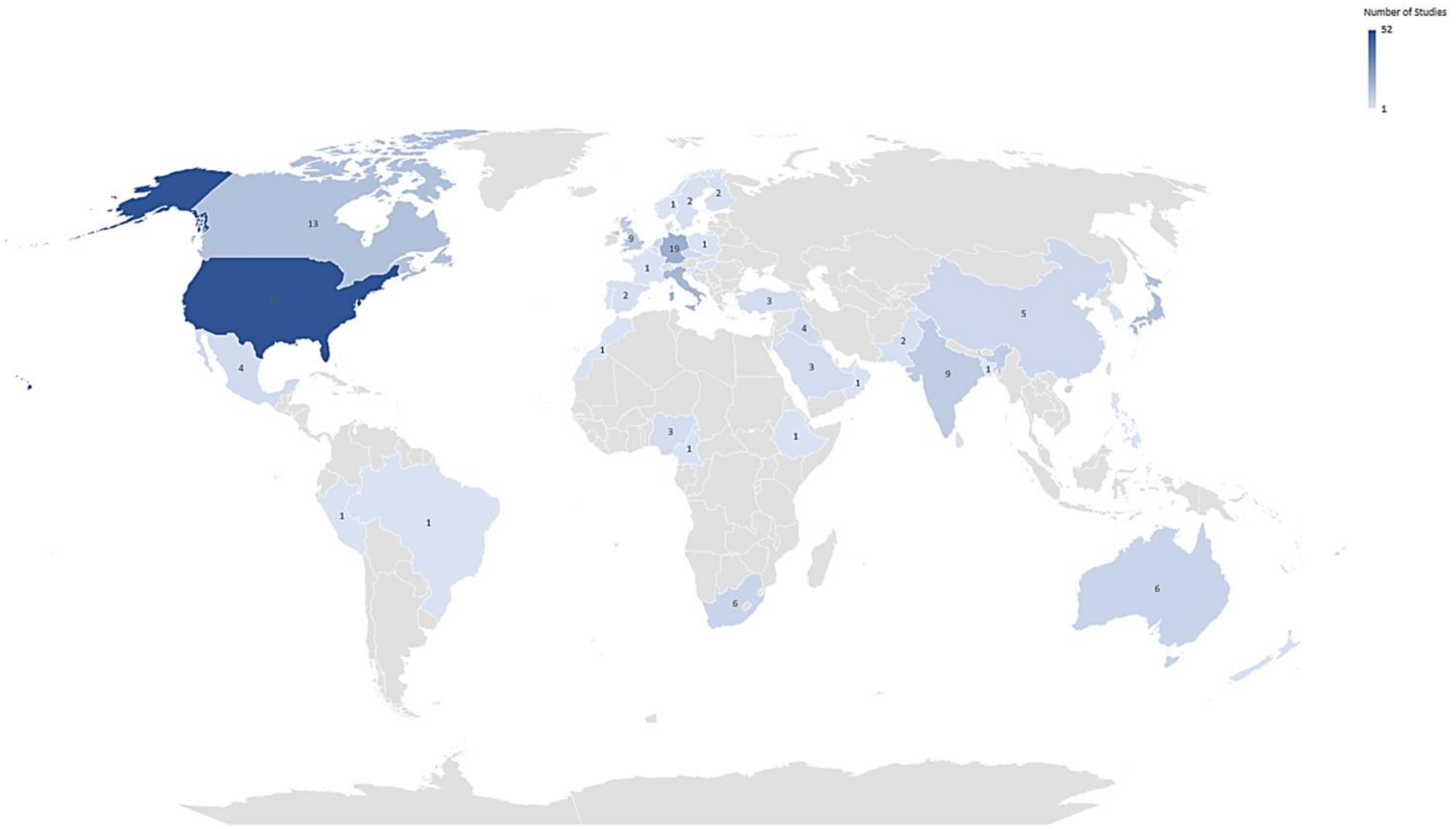

Figure 1.2 Map displaying the global distribution of all studies reporting the presence of one or more opportunistic premise plumbing pathogen in residential properties. Number of studies conducted in each country is indicated by colour intensity

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1.2.7.2. Pathogens identified and prevalence

One hundred and forty-nine studies detected OPPP solely from potable water samples, 27 from only biofilm samples and 17 from both water and biofilm samples. Seventy studies reported the concentration of one or more OPPP in potable water and 12 studies reported the concentration of OPPPs in biofilm samples (Table 1.2). Twenty-one studies identified OPPP within environmental samples, however, they did not report the prevalence from potable water or biofilm samples specifically. Eight studies isolated OPPPs from private well water sources, three studies found higher rates of contamination in well water when compared to municipal water (Abdel Haleem et al., 2016; Dai et al., 2019; Hultén et al., 1998; Katz et al., 2015; Mapili et al., 2020; Mukhopadhyay et al., 2012; Oluyege et al., 2011; Xue et al., 2020).

Sixty-four studies identified bacterial species other than the designated OPPP such as *Achromobacter* spp., *Agrobacterium* spp., *Alcaligenes* spp., *Bacillus* spp., *Bosea* spp*., Brevibacillus* spp., *Brevundimonas* spp., *Campylobacte*r spp., *Chlamydiales* spp., *Chromobacterium* spp., *Desulfovbrio* spp., *Enterobacter* spp., *Enterococcus* spp., *Escherichia* spp., *Flavobacterium* spp., *Gallionella* spp., *Klebsiella* spp., *Kocuria* spp*., L. monocytogenes*., *Lysobacter* spp., *Microbacterium* spp., *Micrococcus* spp., *Moraxella* spp., *Nocardia* spp*., Paenibacillus* spp., *Pasteirella* spp*., Plesiomonas* spp., *Polaromonas* spp., *Rhodococcus* spp., *Salmonella* spp., *Serratia* spp., *Shigella* spp., *Staphylococcus* spp., *Streptococcus* spp., *Sulfuricurvum* spp., *Tatumella* spp., *V. cholerae*., *Xenophilus* spp., and *Yersinia* spp. (Table 11.1).

Thirty studies investigated clinical cases where the infection was linked to contaminated residential drinking water (Table 11.1). This included 20 papers investigating *Legionella* spp. infection, seven papers investigating *Mycobacterium* spp. infection, two papers investigating *Pseudomonas* spp. infection and one paper investigating a case of *Aeromonas* spp. infection. All cases of *Legionella* spp. and *Mycobacterium* spp. were respiratory infections, there was investigation of enteric and bacteraemia *Aeromonas* spp. infection respectively and one investigation of dermal *Pseudomonas* spp. infection. An epidemiological investigation in response to a community acquired Legionnaires' disease case found that the patient did not have their hot water tanks sustained above the recommended 60°C temperature (Laverdière et al., 2001). *Pseudomonas* spp. has caused an estimated 15,000 pneumonia hospitalisations and 730 deaths in the US in 2014 (Collier et al., 2021). However, only two studies included in this review investigated and identified the patients' residence as the source of infection.

Table 1.2 Summary of reported prevalence and concentration of Opportunistic Premise Plumbing Pathogens detected in residential drinking water infrastructure

Opportunistic premise plumbing pathogen	Number of studies	Clinical case investigations	Prevalence and pathogen concentration	
			Drinking water	Biofilm
Legionella spp.	93	20	2.4% to 86.7% (1 to 10^6 CFU/mL)	1.1% to 100% ($5.4x10^2$ to $28.6x10^3$ CFU/swab)
Mycobacterium spp.	60	7	0.6% to 100% (1 to $1.7x10^4$ CFU/mL)	2.5% to 100% ($<10^1$ to 10^7 cells/cm^2)
Pseudomonas spp.	60	2	7.14% to 100% (1 to 640 CFU/mL)	1.2% to 100% in biofilm samples ($1x10^2$ to $1.5x10^5$ CFU/swab)
Aeromonas spp.	20	1	0.7% to 32.4% (5 to 333.3 CFU/mL)	3.9% to 77.5% (concentrations not reported)
Acinetobacter spp.	14	0	4.4% to 80% (concentrations not reported)	1.6% to 2.2% (concentrations not reported)
Stenotrophomonas spp.	8	0	1.5% to 100% (concentrations not reported)	11% to 100% (concentrations not reported)
Methylobacterium spp.	7	0	12% and 46% (concentrations not reported)	46% (>10 CFU/mL)

Helicobacter spp.	5	0	7% to 12% (concentrations not reported)	N/A

1.2.7.3. Antimicrobial resistance

Several antimicrobial resistant (AMR) OPPP strains were identified by studies included for review. Nine studies performed disc diffusion tests following European Committee on Antimicrobial Susceptibility Testing and Clinical and Laboratory Standards Institute Guidelines (Abera et al., 2014; Adday et al., 2019; Koksal et al., 2007; Mathias et al., 2007; Mombini et al., 2019b; Mukhopadhyay et al., 2012; Obi et al., 2007b; Oluyege et al., 2011; Schiavano et al., 2017). Two studies performed broth microdilution (Samie et al., 2012; Zupančič et al., 2019), two studies used VITEK-2 ID cards (Lucassen et al., 2019; Maki, 2019), two studies identified antibiotic resistance genes (Ma et al., 2019; Schages et al., 2020) and three studies did not specify which method they employed (Alavandi et al., 1999; Huminer et al., 1989; Watando et al., 2001). Antimicrobial resistant *Pseudomonas* spp. was detected in nine studies, most commonly from taps (four studies), water (two studies), showers (one study) and a private well (one study) (Abera et al., 2014; Huminer et al., 1989; Lucassen et al., 2019; Ma et al., 2019; Mathias et al., 2007; Mombini et al., 2019b; Mukhopadhyay et al., 2012; Oluyege et al., 2011; Schiavano et al., 2017). Six of these studies found *Pseudomonas* spp. strains that were resistant to two or more antibiotics tested, with two of these studies reporting isolates resistant to 5 or more antibiotics such as the antibiotic combination amoxicillin/clavulanic acid and the broad-spectrum antibiotic chloramphenicol. Antibiotic resistance gene carrying fragments identified in *P. aeruginosa* isolated from residential drinking water was found to carry the *aph*(3')-I determinant which encodes resistance to aminoglycoside antibiotics such as kanamycin and neomycin (Ma et al., 2019).

Four studies reported AMR *Aeromonas* spp. with the highest resistance to the broad-spectrum β-lactam antibiotic ampicillin compared to other antibiotics tested (Alavandi et al., 1999; Koksal et al., 2007; Maki, 2019; Obi et al., 2007b). A shower and a bath were linked to a case of clinical AMR *P. aeruginosa* and *M. avium* infection respectfully, however, the specific AMR profiles were not described (Huminer et al., 1989; Watando et al., 2001). AMR *Acinetobacter* spp*., L. pneumophila* and *S. maltophila* were identified in one study respectively (Adday et al., 2019; Lucassen et al., 2019; Mukhopadhyay et al., 2012; Watando et al., 2001). Two studies identified multiple AMR OPPP, however it is unclear

which antibiotics each isolate was resistant to as results were presented as total resistance (Samie et al., 2012; Schages et al., 2020). The antibiotic resistance genes *bla* CMY-2, *bla* ACT/MIR and *bla* OXA-48 were identified in shower drain biofilm samples containing the OPPP *P. aeruginosa*, *S. maltophila*, *A. lwoffii* and *A. hydrophila* (Schages et al., 2020).

Total chlorine levels were found to be below regulatory values in several studies included for review, including all residential drinking water samples taken from the Limassol DWDS returned residual chlorine levels below the assay detection limit (0.01 mg/L) (Botsaris et al., 2015; Gora et al., 2020). *Legionella* spp. was found to persist in contaminated water related devices despite repeated hyperchlorination at 50 mg/L (Huerta et al., 2003). Attempts to reduce *Pseudomonas* spp. and *Acinetobacter* spp. contamination on a water filtration unit using household bleach and chlorine was unsuccessful and re-emergence was seen after a few days (Payment, 1989).

1.2.7.4. Detection methods

There was significant variation in the methods used to detect target OPPP from environmental water samples (Table 11.1). Culture was the most used isolation technique (162 studies). Specifically, 71 studies performed membrane filtration followed by inoculation of selective broth or agar. Eleven studies enriched the environmental sample by inoculating broth followed by subculture on selective agar (Table 11.1). Of the papers that investigated *Pseudomonas* spp., a variety of selective media were used for isolation such as blood, MacConkey, cetrimide, sabouraud dextrose, m-PAC, phenol red, MPA, NAC and R2A. Middlebrook 7H10 agar was the most commonly used selective agar for the isolation of *Mycobacterium* spp., however, other selective media such as Lowenstein-Jensen slopes (eight studies) (Bullin et al., 1970; Kaustova et al., 1993; Marciano-Cabral et al., 2010; Parashar et al., 2009; Perez-Martinez et al., 2013; Peters et al., 1995; Slosarek et al., 1993; Slosarek et al., 1994), Ogawa media (four studies) (Kaustova et al., 1993; Kobayashi et al., 2014; Slosarek et al., 1993; Slosarek et al., 1994), Herrolds egg yolk (one study) (Klanicova et al., 2013), modified Stonebrink (one study) (Klanicova et al., 2013) and R2A agar (two studies) (Falkinham et al., 2016; Zhang et al., 2021) were used. *Aeromonas* spp. was inconsistently cultured on a variety of media such as blood, nutrient, IBB, ampicillin-dextrose, MacConkey, Aeromonas, phenol red, Xylose deoxycholate citrate, m-endo and eosin methylene blue agar. *Helicobacter* spp. was isolated by culture on blood agar (two studies) (Mulchandani et al., 2013; Watson et al., 2004) and HP medium (one study) (Richards et al., 2018). Fourteen studies referenced specific International Organisation for Standardisation protocols, most commonly ISO 11731 – Water quality enumeration of *Legionella* (11 studies) (Al-Bahry et al., 2011; Boudouaya et al., 2017; Byrne et al., 2018; Collins et al., 2017; Rakic

et al., 2017; Rakić et al., 2012; Rakić et al., 2013; Scaturro et al., 2015; Schiavano et al., 2017; Simmons et al., 2008; Totaro et al., 2020; Totaro et al., 2017). Molecular techniques such as PCR, FISH, whole genome sequencing and 16S RNA sequencing were used in 34 studies (Table 11.1). A combination of culture and molecular techniques for OPPP detection was used in 16 studies. Nine of these studies investigated the presence of *Legionella* spp. in viable and viable but not culturable states (VBNC) (Table 11.1). Three studies conducted in response to clinical cases did not specify the methods used for OPPP bacterial detection (Pastoris et al., 1988; Ristola et al., 2015; Ventura et al., 2015).

1.2.8. Discussion

The designation of a bacterial species as an OPPP is an arbitrary classification based on the presence of shared characteristics that increase their growth and proliferation in drinking water and premise plumbing. These include features such as disinfectant resistance, biofilm formation, amoeba resistance and growth under low nutrient conditions (Falkinham, 2015). The classification of OPPPs has expanded beyond the model organisms *Legionella* spp., *Pseudomonas* spp., and *Mycobacterium* spp., to include species such as *A. baumannii*, *A. hydrophila*, *H. pylori*, *Methylobacterium* spp. and *S. maltophila* in response to epidemiological studies (Falkinham et al., 2015). These additional waterborne pathogens have been highlighted as they meet the previously described arbitrary characteristics and have been found at numerous points throughout plumbing infrastructure (Abdel Haleem et al., 2016; Donohue et al., 2019; Hayes-Phillips et al., 2019; Peter et al., 2018). However, the focus on the ability of a pathogen to grow ubiquitously from the treatment facility through to the point of consumption has overlooked numerous clinically relevant species.

Several WHO designated critical and high priority pathogens, including *Enterobacter* spp., *Klebsiella pneumoniae* and *Staphylococcus aureus* were identified along with OPPPs included in this review with frequencies of up to 83%, 33% and 70% respectively (Table 11.1). Studies have shown that it is not only possible for these pathogens to contaminate water related devices, such as shower heads, tap faucets and drains, but these contaminated sources can also be responsible for HAI outbreaks (French et al., 2004; Hayward et al., 2020; Sexton et al., 2011; Ziwa et al., 2019). *S. aureus* is commonly omitted from consideration as an OPPP as it is more often associated with surfaces such as light switches and doorknobs despite possessing many OPPP characteristics. Clinical and environmental *S. aureus* isolates have demonstrated strong biofilm formation particularly under disinfectant stress (B.-R. Kim et al., 2016; Neopane et al., 2018), resistance to chlorine compounds (Buzón-Durán et al., 2017; Speck et al., 2020), survival under multiple-nutrient limiting conditions in high cell densities (Diaper et al., 1994; Watson Sean et al.,

1998), and proliferation in *Acanthamoeba polyphaga* (Huws et al., 2006). Source water and distribution system tracking will likely miss the presence of these human flora pathogens as it has been suggested that the contamination is likely to be occurring at the point of use via contaminated users and cross contamination from surrounding environmental surfaces (Abubakar et al., 2013; Maki, 2019). One study that investigated the microbiological diversity of domestic and food service business ice cubes found 31 different species dominated by the OPPPs *Acinetobacter* spp. and *Pseudomonas* spp. in addition to pathogenic *Staphylococcus* spp. and *Bacillus* spp. (Settanni et al., 2017). Complex biofilm communities can confer protection to pathogenic species that are not ideally adapted to the premise plumbing environment. A study investigating the microbial quality of urban DWDS in Cyprus found 85% of drinking water samples were contaminated with one or more genera of bacteria including *Pseudomonas* spp., *Staphylococcus* spp., *Bacillus* spp., *Acinetobacter* spp., *Enterococcus* spp., *Enterobacter* spp. and *Aeromonas* spp. (Botsaris et al., 2015). These highly heterogeneous communities promote the transfer of AMR via horizontal gene transfer and/or vertical transmission (Abe et al., 2020; Soto-Giron et al., 2016). When considering future drinking water disinfection protocols and infection control guidelines, it is essential to understand how different environmental niches may be favorable to different pathogens.

1.2.8.1. Control of opportunistic premise plumbing pathogens

A multi-barrier approach has been suggested as the most effective approach to control the growth and proliferation of OPPPs. This risk-based approach allows for the failure of one barrier to be compensated for by the effective maintenance of the additional barriers (Summerscales et al., 2010). Barriers used in the production of safe drinking water include protection of source water, maintenance of infrastructure, filtration and disinfection (Prest et al., 2016). However, the biological stability of drinking water is dynamic and can be affected by variables such as nutrient availability, disinfectant selective pressure and temperature that are unique to each distribution system. Identification of barriers where interventions can be applied is a pre-requisite for this approach. The singular, complex, and diverse nature of building water system environments may compromise the successful implementation of strategies aimed at reducing microbial load (Neu et al., 2020). Reducing levels of biodegradable organic matter and assimilable organic carbon in drinking water prior to distribution has been shown to reduce biofilm formation and growth in premise plumbing (Liu et al., 2015). Ironically disinfection agents such as monochloramine and chlorine aimed at reducing microbial load may cause an increase in assimilable organic carbon due to the oxidation of organic carbon, resulting in potential re-growth of microorganisms in DWDS (Liu

et al., 2016; Liu et al., 2015). It is essential to monitor the microbial, engineering, and chemical parameters of drinking water on a routine and high-frequency basis throughout the distribution system to validate the efficacy of current barriers, particularly at the point of use. If these barriers are found to be inadequate, additional stages of disinfection or re-evaluation of identified barriers can be instigated by water utilities and homeowners to minimise the uncontrolled growth of OPPPs.

Current drinking water treatment principles are tailored to waterborne pathogens that primarily originate from human and animal faecal contamination. Disease from these organisms is generally contracted via ingestion. However, the diseases with the largest number of deaths attributed to waterborne transmission in the US were infections with NTM, *Pseudomonas* spp., and Legionnaires' disease (Collier et al., 2021). In the majority of these cases ingestion is not the route of infection. OPPPs are characterised by their resistance to commonly used disinfectants such as chlorine. When primary disinfection strategies are developed for faecal indicator bacteria, the premise plumbing environment will select for the dominance of disinfectant resistant pathogens (Falkinham et al., 2015). Consequently, these strategies may select for diseases acquired by means other than the faecal–oral route. The US EPA National Primary Drinking Water Regulations state that chloramines (4 mg/L), chlorine (4 mg/L) and chlorine dioxide (0.8 mg/L), are added to drinking water to control microorganisms. The WHO reviewed the national drinking water quality guidelines of 104 countries and found that 66 countries had set a regulatory value for chlorine in municipal drinking water. This value ranged from 0.1-5mg/L and it was not always clear if this value referred to free or total chlorine (World Health Organization, 2018). As a consequence of this variation in additional to other environmental variables such as climate, the persistence of OPPPs in DWDS and subsequent risk of infection will differ between counties. It is difficult to maintain disinfectant residual throughout the distribution system due to reactions with dissolved nutrients, secreted protective exopolysaccharides and sediments. Water utility companies are responsible for managing water treatment throughout the distribution network to the property meter. Once the water enters a premises water quality is the responsibility of the property owner. Larger commercial buildings such as hospitals may opt to conduct additional onsite water treatment to manage waterborne healthcare acquired infections, this rarely happens in residential properties (Prest et al., 2016). Often residential homeowners are not aware of water quality changes that may occur from the water meter to their tap and the infrastructure that may be contributing to this change. When present in premise plumbing biofilms, OPPPs may become more tolerant to disinfection methods. Not all OPPPs are resistant to the same levels of residual disinfection and maintaining a residual level high enough to control highly resistant pathogens such as *Mycobacterium* spp. and *Legionella*

spp. would be problematic. Factors such as water stagnation, temperature fluctuations and the physical integrity of premise plumbing infrastructure can influence the efficacies of residual disinfectants. Sub-lethal concentrations of disinfectants such as chlorine may reduce population diversity and select for the growth of disinfectant resistant OPPP (Falkinham, 2015). Maintaining water temperature has been highlighted in global drinking water guidelines as a factor that can be manipulated to minimise pathogen growth. The WHO guidelines recommend cold water to be stored below 20°C and hot water to be stored above 60°C (World Health Organization, 2017). However, both hot and cold water temperatures can be difficult to maintain during seasonal changes and in large or old buildings. For example, larger building sizes in Flint, Michigan have been linked to higher levels of recoverable *Legionella* spp. when compared to single story buildings due to zones of warm stagnant water favourable to bacterial growth (Schwake et al., 2016). Once pathogens have colonised premise plumbing, particularly when present as a biofilm, hot water flushing may be rendered ineffective. *L. pneumophila* was isolated from the bathroom and kitchen hot water taps of a 1972 apartment after a case of potentially domestically acquired Legionnaires disease was reported in a one week old newborn. Epidemiological investigations found that the water leaving and returning to the heat exchanger were below recommended hot water temperatures at 53^{o}C and 40^{o}C respectively. The hot water temperatures were subsequently increased, and no *Legionella* spp. were detected from the water leaving the hot water exchanger. However, *Legionella* spp. were still detected in hot water returning to the heat exchanger, indicating colonisation of the plumbing infrastructure (Skogberg et al., 2002). The heating element of electrical heated water storage tank is suspended in the water not reaching any sediment at the bottom of the tank that is likely to harbor OPPPs (Bates et al., 2000). Instantaneous hot water systems have been suggested as an appropriate alternative to continuous flow or water storage tanks to minimise warm water remaining stagnant in residential properties (Martinelli et al., 2000).

Point of use (POU) filters have been suggested as a method to reduce exposure to OPPPs from a contaminated water source or device (Bielefeldt et al., 2009). These POU filters may be used in conjunction with point of entry filters that can be installed at the properties main water intake to address water quality degradation from the municipal DWDS (Wu et al., 2021). This intervention has been effective in healthcare settings at eradicating *Legionella* spp. and *P. aeruginosa* resulting in elimination of infection (Barna et al., 2014; Mathys et al., 2008). A cost-benefit assessment estimated that installation of POU devices as the final stage of water treatment could prevent 3.4 million cases of disease and mortality due to waterborne pathogens resulting in $1,814USD of averted costs per disease case (Verhougstraete et al., 2020).As with other barriers ,maintenance of POU filters is critical.

Bacterial numbers may amplify in the POU filter if they are not maintained and operated properly (Chaidez et al., 2004). Biofilms on plumbing fixtures such as tap faucets and shower heads provide a source of nutrients and protection for pathogens such as *S. aureus* and *A. baumannii* that can disseminate AMR. Cross contamination between the kitchen environment and water related devices during inappropriate cleaning practices has been identified as a source of bacterial transmission (Josephson et al., 1997b). Beta-lactam resistant genes were detected in *S. maltophila* and *P. aeruginosa* shower drain isolates (Schages et al., 2020). This colonisation may occur if an individual is a carrier of the antimicrobial resistant infection and is of particular concern when considering the colonisation of shared plumbing fixtures. Significant growth of *P. aeruginosa* was found in a nursing home whirlpool bath after a resident with a known *P. aeruginosa* toe infection used the shared facility daily along with other residents irrespective of incontinence, infection or skin problems (Hollyoak et al., 1995). Carbapenem resistant OPPPs such as *Acinetobacter* spp. and *P. aeruginosa* have been identified as antibiotic resistant threats by the CDC resulting in 41,100 infections and 3,400 deaths combined in 2017 (Centers for Disease Control and Prevention, 2019a). If installed and maintained properly, POU filters may be an appropriate and affordable additional protection barrier for the increasing vulnerable population receiving healthcare at home (Nriagu et al., 2018).

The growth of OPPPs in drinking water and water related devices may be unavoidable, but their impact is manageable. Although OPPPs are identified based on their shared characteristics, they are members of widely different taxonomic groups and therefore react to prevention measures differently. Current drinking water guidelines must acknowledge the growing complexity of plumbing infrastructure and the limitations of disinfection procedures on dynamic bacterial communities. It is not sufficient to rely solely on the water industry to provide and maintain safe drinking water from treatment to point of use. Additional preventative measures should be considered on an individual basis for people considered to be at particular risk of developing a waterborne HAI such as the elderly, infants, and those with weakened immune systems (Oluyege et al., 2011; Von Baum et al., 2010).

1.2.8.2. Pathogen detection from environmental sources

Culture based methods for the detection of indicator bacteria have long been held as the ‘gold standard’ as they detect viable target organisms. However, examining the full spectrum of potentially pathogenic microorganisms is not a feasible part of routine monitoring protocols (Deshmukh et al., 2016). The number of pathogens targeted by culture based epidemiological studies is limited by the selective media chosen prior to sampling and the time required to handle the samples. One of the defining characteristics of an OPPP is the

ability to adapt and proliferate in nutrient poor environments, often resulting in slowed growth rates or conversion to a VBNC state (Nisar et al., 2020a). For example, OPPPs such as *Legionella* spp., *Mycobacterium* spp. and *Methylobacterium* spp. may take up to 14 days before the first appearance of colonies on agar (Falkinham, 2015). Nutrient rich selective agars and pre-treatment steps such as heating or acidification are typically used to combat competitive overgrowth by faster growing organisms (Thomson et al., 2008). These selective media have some drawbacks as they may inhibit or restrict growth of the target organisms and may also induce the VBNC state (Nisar et al., 2020a).

Challenges may arise when trying to enumerate VBNC bacteria using culture-based methods (Marshall et al., 2011; Prévost et al., 1998; Shih et al., 2006; Whiley, 2016). VBNC bacteria are stressed or injured cells that are characterised by their lack of proliferation on agar leading to underestimation of viable cells in a sample. Although they are difficult to enumerate on routine agar, VBNC cells are not considered dead as they have an intact membrane, contain undamaged genetic material and are metabolically active. Nutrient depleted media such as R2A agar have been recommended to enhance the recovery of environmental waterborne pathogens (Gibbs et al., 2008). Growth of OPPPs on selective media was used by 162 studies in this review, including 11 different types of media for *Pseudomonas* spp. International standard methods have been published for the enumeration of the OPPP *L. pneumophila* and *P. aeruginosa* from environmental water samples (International Organization for Standardization, 2017, 2018). Only 15% and 3% of studies that investigated the presence of *Legionella* spp. and *Pseudomonas* spp., respectively, referenced ISO protocols. The US Environmental Protection Agency recommends ISO 11731 and CDC standard culture methods to monitor the presence of *Legionella* spp. in premise plumbing. It is valuable to maintain consistent sampling and testing protocols to understand and implement effective risk assessment protocols. To date such international standards have not been published for the enumeration of OPPPs *Acinetobacter* spp., *Aeromonas* spp., *Helicobacter* spp., *Methylobacterium* spp., *Mycobacterium* spp. and *Stenotrophomonas* spp. from environmental water samples. This lack of standardisation has resulted in significant variation between the sampling techniques and enumeration protocols employed by studies included in this review.

Several nucleic acid and immunology based protocols have been developed to address the limitations associated with traditional culture dependant methods. This includes techniques such as polymerase chain reaction (PCR), microarrays and fluorescence *in situ* hybridisation (FISH) (Benowitz et al., 2018; R. Dey et al., 2019; Pellizari et al., 1995). The CDC have recommended PCR methods for routine *Legionella* spp. testing in conjunction with spread

plate culture techniques. Like many OPPPs, *Legionella* spp. have been shown to replicate intracellularly within macrophagic hosts resulting in a thickened outer membrane, greater resistance to environmental stress and the ability to readily enter a VBNC state. Seven studies included in this review used both culture and PCR methods for the detection of *Legionella* spp. (Table 11.1). Although PCR techniques are considered to be more sensitive than culture-based techniques, commonly used protocols do not distinguish between DNA from viable, injured, or dead cells that persists in the environment, which may contribute to the overestimation of pathogens in a sample (Cangelosi et al., 2014). Propidium monoazide (PMA) quantitative PCR is a practical alternative that can differentiate between live, dead and membrane damaged cells. PMA is an intercalating molecule that selectively binds to the DNA of viable and membrane damaged cells. This bond inhibits PCR amplification of dead bacterial DNA, therefore reducing the likelihood of false positive results and overestimation of pathogen concentration (Golpayegani et al., 2019). Alternative techniques such as FISH have been proposed to bridge the gap between underestimation of contamination by culture and potential overestimation by PCR (Tossa et al., 2006). Buchbinder et al (2002) compared the specificity and sensitivity of culture, PCR and FISH for the detection of *Legionella* spp. in residential drinking water (Buchbinder et al., 2002). It was found that although PCR was significantly more sensitive than FISH, FISH was more specific (72% compared to 47% for PCR). It was suggested that because the FISH assay was able to detect VBNC cells, it is potentially a better alternative than PCR for future routine testing protocols. However, this approach is limited by the high costs associated with user training, protocol optimisation and need for high pathogen densities that may not be present in many environmental samples (Frickmann et al., 2017)

1.2.8.3. Epidemiological investigations

There are several drinking water quality guidelines referenced globally such as the US EPA Safe Water Drinking Act, the EU Drinking Water Directive, and the WHO Guidelines for Drinking Water Quality (The Council of the European Union, 2020; U.S. Environmental Protection Agency, 1996; World Health Organization, 2017). These guidelines suggest testing for microbial and chemical contaminants should be used as an indication of water quality. Countries such as Japan, Singapore, Malaysia, Australia and South Africa have cited these guidelines when developing national drinking water quality standards (Wen et al., 2020). The US EPAs revised total coliform rule was released to identify and reduce potential pathways for faecal DWDS contamination. This rule states that total coliform samples must be collected at sites throughout the DWDS. The frequency of routine sampling is dependent on the number of people that are served by the public water system and the type of

contaminant being tested. This is supported by the WHO Guidelines for Drinking Water Quality that state that the drinking water legislation should be informed by system specific risk assessments. For example, Singapore relies heavily on reservoir water as a source of drinking water and recreational use, resulting in 28 waterborne microbial indicators used in the monitoring of water quality (Wen et al., 2020). The drinking water infrastructure in many African countries is struggling to keep up with the increasing population and urbanisation which may lead to water shortages and contamination. This significant threat to public health is exacerbated by the fact that there are currently no water quality guidelines for many of these countries (Wen et al., 2020). Water quality testing within private residences is not routinely performed by water service providers. However, if a residence is supplied by a private water supply, such as well water or rainwater, the US EPA recommends regular testing by a certified laboratory. Legionnaire's disease is the only OPPP caused disease that is on the national notifiable diseases register in the USA, Australia, Hong Kong, the Netherlands and the UK (Parr et al., 2015). Many countries, including China, India and Malaysia do not have any OPPP related diseases in their national infectious disease surveillance systems (Vlieg et al., 2017). Only 30 studies included in this review were linked to clinical cases of infection despite the US CDC estimating that approx. 96,000 waterborne respiratory illnesses were acquired domestically (Collier et al., 2021) (Table 11.1). Dose and exposure response models are an essential aspect to quantifying the human health risks of a pathogen and can be used to inform future regulatory policies (Chandrasekaran et al., 2019). Of the reviewed studies investigating clinical infection, only 13 reported the concentration of the pathogen found at the exposure site including 12 studies reporting *Legionella* spp. infection and only one reporting *Mycobacterium* spp. infection (Erdoğan et al., 2016; Falkinham Iii et al., 2008; Huerta et al., 2003; Laverdière et al., 2001; Leverstein van Hall et al., 1994; Lück et al., 1993; Moran-Gilad et al., 2012; Ryu et al., 2017; Sax et al., 2002; Schumacher et al., 2020; Stout et al., 1987; Stout et al., 1992a; Young et al., 2005). These cases of clinical infection are typically only published as case reports due to the unique nature of the case including persistent re-infection, antimicrobial resistance or unique a patient demographic. Risk based modelling has been conducted on many OPPPs in the past, for the most part key exposure pathways such as water aspiration have not been considered and will continue to be overlooked by water quality guidelines until there is more consistent published data (Dean et al., 2020; Hamilton et al., 2019; Hamilton et al., 2017).

1.2.9. Conclusions

Residential drinking water and water related devices have been neglected as a source of OPPP infection in national drinking water and infection control guidelines. Although some potential OPPPs may not be as ubiquitous in premise plumbing as model waterborne pathogens, their presence at point of use outlets presents a significant infection risk that must not be underestimated by infection control and prevention guidelines. This review examined previously defined OPPPs in residential plumbing, however, it was also found that many of these studies identified pathogens such as *Staphylococcus* spp. and *Enterobacter* spp. that do not meet the current definition but have been linked to clinical infection resulting from contaminated plumbing infrastructure. The tailoring of currently ineffective water quality treatment plans to address the growth of identified waterborne pathogens is essential to provide safe drinking water throughout the entire distribution system. Water utility providers must consider the potential selective impacts these measures may have on other clinically relevant species that can adapt to high stress environments and may lead to a change in the current OPPP definition. Effective and reproducible microbial water quality surveillance protocols are essential to understand risk factors, monitor the chemical and biological stability of the drinking water and therefore predict and prevent future public health threats. Relying on a single water quality control measure to consistently reduce the total microbial load of drinking water may be ineffective against diverse OPPPs. Despite the literature clearly identifying residential plumbing infrastructure as a consistent reservoir for OPPPs, these sources continue to be overlooked as public health risks to vulnerable populations receiving healthcare at home. This is reflected by the limited information provided by many infection control guidelines (Centers for Disease Control and Prevention, 2020a; National Health and Medical Research Council, 2019; World Health Organization, 2017, 2018). The growth of bacterial pathogens in premise plumbing may be unavoidable, however, it can be managed with an appropriate multi barrier approach that minimises disinfectant resistance, reservoirs, biofilm formation and thermotolerance. Comprehensive and consistent epidemiological investigations of suspected domestically acquired OPPP infections are essential to develop quantitative microbial risk assessment models and inform infection control and prevention guidelines which are lagging behind the demand for healthcare at home services

2. AIMS AND OBJECTIVES

2.1. Aims

The aims of this study were:

- To investigate the prevalence and relationships of OPPPs in drinking water plumbing systems;
- To identify and characterize AMR pathogens within drinking water plumbing biofilms;
- To determine factors that may influence the persistence and growth of these OPPPs;
- To investigate the effect of product design on aerosol generation and OPPP transmission.

2.2. Objectives

To achieve these aims, the objectives of this study were to:

1. Examine the literature and determine the potential role of water as a source of HAIs;
2. Investigate the prevalence of multiple OPPPs, the associations between their prevalences, and the potential influence of building properties on their prevalence using qPCR assays;
3. Characterize the presence of AMR pathogens and AMR genes from residential and healthcare biofilm samples using selective culture and qPCR assays;
4. Use 16S rRNA sequencing analysis to compare the microbial community diversity of faucet and drain biofilms;
5. Investigate the effect of different flow rate restrictors on the number, size and distribution of bioaerosols produced by a handwashing basin by development and implementation of a laboratory model;
6. Evaluate the antimicrobial activity of different plumbing materials in stagnant water systems using a laboratory scale bioreactor model.

3. MATERIALS AND METHODS

This chapter describes the general experimental procedures used throughout the research detailed in this thesis.

3.1. Ethical clearance

This study was approved by the Flinders University Social and Behavioural Research Ethics Committee (SBREC Project Number 7291) as per the recommendations of the National Statement on Ethical Conduct in Human Research, Australia. The study was also approved by Institutional Biosafety Committee, Flinders University (IBC ID: 6985 and 7406).

3.2. Sample collection and processing

Drinking water and biofilm samples were collected from private residential properties, university accommodation and hospital premise plumbing systems and transported according to the Centers for Disease Control and Prevention guidelines (Centers for Disease Control and Prevention, 2019b).

3.2.1. Water and biofilm sampling

Drinking water samples were collected from handwashing basins and showers. Briefly, 1 L of potable water samples were collected in sterile screw capped wide mouth plastic bottles (2105-0032 Nalgene) containing 1 mL of 0.1 N $Na^2S^2O^3$ (124270010, ACROS Organics™) to neutralize residual chlorine-based disinfectants.

Biofilm samples were collected from handwashing basin tap faucets, handwashing basin drains, showerheads, shower drains, bath faucets, bath drains and the internal surfaces of biofilm reactors. Briefly, sterile polyurethane-tipped swabs (CleanFoam®TX751B, Texwipe®) were used to collect biofilms. These swabs were moistened with sterile water and the surface of the faucet aerator or drain was swabbed for 10 s. The swab was then placed in a 10 mL screw capped vial with 5 mL of 1X sterile phosphate buffered saline (PBS).

3.2.1.1. Sample transport and storage

Drinking water and biofilm samples were transported from the collection site to the laboratory at room temperature, stored at 5^{o}C and processed within 72 hrs.

3.2.1.2. Sample processing

Collected biofilm was dislodged from the swab and into the PBS by 5 min of shaking (Griffin Flask Shaker), vortexing (SEM® Vor Mix) and sonication (CooperVision® 895 Ultrasonic Cleaner). The suspended biofilm samples, and 1 L drinking water samples, were vacuumed filtered onto 47 mm diameter 0.2 µm polycarbonate membranes (GTTP04700, Isopore™). Sterile tweezers were used to remove the membrane and transfer it into a sterile tube containing 3 mL of sterile PBS followed by 5 min of shaking (Griffin Flask Shaker), vortexing (SEM® Vor Mix) and sonication (CooperVision® 895 Ultrasonic Cleaner). This suspension was used for further molecular and microbiological testing.

3.3. Microbiological isolates

3.3.1. Heterotrophic plate count

Standard protocols i.e. ISO 9308-1:2014 and AS 4276.3.1 (AS/NZS, 2007; International Organization for Standardization, 2014) were followed to determine heterotrophic plate count (HPC). Heterotrophic bacteria were cultured on plate count agar (CM0325B) (ThermoFisher Scientific: Adelaide, Australia) prepared as per the guidelines provided by the manufacturer. Briefly, 8.75 g of plate count agar was resuspended in 500 mL of distilled water and steam sterilized by autoclaving at 121^{o}C. Sterile plate count agar was cooled in a water bath at 50^{o}C, poured in sterile disposable petri plates and stored at 4^{o}C for 1 week. One hundred microlitres and 10 µL of resuspended environmental sample (See Section 3.2.1.2) was spread on plate count agar plates and incubated under aerobic conditions at 37 +/- 1^{o}C for 24 hours. Colonies were counted and calculated to colony forming units (CFU) per L.

3.3.2. *Acanthamoeba polyphaga*

Heat inactivated fetal bovine serum (HI-FBS) supplemented peptone yeast glucose (PYG) broth was used for amoeba axenic cultures. PYG broth was prepared by dissolving 2 g of peptone (LP0037B, Oxoid Ltd.) and 0.2 g yeast extract (LP—21, Oxoid Ltd.) in 90 mL of distilled water and steam sterilised by autoclaving at 121^{o}C. Once cooled at room temperature, 1.8 g D-glucose was dissolved in 10 mL of distilled water and sterilised via filtration using a 0.2 µm syringe filter. Once filtered, the sterile glucose solution was added to the sterile broth. To prepare FBS-PYG broth, 5 mL HI-FBS (10100139, Gibco™ Thermo Fisher Scientific) was mixed in 45 mL PYG broth and stored at 4°C for 1 week. *A. polyphaga* (Puschkarew) Page ATCC® 30461™ was used as a reference strain and was cultured by adding 500 µL *A. polyphaga* suspension to 4 mL of FBS-PYG broth in a T25 (156367, Nunc™ EasYFlash™, Thermo Fisher Scientific) culture flask and incubated under aerobic conditions at 25 +/- 1^{o}C for 5 to 7 days. *A. polyphaga* cells were decanted from the T25

flasks using a cell scraper into a sterile 10 mL tube and centrifuged at 1500 g for 10 min. The FBS-PYG broth supernatant was discarded and the cell pellet was resuspended in 2 mL of sterile 1X PAGE saline (0.12 g NaCl, 0.004 g $MgSO_4.5H_2O$, 0.004 g $CaCl_2.2H_2O$, 0.142 g Na_2HPO_4 and 0.136 g KH_2PO_4 per litre distilled water, pH 6.8 ± 0.2) for further analyses and biofilm reactor inoculation.

3.3.3. *Acinetobacter baumannii*

Previously published methods were followed to isolate *A. baumannii* (Ajao et al., 2011). *A. baumannii* was isolated on MacConkey agar (CM0007B) (ThermoFisher Scientific: Adelaide, Australia) prepared as per the guidelines provided by the manufacturer. Briefly, 26 g of MacConkey agar was resuspended in 500 mL of distilled water and steam sterilised by autoclaving at 121°C. Sterile plate count agar was cooled in a water bath at 50°C, poured in sterile disposable petri plates and stored at 4°C for 1 week. *A. baumannii* ATCC® 17978™ was used as a control strain for selective culture, and developed shiny, circular, and light pink coloured colonies with a raised elevation. One hundred microlitres of resuspended environmental sample (See Section 3.2.1.2) was spread on MacConkey agar plates and incubated under aerobic conditions at 37 +/- 1°C for 48 hours. Presumptive *A. baumannii* colonies were subcultured on fresh MacConkey agar plates for molecular identification (See 3.4.2).

3.3.4. *Legionella pneumophila*

Standard protocol i.e. ISO11731:2017-05 was followed to isolate *L. pneumophila* (International Organization for Standardization, 2017). *L. pneumophila* was isolated on buffered charcoal yeast extract agar (BCYE) supplemented with glycine, vancomycin, polymyxin B and cycloheximide (GVPC) and growth (buffer/potassium hydroxide, ferric pyrophosphate, Lcysteine and α-ketoglutarate) supplements as per the guidelines provided by the manufacturer. Briefly, 12.5 g CYE agar (CM0655) (ThermoFisher Scientific: Adelaide, Australia) was resuspended in 450 mL of distilled water and steam sterilised by autoclaving at 121°C. Sterile CYE agar was cooled in a water at 50°C. GVPC (SR0152, Oxoid Ltd.) and *Legionella* growth supplement (SR0110C, Oxoid Ltd.) were dissolved each in 20 mL and 30 mL sterile water and added to the cooled CYE agar. The agar pH was adjusted to 6.9 +/- 0.1 with 0.2 M potassium hydroxide or 0.5 M hydrochloric acid. Once adjusted, the agar was poured in disposable petri dishes and stored at 4°C for 1 week. *L. pneumophila* subsp. *pneumophila* Philadelphia sg1 ATCC® 33152™ was used as a control strain for selective culture and serotyping, and developed grey/white opalescent circular colonies. One hundred microlitres of resuspended environmental sample (See Section 3.2.1.2) was spread on BCYE-GVPC agar plates and incubated under aerobic conditions at 37 +/- 1°C for 3-5 days.

Presumptive *L. pneumophila* colonies were subcultured on fresh BCYE-GVPC agar plates for serotyping and molecular identification (See 3.4.2). A latex agglutination test (DR0800, Oxoid Ltd.) was performed on presumptive colonies to identify *Legionella, L. pneumophila* sg1, and *L. pneumophila* sg2-14 as per the manufacturers instructions. Briefly, a single colony was placed on the test card using a disposable loop and resuspended in the provided suspension buffer. One drop of test reagent was added to this suspension and mixed. A positive reaction determined by visible agglutination of the mixture after 30 s.

3.3.5. *Pseudomonas aeruginosa*

Standard protocol i.e. ISO 16266-2:2018 was followed to isolate *P. aeruginosa* (International Organization for Standardization, 2018). *P. aeruginosa* was isolated on Cetrimide agar with glycerol as per the guidelines provided by the manufacturer. Briefly, 45.3 g of Cetrimide agar (CM0579B) (ThermoFisher Scientific: Adelaide, Australia) was resuspended in 500 mL of distilled water with 10 mL of glycerol and steam sterilised at 121^{o}C. Sterile Cetrimide agar was cooled in a water at 50^{o}C and poured in disposable petri dishes and stored at 4^{o}C for 1 week. *P. aeruginosa* PAO1 was used as a control strain for selective culture, and developed dry or mucoid, circular and bright green colonies. Under UV light, colonies fluoresced. One hundred microlitres of resuspended environmental sample (See Section 3.2.1.2) was spread on Cetrimide agar plates and incubated under aerobic conditions at 37 +/- 1^{o}C for 24 hrs. Presumptive *P. aeruginosa* colonies were subcultured on fresh Cetrimide agar plates molecular identification (See Section 3.4.2).

3.3.6. *Staphylococcus aureus*

Standard protocol i.e. ISO/DIS 6888-1 was followed to isolate *S. aureus* (International Organization for Standardization, 2020). *S. aureus* was isolated on Baird Parker agar with egg yolk tellurite emulsion as per the manufacturers guidelines. Briefly, 31.5 g of Baird Parker agar (CM0275B) (ThermoFisher Scientific: Adelaide, Australia) was resuspended in 500 mL of distilled water and steam sterilized by autoclaving at 121^{o}C. Sterile Baird Parker agar was cooled in a water bath at 50^{o}C and 25 mL of egg yolk-tellurite emulsion (SR0054C) (ThermoFisher Scientific: Adelaide, Australia) was added. The mixed agar was then poured into sterile petri dishes and stored at 4^{o}C for 1 week. *S. aureus* ATTC® 6538™ was used as a reference strain for selective culture and latex agglutination, and developed shiny, circular black colonies with a clear zone. One hundred microlitres of resuspended environmental sample (See Section 3.2.1.2) was spread on Baird Parker agar plates and incubated under aerobic conditions at 37 +/- 1^{o}C for 24 hrs. Presumptive *S. aureus* colonies were subcultured on fresh Baird Parker agar plates for serotyping and molecular identification (See 3.4.2). A Staphaurex™ latex agglutination test (R30859902) (ThermoFisher Scientific: Adelaide,

Australia) was performed on presumptive colonies to identify *S. aureus* as per the manufacturer's instructions. Briefly, a drop of test reagent was placed on the test card, and a single colony was mixed using a disposable loop. A positive reaction was determined by visible agglutination of the mixture after 30 s.

3.3.7. *Escherichia coli*

Previously published methods were followed to isolate *E. coli* (Percival et al., 2014). *E. coli* was isolated on MacConkey agar (CM0007B) (ThermoFisher Scientific: Adelaide, Australia) (See Section 3.3.3). *E. coli* ATCC® 700891™ was used as a control strain for selective culture and developed dry, circular and pink coloured colonies surrounded by an area of precipitated bile salts.

3.3.8. *Staphylococcus epidermidis*

MacConkey agar without crystal violet was used to isolate *S. epidermidis* (CM0007B) (ThermoFisher Scientific: Adelaide, Australia) (See Section 3.3.3). *S. epidermidis* ATCC® 14990 was used as a control strain for selective culture and developed small, pale pink and rounded colonies.

3.3.9. Preservation and storage

Bacteria were preserved in water and glycerol at a 1:1 ratio for long term storage. Briefly, 1 mL of water and glycerol mixture was added to a 1.5 mL cryotube with 10-20 bacterial colonies and stored at -80^{o}C.

3.4. DNA extraction and amplification

3.4.1. Genomic DNA extraction and storage

Total genomic DNA was extracted from water samples using the Aquadien™ kit (3578121, BIO-RAD Laboratories Ltd.) following the manufacturers guidelines. Briefly, the filter membrane and 1 mL of the resuspended environmental sample (See Section 3.2.1.2) were placed into the provided 4.5 mL cryotubes containing 2 mL of R1 buffer. Ten µL of Lysozyme (89833) (ThermoFisher Scientific: Adelaide, Australia), (25 mg/mL in 1X PBS) was added to the extraction sample and incubated at 37^{o}C for 15 min Each tube was vortexed for 20 s and incubated in a 90 +/- 5^{o}C water bath for 15 min. Once removed, the cryotube was vortexed again for 20 s and the membrane filter was discarded. The cryotube was left at room temperature for 15 min to settle the precipitate. Five hundred microlitres of supernatant was added to the provided purification column and centrifuged at 6000 g for 10 min, and the eluent was discarded. This step was repeated for the remaining supernatant. Once

completed, 100 µL of R2 buffer was applied to the column, and the column was placed upside down into the collection tube, and centrifuged at 1000 g for 3 min and once finished, the column was discarded. The purified DNA was stored at -20°C for downstream molecular analysis.

Total genomic DNA was extracted from biofilm samples using the DNeasy® PowerBiofilm® kit (24000-50, QIAGEN) following the manufacturer's guidelines. Briefly, 1 mL of the resuspended biofilm sample was centrifuged at 13,000 g for 1 min and supernatant was discarded. The biofilm sample was resuspended in MBL solution and transferred to the PowerBiofilm Bead Tube® with 100 µL of FB solution and vortexed briefly. The PowerBiofilm Bead Tube® was incubated at 65°C for 5 min in an Eppendorf ThermiMixer®C. Once removed, the PowerBiofilm Bead Tube® was secured and vortexed for 10 min (SEM® Vor Mix). The PowerBiofilm Bead Tube® was centrifuged at 13,000 g for 1 min, yielding approx. 400-450 µL of supernatant that was transferred to a 2 mL collection vial with 100 µL of IRS solution and incubated at 4°C for 5 min. The collection tube was centrifuged at 13,000 g for 1 min and the supernatant was transferred to a clean collection tube with 900 µL of MR solution. 650 µL of supernatant was applied to a MB Spin Column® and centrifuged at 13,000 g for 1 min. The eluent was discarded and the previous step was repeated until all of the supernatant was processed. The MB Spin Column® was placed in a clean collection tube, and 650 µL of PW solution was applied followed by further centrifugation at 13,000 g for 1 min. The flow through was discarded and 650 µL of ethanol was applied to the MB Spin Column® and centrifuged twice at 13,000 g for 1 min. The MB Spin Column® was transferred to a clean collection tube, 100 µL of EB solution was applied to the centre of column and was centrifuged at 13,000 g for 1 min. The eluted purified DNA was stored at -20°C for downstream molecular analysis.

3.4.2. Quantitative polymerase chain reaction

Extracted genomic DNA was used for qPCR to detect and quantify *Acanthamoeba* spp., *Vermamoeba vermiformis*, *A. baumannii, Legionella* spp., *L. pneumophila, P. aeruginosa* and *S. aureus*. All reactions were conducted using a Rotor-Gene Q thermal cycler (QIAGEN Ltd.). Each qPCR reaction mix consisted of 10 µL 2X SsoAdvanced™ universal probes supermix (172-5281, BIO-RAD Laboratories Ltd.), 1 µL primers and fluorogenic probe mix (See 3.4.3.1 to 3.4.3.12) 4 µL double autoclaved Milli-Q® water, and 5 µL DNA template. For detection of *Mycobacterium avium* complex (MAC), each qPCR reaction mix consisted of 1 X PCR buffer (Invitrogen), 2.5 mM $MgCl_2$ (Invitrogen), 2.5 mM SYTO9 fluorescent dye (Invitrogen), 0.2 mM deoxynucleoside triphosphate mix (Invitrogen), IU platinum Taq DNA

polymerase (Invitrogen), 0.3 µM MACF primer, 0.3 µM MACR primer and 5 µL of template DNA.

3.4.3. DNA standard curve

gBlock gene fragments (IDT™) were used as standard DNA to plot the standard curve. The DNA was dissolved in double autoclaved Milli-Q® water to a final concentration of 10 ng/mL stock solution. Eight 10-fold serial dilutions were performed to obtain 100 pg/µL, 10 pg/µL, 1 pg/µL, 0.1 pg/µL, 0.01 pg/µL, 0.001 pg/µL, 0.0001 pg/µL and 0.00001 pg/µL concentrations to be used for the standard curve.

3.4.3.1. *Acanthamoeba* 18S rDNA gene

The *Acanthamoeba* 18S rDNA gene was detected using primers, probes and protocols as previously described (Qvarnstrom et al., 2006). Sequences of primers and probes used were:

Forward primer: 5'-CCCAGATCGTTTACCGTGAA-3'

Reverse primer: 5'-TAAATATTAATGCCCCCAACTATCC-3'

Fluorogenic probe: 5'-FAM-CTGCCACCGAATACATTAGCATGG-Q-3'

FAM: 6-carboxyfluorescein $\lambda_{(excitation)}/\lambda_{(emission)}$ 495/520 nm, channel for qPCR: $\lambda_{(source)}$ 470 nm and $\lambda_{(detector)}$ 510 nm

Q: Iowa Black® FQ quencher with absorbance spectrum range λ 420 nm to 620 nm with $\lambda_{(max)}$ 531 nm

Accession number U07413 was used to design gBlock sequence and was as follows (underlined sequences show primers and bold sequence shows probe binding regions):

5’GCGGCGGTGGGTCCCTGGGGCCCAGATCGTTTACCGTGAAAAAATTAGAGTGTTCAA AGCAGGCAGATCCAATTTT**CTGCCACCGAATACATTAGCATGG**GATAATGGAATAGGA CCCTGTCCTCCTATTTTCAGTTGGTTTTGGCAGCGCGAGGACTAGGGTAATGATTAATA GGGATAGTTGGGGGCATTAATATTTAATTGTCAGAGGTGAAATTCT3’

The qPCR assay conditions were: 95°C for 3 min, followed by 40 cycles of 95°C for 15 s and 63°C for 60 s. The standard curves demonstrated 81% efficiency of the qPCR assay with a 40 GU/reaction limit of detection (Figure 3.1).

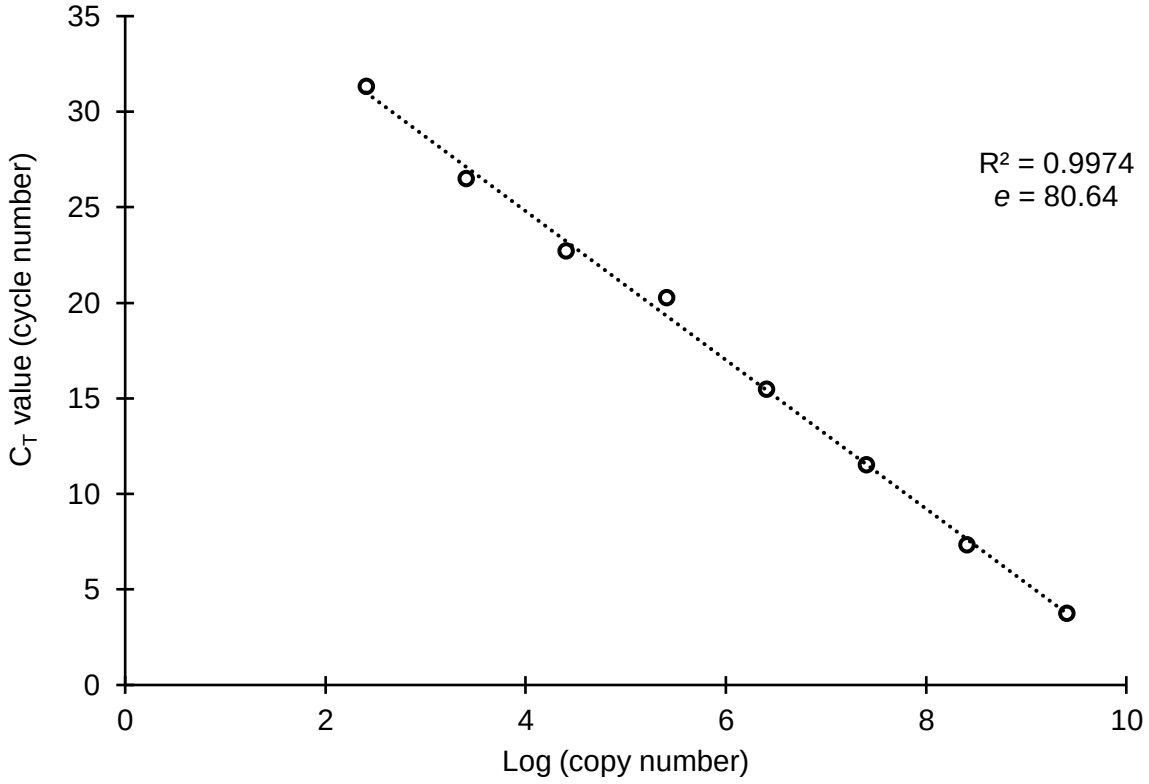

Figure 3.1 *Acanthamoeba* 18S rDNA standard curve

3.4.3.2. *Vermamoeba vermiformis* 18S rDNA gene

The *V. vermiformis* 18S rDNA gene was detected using primers, probes and protocols as previously described (Scheikl et al., 2016). Sequences of primers and probes used were:

Forward primer: 5'-TAACGATTGGAGGGCAAGTC-3'

Reverse primer: 5'-ACGCCTGCTTTGAACACTCT-3'

Fluorogenic probe: 5'-FAM-TGGGGAATCAACCGCTAGGA-Q-3'

FAM: 6-carboxyfluorescein $\lambda_{(excitation)}/\lambda_{(emission)}$ 495/520 nm, channel for qPCR: $\lambda_{(source)}$ 470 nm and $\lambda_{(detector)}$ 510 nm

Q: Iowa Black® FQ quencher with absorbance spectrum range λ 420 nm to 620 nm with $\lambda_{(max)}$ 531 nm

Accession Number KT185625 was used to design gBlock sequence and was as follows (underlined sequences show primers and bold sequence shows probe binding regions):

5’AATTTAAATCCCTTAACGAGTAACGATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCG
GTAATTCCAGCTCCAATAGCGTATATTAAAGTTGTTGCAGTTAAAAAGCTCGTAGTTGGA
TTTCGGAAGGTCTTTAGCAGTCCGCCCCTTCGGGGAGCGGGTTGCTGGCCTCCTATGT
TCCTAACGGTCCTCATCCGCGAGGG**TGGGGAATCAACCGCTAGGA**TCGTTTACTTTGA
GG AAATTAGAGTGTTCAAAGCAGGCGTAACTCGCCTCCGAATACGTT3’

The qPCR assay conditions used were 95°C for 5 min, followed by 40 cycles of 95°C for 20 s and 60°C for 60 s. The standard curves demonstrated 75% efficiency of the qPCR assay with a 44 GU/reaction limit of detection (Figure 3.2).

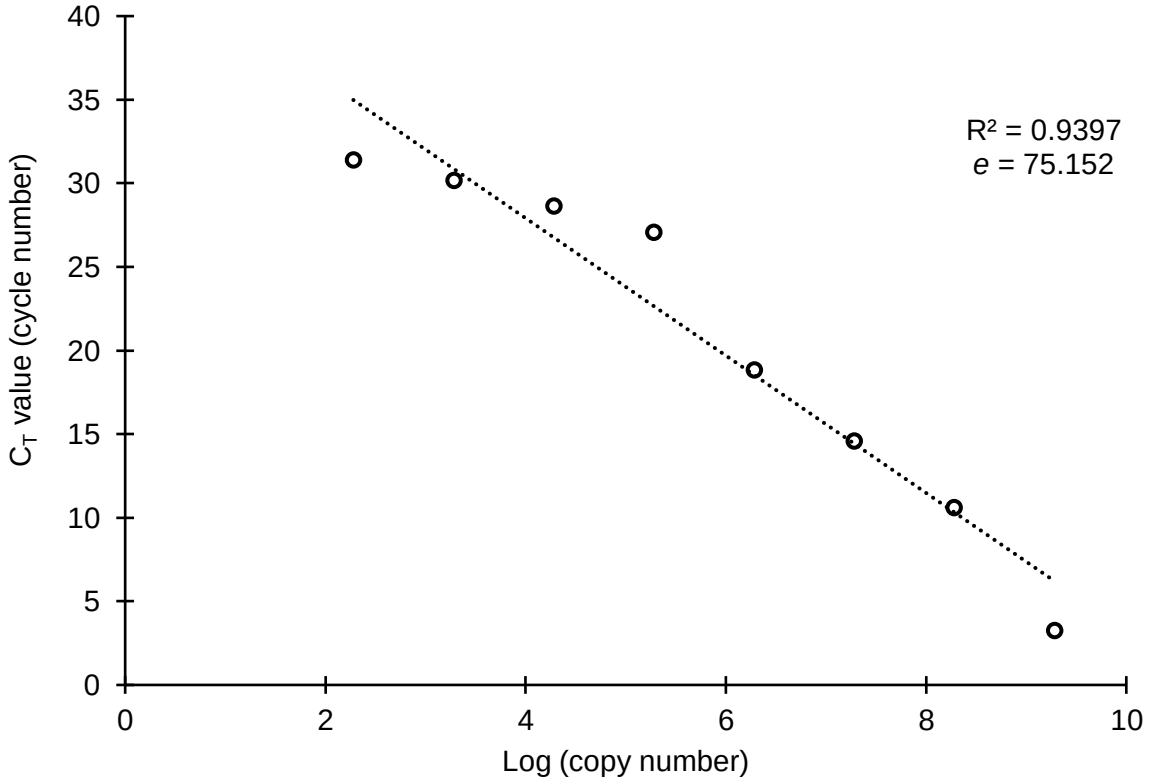

Figure 3.2 *Vermamoeba vermiformis* standard curve

3.4.3.3. *Acinetobacter baumannii ompA* gene

The *A. baumannii ompA* gene was detected using primers, probes and protocols as previously described (McConnell Michael et al., 2012). Sequences of primers and probes used were:

Forward primer: 5’-TCTTGGTGGTCACTTGAAGC-3’

Reverse primer: 5’-ACTCTTGTGGTTGTGGAGCA-3’

Fluorogenic probe: 6FAM-AAGTTGCTCCAGTTGAACCAACTCCA- Q

FAM: 6-carboxyfluorescein $\lambda_{(excitation)}/\lambda_{(emission)}$ 495/520 nm, channel for qPCR: $\lambda_{(source)}$ 470 nm and $\lambda_{(detector)}$ 510 nm

Q: Iowa Black® FQ quencher with absorbance spectrum range λ 420 nm to 620 nm with $\lambda_{(max)}$ 531 nm

Accession Number OL347635.1 was used to design gBlock sequence and was as follows (underlined sequences show primers and bold sequence shows probe binding regions):

5'CTTGCTGGCTTAAACGTAGTTCTTGGTGGTCACTTGAAGCCTGCTGCTCCTGTAGTAG **AAGTTGCTCCAGTTGAACCAACTCCA**GTTGCTCCACAACCACAAGAGTTAACTGAAGA CCTTAACATG3'

The qPCR assay conditions used were 95°C for 5 min, followed by 45 cycles of 95°C for 15 s and 60°C for 30 s. The standard curves demonstrated 84% efficiency of the qPCR assay with a 29 GU/reaction limit of detection (Figure 3.3).

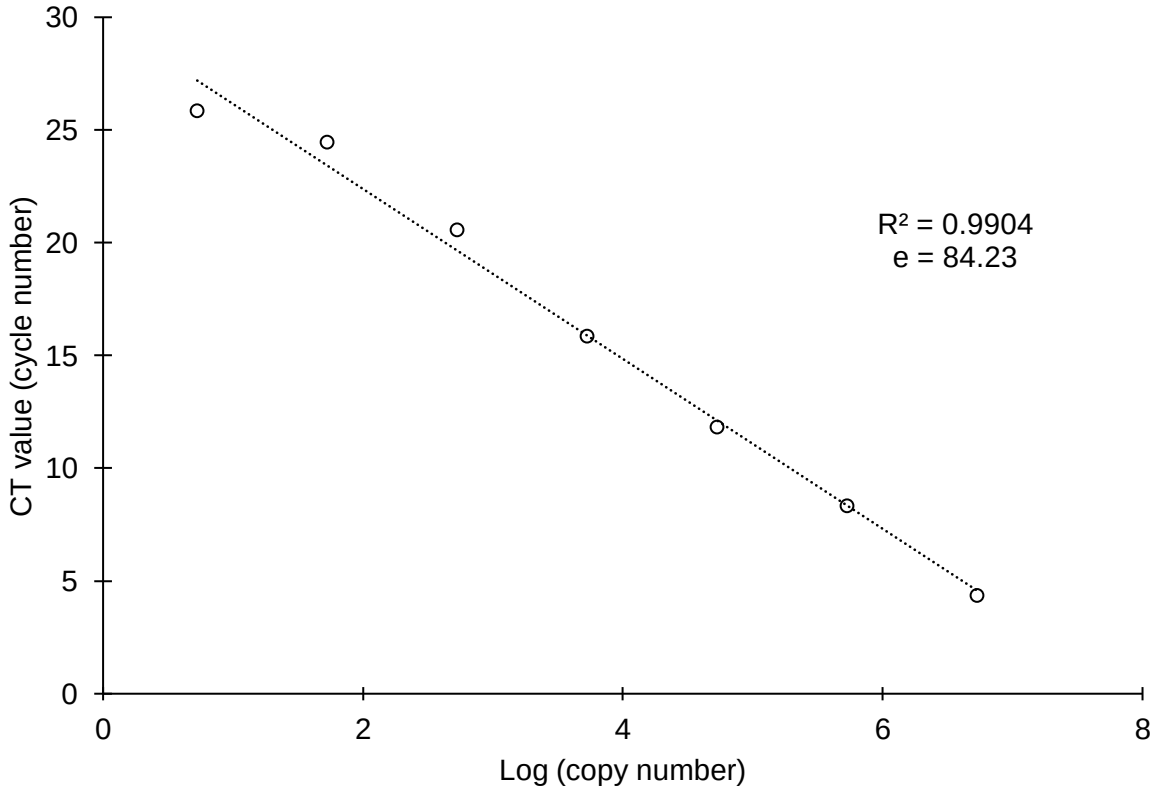

Figure 3.3 – *Acinetobacter baumannii ompA gene* standard curve

3.4.3.4. *Legionella* 16S rDNA gene

The *Legionella* 16S rDNA gene was detected using primers, probes and protocols as previously described by ISO/TS12869:2019 (International Organization for Standardization, 2019). Sequences of primers and probes used were:

Forward primer: 5'-GGAGGGTTGATAGGTTAAGAGCT-3'

Reverse primer: 5'-CCAACAGCTAGTTGACATCGTTT-3'

Fluorogenic probe: 5'-FAM-AGTGGCGAAGGCGGCTACCT-Q-3'

FAM: 6-carboxyfluorescein $\lambda_{(excitation)}/\lambda_{(emission)}$ 495/520 nm, channel for qPCR: $\lambda_{(source)}$ 470 nm and $\lambda_{(detector)}$ 510 nm

Q: Iowa Black® FQ quencher with absorbance spectrum range λ 420 nm to 620 nm with $\lambda_{(max)}$ 531 nm

Accession Number CP021281 was used to design gBlock sequence and was as follows (underlined sequences show primers and bold sequence shows probe binding regions):

5’TAAAGCACTTTCAGTGGGGAGGAGGGTTGATAGGTTAAGAGCTGATTAACTGGACGTTA
CCCACAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTG
CGAGCGTTAATCGGAATTACTGGGCGTAAAGGGTGCGTAGGTGGTTGATTAAGTTATCT
GTGAAATTCCTGGGCTTAACCTGGGACGGTCAGATAATACTGGTTGACTCGAGTATGG
GAGAGGGTAGTGGAATTTCCGGTGTAGCGGTGAAATGCGTAGAGATCGGAAGGAACAC
C**AGTGGCGAAGGCGGCTACCTGGCCT**AATACTGACACTGAGGCACGAAAGCGTGGGG
AGCAAACAGGATTAGATACCCTGGTAGTCCACGCTGTAAACGATGTCAACTAGCTGTTG
GTTATATGAAAATAATTAGTG3’

The qPCR assay conditions used for the amplification were: 95°C for 3 min, followed by 43 cycles of 95°C for 20 s and 60°C for 60 s. The standard curves demonstrated 89% efficiency of the qPCR assay with a 35 GU/reaction limit of detection (Figure 3.4).

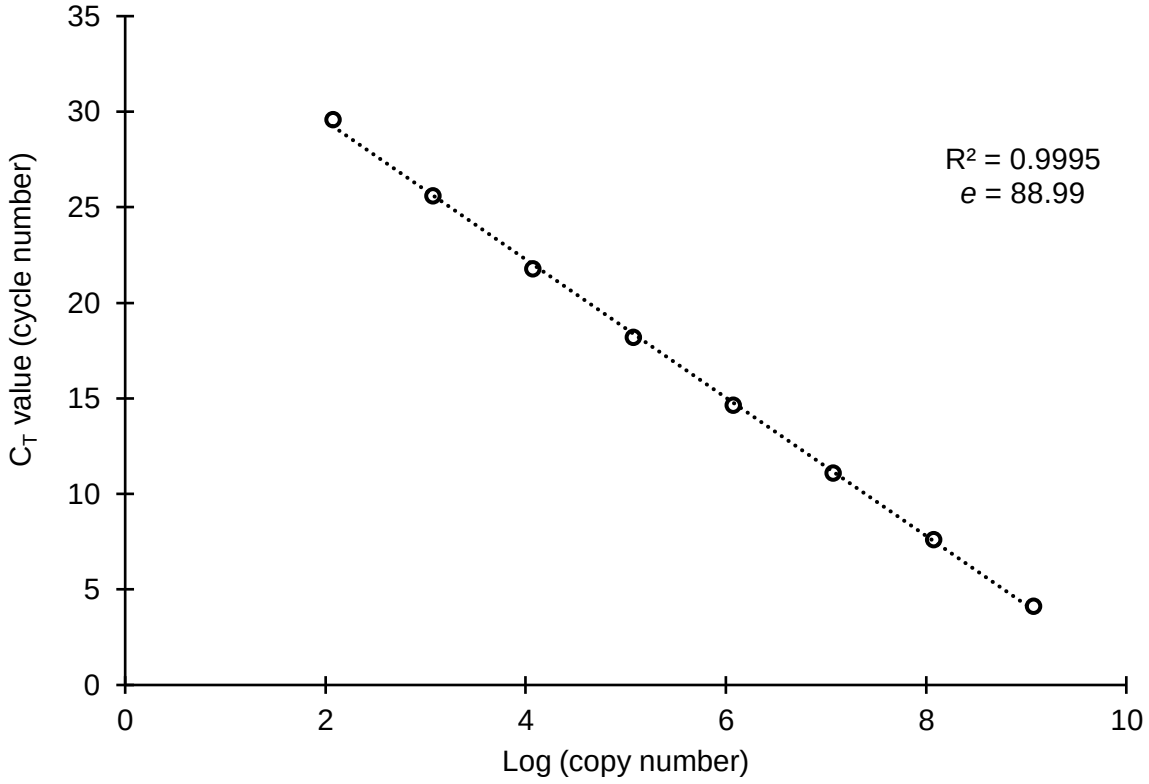

Figure 3.4- *Legionella* 16S rDNA standard curve

3.4.3.5. *Legionella pneumophila mip* gene

The *L. pneumophila mip* gene was detected using primers, probes and protocols as previously described by ISO/TS12869:2019 (International Organization for Standardization, 2019). Sequences of primers and probes used were:

Forward primer: 5'-CCGATGCCACATCATTAGC-3'

Reverse primer: 5'-CCAATTGAGCGCCACTCATAG-3'

Fluorogenic probe: 5'-FAM-TGCCTTTAGCCATTGCTTCCG-Q-3'

FAM: 6-carboxyfluorescein $\lambda_{(excitation)}/\lambda_{(emission)}$ 495/520 nm, channel for qPCR: $\lambda_{(source)}$ 470 nm and $\lambda_{(detector)}$ 510 nm

Q: Iowa Black® FQ quencher with absorbance spectrum range λ 420 nm to 620 nm with $\lambda_{(max)}$ 531 nm

Accession Number KR902705 was used to design gBlock sequence and was as follows (underlined sequences show primers and bold sequence shows probe binding regions):

5’GTCAACAGCAATGGCTGCAACCGATGCCACATCATTAGCTACAGACAAGGATAAGTT
GTCTTATAGCATTGGTGCCGATTTGGGGAAGAATTTTAAAAATCAAGGCATAGATGTTAA
TC**CGGAAGCAATGGCTAAAGGCA**TGCAAGACGCTATGAGTGGCGCTCAATTGGCTTTA
ACCGAACAGCAAATG3’

The qPCR assay conditions used were: 95°C for 3 min, followed by 43 cycles of 95°C for 20 s and 60°C for 60 s. The standard curves demonstrated 99% efficiency of the qPCR assay with a 35 GU/reaction limit of detection (Figure 3.5).

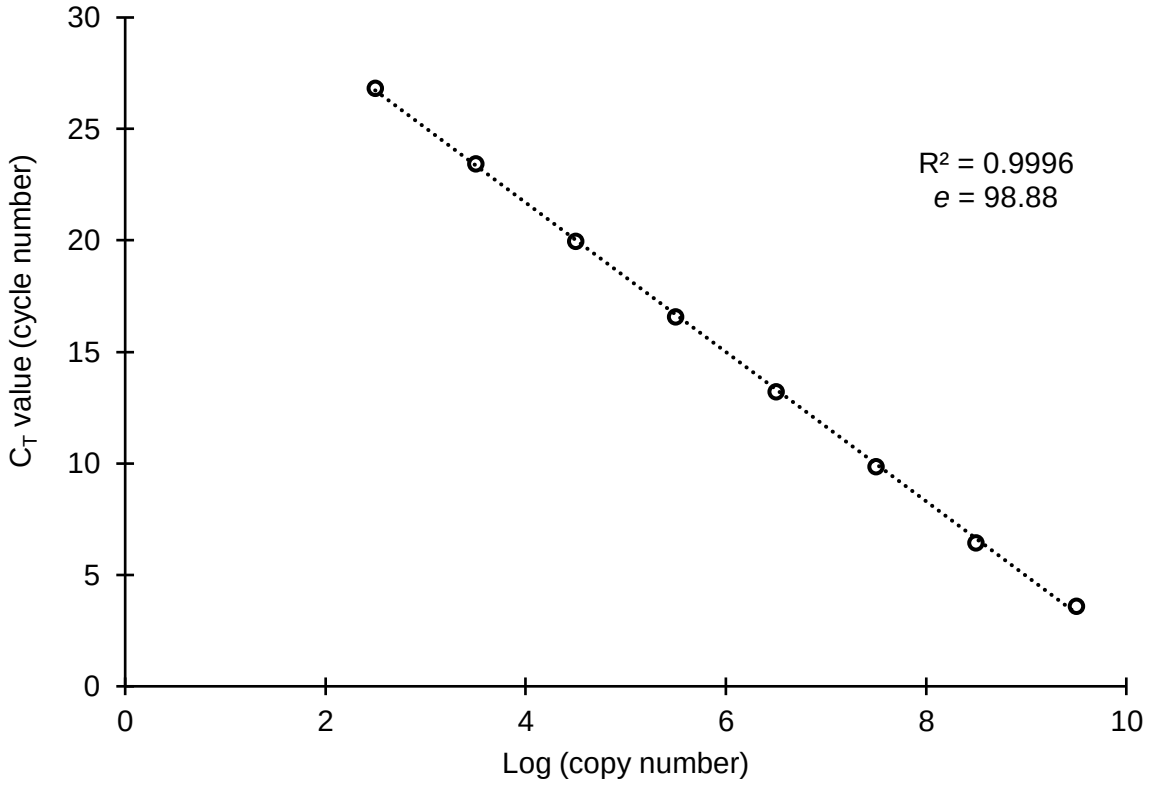

Figure 3.5 – *Legionella pneumophila mip* gene standard curve

3.4.3.6. *Pseudomonas aeruginosa gyrB* gene

The *P. aeruginosa gyrB* gene was detected using primers, probes and protocols as previously described (Lee et al., 2011). Sequences of primers and probes used were:

Forward primer: 5’- GGCGTGGGTGTGGAAGTC- 3’

Reverse primer: 5’- TGGTGGCGATCTTGAACTTCTT-3’

Fluorogenic probe: 6FAM-TGCAGTGGAACGACA- Q 3’

FAM: 6-carboxyfluorescein $\lambda_{(excitation)}/\lambda_{(emission)}$ 495/520 nm, channel for qPCR: $\lambda_{(source)}$ 470 nm and $\lambda_{(detector)}$ 510 nm

Q: Iowa Black® FQ quencher with absorbance spectrum range λ 420 nm to 620 nm with $\lambda_{(max)}$ 531 nm

Accession Number HQ425720.1 was used to design gBlock sequence and was as follows (underlined sequences show primers and bold sequence shows probe binding regions):

5'TCCACTTCAACGTCCAGCGTGAAGAGGACGGCGTGGGTGTGGAAGTCGCCT**TGCAG TGGAACGACA**GCTTCAACGAGAACCTGCTCTGCTTCACCAACAACATCCCGCAGCGTG ACGGCGGCACC3'

The qPCR assay conditions used were: 95°C for 5 min, followed by 45 cycles of 95°C for 15 s, 60°C for 30 s and 72ºC for 20 s. The standard curves demonstrated 84% efficiency of the qPCR assay with a 46.4 GU/reaction limit of detection (Figure 3.6).

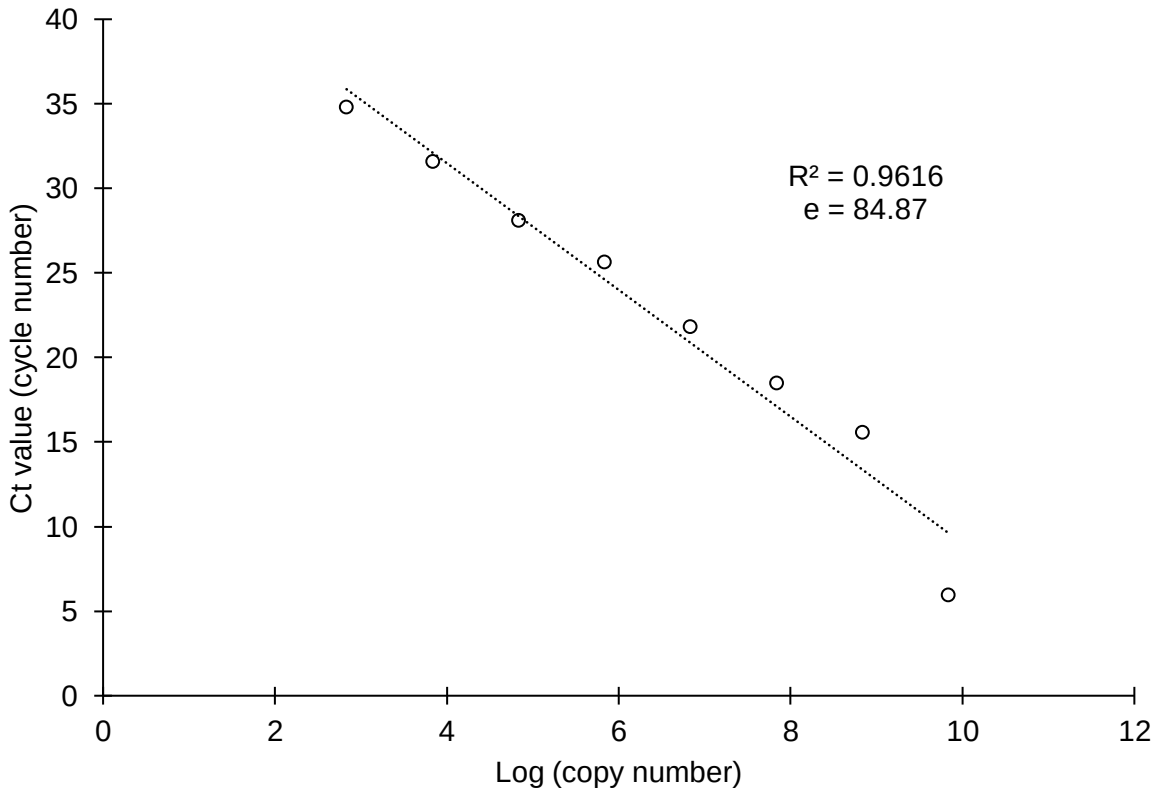

Figure 3.6 - *Pseudomonas aeruginosa gyrB* gene standard curve

3.4.3.7. *Pseudomonas aeruginosa bla*$_{NDM-1}$ resistance gene

The *P. aeruginosa bla*$_{NDM-1}$ carbapenem resistance gene was detected using primer and protocols as previously described (Gondal et al., 2024). Sequences of primers used were:

Forward primer: 5'-GGGCAGTCGCTTCCAACGGT-3'

Reverse primer: 5'-GTAGTGCTCAGTGTCGGCAT-3'

Fluorescent dye: SYBR® Green

Accession Number NG_242571.1 was used to design gBlock sequence and was as follows (underlined sequences show primers binding regions):

5'ACATGCCGGGTTTCGGGGCAGTCGCTTCCAACGGTTTGATCGTCAGGGATGGCGGC
CGCGTGCTGGTGGTCGATACCGCCTGGACCGATGACCAGACCGCCCAGATCCTCAAC
TGGATCAAGCAGGAGATCAACCTGCCGGTCGCGCTGGCGGTGGTGACTCACGCGCAT
CAGGACAAGATGGGCGGTATGGACGCGCTGCATGCGGCGGGGATTGCGACTTATGCC
AATGCGTTGTCGAACCAGCTTGCCCCGCAAGAGGGGATGGTTGCGGCGCAACACAGC
CTGACTTTCGCCGCCAATGGCTGGGTCGAACCAGCAACCGCGCCCAACTTTGGCCCG
CTCAAGGTATTTTACCCCGGCCCCGGCCACACCAGTGACAATATCACCGTTGGGATCG
ACGGCACCGACATCGCTTTTGGTGGCTGCCTGATCAAGGACAGCAAGGCCAAGTCGCT
CGGCAATCTCGGTGATGCCGACACTGAGCACTACGCCGCGTCA3'

The qPCR assay conditions used were: 98^{o}C for 3 min, followed by 40 cycles of 98^{o}C for 15 s and 60^{o}C /30 s with a melt of 65-95^{o}C at 0.5^{o}C increments at 5 s per step. The standard curves demonstrated 90% efficiency of the qPCR assay with a 35 GU/reaction limit of detection (Figure 3.7).

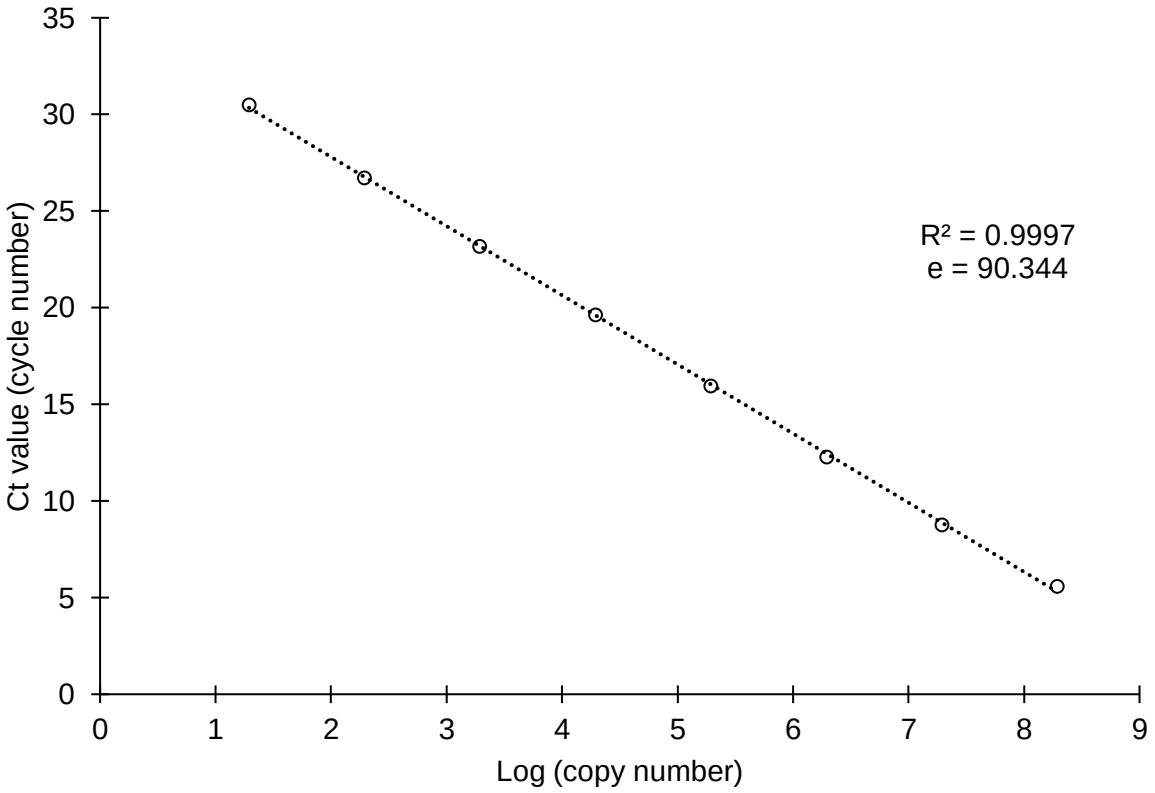

Figure 3.7 – *Pseudomonas aeruginosa bla*$_{NDM-1}$ resistance gene standard curve

3.4.3.8. *Pseudomonas aeruginosa bla*$_{OXA-48}$ resistance gene

The *P. aeruginosa bla*$_{OXA-48}$ carbapenem resistance gene was detected using primer and protocols as previously described (Gondal et al., 2024). Sequences of primers used were:

Forward primer: 5'- GCGTGGTTAAGGATGAACAC-3'

Reverse primer: 5'-CATCAAGTTCAACCCAACCG-3'

Fluorescent dye: SYBR® Green

Accession Number KJ620497.1 was used to design gBlock sequence and was as follows (underlined sequences show primers binding regions):

5'TGGGCGTGGTTAAGGATGAACACCAAGTCTTTAAGTGGGATGGACAGACGCGCGATA
TCGCCACTTGGAATCGCGATCATGATCTAATCACCGCGATGAAATATTCAGTTGTGCCT
GTTTATCAAGAATTTGCCCGCCAAATTGGCGAGGCACGTATGAGCAAGATGCTACATGC
TTTCGATTATGGTAATGAGGACATTTCGGGCAATGTAGACAGTTTCTGGCTCGATGGTG
GTATTCGAATTTCGGCCACTGAGCAAATCAGCTTTTTAAGAAAGCTGTATCACAATAAGT
TACACGTATCGGAGCGCAGTCAGCGCATCGTGAAACAAGCCATGCTGACCGAAGCCAA

TGGCGACTATATTATTCGGGCTAAAACGGGATACTCGACTAGAATCGAACCTAAGATTG
GCTGGTGGGTCGGTTGGGTTGAACTTGATGATAATGTGTG3’

The qPCR assay conditions used were: 98 ^{o}C for 3 min, followed by 40 cycles of 98^{o}C for 15 s and 60^{o}C /30 sec with a melt of 65-95^{o}C at 0.5^{o}C increments at 5 s per step. The standard curves demonstrated 88% efficiency of the qPCR assay with a 32 GU/reaction limit of detection (Figure 3.8).

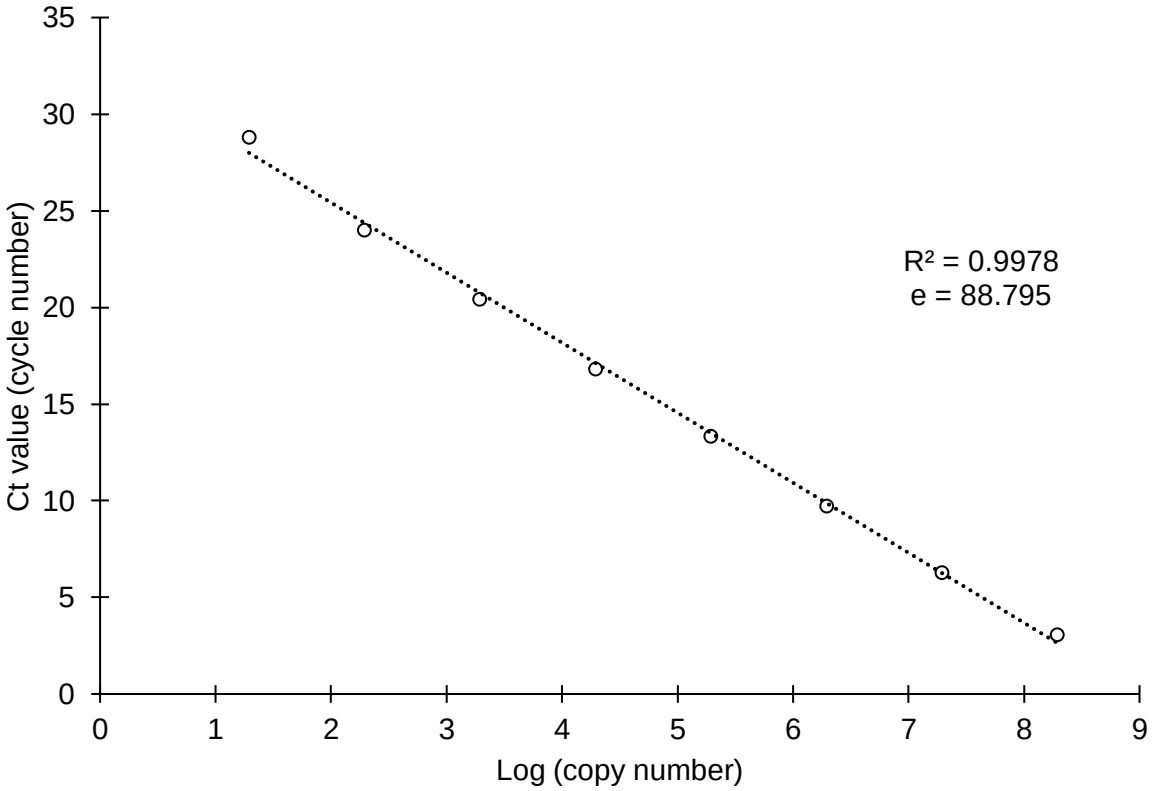

Figure 3.8 – *Pseudomonas aeruginosa bla*$_{OXA-48}$ resistance gene standard curve

3.4.3.9. *Pseudomonas aeruginosa bla*$_{KPC-2}$ resistance gene

The *P. aeruginosa bla*$_{KPC-2}$ carbapenem resistance gene was detected using primer and protocols as previously described (Gondal et al., 2024). Sequences of primers used were:

Forward primer: 5’-GCTACACCTAGCTCCACCTTC-3’

Reverse primer: 5’-ACAGTGGTTGGTAATCCATGC-3’

Fluorescent dye: SYBR® Green

Accession Number KJ620497.1 was used to design gBlock sequence and was as follows (underlined sequences show primers binding regions):

5’TGATTACATCCGGCCGCTACACCTAGCTCCACCTTCAAACAAGGAATATCGTTGATGT
CACTGTATCGCCGTCTAGTTCTGCTGTCTTGTCTCTCATGGCCGCTGGCTGGCTTTTCT
GCCACCGCGCTGACCAACCTCGTCGCGGAACCATTCGCTAAACTCGAACAGGACTTTG
GCGGCTCCATCGGTGTGTACGCGATGGATACCGGCTCAGGCGCAACTGTAAGTTACCG
CGCTGAGGAGCGCTTCCCACTGTGCAGCTCATTCAAGGGCTTTCTTGCTGCCGCTGTG
CTGGCTCGCAGCCAGCAGCAGGCCGGCTTGCTGGACACACCCATCCGTTACGGCAAA
AATGCGCTGGTTCGGTGGTCACCCATCTCGGAAAAATATCTGACAACAGGCATGACGG
TGGCGGAGCTGTCCGCGGCCGCCGTGCAATACAGTGATAACGCCGCCGCCAATTTGTT
GCTGAAGGAGTTGGGCGGCCCGGCCGGGCTGACGGCCTTCATGCGCTCTATCGGCGA
TACCACGTTCCGTCTGGACCGCTGGGAGCTGGAGCTGAACTCCGCCATCCCAGGCGA
TGCGCGCGATACCTCATCGCCGCGCGCCGTGACGGAAAGCTTACAAAAACTGACACTG
GGCTCTGCACTGGCTGCGCCGCAGCGGCAGCAGTTTGTTGATTGGCTAAAGGGAAACA
CGACCGGCAACCACCGCATCCGCGCGGCGGTGCCGGCAGACTGGGCAGTCGGAGAC
AAAACCGGAACCTGCGGAGGGTATGGCACGGCAAATGACTATGCCGTCGTCTGGCCC
ACTGGGCGCGCACCTATTGTGTTGGCCGTCTACACCCGGGCGCCTAACAAGGATGACA
AGTACAGCGAGGCCGTCATCGCCGCTGCGGCTAGACTCGCGCTCGAGGGATTGGGCG
TCAACGGGCAGTAAGGCTCTGAAAATCATCTATTGGCCCACCACCGCCGCCCTTGCGG
GCGGCATGGATTACCAACCACTGTCACATTTAGGCTAGGA3’

The qPCR assay conditions used were: 98^{o}C for 3 min, followed by 40 cycles of 98^{o}C for 15 s and 60^{o}C /30 s with a melt of 65-95^{o}C at 0.5^{o}C increments at 5 s per step. The standard curves demonstrated 94% efficiency of the qPCR assay with a 34 GU/reaction limit of detection (Figure 3.9).

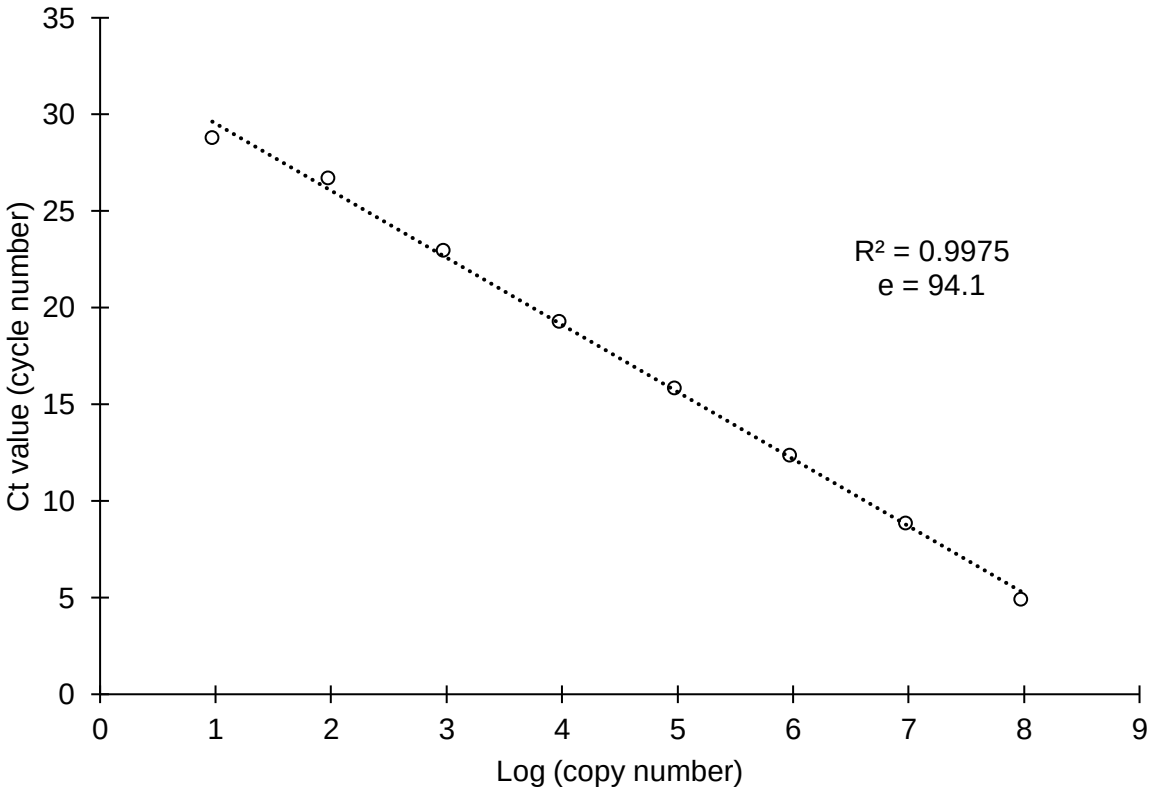

Figure 3.9 – *Pseudomonas aeruginosa bla*$_{KPC-2}$ resistance gene standard curve

3.4.3.10. *Pseudomonas aeruginosa bla*$_{VIM}$ resistance gene

The *P. aeruginosa bla*$_{VIM}$ carbapenem resistance gene was detected using primer and protocols as previously described (Gondal et al., 2024). Sequences of primers used were:

Forward primer: 5'- GATGGTGTTTGGTCGCATA-3'

Reverse primer: 5'- CGAATGCGCAGCACCAG-3'

Fluorescent dye: SYBR® Green

Accession number NG_064786.1 was used to design gBlock sequence and was as follows (underlined sequences show primers binding regions):

5'CTTTACCAGATTGCCGATGGTGTTTGGTCGCATATCGCAACGCAGTCGTTTGATGGC
GCAGTCTACCCGTCCAATGGTCTCATTGTCCGTGATGGTGATGAGTTGCTTTTGATTGA
TACAGCGTGGGGTGCGAAAAACACAGCGGCACTTCTCGCGGAGATTGAGAAGCAAATT
GGACTTCCTGTAACGCGTGCAGTCTCCACGCACTTTCATGACGACCGCGTCGGCGGCG
TTGATGTCCTTCGGGCGGCTGGGGTGGCAACGTACGCATCACCGTTGACACGCCGGC
TAGCCGAGGTAGAGGGGAACGAGATTCCCACGCACTCTCTAGAAGGACTCTCATCGAG

CGGGGACGCAGTGCGCTTCGGTCCAGTAGAACTCTTCTATCCTGGTGCTGCGCATTCG
ACCGACAACTTAGTT3’

The qPCR assay conditions used were: 98ºC for 3 min, followed by 40 cycles of 98ºC for 15 s and 60ºC/30 sec with a melt of 65-95ºC at 0.5ºC increments at 5 s per step. The standard curves demonstrated 82% efficiency of the qPCR assay with a 41 GU/reaction limit of detection (Figure 3.10).

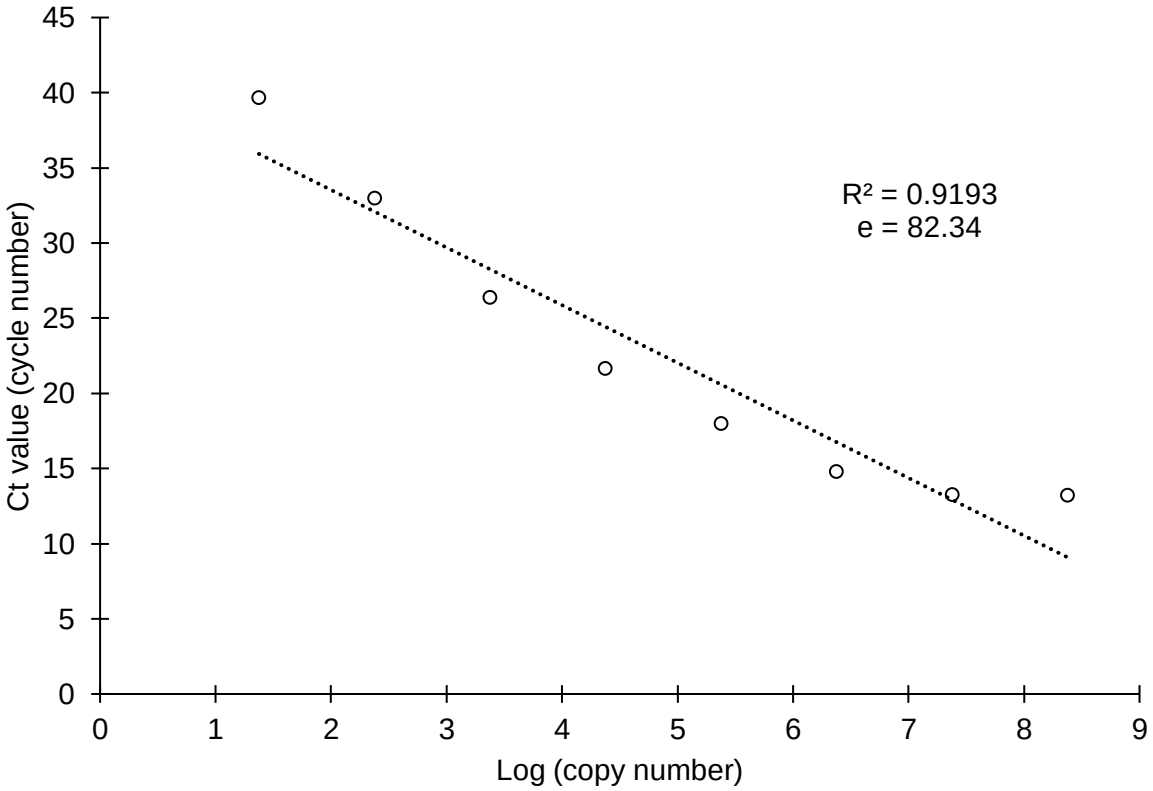

Figure 3.10 – *Pseudomonas aeruginosa bla*$_{VIM}$ resistance gene standard curve

3.4.3.11. *Staphylococcus aureus nuc* gene

The *S. aureus nuc* gene was detected using primers, probes and protocols as previously described (Galia et al., 2019). Sequences of primers and probes used were:

Forward primer: 5’-AAATTACATAAAGAACCTGCGACA-3’

Reverse primer: 5’-GAATGTCATTGGTTGACCTTTGTA-3’

Fluorogenic probe: 6FAM-AATTTAACCGTATCACCATCAATCGCTTT-Q 3’

FAM: 6-carboxyfluorescein $\lambda_{(excitation)}/\lambda_{(emission)}$ 495/520 nm, channel for qPCR: $\lambda_{(source)}$ 470 nm and $\lambda_{(detector)}$ 510 nm

Q: Iowa Black® FQ quencher with absorbance spectrum range λ 420 nm to 620 nm with $\lambda_{(max)}$ 531 nm

Accession number GQ370471.1 was used to design gBlock sequence and was as follows (underlined sequences show primers and bold sequence shows probe binding regions):

5'TGCAACTTCAACTAAAAAATTACATAAAGAACCTGCGACATTAATT**AAAGCGATTGATG GTGATACGGTTAAATT**AATGTACAAAGGTCAACCAATGACATTCAGACTATTATTAGTTG ATACACC3'

The qPCR assay conditions used were: 95°C for 5 min, followed by 45 cycles of 95°C for 15 s, 60°C for 30 s and 72ºC for 20 s. The standard curves demonstrated 91% efficiency of the qPCR assay with a 44.4 GU/reaction limit of detection (Figure 3.11).

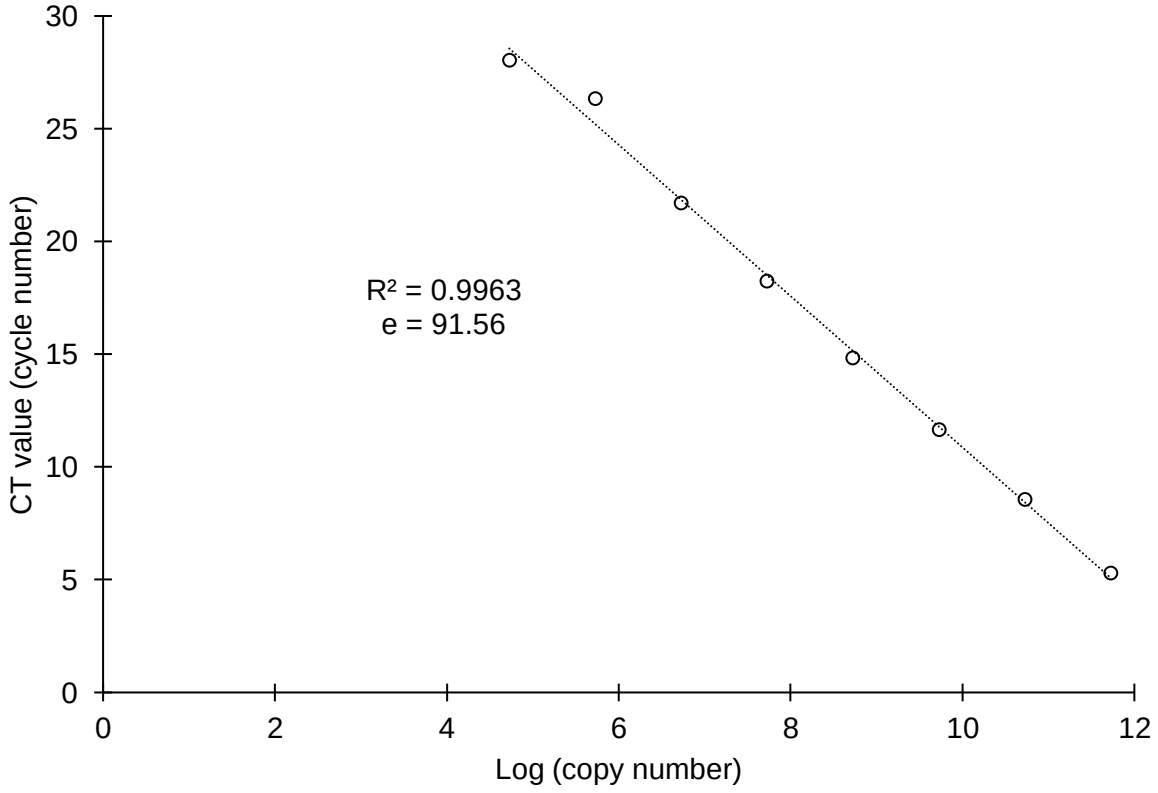

Figure 3.11 - *Staphylococcus aureus nuc* gene standard curve

3.4.3.12. *Mycobacterium avium* complex 23S rRNA

The *M. avium* complex (MAC) 23SrRNA gene was detected using primers and protocols as previously described (Park et al., 2000; Whiley et al., 2014). Sequences of primers used were:

Forward primer: 5'-CCCTGAGACAACACTCGGTC-3'

Reverse primer: 5'-ATTACACATTTCGATGAACGC-3'

Fluorescent dye: SYTO9

Accession number X74494.1 was used to validate primer design and was as follows (underlined sequences show primers):

5’TGCGGTTGGATCACCTCCTTTCTAAGGAGCACCACGAAAAGCACCCCAACTGGTGGG
GTGCGAGCCGTGAGGGGTTCCCGTCTGTAGTGGACGGGGGCCGGGTGCGCAACAGC
AAATGATTGCCAGACACACTATTGGGCCCTGAGACAACACTCGGTCCGTCCGTGTGGA
GTCCCTCCATCTTGGTGGTGGGGTGTGGTGTTTGAGTATTGGATAGTGGTTGCGAGCA
TCTAGATGAGCGCATGGTCTTGGTGGCCGGCGTTCATCGAAATGTGTAATTTCTTTTTT
AACTCTTGTGTGTAAGTAAGTGTTTAAGGGCGCATGGTGGATGCCTTGGCATCGAGAG
CCGATGAAGGACGTGGGAGGCTGCGATATGCCTCGGGGAGCTGTCAACCGAGCATTG
ATCCGAGGATTTCCGAATGGGGGA3;

A purified PCR product was using to create the standard curve from 10^9 – 10^0 copies (Whiley et al., 2014). The qPCR assay conditions used were: 95°C for 5 min, followed by 45 cycles of 95°C for 15 s, 50°C for 30 s and 72°C for 20 s. The standard curves demonstrated 91% efficiency of the qPCR assay with a 25 GU/reaction limit of detection (Figure 3.12).

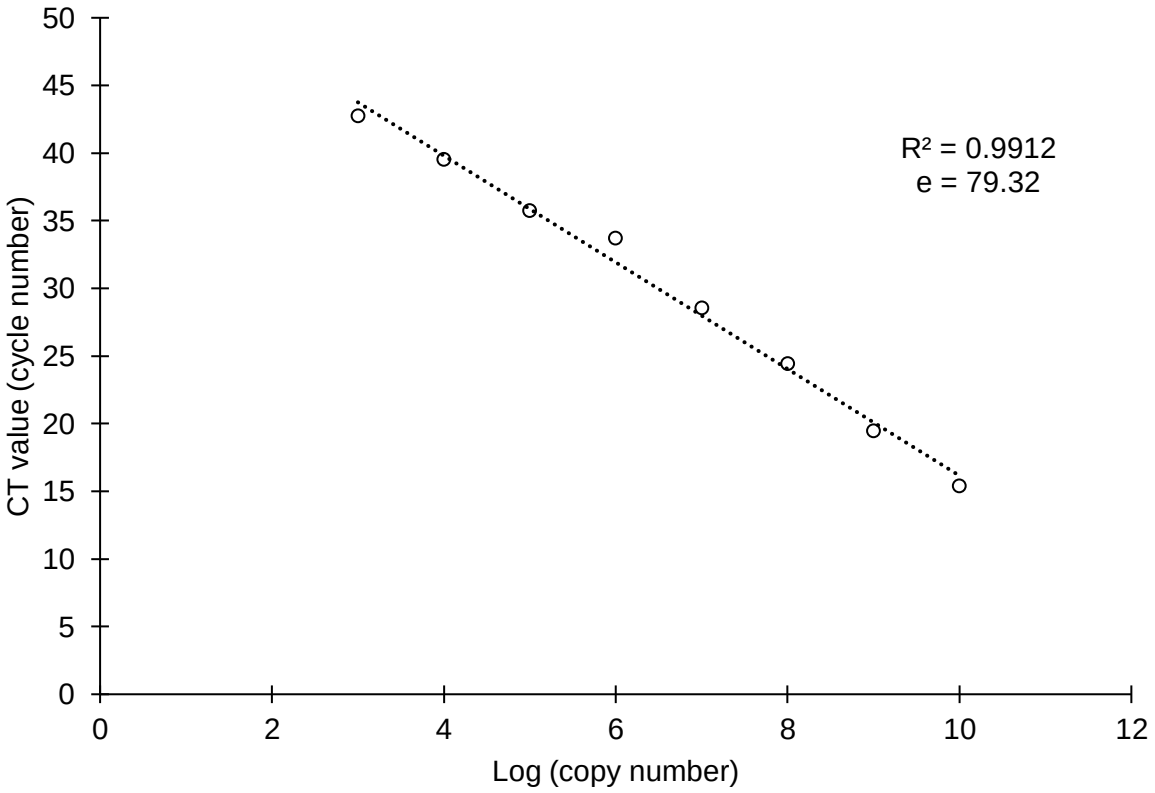

Figure 3.12 – *Mycobacterium avium* complex 23S rRNA gene standard curve

3.5. Viability based flow cytometry

A viability-based flow cytometry-cell sorting and qPCR (VFC+qPCR) assay was used to detect and quantify viable but not culturable bacteria.

3.5.1. Sample processing and staining

The BD™ cell viability kit (349480, BD™)(Becton Dickinson, Franklin Lakes, NJ, USA) was used to stain and quantify alive, injured and dead bacterial cell populations (Nisar et al., 2023a). Briefly, 300 µL of drinking water and biofilm suspensions (See Section 3.2.1.2) were mixed with 200 µL of filtered staining buffer (1 mM EDTA and 0.01% tween-20 in 1X PBS, pH 7.4 ± 0.1) and 420 nm thiazole orange (TO; λ(excitation)/λ(emission): 512/533 nm) and 48 µM propidium iodide (PI; λ(excitation)/λ(emission): 537/618 nm) were added to the mixture and vortexed. Samples were incubated at 5^{o}C for 15 min when 50 µL of counting beads were added.

3.5.2. Gaiting

Analysis and cell sorting were performed on a FACSAria Fusion flow cytometer (Becton Dickinson) under sterile conditions. TO and PI fluorescence plots were used to discern alive, injured, and dead bacterial cells. The bacterial populations were first gated based on their

forward (FSC) and side scatter (SSC) properties. To optimise the gaiting, control samples containing killed or alive *A. baumannii, P. aeruginosa* and MAC were used to position the gates. Control suspensions of *P. aeruginosa*, *A. baumannii* and MAC were prepared to develop VFC+qPCR assays. Briefly, overnight cultures of *P. aeruginosa* and *A. baumannii* were prepared in tryptone soya broth and adjusted to an optical density (OD) 600 nm measured using a UV-1800 Spectrophotometer (Shimadzu UV-1800). A MAC suspension was prepared by pipetting 5 mL of 1X PBS onto R2A agar plate containing MAC colonies. The colonies were harvested by gently scraping with a spreader to resuspend them into the PBS. The suspension was transferred to a sterile centrifuge tube (15 mL) containing 5 mL of sterile PBS and homogenized on a vortex mixer (SEM® Vor·Mix) for one min. The turbidity of the suspension was adjusted to an OD_{600} 0.1 (1.5×10^8 CFU/mL) (Sattar et al., 2018; Thomson et al., 2008). A blank control of the buffer + dyes were run to confirm no auto fluorescence from debris. Approximately 10^2 to 10^6 cells were sorted from each of the alive, injured, and dead populations.

4. MICROBIAL RISKS AND PATHOGEN PREVALENCE IN DRINKING WATER AND PLUMBING SYSTEMS

In this chapter the prevalence and distribution of OPPPs in drinking water and plumbing biofilms across healthcare and residential settings is explored. This chapter addresses Objective 2 and includes an unpublished manuscript that is currently under review. This manuscript examines how the drinking water plumbing environment serves as a reservoir for OPPPs and explores the associated microbial risks. The findings contribute to understanding the complex interactions between pathogens, their environments, and factors such as building type, water quality, and plumbing materials, informing strategies to mitigate the risks posed by OPPPs in diverse built environments.

CITATION: Microbial risks associated with drinking water and plumbing biofilms: Prevalence of opportunistic premise plumbing pathogens in healthcare and residential settings

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This article is currently under review in Water Research

Keywords: drinking water, opportunistic premise plumbing pathogens, drinking water associated pathogens, biofilm, healthcare associated infection

4.1. Abstract

The persistence of opportunistic premise plumbing pathogens (OPPPs) in drinking water plumbing systems poses a significant public health risk that is receiving increasing attention yet remains poorly understood. This study investigated the co-occurrence of OPPPs and the influence of building infrastructure properties on their prevalence. Drinking water and biofilm samples were collected from hospitals and private residences across Australia to investigate the abiotic and biotic factors contributing to the growth and proliferation of OPPPs. Quantitative polymerase chain reaction assays revealed that 41% of samples tested positive for *Pseudomonas aeruginosa*, 26% for *Staphylococcus aureus*, 26% for *Legionella* spp., 24% for *Legionella pneumophila*, and 14% for *Acinetobacter baumannii*. Furthermore, free-living amoebae, including *Vermamoeba vermiformis* (46%) and *Acanthamoeba* spp. (25%), were frequently detected, with *Acanthamoeba* spp. demonstrating a significant positive correlation with all bacterial OPPPs. Overall, results indicated a statistically higher prevalence of OPPPs in residential properties and in biofilms. However, building characteristics, including stagnation, hot water system type, and building age, had inconsistent influences on individual OPPP prevalence. These results emphasize the need to incorporate risk assessments regarding the complex factors within the premise plumbing environment that contribute to pathogen persistence, to inform evidence based targeted preventative strategies for at-risk populations. These findings are particularly critical for individuals receiving healthcare at home, as inconsistent water treatment and monitoring in residential settings may increase their risk of exposure to OPPPs.

4.2. Introduction

Opportunistic premise plumbing pathogens (OPPPs), such as *Legionella pneumophila, Pseudomonas aeruginosa* and *Acinetobacter baumannii* can persist in drinking water plumbing environments (Falkinham et al., 2015). These pathogens are capable of surviving under low-nutrient conditions, in protozoan hosts and in biofilms formed on the surface of plumbing systems (Hayward et al., 2022b). The presence of OPPPs in drinking water plumbing in receiving increasing attention, particularly in the healthcare space (Perkins et al., 2019). The incidence of OPPP related infection has overtaken enteric pathogen infections as the leading cause of water related outbreaks (Beer et al., 2015; Collier et al., 2021). It is estimated that approximately 7.15 million waterborne illnesses occur each year in the United States (US), and most of hospitalisations and deaths were caused by biofilm-associated pathogens including *Pseudomonas* spp., *Legionella* spp. and nontuberculous mycobacteria (Collier et al., 2021). The significance of OPPPs is exacerbated by antimicrobial resistance and virulence factors (Hayward et al., 2022a; LeChevallier et al., 2024). The burden of disease from OPPPs such as *P. aeruginosa* and *A. baumannii* is unclear as these infections

are rarely nationally notifiable (Centers for Disease Control and Prevention, 2024b; Commonwealth of Australia, 2024). Furthermore, the estimates of OPPP HAIs are an underestimation as the incidence of community acquired infection remains unclear (Collier et al., 2021; Hayward et al., 2022b).

Traditionally, drinking water plumbing systems were considered a low risk for diverse microbial communities due to the harsh environmental conditions (LeChevallier et al., 2024). However, recent literature has demonstrated that clinically relevant pathogens can survive in drinking water plumbing systems, and that point of use water related devices may be contaminated by the user (Hayward et al., 2024; Huang et al., 2021; Kelly et al., 2014; Nisar et al., 2023b). These pathogens may be transmitted via consumption, inhalation or from contact with a contaminated water source (Dean et al., 2020; World Health Organization, 2016). Previous research has focussed on the aerosolization of *Legionella* spp. from showers and tap faucets (Bollin et al., 1985; Chang et al., 2012; Kanamori et al., 2016). However, it is unclear if the other OPPPs are also primarily transmitted via the same pathways, considering the diverse range of potential infection types including wound, catheter and central line infections (Ayoub Moubareck et al., 2020; Qin et al., 2022; Tong et al., 2015).

Completely preventing the colonisation of drinking water plumbing systems with OPPPs is unrealistic. It is impractical for water utilities to implement major changes in water temperature or residual disinfection throughout an entire drinking water distribution system. Particularly when these pathogens can be protected from traditional treatment methods by residing in biofilms (Falkinham, 2015). This is further complicated by their survival in free living amoeba hosts, a role that is not well understood for many clinically relevant bacteria (Gomez-Alvarez et al., 2023; Tanya S. Isaac et al., 2020; Nisar et al., 2022; Shaheen et al., 2019). Consequently, effective management of building water systems is crucial to control the risk posed by the growth and proliferation of these pathogens at the point of use. The prevalence of OPPPs in building water systems is influenced by a number of factors such as building size, plumbing system age, flow rate, temperature and water storage (Brazeau et al., 2011; Dai et al., 2018; Leslie et al., 2021; Nisar et al., 2023b). Buildings without evidence-based water management protocols can support OPPP growth and proliferation in drinking water by increasing stagnation, inadequate temperature control and reduction in disinfectant residual (Ley et al., 2020; William J Rhoads et al., 2016). Therefore, a multi-barrier approach is required to control the growth and proliferation of these functionally diverse pathogens (Hayward et al., 2022b; Leslie et al., 2021). Before an appropriate combination of barriers can be determined, it is important to know what factors, both biotic

and abiotic, contribute to the persistence of these pathogens in complex drinking water plumbing systems.

There are limited studies comprehensively investigating the presence of OPPPs in Australia drinking water. More broadly, there are limited studies investigating the relationships between OPPPs and their protozoan hosts. In the present study, water and biofilm samples were collected from hospital and residential building drinking water plumbing systems and were screened for the presence of *Legionella, L. pneumophila, P. aeruginosa, A. baumannii, S. aureus, Acanthamoeba* and *V. vermiformis*. This is the first comprehensive study which used molecular tools for the screening of OPPPs in Australian domestic and hospital drinking water plumbing systems. The results of this study provide critical insights into the environmental factors influencing the prevalence of OPPPs, contributing to the development of more effective water management and public health strategies.

4.3. Methods

4.3.1. Sample collection and processing

This study was approved by the Flinders University Social and Behavioural Research Ethics Committee (SBREC Project Number 7291). From February 2019 to May 2024, 218 water and 182 biofilm samples were collected from showers, faucets, drains, baths, basins and overflows from domestic and healthcare facility water systems. There were 154 domestic samples and 246 hospital samples collected across New South Wales and South Australia. Due to ethical policies, the authors cannot disclose the geographic location of these premises. The physical and environmental parameters of the sampling site were recorded upon sampling where possible including outlet usage frequency, water source, building age, plumbing system age, water heating system and hot water storage (Table 12.1 & Table 12.2). Water and biofilm samples were transported according to the Centers for Disease Control and Prevention guidelines (Centers for Disease Control and Prevention, 2019b). Briefly, 1 L potable water samples were collected in sterile screw capped wide mouth plastic bottles (2105-0032 Nalgene) containing 1 mL 0.1 M $Na_2S_2O_3$ (124270010, ACROS Organics™) to neutralize residual chlorine-based disinfectants. Sterile polyurethane-tipped swabs (CleanFoam®TX751B, Texwipe®) were used to collect biofilms. These swabs were moistened with sterile water and the surface of the faucet aerator or drain was swabbed for 10 sec. The swab was then placed in a 10 mL screw capped vial with 5 mL of 1X sterile phosphate buffered saline (PBS). All samples were stored at 5°C and analysed within 72 h of collection. All water and biofilm samples were vacuum filtered onto a 47 mm diameter 0.2 µm polycarbonate membrane (GTTP04700, Isopore™). The membrane was then transferred to a sterile 10 mL screw top vial containing 3 mL of sterile PBS followed by 5 min

shaking (Griffin Flask Shaker), vortexing (SEM® Vor Mix) and sonication (CooperVision® 895 Ultrasonic Cleaner). This suspension was used for molecular analysis.

4.3.2. Microbial testing

DNA was extracted for quantitative polymerase chain reaction (qPCR) analysis from 1 mL of the concentrated water sample or resuspended biofilm sample using the BIO-RAD Aquadien™ DNA extraction and purification kit following the manufacturer's instructions (Bio-Rad Laboratories, Inc., Sydney, NSW, Australia). Ten µL lysozyme (89833) (ThermoFisher Scientific: Adelaide, Australia) (25 mg/mL in 1X PBS) was added to the extraction sample and incubated at 37^{o}C for 15 min prior to the boiling step.

The ISO/TS12869:2019 standard qPCR assay was used to enumerate the 16S rDNA *Legionella* spp. gene and *L. pneumophila mip* gene (International Organization for Standardization, 2019). The 18S rDNA gene was amplified to quantify *Acanthamoeba* and *V. vermiformis* (Qvarnstrom et al., 2006; Scheikl et al., 2016). The *ompA*, *gyrB* and *nuc* genes were used to quantify *A. baumannii, P. aeruginosa* and *S. aureus,* respectively (Galia et al., 2019; Lee et al., 2011; McConnell Michael et al., 2012). *Legionella* spp.(GenBank Acc CP021281), *L. pneumophila* (GenBank Acc KR902705), *Acanthamoeba castellanii* (GenBank Acc U07413), *V. vermiformis* (GenBank Acc KT185625), *A. baumannii* (GenBank Acc OL347635.1), *P. aeruginosa* (GenBank Acc HQ425720.1) and *S. aureus* (GenBank Acc GQ370471.1) gBlock gene fragments (IDT™) were used to create a standard curve using 10-fold serial dilutions. qPCR reaction mixes consisted of specific oligos (BIO-RAD Laboratories Ltd.), 2X Sso Advanced™ universal probe supermix (172-5281, BIO-RAD Laboratories Ltd.) and template DNA were used in a Rotor-Gene Q thermal cycler (QIAGEN Ltd.). Sequences of oligos and qPCR conditions are described in Table 13.1. All assays were performed in triplicate and mean Ct values were used for estimation of the genomic unit litre of water (GU/L) and genomic unit per mL of biofilm (GU/swab). The qPCR assays for all target bacteria and amoeba yielded a linear relationship between the Ct and log gBlock DNA concentration, and were used to determine the assay efficiency and the limit of detection.

4.3.3. Statistical analysis

Data analyses were performed using SPSS and R software. The results were interpreted at the level of significance $p<0.05$.

4.4. Results

4.4.1. Abiotic factors

Abiotic factors including type of water heating system, hot water storage, building age, plumbing system age and sampled outlet usage frequency were recorded for all domestic water system samples.

Overall, 52% (n = 80) of samples were collected from residential properties with gas hot water heating systems, 25% (n = 39) had electric systems, 1.3% (n = 2) had solar systems and 21% (n = 33) of respondents did not know what system their property had. Half (50%, n = 78) of samples were collected from properties that did not have hot water storage (instantaneous hot water heating), 19% (n = 29) did have hot water storage and 31% (n = 47) of samples were collected from properties where the resident did not know if they had hot water storage or not.

Overall, 64% (n = 99) of samples were collected from properties more than 20 years old, 12% (n = 19) were from buildings less than 5 years old, 5.8% (n = 9) were taken from buildings that were 5-9 years old and 18% (n = 27) were collected from buildings where the resident could not estimate how old the building was. Regarding plumbing system age, 28% (n = 43) of samples were collected from outlets that were less than 5 years old, 25% (n = 38) were collected from outlets that were more than 20 years old, 19% (n = 29) were collected from outlets that were 5-9 years old, 10% (n = 16) from outlets 10-14 years old and 18% (n = 28) were collected from outlets where the residents did not know how old it was.

Most samples, 61% (n = 94), were collected from outlets used 2-10 times per day. 15% of samples were collected from outlets used more than 10 times per day, 5.2% (n = 8) from outlets used less than once per month, 1.9% (n = 3) from outlets used once per week, 1.3% (n = 2) from outlets used once per fortnight, and, 15% (n = 24) were collected from outlets where the resident did not know how frequently it was used.

4.4.2. Amoeba

4.4.2.1. Vermamoeba vermiformis

Overall, 46% (n=183/400) of total (residential and hospital) samples were positive for *V. vermiformis* (18S rDNA gene) with a concentration range of 2.7 x 10^2 to 7.47 x 10^7 GU/L and 1.2 x 10^2 to 3.45 x 10^8 GU/swab (Table 12.3). There was no statistically significant difference in *V. vermiformis* prevalence between residential and hospital buildings (p=0.261) (Table 4.1). Furthermore, there was no statistically significant difference in prevalence between water or biofilm samples (p=0.197) or between outlet types (p=0.065) (Table 4.1).

In residential water, *V. vermiformis* was significantly positively correlated with *Legionella* spp. (ρ = 0.328, p = 0.011), *L. pneumophila* (ρ = 0.316, p = 0.015) and *Acanthamoeba* spp. (ρ = 0.333, p = 0.01) (Figure 4.1A), however, its presence it was not significantly correlated with any of the target OPPPs in residential biofilms (Figure 4.1B). In hospital water, *V. vermiformis* was significantly positively correlated with *L. pneumophila* (ρ = 0.389, p = 0.001) (Figure 4.2A), and in biofilms it was significantly positively correlated with *L. pneumophila* (ρ = 0.319, p = 0.003) and *Acanthamoeba* spp. (ρ = 0.305, p = 0.004) (Figure 4.2B).

Prevalence of *V. vermiformis* increased as outlet usage decreased in both hospitals (ρ=-0.231, p=0.001) and residential buildings (-ρ=0.197, p=0.024) (Figure 4.3).

Table 4.1 Prevalence of target opportunistic premise plumbing pathogens in residential and hospital water systems detected by quantitative polymerase chain reaction.

	Vermamoeba vermiformis (18S rDNA gene)		*Acanthamoeba* spp. (18S rDNA gene)		*Pseudomonas aeruginosa* (*gyrB* gene)		*Staphylococcus aureus* (*nuc* gene)		*Legionella* spp. (16S rDNA gene)		*Legionella pneumophila* (*mip* gene)		*Acinetobacter baumannii* (*ompA* gene)	
Water														
Domestic	38.98% (n=23/59)	P=0.932	11.86% (n=7/59)	P=1	45.09% (n=23/51)	P=0.394	27.08% (n=13/48)	P=1	59.32% (n=35/59)	P=0.001	40.67% (n=24/59)	P=0.3	2.12% (n=1/47)	P=0.825
Hospital	49.36% (n=78/158)		14.46% (n=23/159)		33.02% (n=36/109)		24.13% (n=21/87)		27.04% (n=43/159)		23.27% (n=37/159)		3.7% (n=4/108)	
Total	**46.54% (n=101/217)**		**13.76% (n=30/218)**		**36.88% (n=59/160)**		**25.19% (n=34/135)**		**35.77% (n=78/218)**		**27.98% (n=61/218)**		**3.23% (n=5/155)**	
Biofilm														
Domestic	46.31% (n=44/95)	P=1	58.94% (n=56/95)	P=0.001	63.04% (n=58/92)	P=0.001	34.06% (n=31/91)	P=0.101	36.31% (n=25/95)	P=0.199	18.94% (n=18/95)	P=0.219	34.06% (n=31/91)	P=0.002
Hospital	43.67% (38/87)		14.94% (n=13/87)		12.76% (n=6/47)		12.76% (n=6/47)		11.49% (n=10/87)		8.04% (n=7/87)		12.76% (n=6/47)	
Total	**45.05% (n=82/182)**		**37.91% (n=69/182)**		**46.04% (n=64/139)**		**26.81% (n=37/138)**		**19.23% (n=35/182)**		**13.73% (n=25/182)**		**26.81% (n=37/138)**	

Note: Statistically significant relationships defined as $p<0.05$ and denoted in green. Relationships not statistically significant ($p>0.05$) denoted in red.

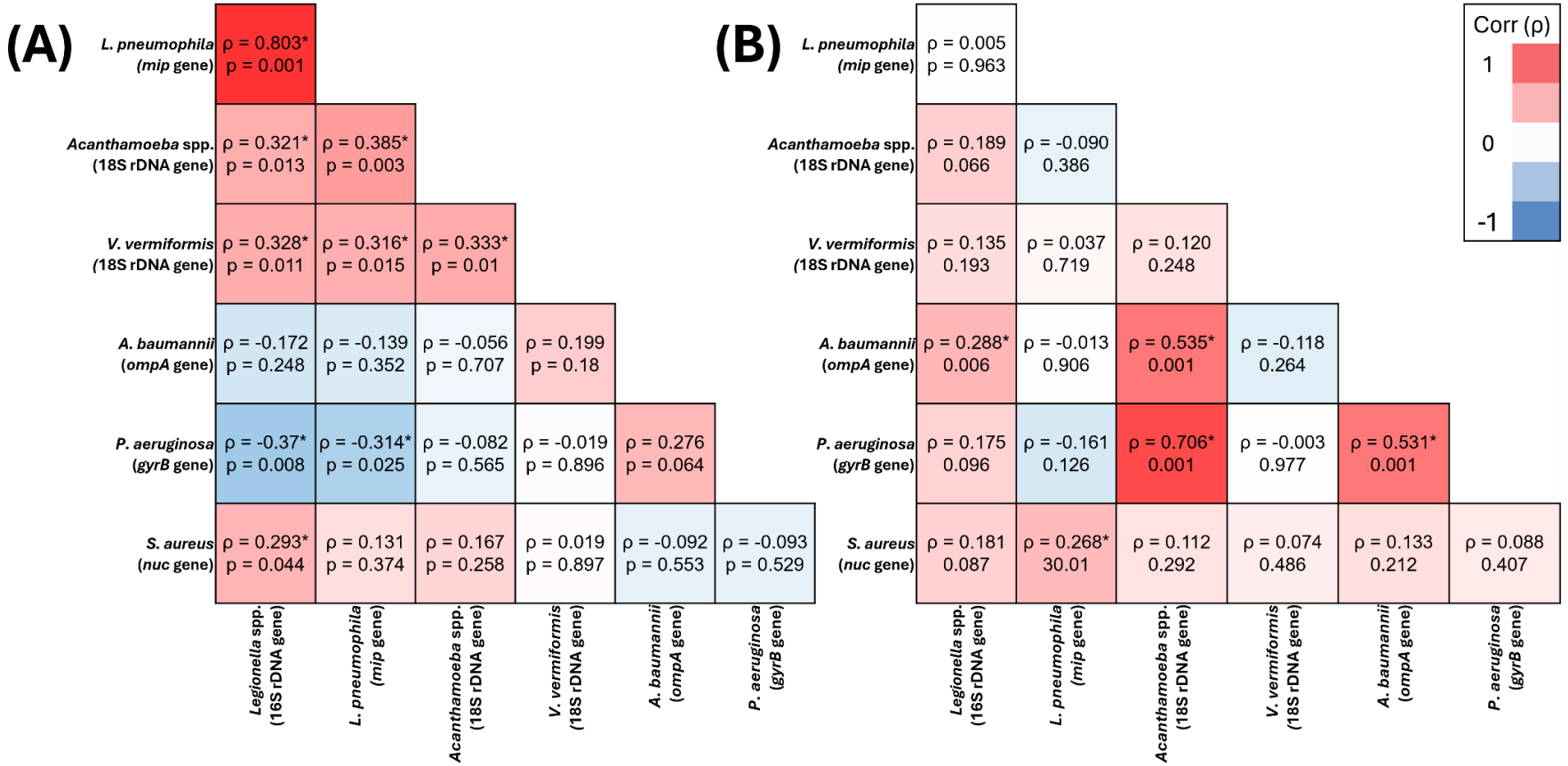

Figure 4.1 - Spearman's correlation analysis of the presence of target opportunistic premise plumbing pathogens in residential water (A) (n=59) and residential biofilm (B) (n=95) samples

The heat map values show the Spearman's correlation coefficient (ρ) to a significance threshold of $p < 0.05$ (* indicates significant relationships), ranging from −1.0 (blue) to 1.0 (red). A minus value demonstrates a negative association, whereas a positive value demonstrates positive association.

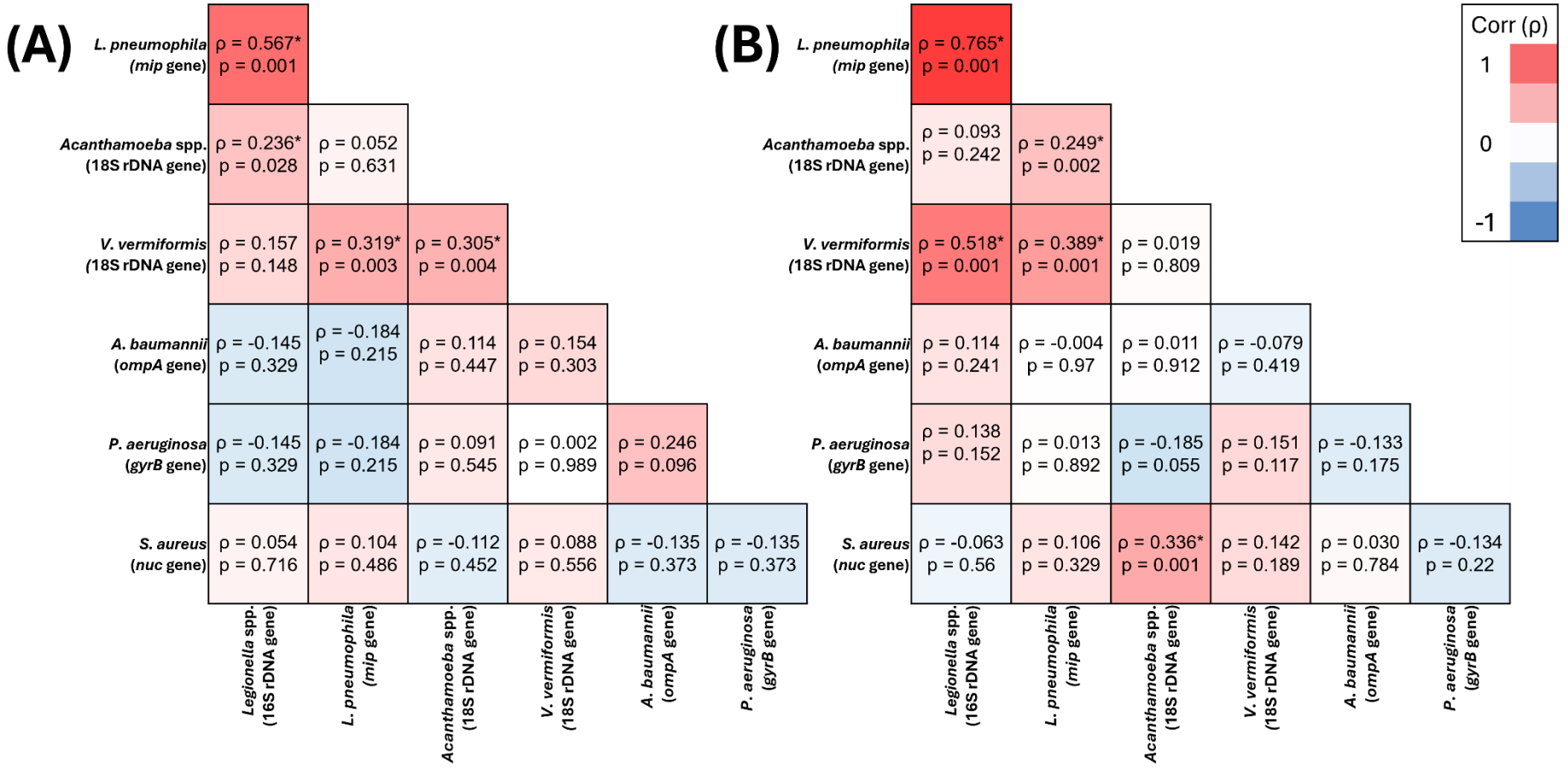

Figure 4.2 - Spearman's correlation analysis of the presence of target opportunistic premise plumbing pathogens in hospital water (A) (n=159) and hospital biofilm (B) (n=87) samples

The heat map values show the Spearman's correlation coefficient (ρ) to a significance threshold of p < 0.05 (* indicates significant relationships),, ranging from −1.0 (blue) to 1.0 (red). A minus value demonstrates a negative association, whereas a positive value demonstrates positive association.

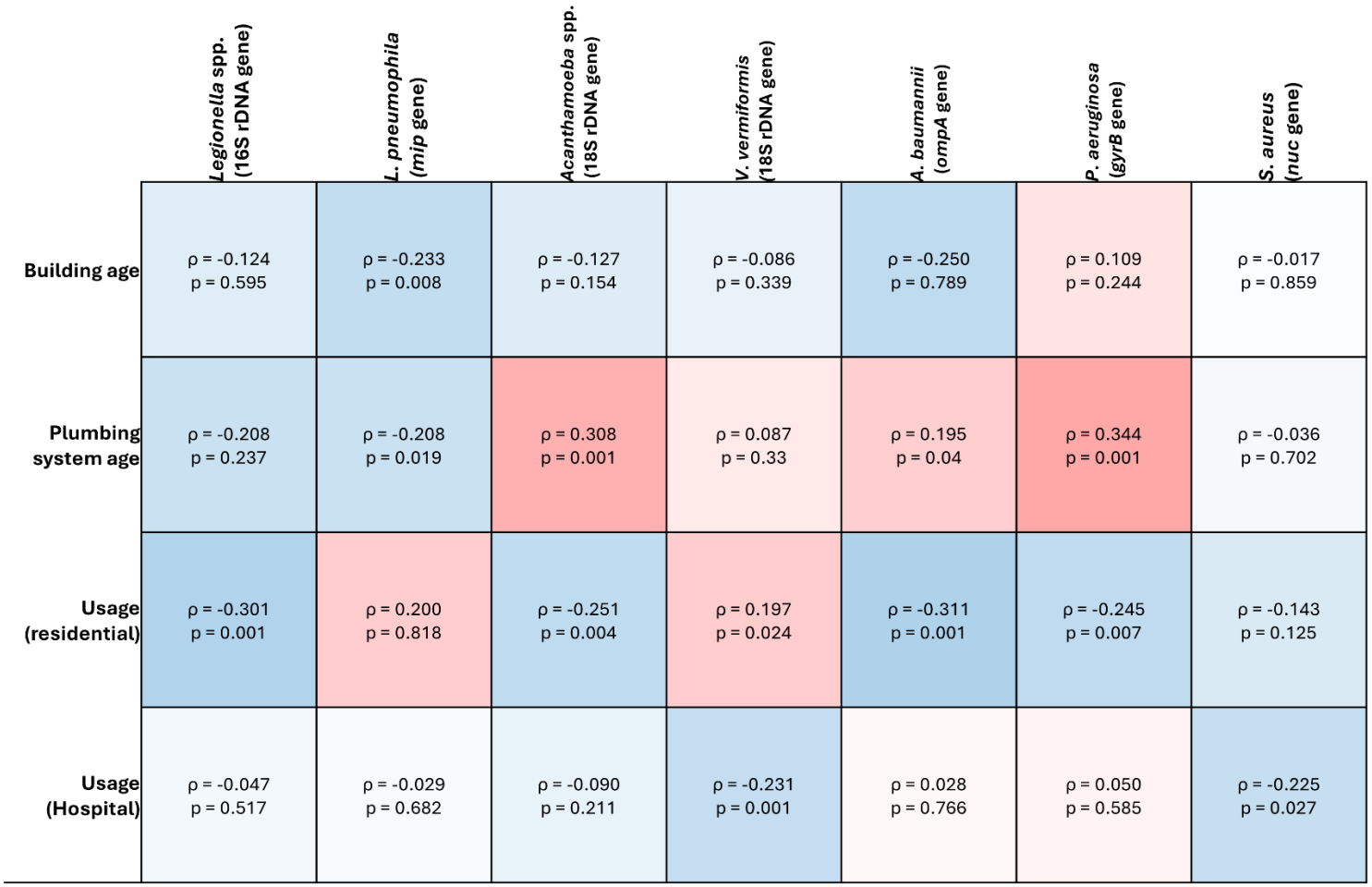

Figure 4.3 - Spearman's correlation analysis of the target opportunistic premise plumbing pathogens against abiotic factors in drinking water plumbing systems.

The heat map value shows the Spearman's correlation coefficient (ρ) to a significance threshold of $p<0.05$, ranging from −1.0 (blue) to 1.0 (red). A minus value demonstrates a negative association, whereas a positive value demonstrates positive association

4.4.2.2. *Acanthamoeba* spp.

Overall, 25% (n = 99/400) of total samples were positive for *Acanthamoeba* spp. (18S rDNA gene) with a concentration range of 1.4 x 10^2 to 2.33 x 10^6 GU/L and 1.16 x 10^2 to 3.63 x 10^8 GU/swab (Table 12.3). The prevalence of *Acanthamoeba* spp. was statistically significantly higher in residential samples than hospital samples (p=0.001) (Table 4.1). There was also significantly higher prevalence in biofilm samples than water (p=0.001), specifically residential biofilms (p=0.001) (Table 4.1).

Acanthamoeba spp. was the only target OPPP whose presence was significantly positively correlated with all other target OPPPs in one or more sample sites. In residential water, *Acanthamoeba* spp. significantly positively correlated with *Legionella* spp. (ρ = 0.321, p = 0.013), *L. pneumophila* (ρ = 0.385, p = 0.003) and *V. vermiformis* (ρ = 0.333, p = 0.01) (Figure 4.1A), and in biofilm *Acanthamoeba* spp. was significantly positively correlated with *P. aeruginosa* (ρ = 0.706, p = 0.001) and *A. baumannii* (ρ = 0.535, p = 0.001) (Figure 4.1B). In hospital water, *Acanthamoeba* spp. was significantly positively correlated with *L. pneumophila* (ρ = 0.249, p = 0.002) and *S. aureus* (ρ = 0.336, p = 0.001) (Figure 4.2A), and in hospital biofilm *Acanthamoeba* spp. was significantly positively correlated with *Legionella* spp. (ρ = 0.236, p = 0.028) and *V. vermiformis* (ρ = 0.305, p = 0.004) (Figure 4.2B).

Within residential buildings, *Acanthamoeba* spp. prevalence was significantly higher in premises that had electric and solar hot water systems compared with gas (p=0.001). However, there was no difference between buildings that did or did not have hot water storage. *Acanthamoeba* spp. prevalence was positively correlated with plumbing system age (ρ=0.308, p=0.001). Prevalence also increased as outlet usage decreased in residential buildings (ρ=-0.251, p=0.004), however there was no significant correlation between usage and prevalence in hospitals (ρ=-0.09, p=0.211) (Figure 4.3).

4.4.3. Bacteria

4.4.3.1. Pseudomonas aeruginosa

Overall, 41% (n=123/299) of total samples were positive for *P. aeruginosa* (*gyrB* gene) with a concentration range of 1.08 x 10^3 to 1.3 x 10^7 GU/L and 1.36 x 10^2 to 1.67 x 10^{10} GU/swab (Table 12.3). The prevalence of *P. aeruginosa* was statistically significantly higher in residential samples compared with hospital samples (p=0.001) (Table 4.1). There was also significantly higher prevalence in biofilm samples than water (p=0.001) (Table 4.1). This was driven by the prevalence in residential biofilm samples, as *P. aeruginosa* prevalence was higher in hospital water than hospital biofilm (p=0.035) (Table 4.1).

In residential water, *P. aeruginosa* prevalence was significantly negatively correlated with *Legionella* spp. (ρ = -0.37, p= 0.008) and *L. pneumophila* (ρ = -0.314, p = 0.025) (Figure 4.1A), and in biofilm *P. aeruginosa* prevalence was significantly positively correlated with *Acanthamoeba* spp. (ρ = 0.706, p = 0.001) and *A. baumannii* (ρ = 0.531, p = 0.001) (Figure 4.1B). Conversely, there were no significant relationships between *P. aeruginosa* and any other target OPPP in hospital water or biofilm (Figure 4.2A & B).

Within residential buildings, *P. aeruginosa* prevalence was significantly higher in buildings with electric hot water heaters compared to gas (p=0.001). However, there was no significant difference between buildings that did or did not have hot water storage (p=0.272). As plumbing system age increased, *P. aeruginosa* prevalence increased significantly (ρ=0.344, p=0.001), however, there was no significant correlation with building age (ρ=0.109, p=0.244). Prevalence also increased as outlet usage decreased in residential buildings (ρ=-0.245, p=0.007) but not in hospitals (ρ=0.05, p=0.585) (Figure 4.3).

4.4.3.2. Staphylococcus aureus

Overall, 26% (n=71/273) of total samples were positive for *S. aureus* (*nuc* gene) with a concentration range of 4.73 x 10^3 to 3.27 x 10^9 and 1.5 x 10^2 to 2.62 x 10^8 GU/swab (Table 12.3). There was no significant difference in *S. aureus* prevalence between residential or hospital samples (p=0.084), or between water and biofilm samples (p=0.634) (Table 4.1).

In residential water, *S. aureus* prevalence was significantly positively correlated with *Legionella* 16S (ρ = 0.293, p = 0.044) (Figure 4.1A), and in biofilms *S. aureus* prevalence significantly positively correlated with *L. pneumophila* (ρ = 0.268, p = 0.01) (Figure 4.1B). In hospital water, *S. aureus* prevalence was significantly positively correlated with *Acanthamoeba* spp. (ρ = 0.336, p = 0.001) (Figure 4.2A), however no significant correlations were seen in hospital biofilms (Figure 4.2B).

Unlike *P. aeruginosa* there was no significant difference in *S. aureus* prevalence between residential hot water systems (p=0.919) or storage (p=1). Similarly, there was no significant correlation between *S. aureus* prevalence and building age (ρ=-0.017, p=0.859) or plumbing system age (ρ=-0.036, p=0.702). In both residential and hospital samples, *S. aureus* prevalence increased as usage decreased ((ρ=-0.143, p=0.125) and (ρ=-0.225, p=0.027) respectively) (Figure 4.3).

4.4.3.3. *Legionella* spp.

Overall, 26% (n = 104/400) of total samples were positive for *Legionella* spp. (16S rDNA gene) with a concentration range of 1 x 10^2 to 2.8 x 10^6 GU/L and 1.3 x 10^1 to 7.7 x 10^4

GU/swab (Table 12.3). The prevalence of *Legionella* spp. was statistically significantly higher in residential samples than hospital samples (p=0.001). There was also significantly higher prevalence in water than in biofilm (p=0.001) (Table 4.1).

In residential water, *Legionella* spp. prevalence was significantly positively correlated with *L. pneumophila* (ρ = 0.803, p = 0.001), *Acanthamoeba* spp. (ρ = 0.321, p = 0.013), *V. vermiformis* (ρ = 0.328, p = 0.011), *S. aureus* (ρ = 0.293, p = 0.044) and significantly negatively correlated with *P. aeruginosa* (ρ = -0.37, p = 0.008) (Figure 4.1A), and in residential biofilms *Legionella* spp. prevalence was significantly positively correlated with *A. baumannii* (ρ = 0.288, p = 0.006) (Figure 4.1B). In hospital water, *Legionella* spp. prevalence was significantly positively correlated with *L. pneumophila* (ρ = 0.765, p = 0.001) and *V. vermiformis* (ρ = 0.518, p = 0.001) (Figure 4.2A), and in hospital biofilms *Legionella* spp. prevalence was significantly positively correlated with *L. pneumophila* (ρ = 0.567, p = 0.001) (Figure 4.2B).

Within residential buildings, *Legionella* spp. prevalence was significantly higher in properties with electric or gas hot water systems when compared with solar (p=0.06 and 0.002 respectively). However, there was no significant difference between buildings that did or did not have hot water storage (p=1). As plumbing system age decreased, there was a significant increase in *Legionella* spp. prevalence (ρ=-0.208, p=0.01), however, there was no significant correlation with building age (ρ=-0.124, p=0.126). *Legionella* spp. prevalence increased significantly as usage decreased in residential buildings (ρ = -0.301, p = 0.001) however, not in hospitals (ρ=-0.047, p=0.517) (Figure 4.3).

4.4.3.4. Legionella pneumophila

Overall, 24% (n=95/400) of total samples were positive for *L. pneumophila* (*mip* gene) with a concentration range of 4 x 10^1 to 3.5 x 10^5 GU/L and 5 x 10^1 to 1.12 x 10^6 GU/swab (Table 12.3). The prevalence of *L. pneumophila* was statistically significantly higher in residential samples than hospital samples (p=0.019) (Table 4.1). There was also significantly higher prevalence in water than biofilm overall (p=0.001), driven by the prevalence in hospital water (Table 4.1).

In residential water, *L. pneumophila* was significantly positively correlated with *Legionella* spp. (ρ = 0.803, p = 0.001), *Acanthamoeba* spp. (ρ = 0.385, p = 0.003), *V. vermiformis* (ρ = 0.316, p = 0.015) and significantly negatively correlated with *P. aeruginosa* (ρ = -0.314, p = 0.025) (Figure 4.1A), and in residential biofilm *L. pneumophila* was significantly positively correlated with *S. aureus* (ρ = 0.268, p = 0.01) (Figure 4.1B). In hospital water, *L. pneumophila* prevalence was significantly positively correlated with *Legionella* spp. (ρ =

0.765, p = 0.001), *Acanthamoeba* spp. (ρ = 0.249, p = 0.002) and *V. vermiformis* prevalence (ρ = 0.389, p = 0.001) (Figure 4.2A), and in hospital biofilm *L. pneumophila* prevalence was significantly positively correlated with *Legionella* spp. (ρ = 0.567, p = 0.001) and *V. vermiformis* (ρ = 0.319, p = 0.003) (Figure 4.2B).

In residential buildings, *L. pneumophila* prevalence was significantly higher in buildings with solar hot water heating systems compared to electric (p=0.028). As both building age and plumbing system age decreased, *L. pneumophila* prevalence increased ((ρ=-0.233, p=0.008) and (ρ=-0.208, p=0.019) respectively). In both residential and hospital buildings, there was no significant correlation between outlet usage and *L. pneumophila* prevalence ((ρ=0.2, p=0.818) and (ρ=-0.029, p=0.682), respectively (Figure 4.3).

4.4.3.5. Acinetobacter baumannii

Overall, 14% (n=42/293) of total samples (residential and hospital) were positive for *A. baumannii* (*ompA* gene) with a concentration range of 2.67 x 10^2 to 2.4 x 10^3 GU/L and 1.36 x 10^2 to 3.33 x 10^5 GU/swab (Table 12.3). The prevalence of *A. baumannii* was statistically significantly higher in residential samples compared to hospital samples (p=0.001) (Table 4.1). Prevalence was also significantly higher in biofilm compared to water samples (p=0.001) (Table 4.1).

In residential water, *A. baumannii* prevalence was not significantly correlated with any other target OPPP prevalence (Figure 4.1A), however in residential biofilms *A. baumannii* prevalence was significantly positively correlated with *Legionella* spp. (ρ = 0.288, p = 0.006), *Acanthamoeba* spp. (ρ = 0.535, p = 0.001) and *P. aeruginosa* prevalence (ρ = 0.531, p = 0.001) (Figure 4.1B). In both hospital water and biofilm, *A. baumannii* was not significantly correlated with any other target OPPP prevalence (Figure 4.2A & B).

Residential buildings with solar hot water heating systems had significantly higher *A. baumannii* prevalence compared with those with gas systems (p=0.007), however, there was no difference between buildings with or without hot water storage (p=1). *A. baumannii* prevalence increased significantly as residential building age decreased (ρ=-0.109, p=0.032), however, there was no significant relationship with plumbing system age (ρ=-0.23, p=0.792). *A. baumannii* was the only target pathogen increased in prevalence as outlet usage increased in residential buildings (ρ=0.311, p=0.001), however, there was no significant relationship between usage and prevalence in hospital buildings (ρ=0.028, p=0.766) (Figure 4.3).

4.5. Discussion

While previous research has studied the occurrence of individual opportunistic premise plumbing pathogens (OPPPs) such as *L. pneumophila*, the surveillance for multiple OPPPs and their protozoan hosts across different building types and outlets is still not well understood (Gomez-Alvarez et al., 2023; Tanya S. Isaac et al., 2020; Lee-Masi et al., 2023; Nisar et al., 2022; Shaheen et al., 2019; Waak et al., 2018). This study provides new quantitative information about the distribution of clinically relevant OPPPs across multiple settings. The high detection frequencies of these pathogens in both water and biofilm samples indicate their persistence and growth in drinking water plumbing systems, a niche thought to be a hostile environment for functionally complex microbiological communities (Jeanvoine et al., 2019).

4.5.1. Prevalence of opportunistic premise plumbing pathogens

The relationships between OPPPs in drinking water and biofilm samples in this study were found to be inconsistent, suggesting complex interactions between different microbial species. Notably, the presence of *L. pneumophila* negatively correlated with *P. aeruginosa* in residential water ($\rho = -0.314$, $p = 0.025$) and biofilm samples ($\rho = -0.161$, $p = 0.126$) (Figure 4.1), indicating that the presence of one pathogen may suppress the other under certain environmental conditions. Previous research has demonstrated that *P. aeruginosa* may antagonise *L. pneumophila* when in biofilms due to the production of bacteriocins or homoserine lactone quorum sensing (Abu Khweek et al., 2018; Mallegol et al., 2012). However, this inhibition deteriorates when *Klebsiella pneumonia* is also present in the biofilm (Stewart et al., 2012). Conversely, significant positive correlations were observed between *P. aeruginosa* and *A. baumannii* ($\rho=0.535$, $p=0.001$), and between *L. pneumophila* and *S. aureus* ($\rho=0.268$, $p=0.01$) in residential biofilm samples (Figure 4.1). Interestingly, these relationships were not seen in hospital biofilm samples. Carbapenem resistance genes have been shown to play a critical role in enhancing biofilm formation and strength for those made by both *P. aeruginosa* and *A. baumannii* (Azizi et al., 2015; Heydari et al., 2015; Sherif et al., 2021). However, the extent to which the presence of these AMR genes contributes to the overall survival and virulence of multispecies biofilms in environments, like drinking water plumbing, is yet to be explored. Recent research has found a high prevalence of *Staphylococcus* spp. at the end points of DWDS and that they may remain dormant deep within biofilm matrices (Batista et al., 2022; Li et al., 2021). This physical protection from other OPPPs affords increased resistance to disinfection when compared to planktonic cells (Gholipour et al., 2024; Li et al., 2021). In the present study, *S. aureus* was found ubiquitously throughout both hospital and residential samples. Despite the growing body of evidence demonstrating that it is possible for *S. aureus* to contaminate drinking water

plumbing systems and cause HAI outbreaks, it continues to be overlooked in drinking water treatment protocols (French et al., 2004; Hayward et al., 2024; Sexton et al., 2011; Ziwa et al., 2019). *Acanthamoeba* spp. was the only OPPP whose presence significantly positively correlated with all bacterial pathogens screened in this study. This finding highlights the important role free-living amoebae play in biofilms, where they can act as protective hosts for bacterial pathogens, shielding them from environmental stressors, such as disinfectants, and enhancing their survival (Nisar et al., 2022; Thomas et al., 2011). This protection complicates water treatment efforts that are designed to target planktonic bacteria. The growth of pathogens in biofilms is shaped not only by the diversity and abundance of microorganisms, but also by the type of interactions between them. This finding underscores the potential risks of focusing treatment or disinfection strategies on a single species. Until these environments are recognised as a niche for diverse microbial communities, originating from both the incoming supply water and via end point contamination, treatment methods will remain ineffective.

4.5.2. Influence of building properties on opportunistic premise plumbing pathogens

One objective of this study was to investigate correlations between OPPP prevalence and abiotic factors such as building type, building and plumbing system age, outlet type and usage frequency. With healthcare at home increasingly promoted as a viable alternative to in-patient treatment, the residential drinking water plumbing environment must be acknowledged as a potential risk to patient health. *Acanthamoeba* spp., *P. aeruginosa, Legionella* spp., *L. pneumophila* and *A. baumannii* prevalence was significantly higher in residential samples compared to hospital samples (Table 4.1). Water utilities manage water treatment up to the property meter, but once water passes the meter, its quality becomes the responsibility of the property owner. While larger commercial buildings, including hospitals, often implement additional onsite water treatment to reduce the risk of waterborne healthcare-associated infections, this is rarely done in residential properties (Prest et al., 2016).

Prolonged water stagnation, often occurring in low-use fixtures or during periods of inactivity, creates conditions where disinfectant levels diminish, allowing OPPPs to thrive (Falkinham, 2015; Lautenschlager et al., 2010; Nisar et al., 2020b). Regular flushing of outlets is recommended in healthcare premise plumbing management guidelines to mitigate this risk (National Health and Medical Research Council, 2011; World Health Organization, 2017). In the present study, *Acanthamoeba* spp., *V. vermiformis*, *P. aeruginosa* and *S. aureus* prevalence increased as outlet usage decreased (Figure 4.3). Conversely, *A. baumannii*

prevalence increased as outlet usage increased (Figure 4.3). Considering *A. baumannii* prevalence was significantly higher in biofilm samples compared to water, this may be a result of end point contamination. As *A. baumannii* is an emerging AMR threat (Centers for Disease Control and Prevention, 2019a; World Health Organization, 2015), highlighting the need for updated strategies to manage this pathogen (Centers for Disease Control and Prevention, 2019a; World Health Organization, 2015). In addition to flushing, maintaining hot water storage above 60^{o}C has been suggested as an accessible control mechanism for property owners. Electric and solar hot water heating systems strongly correlated with the prevalence of *Acanthamoeba* spp., *P. aeruginosa*, *L. pneumophila* and *A. baumannii* in the present study (Figure 4.3). Interestingly, there was no significant correlation between OPPP prevalence and if the residence had hot water storage or instantaneous water heating. The heating element in an electrically heated water-storage tank is suspended in the water and does not come into contact with sediment at the bottom, which may harbor OPPPs (Bates et al., 2000; Martinelli et al., 2000). Instantaneous hot-water systems have been proposed as a better alternative to traditional continuous-flow or water-storage tanks, as they reduce the amount of warm water that remains stagnant in residential properties (Martinelli et al., 2000). Concerningly, 21% of sampled residents did not know what type of hot water system they had at their properties, and 30% did not know if they had hot water storage or an instantaneous system (Table 12.1). Given that water temperature is considered one of the foundational barriers to control the growth and proliferation of these pathogens, this is an area that requires attention for future research and must be addressed when vulnerable individuals are receiving healthcare at home. Future research is needed to improve awareness of the role that residential water systems may play in the prevalence of OPPPS, particularly as vulnerable individuals receive healthcare at home.

4.6. Conclusion

Whilst previous research has studied the prevalence of individual opportunistic pathogens, there is limited knowledge understanding co-occurrence of multiple OPPPs and how building properties may influence their risk. Key findings include the prevalence of OPPPs in both water and biofilms from hospitals and residences, with *Acanthamoeba* showing a significant positive correlation with all bacterial OPPPs. Notably, a higher prevalence of OPPPs was found in biofilms compared to water, and residential properties exhibited a greater occurrence of these pathogens compared to hospitals. This study highlights how microbial drinking water quality can vary significantly between residential and hospital water systems, and how poor water system management may exacerbate OPPP persistence. The high frequencies of detection of *Legionella* spp., *L. pneumophila*, *P. aeruginosa*, *S. aureus* and *A. baumannii* along with pathogenic protozoan hosts *Acanthamoeba* spp. and *V. vermiformis*

particularly in residential properties, indicates their growth and persistence in treated drinking water plumbing systems. This research suggests that even in well treated drinking water systems, conditions may still permit the proliferation of diverse OPPPs, particularly in biofilms. The growth of pathogens within biofilms was influenced not only by the diversity and abundance of microorganisms, but also by the nature of their interactions. For example, *P. aeruginosa* and *L. pneumophila* were significantly negatively correlated with one another in drinking water. This relationship has implications on water treatment approaches, as targeting one OPPP may inadvertently exacerbate the risk of another. Current drinking water guidelines must recognize the increasing complexity of plumbing systems and the limitations of disinfection methods on dynamic bacterial communities. It is inadequate to depend solely on the water industry to ensure safe drinking water from treatment through to the point of use.

4.7. Acknowledgements

C.H., K.E.R, M.H.B., R. B. and H.W. conceived and designed the experiments. C.H. performed the experiments. M.A.N and J. X. assisted with data analysis. C.H. and H.W. drafted and edited the manuscript. K.E.R, M.H.B, M.A.N, R.B., J.H., J.X. and H.W corrected and contributed to the manuscript. All authors approved the final manuscript.

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5. HEALTHCARE WATER SYSTEMS AND ANTIMICROBIAL RESISTANCE INFECTION RISK

In this chapter, the role of drinking water plumbing systems in disseminating antimicrobial resistance is explored. This chapter addresses Objectives 2 and 3, and includes two manuscripts, one published and one currently under review. The first manuscript provides a critical overview of the how hospital and residential drinking water systems contribute to HAIs, highlighting their role as reservoirs for AMR pathogens. The second manuscript complements this narrative by presenting surveillance data on water and biofilm samples, identifying key AMR threats, and evaluating their prevalence in drinking water plumbing. Together, these studies demonstrate the urgent need for enhanced surveillance, targeted mitigation strategies, and multidisciplinary approaches to address the proliferation of AMR pathogens within drinking water systems.

CITATION: Hospital water as the source of healthcare associated infection and antimicrobial resistant organisms

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5.1. Abstract

Purpose of review

Drinking water is considered one of the most overlooked and underestimated sources of healthcare associated infections (HAIs). Recently, the prevention and control of opportunistic premise plumbing pathogens (OPPPs) in healthcare water systems has been receiving increasing attention in infection control guidelines. However, these fail to address colonisation of pathogens that do not originate from source water. Subsequently, this review explores the role of water and premise plumbing biofilm in HAIs. The potential mechanisms of contamination and transmission of antimicrobial resistant (AMR) pathogens originating both from supply water and human microbiota are discussed.

Recent findings

OPPPs such as *Legionella pneumophila, Pseudomonas aeruginosa* and *Mycobacterium avium* have been described as native to the plumbing environment. However, other pathogens, not found in the source water, have been found to proliferate in biofilms formed on outlets devices and cause HAI outbreaks.

Summary

Biofilms formed on outlet devices such as tap faucets, showers and drains provide an ideal niche for the dissemination of antimicrobial resistance. Thus, comprehensive surveillance guidelines are required to understand the role that drinking water and water related devices play in the transmission of AMR HAIs and to improve infection control guidelines.

5.1.1. Key points

- Biofilms formed on outlet devices can harbour antimicrobial resistant pathogens originating from the supply water and human microbiota via washing contaminated hands.
- Outlet device design can facilitate splashing and aerosolization of water, resulting in contamination of the surrounding environment and cause indirect disease transmission.
- Broad, universal environmental surveillance guidelines are required to understand the role of drinking water and water related devices in transmission of healthcare acquired infections and dissemination of antimicrobial resistance threats.

5.2. Introduction

Healthcare associated infections (HAIs) are one of the most common and preventable patient complications (Haque et al., 2018). The United States Centers for Disease Control and Prevention (US CDC) estimated that approximately 1 in 31 hospital patients acquired at least one HAI, costing US$28-45 billion annually (Collier et al., 2021; Stone, 2009). Concerningly, the duration and severity of HAIs are increasing due to the rise of antimicrobial resistance and multidrug resistant (MDR) organisms (Centers for Disease Control and Prevention, 2019a; Dadgostar, 2019). Water and water related devices remain one of the most overlooked and underestimated sources of HAIs, with reports estimating that approximately 21.6% of all recorded HAIs can be attributed to water (Kanamori et al., 2016; Perkins et al., 2019).

Opportunistic premise plumbing pathogens (OPPPs) are a group of waterborne pathogens that persist in drinking water due to their unique characteristics. This includes disinfection resistance, biofilm formation, growth in amoeba, and growth in low nutrient environments (Falkinham, 2015). Common OPPPs include *Legionella* spp, nontuberculous mycobacteria and *Pseudomonas aeruginosa* (Falkinham, 2015). These microorganisms form biofilms on the surface of pipes and water related devices that can also provide refuge to pathogens not typically associated with drinking water including *Staphylococcus aureus*, Enterobacteriaceae, *Klebsiella pneumoniae* and *Escherichia coli* (El Haddad et al., 2021; Hayward et al., 2020; Jung et al., 2020; Kim et al., 2022; Nakamura et al., 2021; Sharma et al., 2021). Biofilms provide protection against unfavourable environmental conditions including disinfection processes and deliver the ideal environment for the transfer of antimicrobial resistance genetic elements (Kim et al., 2022; Schages et al., 2020). There is limited research investigating the sources and mechanisms of transmission that enable these pathogens to colonise premise plumbing biofilm. To improve future water management

and infection control guidelines, it is essential that we understand the potential transfer of pathogens from human microbiota to premise plumbing biofilm and vice versa.

5.3. Water as a source of HAI infections

The presence of OPPPs in supply water and premise plumbing is slowly gaining attention. For example, the United States (US) Environment Protection Authority's National Primary Drinking Water Regulations provide legally enforceable standards for *Legionella* spp. in public water systems (U.S. Environmental Protection Agency, 1996). However, these guidelines fail to address premise plumbing colonisation by bacterial pathogens that have not originated from the supply water. Species such as *S. aureus, K. pneumoniae, Enterobacter cloacae, Serratia marcescens* and *E. coli* have been found in premise plumbing and linked to waterborne HAI outbreaks (French et al., 2004; Hayward et al., 2020; Sexton et al., 2011).

5.4. Mechanisms of contamination and transmission

Unlike OPPPs that can colonise throughout the drinking water distribution network and premise water system, these pathogens are often found colonising end point water related devices such as shower heads, tap faucets and drains (Hayward et al., 2020). Even when HAI outbreaks are linked to a contaminated water source, the route of bacterial transmission is often unclear. A patient may be directly exposed to contaminated water, have indirect contact via a contaminated intermediary surface, or via the hands of healthcare personnel after improper hand hygiene (Decker et al., 2014; Volling et al., 2021). For example, a hospital in Zambia found that *K. pneumoniae* was frequently isolated from a communal bathtub used by burns patients (Ziwa et al., 2019). *K. pneumoniae* has been found widely in hospital environments including nursing counters, bathroom sinks, dressing trolleys and patient beds (Sharma et al., 2021). As such, it was hypothesised that water outlets were contaminated with *K. pneumoniae* from patients, staff and the surrounding environment rather than from the source water (Franco et al., 2020; Roux et al., 2013). To investigate this theory, a study conducted in a Japanese hospital monitored four clinical isolates and the surrounding environment before and after moving to a new facility (Nakamura et al., 2021). One infection was identified prior to a patient being transferred to the new facility and after 6 months, this isolate was seen in the bathroom sink drains of the new facility, demonstrating the transmission of *K. pneumoniae* from patient to the premise plumbing biofilm.

Once biofilms have established on these plumbing surfaces, there are numerous potential mechanisms for contamination and transmission as shown in Figure 5.1. Subsequently, a patient may be exposed to these pathogens via several routes including inhalation,

aspiration, ingestion, or skin contact (World Health Organization, 2016). This can result in a wide range of HAIs such as surgical site infections, pneumonia, bloodstream infections and gastrointestinal infections (Collier et al., 2021). Many studies have demonstrated that *Legionella* spp. can be aerosolised when showering and using tap faucets (Bollin et al., 1985; Chang et al., 2012; Kanamori et al., 2016). Bollin et al. (1985) reported that approx. 90% of aerosols produced from showers were small enough to enter the respiratory tract and cause disease (Bollin et al., 1985). These aerosols become diluted in air at further distances and have been deemed a low risk to public health (Crimi et al., 2006). However, such conclusions may be misleading as it is possible for viable but non-culturable *Legionella* spp. to be aerosolised but not detected (Prussin et al., 2017). Additionally, aerosol and droplet sizes that are either too large to cause pulmonary infections or settle out of the air before they can be inhaled are commonly overlooked. There is limited research investigating the potential for these aerosols to settle on surrounding surfaces and the concentrations necessary to cause indirect disease transmission (Prussin et al., 2017).

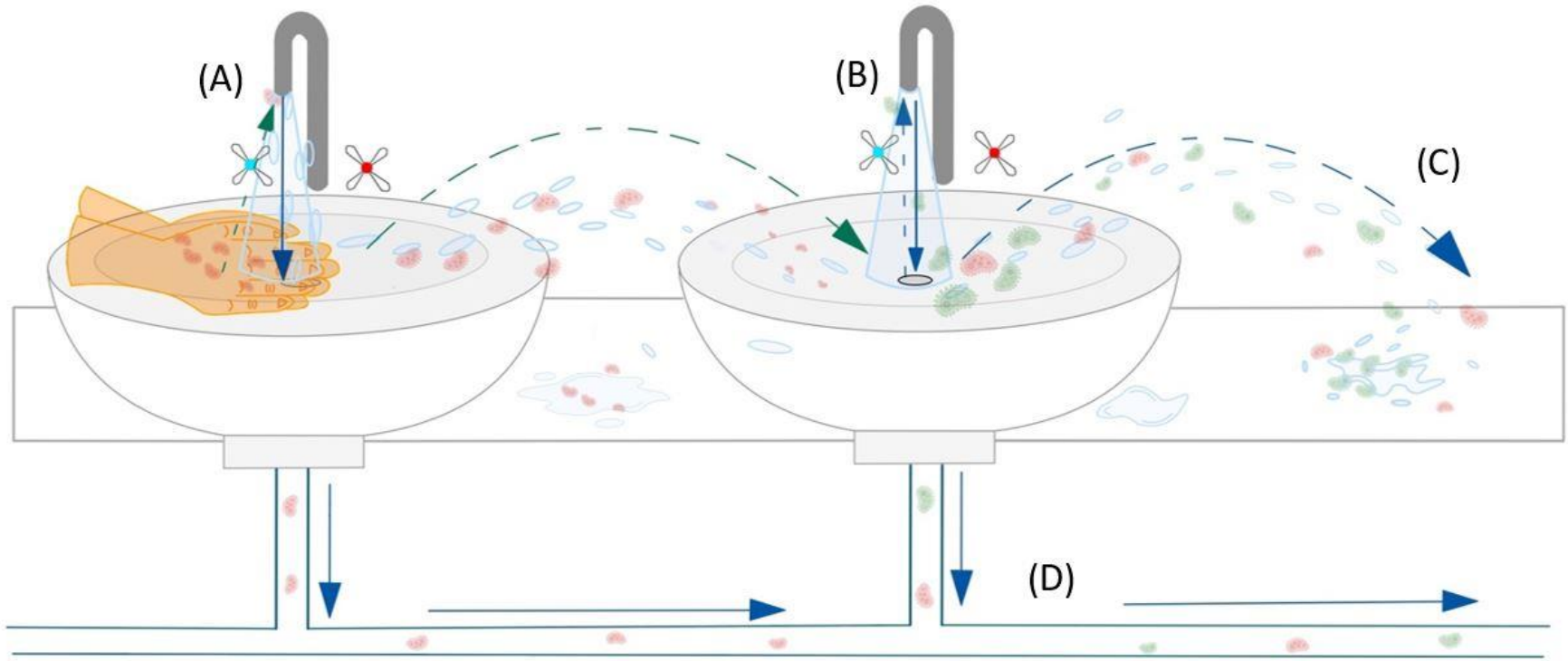

Figure 5.1 - Proposed mechanisms for outlet contamination. Solid arrows indicate intended direction of water flow. Dashed arrows indicate subsequent splashing of potentially contaminated water.

(A) Washing of soiled hands resulting in splashing to the surrounding environment and neighbouring basins, and causing retrograde contamination of the tap faucet with human microflora (shown in red), indicated by dashed green arrows. (B) Water flow hitting the contaminated drain located directly below the faucet, causing retrograde contamination of the tap faucet with opportunistic premise plumbing pathogens (shown in green), indicated by the dashed blue arrow. (C) Splashing from handwash basin contaminating the surrounding environment with both human flora (shown in red) and opportunistic premise plumbing pathogens (shown in green) (D) Colonisation of the deeper premise plumbing infrastructure with both human flora (shown in red) and opportunistic premise plumbing pathogens (shown in green).

Basin splashing via handwashing or splashing off the basin drain to the surrounding environment is a proposed, but under investigated route of HAI transmission (Figure 5.1). An outbreak of MDR *P. aeruginosa* was linked to multiple handwashing sinks (Hota et al., 2009). When investigated, it was found that the water flow directly hit the basin drain resulting in splashing onto surfaces up to 1 metre away, including medication and sterile dressing preparation areas. This study also acknowledged that microparticles and aerosols not visible may travel further than 1 metre. Similarly, an outbreak of carbapenem-resistant Enterobacteria (CRE) in a cardiology unit was linked to the patient's environment when epidemiological investigations identified *K. pneumoniae* carbapenemase (KPC) producing *E. coli*, New Delhi metallo-beta-lactamase producing *C. freundii* and *E. cloacae* in water dispensers and sink drains (Jung et al., 2020). Only after an extensive investigation, it was found that the pouring of contaminated human waste, such as dialysis fluid, into the handwashing sink was the direct cause of the KPC-producing *E. coli* outbreak. The splashing of droplets and/or aerosols from this sink resulted in the contamination of the adjacent water dispenser. Basin designs that include offset drains to avoid water directly hitting the drain and causing pathogen dispersal have been proposed (Aranega-Bou et al., 2019). The United Kingdom has released guidelines stating that 'tap outlet flow should not discharge directly into the waste aperture' (Department of Health London, 2013). A laboratory study investigated the influence of drain position and drainage rate on the dispersal of CRE (Aranega-Bou et al., 2019). When the drain was situated directly under the faucet, CRE dispersal occurred regardless of the drainage rate compared to minimal dispersal when the drain was located at the rear of the basin. When drainage was impaired, CRE dispersal was almost 30-times greater in drains directly under the faucet than rear facing drains.

5.5. Biofilm formation

Biofilms are heterogenous communities of microorganisms that can grow on plumbing infrastructure surfaces, within water related devices and on sediment deposits (Batté et al., 2003; Sharma et al., 2021). Once cells have initially adhered to the surface, microbial diversity and metabolic activity typically increases (Yu et al., 2010). Favourable conditions for biofilm formation include areas of slow flow rate, warm temperatures and low residual disinfection (Toyofuku et al., 2016). Due to the complex design of modern plumbing infrastructure, multiple species can exploit different niches throughout the building leading to a high microbial biofilm diversity (De Sotto et al., 2020; Douterelo et al., 2020; Lee et al., 2021).

Although outlet plumbing devices such as hand washing basins and showers are receiving greater attention as sources of AMR HAIs, this is typically circumstantial and only investigated in response to an extended outbreak (Volling et al., 2021). Outlet fittings have been shown to facilitate the establishment of biofilms consisting of OPPPs and other bacterial pathogens from human contamination (Falkinham et al., 2015; Franco et al., 2020; French et al., 2004; Kanamori et al., 2016; Nisar et al., 2020a; Roux et al., 2013; Sexton et al., 2011; Xue et al., 2020). Basin drains and descending P-traps are wet, humid, and protected environments that provide an ideal niche for the formation of microbially diverse biofilms from these different contamination routes (Vickery et al., 2012). The use of non-touch electronic faucets to minimise water consumption and reduce touching the tap is increasing in healthcare settings. However, these faucets have been shown to have higher contamination rates of pathogens such as *Legionella* spp. and *P. aeruginosa,* facilitated by low flow rates, low water pressure and warm water remaining stagnant in the tap column (Halabi et al., 2001; Merrer et al., 2005; Moore et al., 2015). These devices are made of materials such as rubber and PVC that have been shown to enhance *P. aeruginosa* adhesion and biofilm formation (Emilie Bédard et al., 2016). When in a biofilm, these native OPPPs can confer protection to pathogenic bacteria that may not otherwise survive premise plumbing environmental conditions in planktonic form (Clayton et al., 2021). For example, *S. aureus* located deep within biofilm matrices have been found to stay 'dormant' with low metabolic activity and increased resistance to bacteriocides and antibiotics, up to 1000-fold, compared to active aerobic cells (Wilson et al., 2022). Some species of *E. coli* unable to attach to solid surfaces can form biofilms with 'adhesive' species such as *Pseudomonas putida* (Castonguay et al., 2006). A recent study found that 75% of clinical *K. pneumoniae* isolates demonstrated biofilm formation and showed a significant correlation between biofilm formation and antibiotic resistance (Karimi et al., 2021). Furthermore, biofilm growth and AMR pathogen selection may be facilitated when waste such as unused antimicrobials, beverages and soaps are disposed of in a sink (Kotay et al., 2017).

5.6. Antimicrobial resistance

The rise of AMR and MDR bacteria has been identified as one of the most significant threats facing global public health (World Health Organization, 2020a). It has been estimated that the annual global GDP could reduce by approx. 1% or $100-210 trillion with MDR tuberculosis alone accounting for $16.7 trillion (Dadgostar, 2019). In 2015, the World Health Assembly adopted a global action plan to improve understanding of antimicrobial resistance via surveillance and reduce infection via effective sanitation and antimicrobial stewardship programs (World Health Organization, 2015). Despite such efforts, the US CDC reported significant increases in central line associated infections, catheter-associated urinary tract

infections, ventilator-associated infections and methicillin-resistant *S. aureus* (MRSA) infections from 2019-2020 despite decreases in surveillance due to the COVID-19 pandemic (Weiner-Lastinger et al., 2021).

Drinking water and water related devices have been linked to outbreaks of 'urgent' and 'serious' threat antibiotic resistant bacteria such as carbapenem-resistant and ESBL-producing Enterobacteria, vancomycin resistant Enterococci (VRE) and MRSA (Arvanitidou et al., 2003; Hayanga et al., 1997; Layton et al., 1993; Nagoba et al., 1997; Perryman et al., 1980; Squeri et al., 2012; Sserwadda et al., 2018; Ziwa et al., 2019). AMR bacteria have been found in higher quantities in taps at the outlet compared to the supply water entering the hospital, indicating that the premise plumbing infrastructure can serve as a reservoir for antimicrobial resistance genes (Zhang et al., 2021). AMR species can proliferate in biofilms found at the outlet in response to the widespread use of antimicrobials used in hospitals creating an environment of selective pressure (Chan et al., 2019; Goel et al., 2021). Currently, the US CDC does not consider contaminated water to be a source of ESBL-producing Enterobacteria infection in the United States due to a lack of data (Evins et al., 2021). However, an epidemiological study conducted in France during a three-year outbreak of ESBL producing *E. cloacae*, found 17 environmental isolates in patient sink and shower drains, six of which had identical pulsotypes to clinical strains.

The COVID-19 pandemic has only heightened the use of disinfectants and sanitisers, particularly in healthcare facilities (Weiner-Lastinger et al., 2021). Although antiseptic soaps may be effective for hand washing, the subsequent run off into sink drains may be at sublethal concentrations to combat established biofilms and instead results in selective pressure for resistant populations (Lineback et al., 2018). Antimicrobial stewardship programs have been implemented to prevent the inappropriate use of antibiotics, however many of these guidelines are inconsistent when it comes to environmental disinfection. The Australian National Safety and Quality Health Service Standards state that fomite surfaces such as door handles and bed rails should be cleaned with 'multi-resistant organism disinfectant' whereas handwashing sinks and baths are to be cleaned with detergent only (National Health and Medical Research Council, 2019). Benzalkonium chloride (BAC) is a quaternary ammonium compound (QAC) commonly used in healthcare settings due to its broad spectrum biocidal activity (Hegstad et al., 2010). Repeated low exposure to BAC biocides has resulted in adaptive resistance via physiological or genetic changes in species such as *P. aeruginosa, E. coli* and *Staphylococcus* spp. (Dashtbani-Roozbehani et al., 2021; Lee et al., 2020; Zhang et al., 2021). Resistance to QACs is typically associated multidrug efflux pump upregulation via gene mutation or acquisition via horizontal gene transfer

(Chitsaz et al., 2017; Kim et al., 2018). Adaptive changes such as decreased growth rates, decreased fatty acid biosynthesis and energy metabolism have also been observed (Kim et al., 2018; Kücken et al., 2000). Concerningly, these resistance mechanisms can confer cross resistance to antibiotics despite the lack of antibiotic selective pressure in the environment (Brauner et al., 2016; Nordholt et al., 2021). The increased use of antimicrobials in healthcare settings is reflected by the high abundances of antimicrobial resistance genes in hospital effluent (Kaur et al., 2020; Zhang et al., 2020). This effluent enters wastewater systems that intersect with residential properties, other healthcare facilities and agricultural farms that may be discharged into rivers, lakes and oceans without effective treatment (Hassoun-Kheir et al., 2020). A wastewater pipeline breakage caused contamination of the municipal water system and resulted in approximately 450 cases of illness including *Campylobacter* Environmental isolates typically present higher minimum inhibitory concentration adaptations to QACs when compared to stepwise laboratory evolution experiments (Nordholt et al., 2021). This suggests there are additional unknown external environmental conditions that favour the emergence of high resistance isolates. If we are to relieve the pressure on the growing antimicrobial resistance crisis and avoid exacerbating the problem, it is essential that we understand the efficacy of different cleaning protocols on diverse microbial communities and introduce stricter disinfectant use.

5.7. Control and interventions

Current healthcare infection control guidelines are inconsistent when it comes to routine environmental disinfection and outlet device remediation methods after a confirmed outbreak. Additionally, there is limited research comparing the effectiveness of cleaning protocols against established biofilms formed on outlet devices (Volling et al., 2021). Disinfection of basins and exposure manipulation is typically achieved by targeted cleaning and/or replacement of all or part of the device (Volling et al., 2021). This cleaning may involve hydrogen peroxide, chlorine products, steam cleaning and/or mechanical brushing (Volling et al., 2021). Despite the fact that immediate post intervention cultures appear sterile, recolonisation over time is often observed (Hota et al., 2009). It has been demonstrated that microorganisms deep within the basin P-trap are able to extend vertically resulting in colonisation of the basin drain (Kotay et al., 2017). Replacing part of or the entire basin drastically reduces or halts identification of new HAI cases (Volling et al., 2021). However, recent studies have shown that premise plumbing is a dynamic and continuous ‘express way’ for microorganisms to spread throughout a building despite no shared patient or personnel contact (Weinbren, 2020). Areas of slow flow rate and high nutrients in hospital wastewater systems provides an ideal niche for antimicrobial pathogen enrichment (Hassoun-Kheir et al., 2020). Model studies have demonstrated that a single contaminated

P-trap enables the spread of organisms along common wastewater pipes resulting in retrograde contamination of neighbouring basin drains in 7 days (Kotay et al., 2017). Transmission of daptomycin resistant strains of VRE were studied in an American cancer centre to understand the potential spread between patients and their environment. It was found that there was horizontal transfer of genetically related strains of VRE between patients on different floors of the hospital and within their room environment (El Haddad et al., 2021). Future research is required to understand the influence of outlet design on the spread of pathogen contamination, and on the effectiveness of different disinfection and control approaches.

5.8. Conclusion

Currently, many water and infection control guidelines focus on managing OPPPs in premise water systems but overlook the potential contamination of plumbing biofilm by other clinically relevant microorganisms. This includes many AMR pathogens identified as serious threats to our healthcare system by the WHO and US CDC.

To reduce the risk from these pathogens, a multiple barrier approach is needed to control both the pathogens originating from the source water and those originating from human microbiota. This includes engineering measures, appropriate product design and effective cleaning, sanitation and hygiene protocols. To inform these necessary changes in guidelines, future research is needed to better understand the mechanisms of transfer and conditions driving AMR in plumbing biofilms.

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5.11. Conflicts of interest

None

CITATION: Drinking water plumbing systems are a hot spot for antimicrobial resistant pathogens

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5.12. Abstract

The rise of antimicrobial resistant (AMR) pathogens in drinking water plumbing systems represents a significant yet underestimated public health threat. This is the first study to use qPCR and culture-based methods to investigate the prevalence of the key AMR threats, methicillin resistant *Staphylococcus aureus* (MRSA) and carbapenem resistant *Pseudomonas aeruginosa* and *Acinetobacter baumannii,* in Australian hospital and residential drinking water and plumbing biofilm samples. Seventy three percent of residential water and biofilm samples were qPCR positive for at least one target pathogen compared with 38% of hospital samples, and 45% of residential plumbing fixtures (hand basins or showers) were found to harbour at least two target pathogens. Thirty seven percent of total water and biofilm samples were qPCR positive for *P. aeruginosa*, 22% for *A. baumannii* and 22% for *S. aureus*. Using culture, 10% of samples were positive for *P. aeruginosa*, 8% for *A. baumannii* and 7% for *S. aureus*. Of these culture isolates, 29% of *P. aeruginosa* and 28% of *A. baumannii* were carbapenem resistant, and 54% of *S. aureus* isolates were identified as MRSA. Drain biofilms were the most common reservoir for AMR *A. baumannii, S. aureus* and *P. aeruginosa*. Carbapenem resistance genes including $bla_{\text{NDM-1}}$, $bla_{\text{OXA-48}}$, $bla_{\text{KPC-2}}$ and bla_{VIM} were found in biofilm samples otherwise negative for *P. aeruginosa*, indicating drinking water plumbing biofilms are acting as an eDNA reservoir. These findings underscore the critical role of drinking water plumbing biofilms as hotspots for diverse AMR pathogens, increasing risks for vulnerable populations, particularly in healthcare at home settings. This study highlights the need for enhanced surveillance and evidence based interventions to control AMR pathogens in drinking water plumbing systems.

5.13. Introduction

Antimicrobial resistance has been identified as a global public health threat (Murray et al., 2022). The World Health Organization (WHO) estimates antimicrobial resistant (AMR) infections will result in an estimated 10 million deaths each year, and overtake cancer as the leading cause of death by 2050 (Naghavi et al., 2024). AMR and multi-drug resistant infections result in prolonged hospital stays, increased associated medical costs and reliance on last line of defence antibiotics (Mestrovic et al., 2022; Murray et al., 2022). The cost of treating an AMR infection can be up to 1.6 times higher than non-resistant infections, adding approximately US$1,400 in treatment costs per infection (Dadgostar, 2019).

The WHO and United States (US) Centers for Disease Control and Prevention (CDC) has created a list of priority AMR pathogens to inform research and development of new clinical and environmental health interventions (Centers for Disease Control and Prevention, 2019a; World Health Organization, 2024). Critical AMR threats pose a significant risk in hospitals,

nursing homes, and patients requiring medical devices such as ventilators and catheters (World Health Organization, 2024). This group includes carbapenem resistant *Acinetobacter baumannii* and *Pseudomonas aeruginosa*, and methicillin-resistant *Staphylococcus aureus* (MRSA) (Murray et al., 2022; World Health Organization, 2024). These pathogens canspread within the built environment, may be resistant to several classes of antibiotics, and are capable of acquiring additional resistance. *P. aeruginosa* is a leading cause of pneumonia in immunocompromised patients, with AMR infections resulting in a 24% higher risk of mortality compared to susceptible strains (Nathwani et al., 2014). *A. baumannii* has is as an emerging critical AMR threat, and is the leading pathogen for mortality attributable to antimicrobial resistance in Southeast Asia, East Asia and Oceania (Jean et al., 2022; Murray et al., 2022). MRSA emerged as an AMR threat in 1961 after the introduction of beta-lactam antibiotics, continues to be a threat due to the increasing number of HAIs with community acquired cases rising worldwide (Mestrovic et al., 2022; Murray et al., 2022).

Despite the recognition of these pathogens, AMR pathogen surveillance remains inconsistent. Many existing systems focus on known clinical cases whilst overlooking environmental reservoirs of AMR, such as drinking water plumbing systems (Bengtsson-Palme et al., 2023; Hayward et al., 2022a). Current surveillance systems often fail to include water-related devices, such as faucets, showers, and water tanks, despite substantial evidence linking contaminated water sources to AMR HAI outbreaks (Anaissie et al., 2002; Berrouane et al., 2000; Hayward et al., 2020; Kanamori et al., 2016; Wendel et al., 2016; Yiallouros et al., 2013). The complex infrastructure of drinking water plumbing systems in hospitals and private residences creates environments conducive to biofilm formation and bacterial growth (Nisar et al., 2023b). Biofilms are complex communities of microorganisms that adhere to surfaces and serve as a protective niche for these bacteria, enhancing their survival and resistance to disinfectants (Flemming et al., 2010). Biofilms have been described as a ‘hot spot’ for AMR pathogens. Drinking water plumbing biofilms in residential properties are also an increasing concern for vulnerable populations due to healthcare at home initiatives (Hayward et al., 2022b).

There are few studies investigating the presence of key AMR threats in drinking water plumbing environments in Australia. Furthermore, there are limited studies investigating the risk of these pathogens in residential properties and the implications for the growing home healthcare industry. This study aimed to investigate the prevalence of *P. aeruginosa*, *A. baumannii*, and *S. aureus* in drinking water plumbing systems. By sampling drinking water and biofilms from hospitals and residential properties, this study provides valuable insights into the prevalence of these critical pathogens, their antimicrobial resistance profiles, and the

associated public health implications. The findings of this research highlight the importance of surveillance systems that cover both healthcare-associated and community-acquired infections. In particular, investigating water-related infections and the risks to patients receiving healthcare at home. By understanding these conditions, we can create more effective management strategies to address the growing threat of AMR.

5.14. Methods

5.14.1. Sample collection

This study was approved by the Flinders University Social and Behavioural Research Ethics Committee (SBREC Project Number 7291). From February 2019 to May 2024, 39 water and 127 biofilm samples were collected from showers, faucets, drains, baths, basins and overflows from residential and healthcare facility drinking water plumbing systems. There were 86 domestic samples and 80 hospital samples collected across New South Wales and South Australia. Due to SBREC constraints, the authors cannot disclose the geographic location of these premises. Water and biofilm samples were transported according to the Centers for Disease Control and Prevention guidelines (Centers for Disease Control and Prevention, 2019b).

Briefly, 1 L potable water samples were collected in sterile screw capped wide mouth plastic bottles (2105-0032 Nalgene) containing 1 mL 0.1 N $Na_2S_2O_3$ (124270010, ACROS Organics™) to neutralize residual chlorine-based disinfectants. Sterile polyurethane-tipped swabs (CleanFoam®TX751B, Texwipe®) were used to collect biofilms. These swabs were moistened with sterile water and the surface of the faucet aerator, showerhead or drain was swabbed for 10 s. The swab was then placed in a 10 mL screw capped vial with 5 mL of sterile 1X phosphate buffered saline (PBS). All samples were stored at 5^{o}C and analysed within 72 h of collection. Collected biofilm was dislodged from the swab and into the PBS by 5 min shaking (Griffin Flask Shaker), vortexing (SEM® Vor Mix) and sonication (CooperVision® 895 Ultrasonic Cleaner). All water and biofilm samples were vacuum filtered onto a 47 mm diameter 0.2 µm polycarbonate membrane (GTTP04700, Isopore™). The membrane was then transferred to a sterile 10 mL screw top vial containing 5 mL of sterile 1X PBS followed by 5 mins of shaking (Griffin Flask Shaker), vortexing (SEM® Vor Mix) and sonication (CooperVision® 895 Ultrasonic Cleaner). This suspension was used for microbiological and molecular analysis.

5.14.2. Microbial testing

5.14.2.1. Selective culture

Stagnant water and resuspended biofilm samples were analyzed for the target pathogens *A. baumannii, S. aureus* and *P. aeruginosa* using previously described culture techniques (Ajao et al., 2011; International Organization for Standardization, 2018; Missiakas et al., 2013). *P. aeruginosa* colonies were identified as green colour or producing fluorescence, *A. baumannii* colonies were identified as non-lactose fermenting and opaque, and *S. aureus* colonies were identified as black surrounded by a clear zone. All suspected isolates were further identified by quantitative polymerase chain reaction (qPCR) for species specific genes.

5.14.2.2. Quantitative polymerase chain reaction

DNA was extracted for qPCR analysis from 1 mL of the stagnant water and resuspended biofilm samples using the BIO-RAD Aquadien™ DNA extraction and purification kit following the manufacturer's instructions (Bio-Rad Laboratories, Inc., Sydney, NSW, Australia). DNA was extracted from the suspected culture positive isolates using the boiling method (Dashti et al., 2009). qPCR for *A. baumannii*, *P. aeruginosa* and *S. aureus* was performed as previously described (Table 13.1).

5.14.3. Antimicrobial susceptibility testing

Antibiotic susceptibility testing was carried out by the Kirby-Bauer disk diffusion method according to the CLSI and EUCAST guidelines (Clinical Laboratory Standards Institute, 2017; European Committee on Antimicrobial Susceptibility Testing, 2024) (Table 13.2). For susceptibility testing, ATCC 29213 (*S. aureus*), ATCC 27853 (*P. aeruginosa*) and ATCC 25922 (*Escherichia coli*) were used as controls.

5.14.4. Detection of *Pseudomonas aeruginosa* carbapenem resistance genes

Genomic DNA extracted from all water and biofilm samples, along with qPCR confirmed *P. aeruginosa* isolates (See Section 5.14.2.2) that were resistant to carbapenem antibiotics, were screened for the presence of carbapenem resistant genes $bla_{\text{NDM-1}}$, $bla_{\text{OXA-48}}$, $bla_{\text{KPC-2}}$ and bla_{VIM} (Table 13.3). Reaction mixes consisted of 1 µL of specific oligos (BIO-RAD Laboratories Ltd.), 10 µL of 2X Sso Advanced™ universal inhibitor tolerant SYBR® Green supermix (1725016, BIO-RAD Laboratories Ltd.), 4 µL of sterile water and 5 µL of template DNA were used in a Rotor-Gene Q thermal cycler (QIAGEN Ltd.).

5.14.5. Statistical analysis

Data analyses were performed using SPSS and R software. The results were interpreted at the level of significance $p<0.05$.

5.15. Results

The target pathogens *P. aeruginosa, A. baumannii* and *S. aureus* were found frequently throughout premise plumbing systems, with an overall higher prevalence in residential settings compared to hospitals, and qPCR demonstrating greater sensitivity than selective culture.

In residential samples, 73% (n=63/86) of samples collected were qPCR positive for at least one target pathogen, and 45% (n=39/86) were positive for multiple target pathogens. However, only 26% (n=23/86) of samples were culture positive for at least one target pathogen, with six of these fixtures positive for two target pathogens. Antimicrobial resistant (AMR) *P. aeruginosa* and *A. baumannii* were the most frequently identified (n=5 isolates), with resistance to carbapenem antibiotics most prevalent. In hospital samples, 38% (n=30/80) of total samples collected were qPCR positive for at least one target pathogen, and three of these were positive for two target pathogens. Additionally, 15% (n=12/80) of plumbing fixtures were culture positive for at least one target pathogen, and one of these fixtures was positive for both *P. aeruginosa* and *S. aureus*. AMR *S. aureus* was the most frequently detected AMR pathogen in hospitals, with four isolates resistant to penicillin and two of these isolates also methicillin resistant. These data indicate that the residential drinking water plumbing environment is a key yet overlooked niche for AMR pathogens when compared to hospitals.

5.15.1. *Acinetobacter baumannii*

Overall, 22% (n=37/166) of total samples were qPCR positive for *A. baumannii* (*ompA* gene). The prevalence of *A. baumannii* was significantly higher in residential properties compared with hospitals (p=0.001). *A. baumannii* prevalence was also significantly higher in biofilm samples compared to water (p=0.002). There was a significant difference in *A. baumannii* prevalence across sampling sites (p=0.002), with detection highest in outlet biofilms compared to drain biofilms. Of the 29 residential biofilm samples positive for *A. baumannii*, 15 were from drains and 14 were from outlets. Conversely in hospital biofilm samples, five positives were from outlets and one was from a drain. Two hospital water samples collected from handwashing basins were qPCR positive for *A. baumannii*.

Only 8.4% (n=14/166) of total samples were culture positive for *A. baumannii*. There was a significant difference in *A. baumannii* culture prevalence between sampling sites, with the highest detection in drain biofilms compared to outlet biofilms. Of the 14 residential biofilm samples that were culture positive for *A. baumannii*, 12 were from drains and two were from outlets. No hospital samples or water samples were culture positive for *A. baumannii*.

When analysed for antimicrobial resistance, 50% (n=7/14) culture isolates were resistant to one or more of the antibiotics tested (Table 5.1). Of these, two isolates were resistant to two antibiotics, and one isolate was resistant to four antibiotics. All resistant isolates were collected from residential properties, six from drains and one from an outlet. Resistance to doripenem (DOR) was the most common (n=3), followed by imipenem (IMI) and trimethoprim-sulfamethoxazole (SXT) (n=2 respectively). Resistance to piperacillin (PIP) and tobramycin (TOB) was also seen (n=1 respectively). All isolates were sensitive to piperacillin + tazobactam (TZP), ceftazidime (CAZ), cefepime (FEP), ciprofloxacin (CIP) and levofloxacin (LEV).

Table 5.1 - Antimicrobial resistance profiles of *Acinetobacter baumannii* isolates

Building type	Sampling site	Sample ID	Antibiotics											
			Penicillins	B-lactam combination agents	Cephems		Carbapenems			Aminoglycosides		Fluoroquinolones		Folate pathway antagonist
			PIP	TZP	CAZ	FEP	DOR	IMI	MER	CN	TOB	CIP	LEV	SXT
Residential	Faucet	R101	S	S	S	S	S	S	S	R	R	S	S	S
	Drain	R109	S	S	S	S	S	S	S	S	S	S	S	R
		R100	S	S	S	S	S	S	S	S	S	S	S	S
		R77	R	S	S	S	R	R	R	S	S	S	S	S
		R27	S	S	S	S	R	S	S	S	S	S	S	S
		R79	S	S	S	S	S	S	S	S	S	S	S	S
		R97	S	S	S	S	S	S	S	S	S	S	S	S
		R94	S	S	S	S	S	S	S	S	S	S	S	S
		R98	S	S	S	S	S	R	S	S	S	S	S	R
		R87	S	S	S	S	S	S	S	S	S	S	S	S
		R104	S	S	S	S	S	S	S	R	S	S	S	S
		R84	S	S	S	S	R	S	S	S	S	S	S	S
		R47	S	S	S	S	S	S	S	S	S	S	S	S
		R90	S	S	S	S	S	S	S	S	S	S	S	S

PIP: Piperacillin; TZP: piperacillin + tazobactam; CAZ: ceftazidime; FEP: cefepime; DOR: doripenem; IMI: imipenem; MER: meropenem; CN: gentamicin; TOB: tobramycin; CIP: ciprofloxacin; LEV" levofloxacin; SXT: trimethoprim-sulfamethoxazole. (S: sensitive, R: resistant (shown in red))

5.15.2. *Staphylococcus aureus*

Overall, 22% (n=36/166) of total samples were qPCR positive for *S. aureus* (*nuc* gene). There was no significant difference in *S. aureus* prevalence when comparing hospital and residential samples (p=0.595), biofilm and water samples (p=0.231) or across sampling site (p=0.105). Of the 28 residential biofilm samples positive for *S. aureus*, 14 were from drains and 14 were from outlets. Conversely in hospital biofilm samples, five positives were from outlets and three were from drains. Fifteen hospital water samples were qPCR positive for *S. aureus*, nine from handwashing basins and six from showers.

Only 7% (n=11/166) of total samples were culture positive for *S. aureus*. There was no significant difference in *S. aureus* culture prevalence between hospital and residential samples (p=0.851). However, there was a significant difference between sampling sites, with culture positive prevalence highest in drain biofilms when compared to outlet biofilms (p=0.002). Of the six residential biofilm samples that were culture positive for *S. aureus*, five were from drains and one was from an outlet. Five hospital biofilm samples were culture positive for *S. aureus*, three from drains and two from outlets.

When analysed for antimicrobial resistance, 72% (n=8/11) culture isolates were resistant to one or more of the seven antibiotics tested (Table 5.2). Of these, five isolates were resistant to two antibiotics and one isolate was resistant to three antibiotics. Seven of the resistant isolates were collected from drains, three from hospitals and four from residential properties, whilst one of the resistant isolates was collected from a hospital outlet. Resistance to penicillin (PEN) was most common (n=7), followed by cefoxitin (CFX) (n=6). Resistance to gentamicin (CN) and tobramycin (TOB) (n=1 respectively) was also seen. All isolates were sensitive to ciprofloxacin (CIP), levofloxacin (LEV) and trimethoprim-sulfamethoxazole (SXT).

Table 5.2 - Antimicrobial resistance profiles of *Staphylococcus aureus* isolates

Building type	Sampling site	Sample ID	Antibiotic						
			Penicillinase-labile penicillins	Penicillinase-stable penicillins	Aminoglycosides		Fluoroquinolones		Folate pathway antagonists
			PEN	CFX	TOB	CN	CIP	LEV	SXT
Hospital	Basin	16	S	S	S	S	S	S	S
		01	R	S	S	S	S	S	S
	Drain	82	R	S	S	S	S	S	S
		87	R	R	S	S	S	S	S
		90	R	R	S	S	S	S	S
Residential	Drain	R90	S	S	S	S	S	S	S
		R100	S	R	R	S	S	S	S
		R47	S	S	S	S	S	S	S
		R25	R	R	S	S	S	S	S
		R35	R	R	S	S	S	S	S
		R80	R	R	S	R	S	S	S

PEN: penicillin; CFX: cefoxitin; TOB: tobramycin; CN: gentamicin; CIP: ciprofloxacin; LEV: levofloxacin; SXT: trimethoprim-sulfamethoxazole

(S: sensitive, R: resistant (shown in red))

5.15.3. *Pseudomonas aeruginosa*

Overall, 37% (n=62/166) of total samples were qPCR positive for *P. aeruginosa* (*gyrB* gene). The prevalence of *P. aeruginosa* was significantly higher in residential properties compared to hospitals (p=0.001). *P. aeruginosa* prevalence was significantly higher in biofilm samples compared to water (p=0.001). There was a significant difference in *P. aeruginosa* prevalence between sampling site (p=0.001), with detection highest in outlet biofilms. Of the 55 residential biofilm samples positive for *P. aeruginosa*, 25 were from drains and 37 were from outlets. Conversely in hospital biofilm samples, only one positive was from a drain and five were from outlets. One water sample collected from a handwashing basin was positive for *P. aeruginosa.*

Only 10% (n=17/166) of total samples were culture positive for *P. aeruginosa*. There was no significant difference in *P. aeruginosa* culture prevalence between hospitals and residential samples (p=0.921) or between water and biofilm samples (p=0.765). *P. aeruginosa* culture prevalence was significantly different between sampling sites, with the highest detection in drain biofilms (p=0.011) compared to outlet biofilms. Of the nine residential biofilm samples that were culture positive for *P. aeruginosa*, eight were from drains and one was from an outlet. Five hospital biofilm samples were culture positive for *P. aeruginosa*, two from drains and three from outlets. Furthermore, three hospital water samples were positive for *P. aeruginosa*, one from a handwashing basin and two from showers.

When analysed from antimicrobial resistance, 35% (n=6/17) culture isolates were resistant to one or more of the 12 antibiotics tested (Table 5.3). Of these, two isolates were resistant to two antibiotics, one isolate was resistant to three antibiotics, and one was resistant to four antibiotics. Four of the resistant isolates were collected from residential drains and one from a residential faucet. Only one hospital shower water isolate was resistant. Overall, resistance to the carbapenem class was most common, with five isolates resistant to one or more carbapenem antibiotics (Table 5.3). Within this class, doripenem (DOR) resistance was the most frequent (n=4), followed by meropenem (MEM) (n=3) and imipenem (IMI) (n=2). Interestingly, the isolate resistant to four antibiotics was not resistant to any of these carbapenems. This isolate was instead resistant to ceftazidime-avibactam (CZA), cefepime (FEP), ciprofloxacin (CIP) and levofloxacin (LEV) (Table 3). All isolates susceptible to piperacillin (PIP), piperacillin-tazobactam (TZP), and tobramycin (TOB), and one isolate was intermediately resistant to gentamicin (CN).

Table 5.3 - Antimicrobial resistance profiles of *Pseudomonas aeruginosa* isolates

Building type	Sampling site	Sample ID	Antibiotics											
			Penicillins	B-lactam combination agents		Cephems		Carbapenems			Aminoglycosides		Fluoroquinolones	
			PIP	TZP	CZA	CAZ	FEP	DOR	IMP	MEM	CN	TOB	CIP	LEV
Hospital	Water	71	S	S	S	S	S	S	S	S	I	S	S	S
	Shower water	33	S	S	S	S	S	S	S	S	S	S	S	S
		48 ◊	S	S	S	S	S	R	S	S	S	S	S	S
	Faucet	77	S	S	S	S	S	S	S	S	S	S	S	S
		80	S	S	S	S	S	S	S	S	S	S	S	S
		80	S	S	S	S	S	S	S	S	S	S	S	S
	Drain	79	S	S	S	S	S	S	S	S	S	S	S	S
		90	S	S	S	S	S	S	S	S	S	S	S	S
Residential	Drain	R31 §	S	S	S	S	S	S	R	S	S	S	S	S
		R73	S	S	S	S	S	S	S	S	S	S	S	S
		R36	S	S	S	S	S	S	S	S	S	S	S	S
		R7	S	S	R	S	R	S	S	S	S	S	R	R
		R10	S	S	S	S	S	S	S	S	S	S	S	S
		R80 §†	S	S	S	S	S	R	R	R	S	S	S	S
		R87 §	S	S	S	S	S	R	S	R	S	S	S	S
		R79	S	S	S	S	S	S	S	S	S	S	S	S
	Faucet	R16	S	S	S	S	S	R	S	R	S	S	S	S

PIP: Piperacillin; TZP: piperacillin + tazobactam; CZA: ceftazidime-avibactam; CAZ: ceftazidime; FEP: cefepime; DOR: doripenem; IMI: imipenem; MER: meropenem; CN: gentamicin; TOB: tobramycin; CIP: ciprofloxacin; LEV levofloxacin

Note: ◊ blaOXA-48, § blaNDM-1, † blaKPC-2 denotes gene detection. (S: sensitive, I: intermediate (shown in yellow), R: resistant (shown in red))

5.15.3.1. Carbapenem resistance gene identification

All water and biofilm samples, and carbapenem resistant culture positive *P. aeruginosa* isolates were screened for the presence of four carbapenem resistance genes (*bla*$_{NDM-1}$, *bla*$_{OXA-48}$, *bla*$_{VIM}$ and *bla*$_{KPC-2}$). Overall, *bla*$_{NDM-1}$ was the most frequently detected carbapenem resistance gene in total environmental samples (9.6%, n=16/166). Seven qPCR *P. aeruginosa* positive samples and three carbapenem resistant culture isolates were positive for *bla*$_{NDM-1}$. *bla*$_{NDM-1}$ was detected in nine samples that were qPCR negative for *P. aeruginosa*. There was no significant difference in *bla*$_{NDM-1}$ prevalence between total hospital and residential samples (p=0.602), between water and biofilm samples (p=1) or across sampling sites (p=0.484).

bla$_{OXA-48}$ was detected in 9% (n=15/166) of total samples. Eight qPCR *P. aeruginosa* positive samples and one carbapenem resistant isolate were positive for *bla*$_{OXA-48}$. *bla*$_{OXA-48}$ was detected in seven samples that were qPCR negative for *P. aeruginosa*. *bla*$_{OXA-48}$prevalence was significantly higher in biofilm samples compared to water (p=0.023). There was no significant difference in *bla*$_{OXA-48}$ prevalence between total hospital and residential samples. There was a significant difference in *bla*$_{OXA-48}$ detection between sampling sites, with detection highest in drains (n=8).

bla$_{VIM}$ was detected in 5% (n=9/166) of total samples. Two qPCR *P. aeruginosa* positive samples were positive for *bla*$_{VIM}$, however, *bla*$_{VIM}$ was not detected in any carbapenem resistant isolates. *bla*$_{VIM}$ was detected in seven samples that were qPCR negative for *P. aeruginosa*. *bla*$_{VIM}$ prevalence was significantly higher in water samples compared to biofilm (p=0.006). There was no significant difference in *bla*$_{VIM}$ prevalence between total hospital and residential samples.

bla$_{KPC-2}$ was the least prevalent carbapenemase resistance gene and was detected in 4% (n=7/166) of total samples. Two qPCR *P. aeruginosa* positive samples and one carbapenem resistant isolate were positive for *bla*$_{KPC-2}$. This isolate was also positive for *bla*$_{NDM-1}$. *bla*$_{KPC-2}$ was detected in five samples that were qPCR negative for *P. aeruginosa*. There was no significant difference in *bla*$_{KPC-2}$ prevalence between hospital and residential samples (p=0.445), between water and biofilm samples (p=689) or across sampling site (p=0.685).

5.16. Discussion

The presence of antimicrobial-resistant (AMR) pathogens in drinking water plumbing biofilms presents a significant public health challenge. However, the control of these biofilms is largely overlooked in infection controls guidelines. This study identified critical AMR threats including methicillin resistant *S. aureus* (MRSA), and carbapenem resistant *A. baumannii*

(CRAB) and *P. aeruginosa* (CRPA) in hospital and residential drinking water and plumbing biofilms. These AMR pathogens were frequently detected, indicating a significant yet overlooked risk to the growing population of vulnerable individuals, particularly those receiving healthcare at home.

5.16.1. The role of biofilms in antimicrobial resistance persistence and spread

Biofilms play a crucial role in the spread and rise of antimicrobial resistance (Flores-Vargas et al., 2021). In the present study, all target pathogens were frequently identified in drinking water plumbing systems, with *P. aeruginosa* and *A. baumannii* significantly more prevalent in biofilms compared to water. Biofilms are diverse communities of microorganisms that adhere to surfaces and produce an extra polymeric substance (EPS) matrix to protect against environmental stresses (Flemming et al., 2010). This protection can render current water treatment protocols that are designed to target planktonic bacteria ineffective (J. Y. Maillard et al., 2023). The EPS structure can adsorb these residual disinfectants, resulting in a subinhibitory concentration reaching the bacteria embedded deep within the biofilm (J. Y. Maillard et al., 2023; Xue et al., 2012). Concerningly, exposure to subinhibitory antimicrobials has been shown to upregulate the expression of antimicrobial resistance mechanisms. For example, previous research has demonstrated that chlorine exposure can result in an increased expression of efflux pumps in *P. aeruginosa* and *A. baumannii* that are also associated with antibiotic resistance (Karumathil et al., 2014; Shrivastava et al., 2004).

Antimicrobial resistance may also spread throughout biofilms via horizontal gene transfer (HGT) due to the close proximity to diverse species (Abe et al., 2020; Michaelis et al., 2023). *P. aeruginosa* can acquire resistance genes from neighbouring cells via mechanisms such as conjugation, transformation and transduction (Johnston Ella et al., 2023; Reem et al., 2024). CRPA has been identified as a key AMR threat by the World Health Organization (WHO) and Centers for Disease Control and Prevention (CDC) due to the ability of these pathogens to easily acquire multiple carbapenemase genes from other species, such as *A. baumannii* (Centers for Disease Control and Prevention, 2019a; Kaluba et al., 2021; World Health Organization, 2024). In the present study, resistance to carbapenem antibiotics was the most common, with multiple *P. aeruginosa* and *A. baumannii* isolates resistant to multiple carbapenem antibiotics. When analysed further, the $bla_{\text{NDM-1}}$ carbapenem resistance gene was the most prevalent gene in both the *P. aeruginosa* resistant culture isolates and in total samples. Carbapenem antibiotics are one of the last lines of defence against multi-drug resistant *P. aeruginosa* (Buehrle et al., 2017). Furthermore, the identification of $bla_{\text{NDM-1}}$ in biofilms highlights the potential for HGT between bacterial species, as this plasmid has been

previously detected among the Enterobacteriaceae family (Jovcic et al., 2011). Concerningly, all carbapenem resistance genes were detected in samples that were qPCR and culture negative for *P. aeruginosa*. Extracellular DNA can persist within biofilms and become embedded within the EPS even after the original host cell has died (Panlilio et al., 2021). The EPS then serves as a genetic reservoir, where these resistance genes can persist and potentially be acquired by other cells in the biofilm (Panlilio et al., 2021; Tang et al., 2013). MRSA was also found in drinking water plumbing biofilms in the present study. Notably, all instances of MRSA were detected in drain biofilms. Outbreaks of MRSA have typically been associated with dry high touch surfaces such as doorknobs and bedrails (Jaradat et al., 2020). This focus in infection control guidelines leads to an oversight of water-related devices, such as faucets, showerheads, and sink drains. However, these devices have been linked to significant HAI outbreaks (Hayanga et al., 1997; Layton et al., 1993; Squeri et al., 2012; Sserwadda et al., 2018). These findings emphasize the need for further research to understand the role of drinking water plumbing biofilms in the spread of antimicrobial resistance, and how these biofilms can provide protection to pathogens not typically considered waterborne.

5.16.2. Public health implications for healthcare at home

The hospital environment is recognised as a niche for AMR due to strong selection pressures from frequent antibiotic use and colonisation by AMR pathogens shed from infected patients (Fletcher, 2015; Mulvey et al., 2009). However, this attention is not extended to the residential environment and in particular drinking water plumbing. Although selective AMR pressures may be reduced, the lack of consistent maintenance and cleaning can result in an environment that allows for the growth and proliferation of AMR bacteria (Hayward et al., 2022b). The inconsistent use of household cleaning products may not be effective against drinking water plumbing biofilms, therefore exerting an underestimated selective pressure (van Dijk et al., 2022; Zhu et al., 2023). Additionally, there is an increasing concern regarding community-acquired AMR infections, particularly amongst individuals receiving healthcare at home (Hayward et al., 2022b; Hidron et al., 2009). The unique risks presented by home healthcare environments are overlooked in current infection control and prevention guidelines that are tailored to healthcare facilities (de Sousa Vale et al., 2019). Patients receiving healthcare at home, especially those with invasive medical devices or those who are immunocompromised, may be at risk of exposure to pathogens in contaminated water, leading to severe health complications. In the present study, prevalence of *A. baumannii* and *P. aeruginosa* was significantly higher in residential properties compared with hospitals. Furthermore, biofilms collected from drains were identified as the most common reservoir for AMR *A. baumannii, S. aureus* and *P. aeruginosa*. Handwashing

basin and shower drains are prone to biofilm formation due to the constant exposure to moisture and nutrients, low flow rates, and multiple colonisation routes (Hayward et al., 2024; McBain et al., 2003a). Routine activities such as bathing, dishwashing and handwashing serve as routes of drain biofilm colonisation, but also can expose vulnerable individuals to the AMR pathogens that have been able persist in this niche (Hayward et al., 2022a; McBain et al., 2003a).

The promotion of healthcare at home services, such as chemotherapy, post-surgical care and chronic disease management, has gained significant support as an alternative to in-patient treatment (de Sousa Vale et al., 2019; Di Mascolo et al., 2017). These services can ease the burden on healthcare systems, reduce associated costs and improve patient comfort. However, this transition is occurring at a faster rate than the understanding of the risks posed by the residential environment, delaying the development of tailored infection control guidelines (Di Mascolo et al., 2017). Continuing to view infection risks as confined to hospitals overlooks the need for tailored infection control in homes, leaving homeowners and caregivers unaware of the risks. Furthermore, the transition to healthcare at home has lacked collaboration between the healthcare providers, infection control professionals and funding bodies, leading to a fragmented understanding of the risks and required infection prevention approaches. This approach can result in inconsistent monitoring and reporting of community acquired HAIs, further hindering the quantification of the risks associated with healthcare at home alternatives (Bertagnolio et al., 2023; Hayward et al., 2022b; Murray et al., 2022). Current estimates of the burden of AMR in communities are limited due to inconsistent reporting and hospital coding, as well as a bias toward hospital acquired infections (Bertagnolio et al., 2023; Murray et al., 2022). To address this gap, there is a need for increased awareness and education about the infection risks associated with residential environments. Homeowners and caregivers should be informed about biofilm-associated AMR and the benefits of adopting infection control practices, such as regular maintenance of drinking water plumbing systems, the use of effective cleaning and disinfection protocols, and ongoing education regarding hygienic practices.

5.17. Conclusion

The rise of antimicrobial resistance is recognised as a growing public health threat, however, there is limited understanding of how the drinking water plumbing environment contributes to this rise. This is the first study to examine the prevalence of AMR pathogens in Australian drinking water plumbing systems. This study found that 73% of residential samples were positive for at least one target pathogen, compared to 38% of hospital samples. Concerningly, 45% of residential drinking water plumbing fixtures harboured at least two

target pathogens. Recognizing the unique risks presented by residential environments is essential to develop effective public health strategies aimed at preventing the spread of antimicrobial resistance and protecting vulnerable populations. Key findings also include the prevalence of critical AMR threats in both hospitals and residential drinking water plumbing, with biofilms formed on drains identified as a frequent reservoir. This study highlights how inconsistent disinfection and a lack of understanding may be contributing AMR pathogen persistence in residential properties. The detection of carbapenem resistance genes in water and biofilm samples that were negative for *P. aeruginosa* indicates the biofilm may be a reservoir for antimicrobial resistance genes to persist and acquired by other cells long after the original host has died. This research suggests that the drinking water plumbing environment serves as a niche for complex AMR bacterial communities, including pathogens not typically considered waterborne. For example, MRSA, an AMR pathogen associated with dry high touch surfaces such as bed rails and door knobs, was found in both water and biofilm samples. Further understanding of biofilm ecology, the selective pressures that promote resistance, and the mechanisms of pathogen transmission in residential environments is essential to develop effective strategies to combat this growing threat. While healthcare at home presents promising alternative to inpatient care, it is important that the unique risks associated with residential environments are thoroughly understood and addressed. This research is required to inform tailored infection prevention for home healthcare settings to safeguard patients' health and contribute to the overall efficacy of this healthcare model.

6. BACTERIAL DIVERSITY IN PREMISE PLUMBING BIOFILMS AND ASSOCIATED INFECTION RISKS

In this chapter, the microbial diversity of handwashing basin biofilms was explored. This chapter addresses Objectives 2 and 4, and includes a published manuscript. This manuscript used 16S rRNA sequencing to characterize biofilm communities from hospital and residential handwashing basins. This research highlighted significant differences in biofilm bacterial diversity and community composition between residential and healthcare environments. It identified potentially biofilm forming, pathogenic and corrosive genera, demonstrating the complex and functionally diverse nature of these microbial communities.

CITATION: Handwashing basins and healthcare associated infections: Bacterial diversity in biofilms on faucets and drains

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6.1. Structured Summary

6.1.1. Background

Increasingly, hospital handwashing basins have been identified as a source of healthcare-associated infections. Biofilms formed on the faucet and drains of handbasins can potentially harbour pathogenic microbes and promote the dissemination of antimicrobial resistance. However, little is known about the diversity of these biofilm communities and the routes of contamination.

6.1.2. Aim

The aim of this paper was to use 16S rRNA gene amplicon sequencing to investigate the diversity of prokaryote communities present in faucet and drain biofilm samples taken from hospital and residential handbasins.

6.1.3. Findings

The biofilm prokaryotes communities were diverse, with high abundances of potentially corrosive, biofilm forming and pathogenic genera, including those that are not typically waterborne. The β-diversity showed statistically significant differences in the variation of bacterial communities on the basis on building type (hospital vs residential p=0.0415). However, there was no statistically significant clustering based on sampling site (faucet vs drain p=0.46). When examining the β-diversity between individual factors, there was a significant difference between drain biofilms of different buildings (hospital drain vs residential drain p=0.0338).

6.1.4. Conclusion

This study demonstrated that biofilms from hospital and residential handbasins contain complex and diverse microbial communities that differ significantly by building type. It also showed biofilms formed on the faucet and drain of a hospital's handbasins were not significantly different. Future research is needed to understand the potential mechanisms of transfer between drains and faucets of hospital handbasins. This information will inform improved infection control guidelines to control this underrecognized source of infections.

6.2. Introduction

Healthcare-associated infections (HAI) have been identified as the most common hospital complication, with 7% high income and 15% of low and middle income acute care patients developing at least one HAI during their hospital stay (World Health Organization, 2022). Point of use biofilms have been identified as the cause of waterborne HAI outbreaks in both hospital and residential settings (Akkina et al., 2020; Bae et al., 2019; Barna et al., 2014; Chaidez et al., 2004). Despite being responsible for approximately 7.15 million infections per year resulting in over US $3.33 billion in associated costs, residential drinking water-related infections continue to be overlooked by infection control and prevention guidelines (Collier et al., 2021).

Handwashing basins can become contaminated via the incoming water supply and by transfer of patient and staff microbiota (Grabowski et al., 2018). Although many water treatment processes target solids and chemical parameters, and the final disinfection step designed specifically to reduce microorganisms, drinking water has still been shown to be colonized by many bacterial species (Huang et al., 2021; Inkinen et al., 2016; Nisar et al., 2023b). Premise plumbing systems (building water systems) are complex, with fluctuations in water temperature and changing water flow dynamics impacting water quality and biofilm formation (Wang et al., 2012). The detachment of biofilms formed on plumbing pipes due to changes in flow rate and shear stress results in further colonisation of the premise plumbing environment, extensive microbially induced corrosion and disease transmission (Khu et al., 2023; Wang et al., 2022). These mobilised biofilms and corrosion products can accumulate at the point of use on water related devices such as tap faucets, drains and showers (E. Bédard et al., 2016). These areas are also subject to contamination of bacterial species from the user that are not typically associated with drinking water such as *Staphylococcus aureus*, *Klebsiella pneumoniae* and *Enterobacter* spp., all strong biofilm formers in their own right (Hayward et al., 2022b). Simultaneous contamination by source water and end point use results in a diverse and complex biofilm community that may provide an ideal niche for disinfection survival, further corrosion of plumbing products and transfer of antimicrobial resistant genes (Franco et al., 2020; Qiao et al., 2020). Biofilms provide protection against unfavourable environmental conditions such as heat treatment and disinfection, rendering remediation attempts often ineffective (Falkinham et al., 2015).

When investigating infection control risks from handwashing basins, it's essential to consider microbial risks in residential buildings, as more healthcare services are now provided in these settings. For example, healthcare services such as chemotherapy, ventilator support and post-surgical care in patients' homes has emerged as an alternative to inpatient hospital

treatment (Houston et al., 2020; Montalto et al., 2020). These schemes are being facilitated by government funds, for example, the United Kingdom, United States and Australia, to reduce the burden on healthcare systems (Landers et al., 2016). However, patients may be exposed to increased environmental risks associated with poor plumbing maintenance, sanitation and ventilation in their home environment (Dion-Fortier et al., 2009; Hayward et al., 2022b). Currently, infection control guidelines that attempt to mitigate the risk of environmental infection exposure largely focus on healthcare settings, without acknowledging the potential of unique risks also posed by the residential environment.

As handwashing basins are increasingly recognised as a source of HAI, there is a need for more research investigating the mechanisms of contamination and transfer to devise appropriate control measures. The aim of this study was to investigate the bacterial diversity of biofilms formed on faucets and drains of handwashing basins present in a hospital and residential buildings. The differences in the communities present in the different building sites and the biofilms formed on different aspects of the handwashing basins will provide insight into this unique niche as a potential source of HAI. Identifying these previously overlooked environments as potential hazards will inform future infection control protocols by allowing for more targeted and effective risk management.

6.3. Methods

6.3.1. Sample collection and processing

Biofilm samples were collected from the faucet and drain of 20 handwashing basins, totalling 40 biofilm samples. Eleven different handwashing basins were sampled from residential properties in South Australia and nine different basins from a hospital in New South Wales (Australia) opportunistically. The residential samples were all taken from bathroom handwashing basins and the hospital samples were taken from six patient room ensuites, two communal hallway basins and one staff room basin. To ensure participants and study locations remained anonymous the exact geographic location of the buildings and information around the building type and size was not collected as this may have resulted in a site becoming identifiable. The samples were collected using sterile nylon swabs (FLOQSwab™; Copan Italia S.p.A., Brescia Italy) moistened with sterile water. The external surface of the faucet or drain was swabbed for ten seconds. The swab was placed in a sterile 10 mL centrifuge tube with 3 mL of phosphate buffered saline (PBS). A negative control was performed with a sterilised nylon swab. All samples were stored at 5 +/- 2°C and processed within three days. Biofilm was removed from the swab and suspended into PBS by 5 minutes of each shaking (Griffin Flask Shaker), vortexing (SEM® Vor Mix) and sonication (CooperVision® 895 Ultrasonic Cleaner).

6.3.2. DNA extraction and 16S rRNA amplification

DNA was extracted from the resuspended biofilm sample using the DNeasy PowerBiofilm kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The extracted DNA was subjected to polymerase chain reaction (PCR) targeting the V4 region of the 16S rRNA gene with a target amplicon size of ~250 base pairs (bp) using primers 515F (5' – GTGYCAGCMGCCGCGGTAA – 3') and 806R (5' – GGACTACNVGGGTWTCTAAT – 3') (Ugarelli et al., 2018). Nextera adapter sequences, specific to the sequencing platform, were connected to the primers. Each PCR reaction contained 10 ng template DNA, 0.32 µM of each primer, 1 U Q5® hot start high-fidelity DNA polymerase (New England Biolabs®), 1 x Q5® reaction buffer (New England Biolabs®), 10mM dNTP mix (Promega®), MilliQ water for a total volume of 50 µL. The cycling conditions included one cycle of initial melting at 98^{o}C for 60 s, followed by 30 cycles consisting of 98^{o}C for 30 s, 51^{o}C for 30 s and 72^{o}C for 30 s with a final extension at 72^{o}C for 10 minutes. Ten µL of amplified PCR product was applied to a 2% agarose gel stained with GelRed® (Biotium), and the molecular mass of the PCR products was estimated using a 100 bp DNA ladder (Promega®). PCR product with amplicons of approximately 291 bp were sent to the Australian Genome Research Facility (AGRF Ltd., Australia) for barcode indexing and pair end sequencing using the Illumina MiSeq^{TM} System.

6.3.3. Bioinformatic analysis

The sequence data generated by the MiSeq^{TM} System were analysed using mothur (version 1.48.0) (Schloss et al., 2009). The raw sequence files were filtered for quality and trimmed to a maximum of 275 bp. Sequences with homopolymers longer than eight bps and greater than two mismatches were removed from analysis. To generate count tables, the selected V4 16S rRNA region was aligned with the SILVA (version 138.1) database (Quast et al., 2012). Chimeric sequences were identified and removed using VSearch (version 2.21.1) (Rognes et al., 2016). The OptiClust algorithm was used to group the sequences into operational taxonomic units (OTUs) based on a 0.03 distance limit. OTUs that occurred as a single count in only one sample were removed. The lowest identifiable taxa level was added to OTUs binned as unclassified or uncultured.

6.3.4. Community and statistical analysis

The total abundances of OTUs were Log(x+1) scale transformed to analyse α-diversity and β-diversity using PRIMER v7 with PERMANOVA+ (Plymouth Routines in Multivariate Ecology Research, United Kingdom) (Anderson et al., 2008). Microbiome Analyst, an online tool based on multiple R packages, was used to determine the core microbiome, and comparative abundance profile pie charts (Chong et al., 2020). The data were transformed

into total sum scaling to address the variability in sampling depth and sparsity of the data and for univariate analysis. The p values <0.05 were considered statistically significant and were designated as follows: *** p<0.001; ** p<0.01; * P<0.05 and 'ns' non-significant.

6.3.5. α-diversity analysis

The α-diversity is an indication of richness and evenness of each microbial taxa within each sample (Whittaker, 1972). This was measured using Pielou's evenness, Species Richness, Simpson and Shannon diversity indices, given the selected environmental parameters i.e. sampling site (faucet or drain), building type (residential or hospital) and building specific sites (HF: hospital faucet; HD: hospital drain; RF: residential faucet and RD: residential drain). The Mann-Whitney test was used as a non-parametric method of determining if the estimated diversity index was significantly ($p<0.05$) different in the selected environmental parameters.

6.3.6. β-diversity analysis

The β-diversity was measured as an indication of variations in bacterial community composition among samples within an environmental parameter (Whittaker, 1972). The environmental parameters considered for β-diversity analysis were sampling site (faucet or drain), building type (residential or hospital) and building specific sites (HF: hospital faucet; HD: hospital drain; RF: residential faucet and RD: residential drain). The Bray-Curtis dissimilarity was used to determine the differences in bacterial community composition. Principal Coordinates Analyses (PCoA) and Canonical Analysis of Principal coordinates (CAP) were used to visualise the Bray-Curtis matrix. A Permutational Multivariate Analysis of Variance (PERMANOVA) method was developed to determine the statistically significant ($p<0.05$) differences in bacterial community composition. The contribution of individual taxa to these differences were determined using similarity percentage (SIMPER) analysis for each environmental parameter. The SIMPER analysis results were displayed on CAP plots to visualise such dissimilarities.

6.3.7. Univariate statistical analysis

The OTU abundance table data was transformed into TSS format for univariate statistical analysis. As the data didn't meet the assumptions of normality and homoscedasticity, non-parametric Mann-Whitney test was performed to test a significant difference between selected parameters. Significant values are presented in logarithmic (log_{10}) scale.

6.3.8. Co-occurrence correlation analysis

Co-occurrence of bacterial genera (specifically pathogenic, corrosive and biofilm forming) in biofilm was identified by performing a non-parametric Spearman's correlation analysis ($p<0.05$) significance threshold. Correlation matrices were visualised using "ggcorplot (version 0.1.4.)" package (Kassambara, 2016).

6.4. Results

Of the 40 biofilm samples (18 from hospitals and 22 from residential properties), the 16S rRNA region of all samples were able to be successfully amplified and sequenced. Bioinformatic processing revealed a total of 90 archaeal OTUs and 4,079 bacterial OTUs at the genus level. The premise plumbing biofilm communities were complex, consisting of up to 250 bacterial phyla. Pseudomonadota was the most abundant phylum in 44/45 samples (70.9% average relative abundance). Other phyla with >1% relative abundance of the total population included Bacteroidota (10.6%), Planctomyetota (5.60%), Actinobacteriota (5.77%) and Verrucomicrobiota (1.68 %) (Figure 14.1). Many 16S rRNA sequences were not classified to a genus, or in some instances at a family or order level. Overall, the most abundant genera present in all biofilm samples were *Cycloclasticus* (10.9%), *Xanthobacteraceae* (8.72 %), an unclassified Rhodobacteraceae (3.61%), *Enhydrobacte*r (3.32%) and an unclassified Sphingomonadaceae (3.16%) (Figure 14.2).

6.4.1. Influence of building type and sampling site on bacterial community composition

The β-diversity was measured as the changes in bacterial community composition when considering different environmental factors (building type and sampling site) using the Bray-Curtis dissimilarity as a resemblance measure, visualised on PCoA plots. When comparing sampling sites (faucet vs drain), there was no statistically significant clustering (p=0.46). However, there was significantly predictable clustering on the basis of building type (hospital vs residential) (p=0.0415; Figure 6.1). When examining the β-diversity between individual factors, there was a significant difference between drain biofilms of different buildings (Hospital drain (HD) vs residential drain (RD); PERMANOVA: p=0.0338). There was no significant difference in β-diversity between faucet and drain biofilms from hospital handwashing basins (PERMANOVA: p=0.303) with an average community similarity of 41.6% at the genus level (Figure 6.2). SIMPER analysis comparing hospital faucet (HF) and HD showed the genera *Methylobacterium-Methylorubrum*, unclassified Alphaproteobacteria, unclassified Sphingomonadaceae, *Pseudomonas* and *Staphylococcus* were key taxa driving this similarity, and contributed ~3% of the similarity between samples. Although the bacterial communities of residential faucets and drains seem dissimilar (Figure 6.2), there was also no

significant difference in β-diversity between sites (PERMANOVA: *p*=0.0915) with samples sharing an average dissimilarity of 63.9%. The genera Nitromonadaceae-DSSD61, unclassified Acetobacteraceae, Sphingomonadaceae-*Novosphiongobium*, *Flavobacterium*, *Chryseobacterium* and *Nubsella* in addition to 22 other taxa were responsible for 5% of variance between residential faucet (RF) and RD samples This indicates that the bacterial community compositions within handwashing basins biofilms differ significantly between building types, particularly in the drain environmental niche.

A

FACTOR		Beta Diversity (Bray Curtis dissimilarity index)
BUILDING		
Hospital (n=18)	Residential (n=22)	p=0.0415
SITE		
Faucet (n=20)	Drain (n=20)	p=0.461
BUILDING X SITE		
HF (n=9)	HD (n=9)	p=0.3037
HF (n=9)	RF (n=11)	p=0.1862
RF (n=11)	RD (n=11)	p=0.0915
HD (n=9)	RD (n=11)	p=0.0338

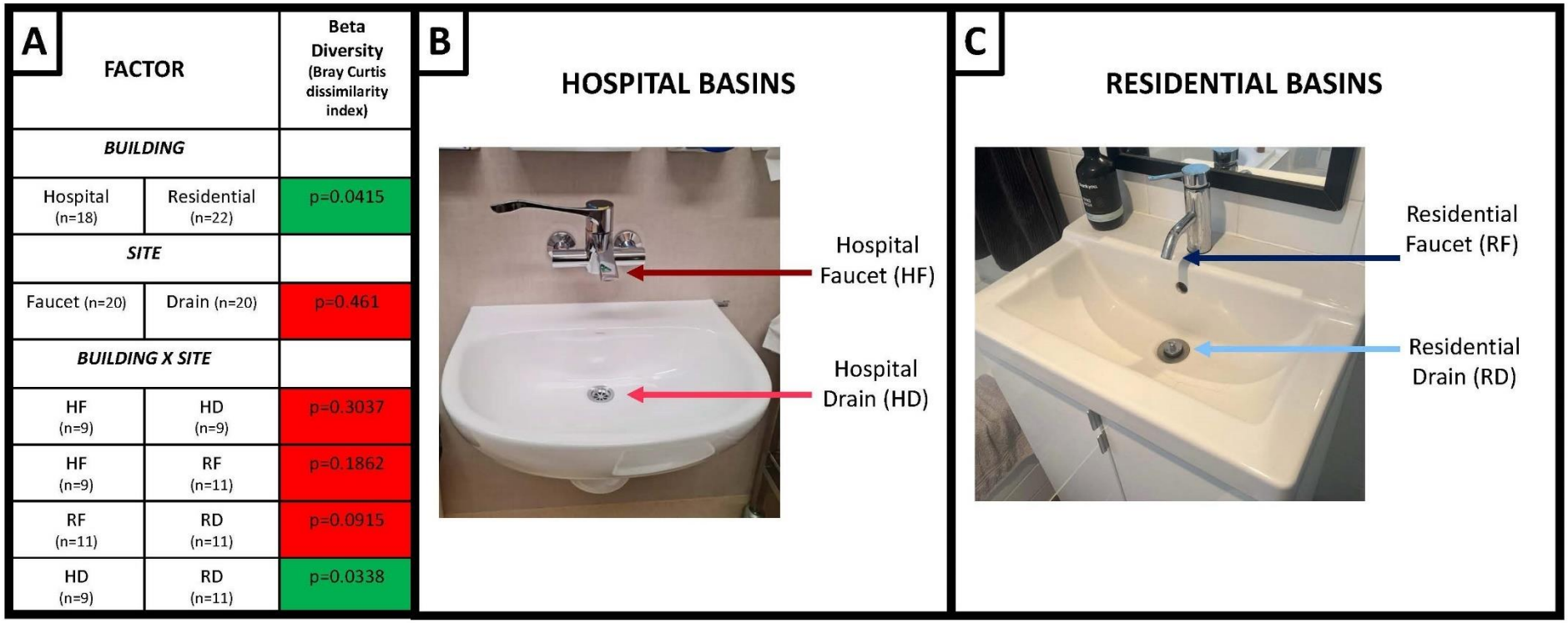
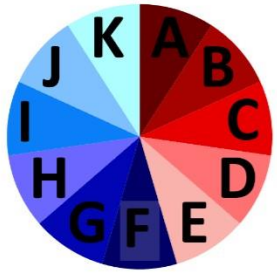


Figure 6.1 - Overview diagram showing significant differences in biofilm microbial diversity between hospital and residential handwashing basins.

(A) Summary table comparing sampling factors (Building, Site and Building X Site) for β-diversity (Bray Curtis dissimilarity index). Statistically significant differences between factors indicated in green ($p < 0.05$) and non-significant values indicated in red ($p > 0.05$). (B) Diagram of a hospital handwashing basin, indicating hospital faucet (HF) and hospital drain (HD) biofilm sampling locations. (C) Image of a residential handwashing basin, indicating residential faucet (RF) and residential drain (RD) sampling locations.



A: *Methylobacterium Methylorubrum*

B: Alphaproteobacteria unclassified

C: Sphingomonadaceae unclassified

D: *Pseudomonas*

E: *Staphylococcus*

F: Nitromonadaceae-DSSD61

G: Acetobacteraceae unclassified

H: Sphingomonadaceae-*Novosphingobium*

I: *Flavobacterium*

J: *Chryseobacterium*

K: *Nubsella*

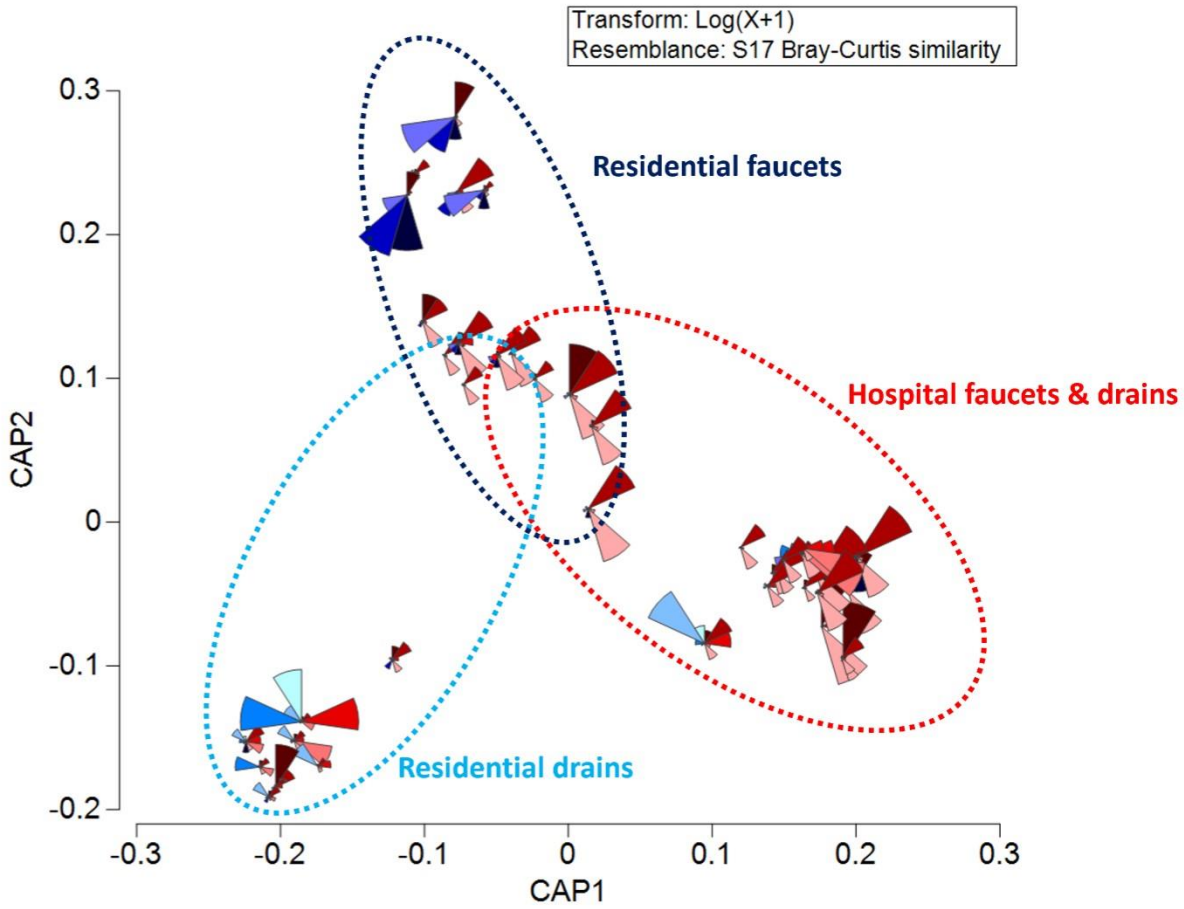


Figure 6.2 - Canonical analysis of principal coordinates (CAP) based on Bray-Curtis dissimilarity displaying the variation in bacterial communities on the basis on building type (hospital or residential) and sampling site (faucet or drain).

The overlayed similarity percentage (SIMPER) analysis shows the 11 bacterial genera responsible for driving 5% variance. The genera (A-E) dominant in hospital buildings were coloured red and the genera (F-K) dominant in residential buildings were coloured blue. The increasing colour intensity indicates increased prevalence in the relevant building type.

The α-diversity was measured as the richness and evenness of bacterial taxa within each sample using the Shannon diversity index. When comparing building types (hospital vs residential) and sampling sites (faucets vs drains), there was no significant difference in α-diversity (p=0.86 and 0.35, respectively). However, when examining the differences between individual factors, there was a significantly difference in α-diversity between residential faucets and drains (p=0.047). There was no significant difference in α-diversity between drain biofilms of different buildings (RD vs HD), between faucet biofilms of different buildings, or between faucet and drain biofilms of hospitals (HF vs HD) (Figure 14.3, Figure 14.4, Figure 14.5).

6.4.2. Composition of bacterial and archaeal taxa in biofilm communities

Univariate analysis of key potentially pathogenic, corrosive and/or biofilm forming genera, demonstrated that drain biofilms harboured significantly higher relative abundances of *Pseudomonas*, *Chryseobacterium*, *Delftia* and *Microbacterium,* whereas *Methylobacterium-Methlylorubrum* was significantly more abundant in faucet biofilms (Figure 14.6). Residential handwashing basins had significantly higher relative abundances of six key taxa including *Legionella*, *Bosea*, *Sphingomonas*, *Flavobacterium*, *Acidovorax* and *Delftia. Staphylococcus* was the only potentially pathogenic genera to be significantly more abundant in a hospital basin biofilm (Figure 6.3). Specifically, residential drains were found to harbour the most key taxa at significantly higher relative abundances when compared hospital drains, included *Chryseobacterium, Delftia* and *Sphingomonas*. Similarly, residential faucets harboured three key taxa, *Acidovorax, Delftia* and *Legionella*, at significantly higher relative abundances than hospital faucets (Figure 6.4).

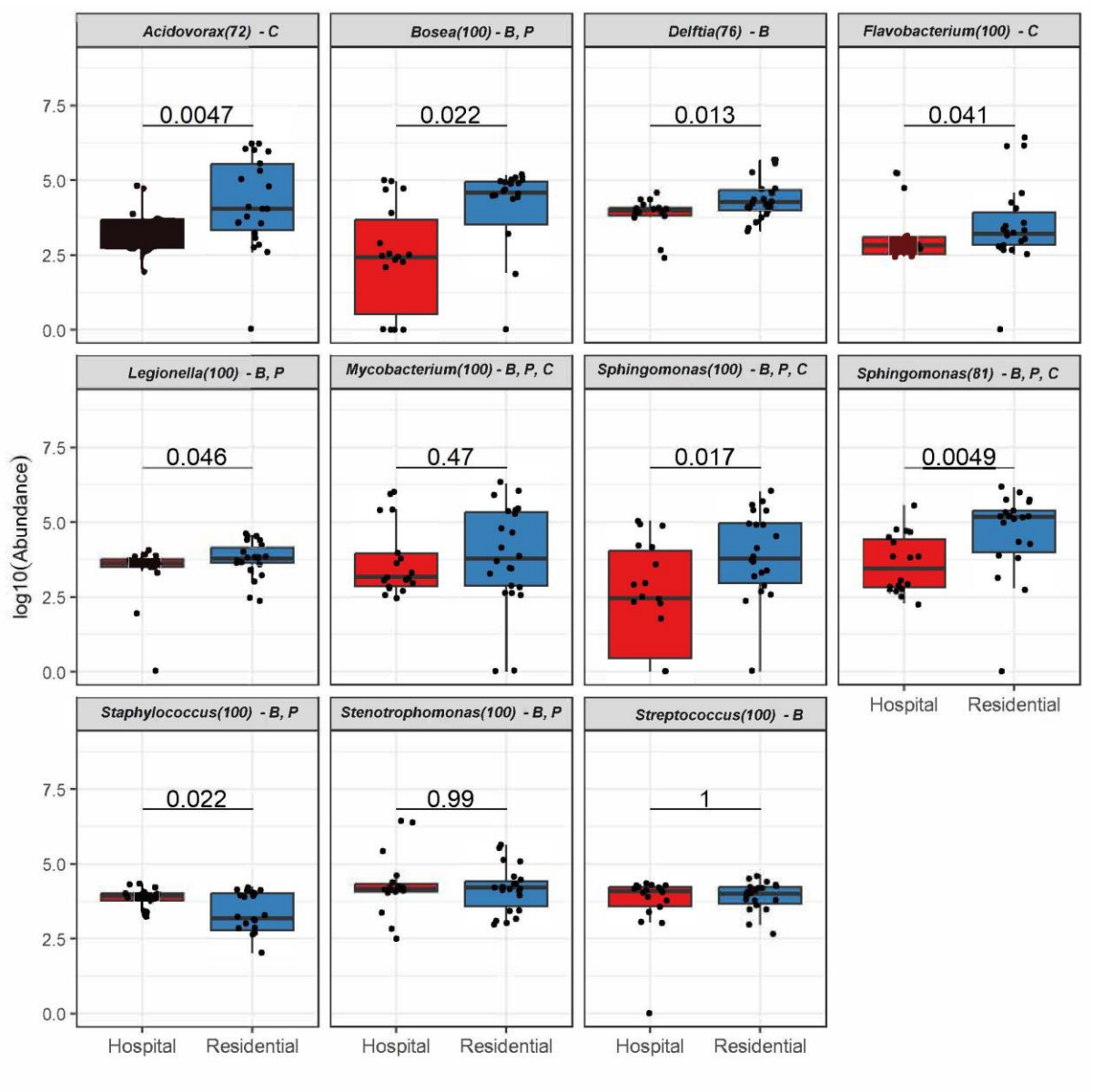


Figure 6.3 - Relative abundance (Log^{10} transformed) of pathogenic, corrosive and biofilm forming bacterial genera (n = 11) that differed significantly between hospital (red) and residential (blue) buildings. B = Biofilm forming, P = pathogenic and C = corrosive genera.

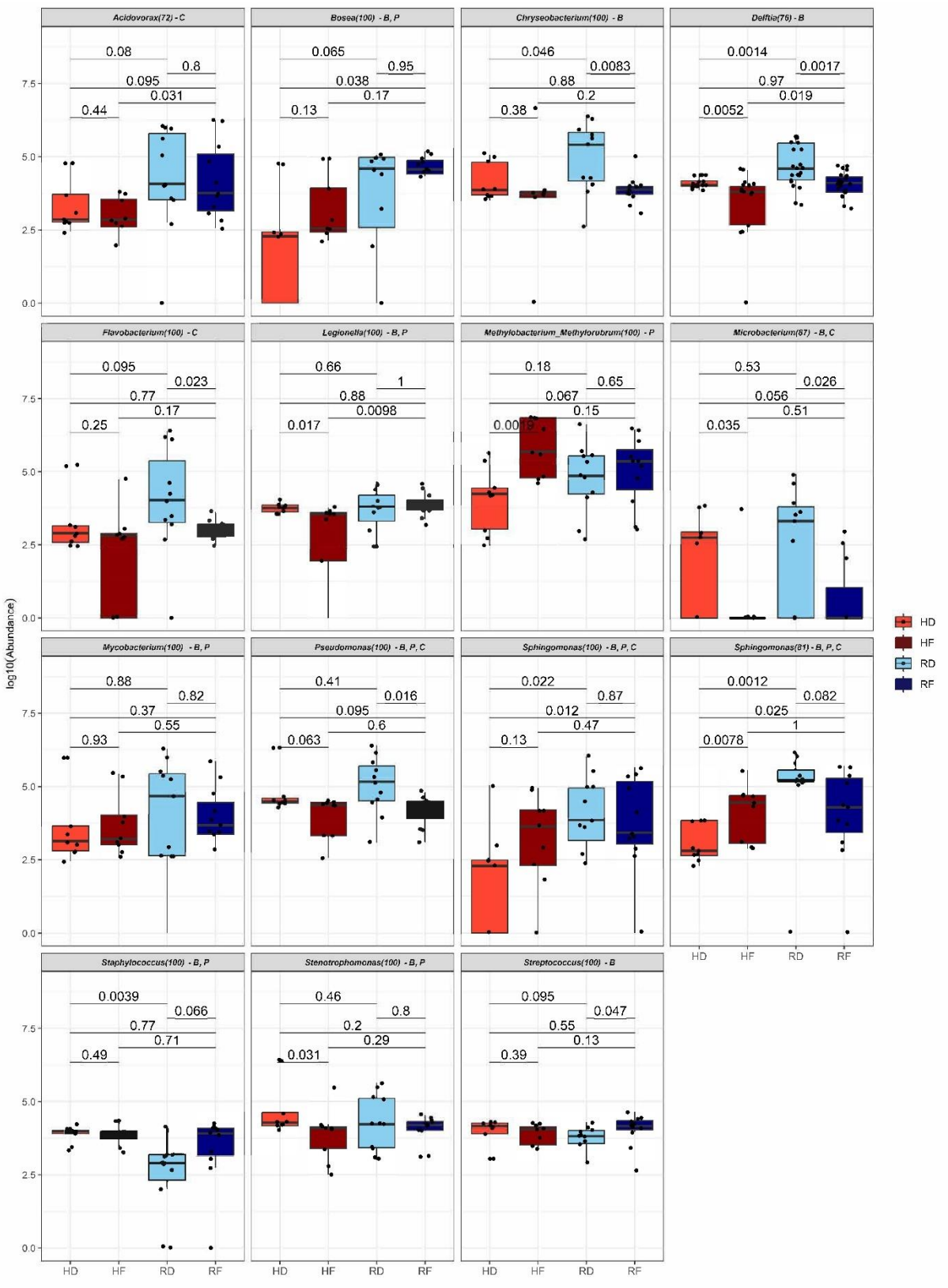


Figure 6.4 - Relative abundance (Log^{10} transformed) of pathogenic, corrosive and biofilm forming bacterial genera (n = 15) that differed significantly between sampling factors.

Sampling factors: hospital faucet (HF: dark red), hospital drain (HD: light red), residential faucet (RF: dark blue) and residential drain (RD: light blue). B = Biofilm forming, P = pathogenic and C = corrosive genera.

6.4.2.1. Potentially pathogenic genera

Bioinformatic analysis identified the presence of 19 potentially pathogenic genera (Table 6.1) (Collier et al., 2021; Falkinham, 2015; Hayward et al., 2022b; Pereira et al., 2017). The relative abundance of each potentially pathogenic genus ranged greatly. From 0.001% for *Campylobacter* and up to 95% for *Methylobacterium-Methylorubrum* (Figure 14.9). Spearman's correlation analysis (ρ) was used to study the co-occurrence of these potentially pathogenic genera (Figure 6.5 and Text 14.1).

6.4.2.2. Corrosive genera

Thirty-eight potentially corrosive bacterial genera were identified in the samples (Table 14.2) (Hernandez-Santana et al., 2022; Kushkevych et al., 2021; Li et al., 2018; Piazza et al., 2019; Rana et al., 2020; Singh et al., 2018). The relative abundance of each potentially corrosive genera ranging from 0.00128% for *Acidovorax* and *Sulfurovum* and to up to 99.896% for *Sphingomonas* (Figure 14.10). Spearman's correlation analysis (ρ) was used to study the co-occurrence of these potentially corrosive genera (Figure 6.6 and Text 14.3).

6.4.2.3. Biofilm forming genera

Twenty bacterial genera with strong biofilm forming capabilities were identified in the samples (Table 14.4) (Khatoon et al., 2018; Maes et al., 2019; Mahapatra et al., 2015). The relative abundance of each biofilm-forming genus ranged greatly from 0.001% for *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* and up to 95.4% for *Sphingomonas* (Figure 14.11). Co-occurrence of these biofilm forming species was determined using Spearman's Correlation (ρ) (Figure 6.7 and Text 14.5).

Table 6.1 - Potentially pathogenic bacterial genera identified in handwashing basin biofilms

Potentially pathogenic genera (Collier et al., 2021; Falkinham, 2015; Hayward et al., 2022b; Pereira et al., 2017)		
Aeromonas	*Enterobacteriaceae*	*Mycoplasma*
Acinetobacter	*Haemophilus**	*Pseudomonas*
*Bacillus**	*Legionella*	*Sphingomonas*
Bosea	*Methylobacterium*	*Staphylococcus**
Campylobacter	*Methylorubrum*	*Stenotrophomonas*
*Coxiella**	*Mycobacteria*	*Vibrio*
	Yersinia	

* Denotes genera that are not typically associated with drinking water.

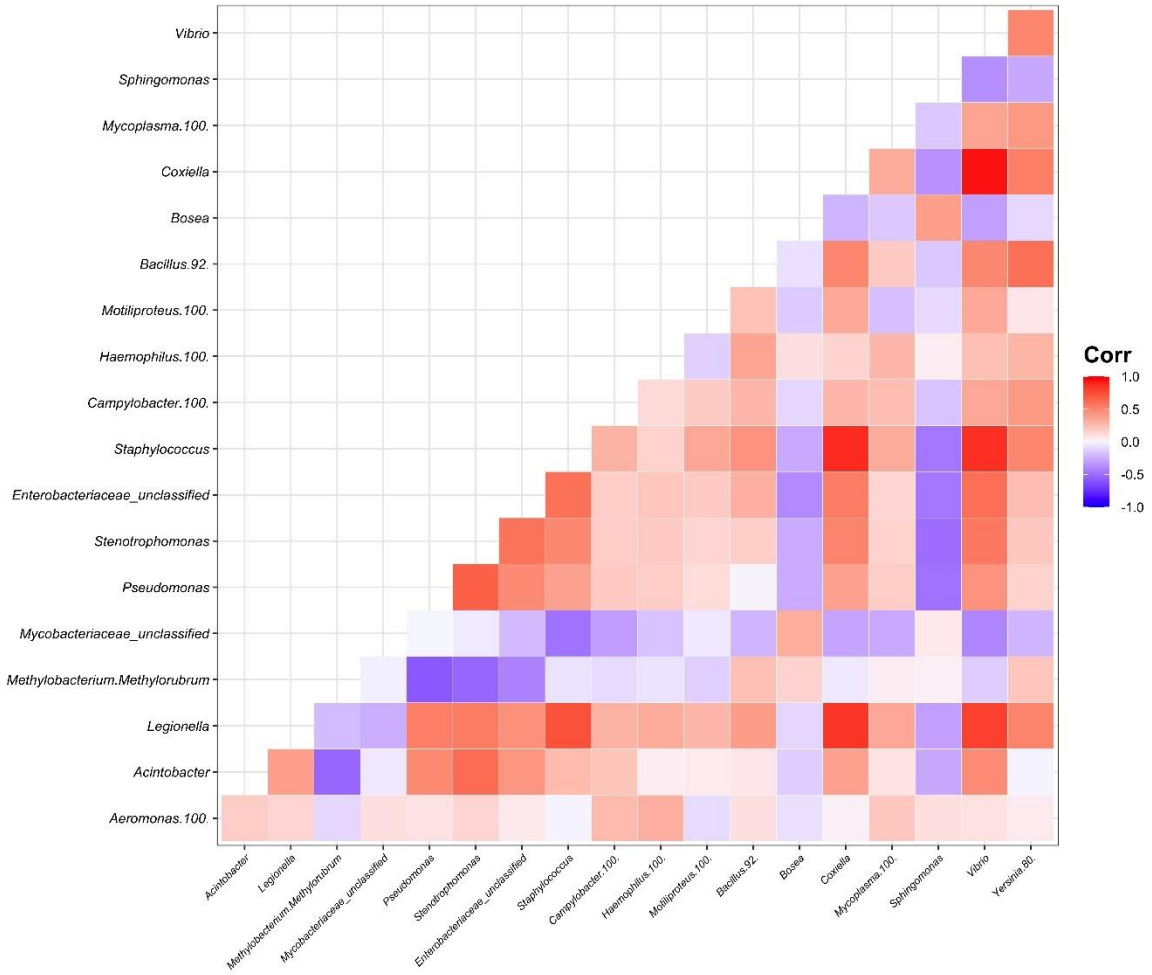


Figure 6.5 - Spearman's correlation analysis of the potentially pathogenic bacterial genera (n = 18) of basin biofilm samples.

The heat map value shows the Spearman's correlation coefficient to a significance threshold of p < 0.05, ranging from −1.0 (blue) to 1.0 (red). A minus value demonstrates a negative association, whereas a positive value demonstrates positive association.

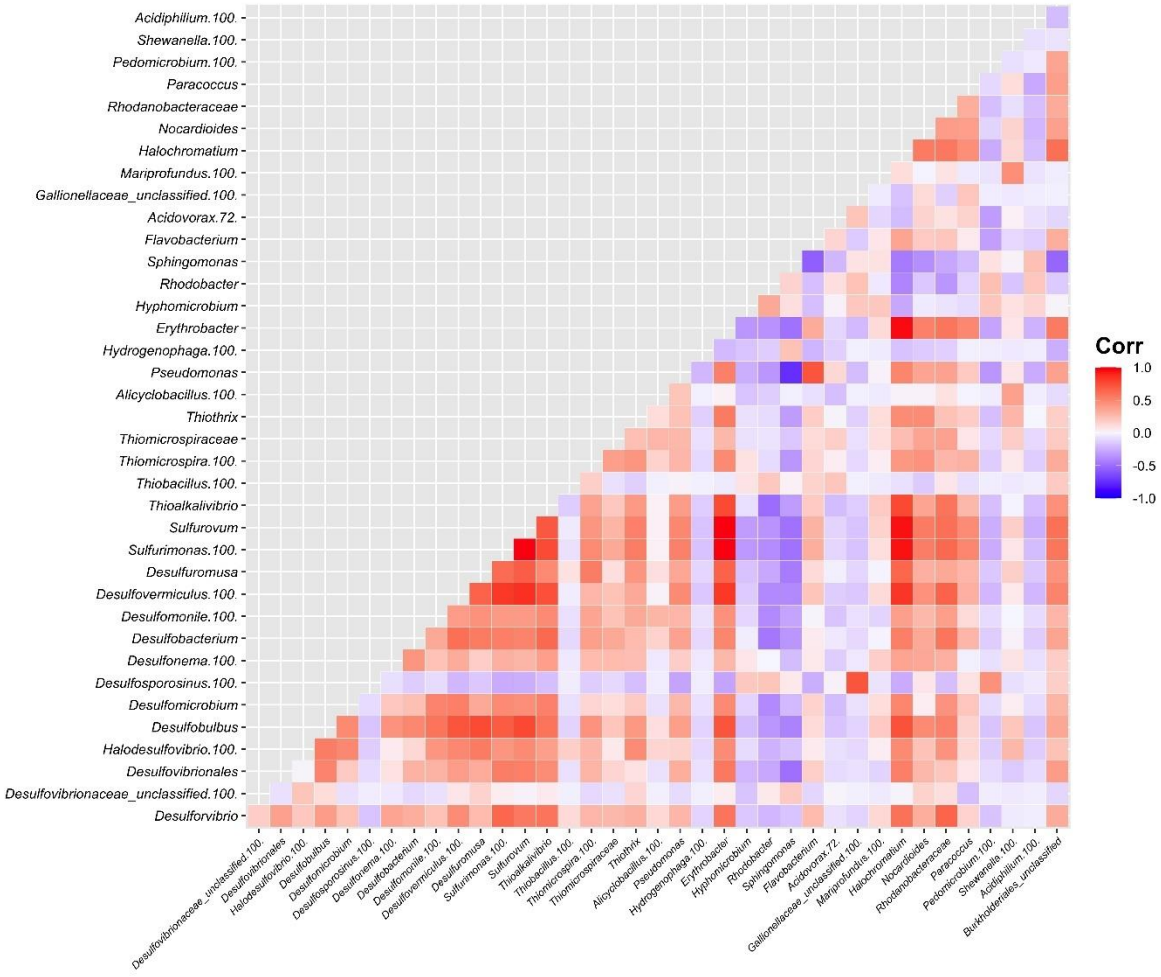


Figure 6.6 - Spearman's correlation analysis of the potentially corrosion related bacterial genera (n = 38) of basin biofilm samples.

The heat map value shows the Spearman's correlation coefficient to a significance threshold of p < 0.05, ranging from −1.0 (blue colour) to 1.0 (red colour). A negative value demonstrates a negative association, and a positive value demonstrates positive association.

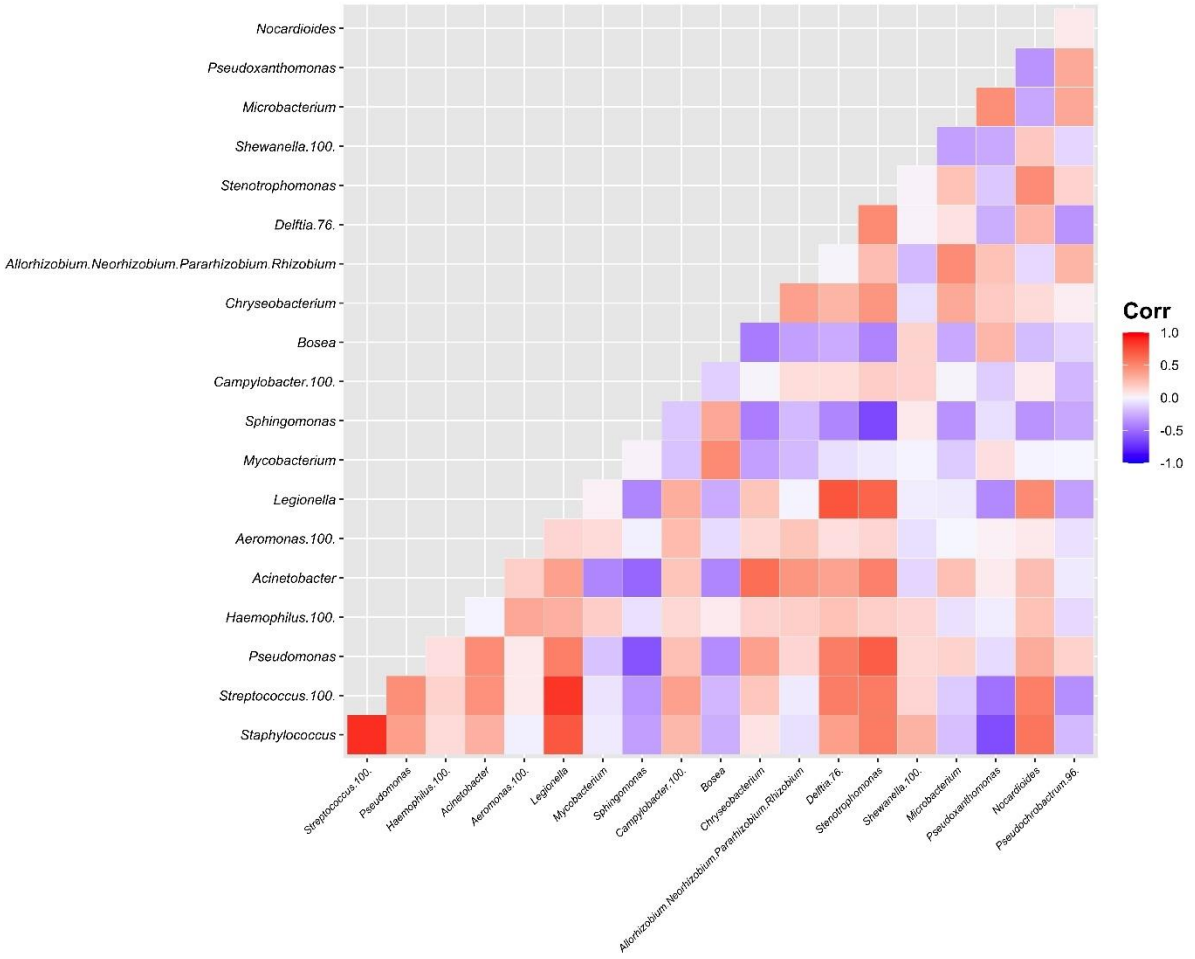


Figure 6.7 - Spearman's correlation analysis of biofilm forming bacterial genera (n = 20) of basin biofilm samples.

The heat map value shows the Spearman's correlation coefficient to a significance threshold of p < 0.05, ranging from −1.0 (blue colour) to 1.0 (red colour). A negative value demonstrates a negative association, and a positive value demonstrates positive association.

6.5. Discussion

6.5.1. Biofilms within the premise plumbing environments

The premise plumbing environment is often considered to have low bacterial diversity due to the low nutrient availability, disinfectant stress and fluctuating temperatures resulting in drinking water being an overlooked source of infection by regulators (Novak Babič et al., 2020). However, bulk water samples may be continuously inoculated by incoming water and previously contaminated downstream networks. Biofilms also provide refuge and protection from environmental conditions for genera that may not survive in planktonic form resulting in the survival and proliferation of diverse communities (Besemer et al., 2012; Douterelo et al., 2019; Huang et al., 2021; Ling et al., 2016; Thom et al., 2022). Recent research has identified the design, physical, and chemical conditions of premise plumbing infrastructure can influence the diversity and relative abundance of taxa within biofilm and bulk water (Huang et al., 2021; Kelly et al., 2014; Nisar et al., 2023b). In this study, the biofilm samples had high relative abundances of genera including *Methylobacterium Methylorubrum, Cycloclasticus, Sphingomonas* and unclassified Rhodobacteraceae that are frequently found in drinking water (Figure 14.2) (Kelly et al., 2014; Nisar et al., 2023b). However, genera such as *Staphylococcus, Cupriavidus, Chryseobacterium* and *Streptococcus* that are not typically associated with drinking water were also identified (Figure 14.2), indicating the point of use contamination plays a role in community development (Avire et al., 2021; Fritz et al., 2014; Mwanza et al., 2022; Vandamme et al., 2004). The presence of these genera, in addition to high relative abundances of unclassified and uncultured taxa, indicate that biofilms formed on point of use devices such as faucets are drains present a unique and understudied environmental niche for functionally complex biofilms (Collier et al., 2021).

6.5.1.1. Pathogenic bacteria of concern

Drinking water as a source of infection is beginning to receive increased attention in the healthcare space (Collier et al., 2021). Opportunistic premise plumbing pathogens (OPPPs) are a group of waterborne microorganisms that are uniquely adept to surviving and growing in drinking water distribution systems (Falkinham et al., 2015). Seven genera of OPPPs were identified in this study including *Stenotrophomonas, Pseudomonas*, an unclassified Mycobacteriaceae, *Methylobacterium-Methylorubrum, Legionella, Acinetobacter* and *Aeromonas* (Figure 14.9). However, due to the large number of taxa identified as uncultured or unclassified, it is unclear whether the relative abundance of OPPPs in the present study is greater than reported. *Legionella* was the only OPPP genera to differ significantly between building types, with a higher relative abundance in residential properties (Figure 6.3). This is attributed to the faucet biofilm communities, where there is a significantly ($p=0.0098$) higher

relative abundance of *Legionella* spp. in residential faucets compared to hospital faucets. Hospitals typically have established water quality risk management teams and protocols that are designed to manage *Legionella* spp. risks in water systems, whereas domestic water systems are typically not managed the same way (Danila et al., 2018). *Pseudomonas* was found in higher relative abundance in drains whereas *Methylobacterium-Methylorubrum* was found higher in faucets (Figure 14.6). In addition to these OPPPs, 12 other potentially pathogenic genera were identified. Seven of these were found in higher abundances in residential properties than hospitals (Figure 6.3). This included Center of Disease Control and Prevention (CDC) and World Health Organisation (WHO) antimicrobial resistant (AMR) pathogens of concern such as *Staphylococcus* and *Enterobacter* (Centers for Disease Control and Prevention, 2019a; World Health Organization, 2020a). AMR threats such as *Staphylococcus* and *Enterobacteriaceae,* and *Acinetobacter* and *Pseudomonas* were found in to be positively correlated in the present study (Figure 6.5). Current infection control guidelines overlook drinking water and water-related devices as a source of these bacteria, focussing instead of dry high touch surfaces and medical devices (Haque et al., 2018). Despite these oversights, the lack of such guidelines for residential properties may be a significant driver in the differences in potentially pathogenic genera abundances between building types. Significant AMR HAI outbreaks of these pathogens have been linked to handwashing basins and showers (Abboud et al., 2016; Chapuis et al., 2016; De Geyter et al., 2017; Feng et al., 2020; Hayanga et al., 1997; Sserwadda et al., 2018). For example, extended spectrum beta-lactamase -producing Enterobacteriaceae and MRSA, have been located in a hospital sink bowl, hospital bathroom sink taps and a hospital bathtub (French et al., 2004; Sexton et al., 2006; Ziwa et al., 2019). Additionally, many of these pathogens have displayed the same characteristics as OPPPs such as biofilm production, disinfectant resistance, and survival in amoeba (Huws et al., 2006; B.-R. Kim et al., 2016; Neopane et al., 2018). To appropriately manage the risk from these pathogens it is important to understand how these key genera interact in premise plumbing biofilms to ensure control and cleaning practices do not inadvertently select for another pathogen. Furthermore, additional studies delving deeper to identify pathogens to the species level is required to appropriately quantify this risk.

6.5.1.2. Corrosive genera of concern

Microbially influenced corrosion (MIC) in premise plumbing is due to corrosive metabolites released by bacteria or the harvesting of electrons from the metal surface, and is exacerbated by water and carbon dioxide (Dou et al., 2021). Corrosion may occur on all types of materials including metals, concrete and plastic pipes . Biofilms can also provide an anerobic niche where sulphate-reducing bacteria are able to proliferate (Gomez-Smith et al.,

2015). Eleven genera of sulphate-reducing bacteria were identified in the present study, found consistently across building types and sample sites (Figure 14.10). Furthermore, MIC creates loose corrosion deposits and corrosion scales which serve as nutrient sources and shelter for further bacterial growth, creating a feedback loop to continue water quality degradation (Prest et al., 2016). Considering such potentially corrosive genera are ubiquitous throughout premise plumbing biofilms and play a pivotal role in biofilm longevity, they may be an effective target for water quality monitoring and treatment (Gomez-Smith et al., 2015). MIC of pipe material is also a costly problem, estimated to cost approx. US $5 million globally each year (Amendola et al., 2022). Understanding their presence in biofilm communities and the role played under changing conditions is essential to determine how these factors can be appropriately manipulated to control bacterial growth.

6.5.2. Community dynamics of biofilms in hospitals and residential properties

Despite all biofilm samples being collected from buildings fed by treated mains water, there was a significant difference in β-diversity between hospital and residential handwashing basin biofilm communities at the genus level (p=0.0415) (Figure 6.1). Due to strict ethical guidelines, it was not possible to collect further data regarding municipal water plant. Furthermore, there was a significant difference in β-diversity of drain biofilm communities between hospitals and residential basins (p=0.0338) but not between faucets (p=0.186) (Figure 6.1). Variations in usage, cleaning and/or design between building types may be responsible for the distinct unique communities observed (Figure 6.8). However, due to the opportunistic nature of the sampling performed in the present study, these details could not be gathered.

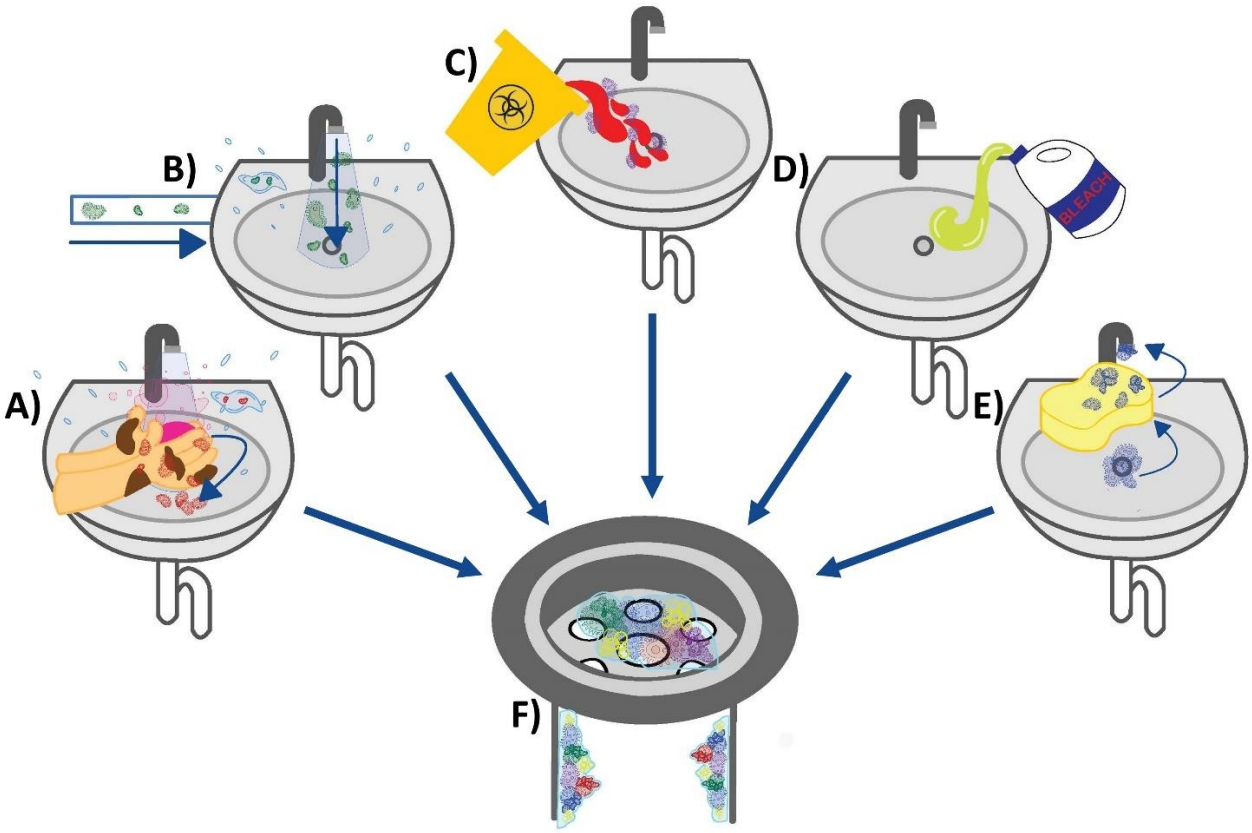


Figure 6.8 - Proposed mechanisms resulting in handwashing basin drain contamination and biofilm formation.

Solid arrows indicate direction of microbial contamination. (A) Washing of soiled hands resulting in transfer of human and environmental flora (microbes shown in red) from hands to drain, and splashing of contaminated water to the surrounding environment. (B) Feed water contaminated with opportunistic premise plumbing pathogens (shown in green) and water flow hitting the contaminated drain located directly below the faucet causing splashing to surrounding environments and retrograde contamination of the faucet. (C) Inappropriate disposal of human waste contaminated with pathogenic bacteria (shown in purple). (D) Treatment of drains with disinfectant chemicals and residual antimicrobial medicines creating a selective environment for antimicrobial resistant populations. (E) Improper cleaning protocols resulting in the spread of microorganisms (shown in blue) from one environmental niche to another (i.e. drain to faucet shown). (F) Resultant diverse drain biofilm community harbouring environmental (red and blue), waterborne (green) and pathogenic (purple) bacterial taxa.

6.5.2.1. Basin usage

Although designated 'handwashing basins', these devices are often used for purposes outside the scope of their intended design. The activities conducted prior to, or in addition to washing hands, may differ significantly between building types. For example, private property owners may tend to their garden or clean soiled clothes in a basin, activities not conducted in healthcare species (Figure 6.8). In this study, SIMPER analysis identified *Qipengyuania* and *Sphingobium*, genera commonly found in soils, were key taxa driving the difference between buildings and were found at higher relative abundances in residential properties. Conversely, healthcare basins may be used to discard unused drugs or to dispose of infected human waste (Franco et al., 2020). An outbreak of carbapenem-resistant *Enterobacteriaceae* in a Korean cardiology ward was linked to water dispensers and sink drains (Jung et al., 2020). Extensive investigation found that disposal on contaminated dialysis fluid and human waste into a handwashing basin followed by subsequent aerosolisation was the direct cause of the outbreak (Jung et al., 2020) (Figure 6.8). Pathogenic genera including *Methylobacterium-Methylorubrum* and *Staphylococcus* were found in greater relative abundance in hospital biofilms (Figure 6.3). The difference in *Methylobacterium- Methylorubrum* relative abundance was driven primarily by the faucet biofilm, whereas the difference in *Staphylococcus* relative abundance was driven by the drain (Figure 6.4). These activities are unique to the building type and influence the communities developed in the basin, yet such nuance is overlooked in water quality and maintenance guidelines.

6.5.2.2. Cleaning

HAIs are receiving increased attention in the wake of the COVID-19 pandemic, with the WHO highlighting gaps in infection control and prevention guidelines as an area that requires rapid strengthening (Gilbert et al., 2022). Although waterborne pathogens, particularly AMR species such as *P. aeruginosa* and *A. baumannii* have been identified as leading causes of severe HAIs, environmental disinfection and water related device remediation guidelines remain inconsistent and often ineffective (Collier et al., 2021; Hayward et al., 2022a). The CDC recommends cleaning the faucet and drain, as well as nearby surfaces at least daily with disinfectant (Centers for Disease Control and Prevention, 2024a). The Australian Guidelines for the Prevention and Control of Infection in Healthcare recommends cleaning handwashing basins up to twice a day with detergent along with other high touch surfaces such as light switches, doorknobs and bedrails with the same frequency (National Health and Medical Research Council, 2019). If a handwashing basin is wiped with a cloth that was previously used to wipe a doorknob, rather than using separate cloths, it may result in the inoculation of the basin biofilm with genera commonly found on high touch

surfaces and vice versa. Similarly, if the same cloth is used to wipe the basin faucet and then the drain, or vice versa, this will cause transfer of the biofilm between niches resulting in similar communities. For example, methicillin resistant *S. aureus* is one of the leading causative agents of HAI and transmission is often associated with colonised hands and contaminated dry environmental surfaces (Hardy et al., 2006). However, in this study *Staphylococcus* was found in high relative abundance in hospital biofilm samples, particularly in hospital drains (Figure 14.6). This supports previous studies that have identified water as the source of diverse AMR HAI outbreaks (Hayanga et al., 1997; Layton et al., 1993; Squeri et al., 2012). The hospital handbasin samples in the present study were collected from patient ensuites, staff bathrooms and communal hallways presenting different usages, however, the faucet and drain communities compositions remained similar. The consistent cleaning of faucets and drains in hospital environments may be responsible for the consistent diversity between across faucet and drain biofilms samples in the present study (Figure 6.8).

Conversely, cleaning of handwashing basins in private properties is up to the discretion of the property occupier and has the potential to remain entirely overlooked. Additionally, the cleaning chemicals used, if any, are also dependant on occupier choice. All residential biofilm samples in the present study were collected from bathroom handbasins, yet the faucet and drain communities diverged. Many disinfectants are not appropriate for hydrated biofilm environments such as handwashing basins, and if a disinfectant is not prepared correctly, used at a subinhibitory concentration, or past its use-by-date, it may select for a resistant population (Lineback et al., 2018; J.-Y. Maillard et al., 2023) (Figure 6.8). Residential properties were found to have significantly higher relative abundances of seven potentially pathogenic or corrosive genera (Figure 6.3). Furthermore, drains were highlighted as a hot spot for harbouring high relative abundances of *Pseudomonas*, *Stenotrophomonas* and *Streptococcus* (Figure 6.4). Resistance to commonly used chlorine disinfectants such as bleach can select for both disinfectant and antibiotic resistant strains (Nordholt et al., 2021; Tong et al., 2021). Environmental isolates typically present higher minimum inhibitor concentrations to quaternary ammonium compound based disinfectants when compared to clinical and laboratory strains (Nordholt et al., 2021). This indicates that there are currently unknown external conditions that favour highly resistant isolates. Although antimicrobial stewardship programs have been implemented to curb this AMR rise, they are inconsistent regarding environmental cleaning and overlook the different external conditions presented by a residential building.

6.5.2.3. Basin design

Handwashing basins present multiple routes of HAI transmission including aerosolization from the faucet, splashing off the drain or direct contact with contaminated water (Hayward et al., 2022a; Kotay et al., 2019) (Figure 6.8). The aerosols and droplets generated can contaminate surrounding surfaces and neighbouring basins, a mechanism that has been linked to numerous HAI outbreaks (Abboud et al., 2016; Chapuis et al., 2016; De Geyter et al., 2017; Jung et al., 2020; Qiao et al., 2020) (Figure 6.8). This is an area needing future research to ensure that evidence-based changes to design and best practice are taken into account. For example, some healthcare facilities are now utilising offset drains to avoid water hitting the drain directly causing splash back and styles that are efficient and easy to clean (Pirzadian et al., 2022). However, offset drain designs can cause pooling of water in the bottom of the basin if not installed level, resulting in additional cleaning to remove residual water (Kotay et al., 2019). These considerations also need to be applied in the residential space to consider the increase in healthcare taking place within the community. Currently, design considerations such as reducing aerosol production, optimising flow rate to reduce stagnation and ease of cleaning are not as prescriptive in residential properties compared to healthcare (Weinbren et al., 2021b). Aesthetic choices such as under mounted basins, water saving low flow faucets and linear trench style drains may be prioritised without considering how these designs will potentially facilitate biofilm growth (Hayward et al., 2022b; Kotay et al., 2019). Not only were residential properties found that have significantly higher relative abundances of key pathogenic and corrosive genera, but residential basin drains were also identified as key niches for *Chryseobacterium, Delftia, Microbacterium, Pseudomonas* and *Stenotrophomonas* (Figure 6.3 and Figure 6.4). Research into the influence of product design on biofilm growth and dispersal is essential before effective disinfection guidelines and infection control protocols for vulnerable residents can be developed.

6.6. Conclusion

There are limited studies investigating bacterial biofilm communities in point of use devices such as handwashing basins, and how building type, product design and disinfection/cleaning methods alter the public health risk. The present study demonstrated that handwashing basin biofilm communities differ significantly between hospital and residential buildings. The biofilm communities were diverse, including potentially pathogenic genera including *Pseudomonas, Legionella, Stenotrophomomas,* and *Acinetobacter* as well as those not typically considered waterborne such as *Staphylococcus* were found in high relative abundance. There was no significant difference in hospital faucet and drain biofilm communities whereas residential handwashing basins serve as an overlooked niche for key

potentially pathogenic and corrosive genera. The driving factors contributing to the similarity between hospital biofilms and diversity between residential biofilms are unclear. This study demonstrates the need for more research to understand how these functionally complex biofilms form on these surfaces and what role they play in disseminating antimicrobial resistance and infection. Complementary studies delving deeper to identify specific pathogenic species in these potentially high risk niches is required. As our population risk factors increase and healthcare at home services are promoted, infection risks presented by residential water sources must be understood to inform lagging infection control guidelines.

6.7. Acknowledgements

C.H., K.E.R, M.H.B., S.L., and H.W. conceived and designed the experiments. C.H. performed the experiments. T.J. and M.A.N assisted with data analysis. C.H. and H.W. drafted and edited the manuscript. K.E.R, M.H.B, M.A.N, R.B., J.H., T.J., S.L. and H.W corrected and contributed to the manuscript. All authors approved the final manuscript.

6.8. Funding statement

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6.9. Conflict of Interest

Jason Hinds (JH) works for Enware, a plumbing manufacturing company. Richard Bentham (RB) works for Built Water Solutions, a water consultancy company. These could be perceived conflicts of interest. JH and R.B. were not involved in the study design or data analysis. All other authors have no conflicts to declare.

7. WATER FLOW DYNAMICS AND THE GENERATION OF BIOAEROSOLS IN HEALTHCARE SETTINGS

In this chapter, the role of water flow rates in facilitating the transmission pathways of OPPPs via bioaerosol production was investigated. This chapter address Objective 5 and includes one manuscript currently under review which used a laboratory model to simulate real-world handwashing basin conditions and quantify bioaerosol generation from source water and contaminated drains. This research revealed that water flow rate significantly influenced the production of bioaerosols, with lower flow rates generating higher levels aerosols from the drain and retrograde contamination of faucet outlets. These results underscored the importance of flow rate as a key factor in OPPP transmission and highlighted the potential risks posed by poorly managed handwashing basins in both healthcare and residential settings. This chapter advocates for the consideration of flow rate in plumbing design and infection control guidelines to reduce the risk of bioaerosol production and pathogen transmission.

CITATION: The impact of water flow rates on bioaerosol production from handwashing basins

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Key words: Healthcare-associated infections, aerosol generation, handwashing basins, product design, flow rate, retrograde contamination, infection control

7.1. Abstract

Bioaerosols within the built environment pose a potential risk to public health. In healthcare settings, aerosols generated from handwashing basins have been receiving increasing attention due to their role in healthcare-associated infection outbreaks. Currently, there is limited knowledge regarding the impact of hand basin design parameters on bioaerosol production and transmission routes to patients. This study used a model hand basin system to investigate the effect of different flow rate restrictors on the number, size and distribution of bioaerosols produced. Source water was spiked with *Escherichia coli* and the hand basin drain seeded with *Staphylococcus epidermidis* as indicator bacteria to facilitate tracking of the bioaerosol source. All three flow restrictors utilised generated bioaerosols of respirable size with no significant difference in the number of aerosols and droplets from the source water across different flow rates. However, lower flow rates led to a higher generation of aerosols from the drain and increased retrograde contamination from the drain to faucet compared to higher flow rates. These findings suggest that modifying design elements, such as aerator design and flow rates, could potentially reduce infection risks, but the optimal configuration remains unclear. This highlights the need for future collaborative research between product designers, engineers and microbiologists to inform optimum design and ensure functional needs are met whilst protecting occupants in healthcare and other high health-risk built environments.

7.2. Highlights

- Aerosols from handwashing basins can cause healthcare-associated infections.
- Lower flow rates increase aerosols and retrograde contamination.
- Findings stress need for research on design to reduce infection risks.

7.3. Introduction

Healthcare-associated infections (HAIs) are a serious public health threat resulting in increased morbidity and mortality, increased length of hospital stays and excess health costs (Russo et al., 2019). This is further exacerbated by the increasing number of antimicrobial resistant infections (Collier et al., 2021; World Health Organization, 2015). The built environment and building design play a critical role in preventing the transmission of HAIs (Zimring et al., 2013). Specifically, handwashing basins have recently been identified as an environmental niche for antimicrobial resistant HAIs microbes (Hayward et al., 2020). Microbes present in the water and on patient hands colonise the faucets and drains of hand basins and form biofilms, a community of microbes embedded in a slimy matrix, these have then been linked to numerous antimicrobial resistant HAI outbreaks (French et al., 2004;

Hota et al., 2009; Jung et al., 2020; Kanamori et al., 2016; Knoester et al., 2014; Lv et al., 2019; Perkins et al., 2019; Takajo et al., 2020). Hand basins are ubiquitous throughout healthcare facilities in patient care rooms, near sterile medical preparation areas, and by nurses' stations (Weinbren et al., 2021a). However, despite ongoing efforts to improve infection control and prevention guidelines there is limited understanding of how the disease-causing microbes from a contaminated hand basin are transmitted to patients, resulting in outbreaks (Benoit et al., 2021; Park et al., 2013; Pirzadian et al., 2022).

A patient may be exposed to contaminated water by direct skin or wound contact during handwashing, by ingestion, or by inhalation of aerosols contaminated with microbes (known as bioaerosols) (Decker et al., 2014). These aerosols may be generated as the water flows through the faucet aerator or when the water stream impacts the basin bowl, causing larger droplets to spread to surrounding surfaces (Kotay et al., 2019). Small aerosols (<5 µm) may linger in the air for hours, exposing many to the risk of inhalation. Larger droplets (>5 µm) settle out of the air quickly and are therefore not typically associated with airborne transmission (Kotay et al., 2019). However, previous research has suggested that these droplets can still travel up to one metre from the handwashing basin and contaminate surrounding surfaces (Hota et al., 2009).

Currently there is limited knowledge on the impact of different design factors on bioaerosol production. This include factors like: aerator design, hydraulic flow, drain location and design, faucet height, location and design, basin bowl design; and even the surrounding environment may impact the sizes and extent of bioaerosol dispersion (Benoit et al., 2021; Succar et al., 2023). Current infection prevention guidelines have begun to recommend specific design elements, such as offset drains and low-flow aerators, to reduce splashing and aerosolization, and to minimise water consumption (2022; National Health Service England, 2013a, 2013b; National Health Sustainability Office, 2020). However, these recommendations are often based on limited or inconclusive evidence, making it difficult to determine the most effective design and operational strategies.

The aim of this study was to investigate the effect that the faucet flow rate has on aerosol generation. A hand basin model, utilising indicator bacteria, was developed and used in this study to quantify the differences in aerosolization and droplet dispersion originating from either the source water or a contaminated drain under three faucet flow rate conditions. By examining the interaction between flow rates, product design, and aerosolization, this research aims to provide valuable insights into optimizing basin designs to minimize the risk of HAIs, therefore improving patient protection in healthcare and other high-risk environments. The finding from this research will inform future investigations into more

complex design variables and interactions between different design factors to ensure that hand basins and bathrooms are designed to both be functional but also to protect patients from bioaerosols.

7.4. Materials and Methods

7.4.1. Experimental set-up

A laboratory model hand basin was designed to evaluate aerosol and droplet generation from hand basins utilising different flow rate restrictors in the faucet (Figure 7.1). A 60 L tank (Behroplast Nv. 12060) was connected to the inlet of a pump (Pump master Australia JSL 60-101) using 15 mm x 700 mm flexible stainless steel braided water pipes with PEX inner tubes. A multi-prong brass manifold and 15 mm x 700 mm flexible stainless steel braided water pipes were connected to the outlet of the pump to deliver water to the model hand basin. The hand basin used (Estilo EST0233) had a width of 480 mm, depth of 370 mm and height of 130 mm with a flick mixer tap (Dorf, 5215.045A) (Figure 7.2). The basin drain was a free-flowing design and 45 mm in diameter, positioned 160 mm below the tap faucet. The basin drain was connected to a 40 mm plastic combination S and P trap that drained into a sealed 60 L tank (Behroplast Nv. 12060).

Air sampling was conducted using a 6-stage Anderson Cascade Impactor (The Staplex Company: Brooklyn, NY, USA). The Anderson Sampler was placed 1500 mm above the ground and 450 mm from the hand basin faucet to simulate ‘head height’ of a user (Figure 7.1).

Hand basin model experiments were conducted in a Physical containment (PC2) laboratory. Temperature and relative humidity were monitored during experiments using a HOBO MX1101 data logger. Potable water was collected from a building distribution system and used as the source water. This source water was then spiked with target bacteria *Escherichia coli* (ATCC 700891) at a final concentration of $1x10^7$ CFU/mL. The hand basin drain was then inoculated with 1 mL of $1x10^{10}$ CFU/mL *Staphylococcus epidermidis* suspension around the exterior of the drain and 0.5 mL was applied to the surface of the drain using a sterile pipette.

For each flow rate restrictor, 60 L of water was run through the model hand basin resulting in a sampling time of 30 min (2 L/min), 10 min (6 L/min), and 6.7 min (9 L/min) with an additional hour to collect residual aerosols. Non-inoculated source water and drains were used as negative controls to monitor for any background microbial presence.

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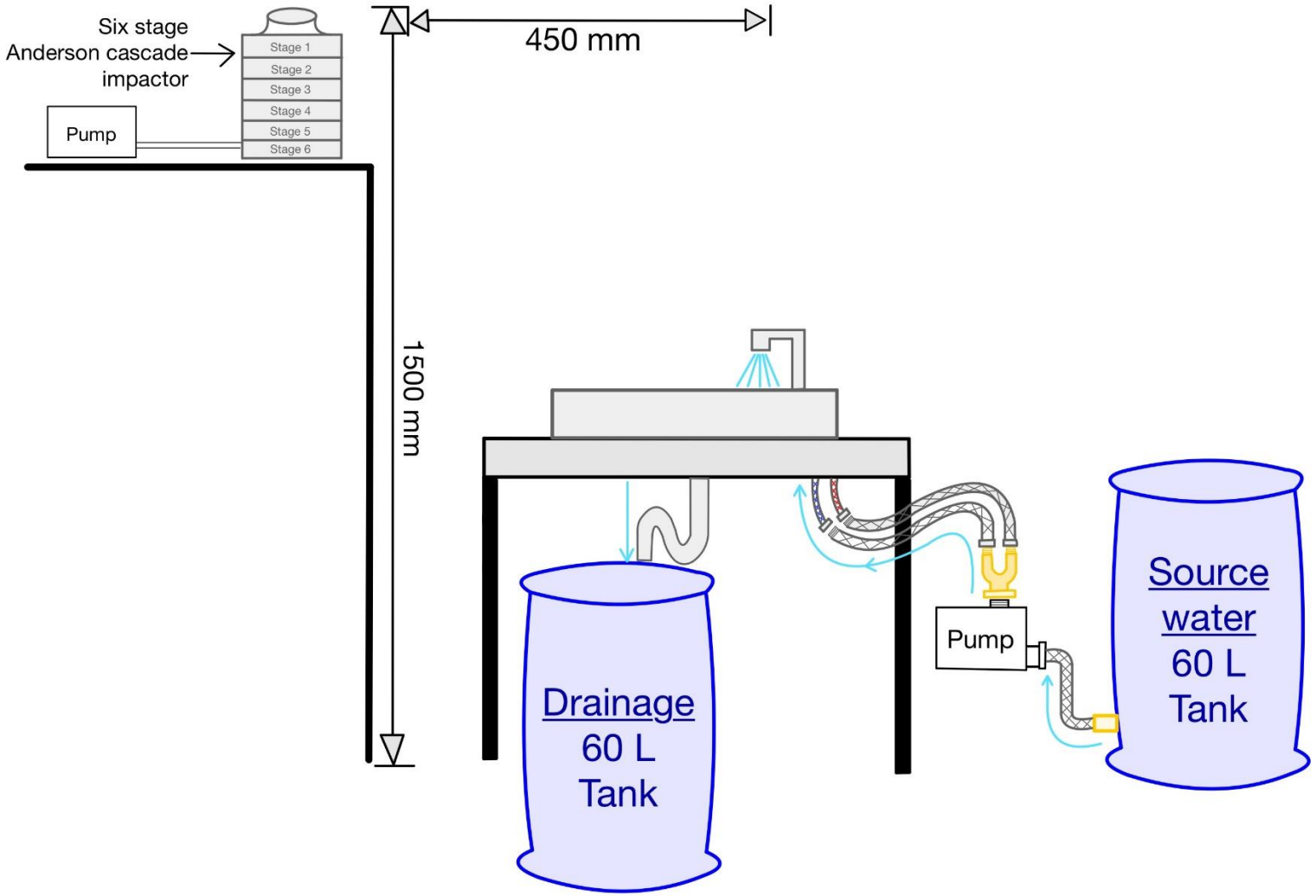

Figure 7.1 - Experimental set-up for the evaluation of restrictor flow rate on aerosol and droplet production. Water flow direction is represented with blue arrows from the source water tank, through the pump and hand basin to the drainage tank. The six stage Anderson Cascade Impactor is shown 1500 mm above the floor and 450 mm from the hand basin faucet.

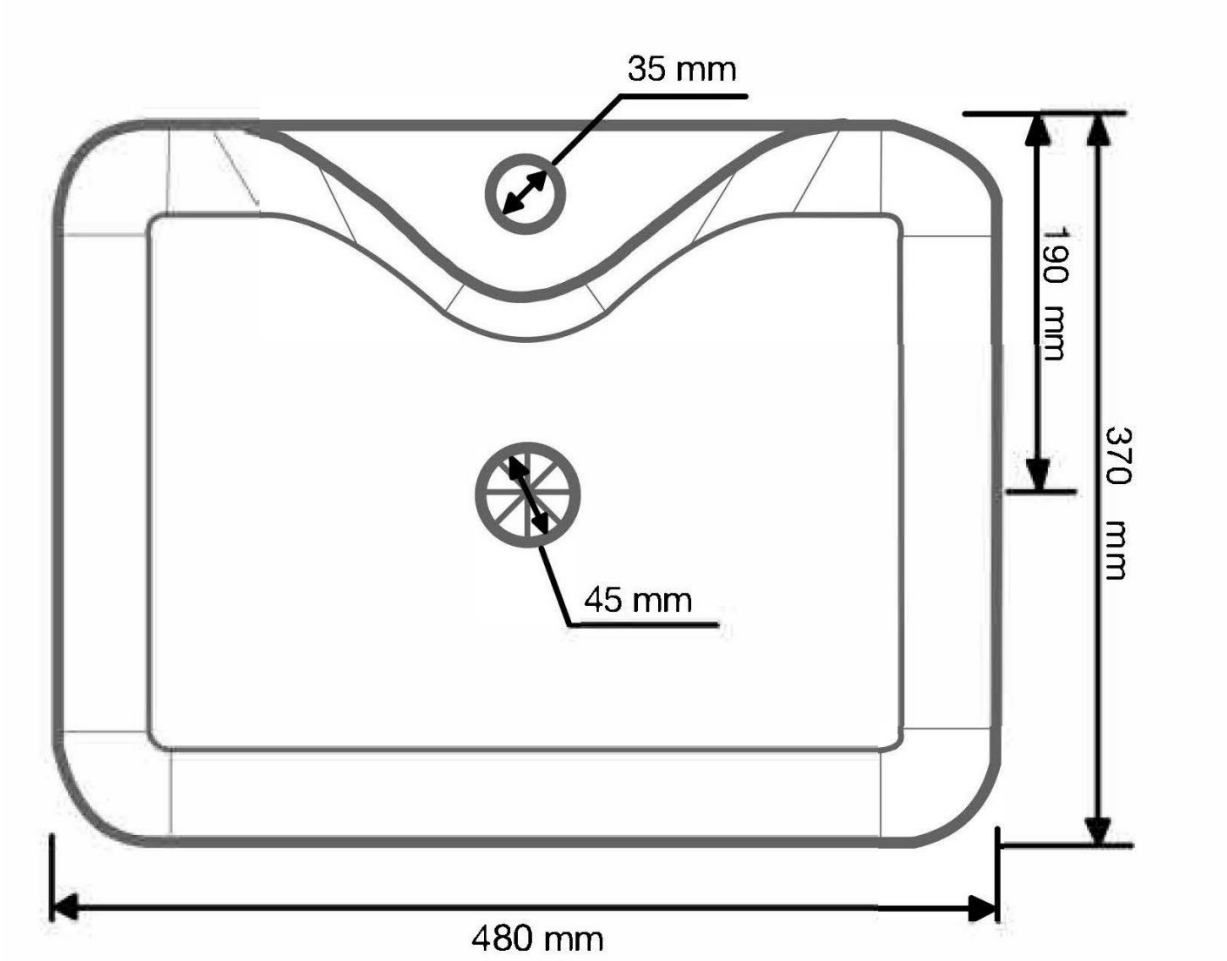

Figure 7.2 - Schematic diagram of the model hand basin showing key dimensions. The basin had an overall width of 480 mm and a depth of 370 mm. The tap hole diameter was 35 mm, and the drain hole diameter was 45 mm, positioned centrally within the basin and 190 mm from the rear of the basin

7.4.2. Enumeration of respirable bioaerosols

The produced bioaerosols were enumerated using a 6-stage Anderson Cascade Impactor and bacterial growth on MacConkey agar plates (The Staplex Company: Brooklyn, NY, USA). Agar plates were incubated at 37 +/- 1 ^{o}C for 24 +/- 1 hours and *S. epidermidis* and *E. coli* colonies were identified based on selective morphology on MacConkey agar. The number of CFU of each target organism were counted and recorded as positive hole corrected (Lidwell et al., 1965; Macher, 1989). The positive hole corrected counts for each of the six stages were analysed individually as well as adding the six stages together for total aerosol analysis

Faucet flow restrictors were selected based on flow rate. Three flow restrictors were selected to represent low (2 L/min), medium (6 L/min) and high (9 L/min) flow rates (Figure 7.3). The testing order was randomised for the three evaluated flow restrictors. All flow restrictors were tested in triplicate.

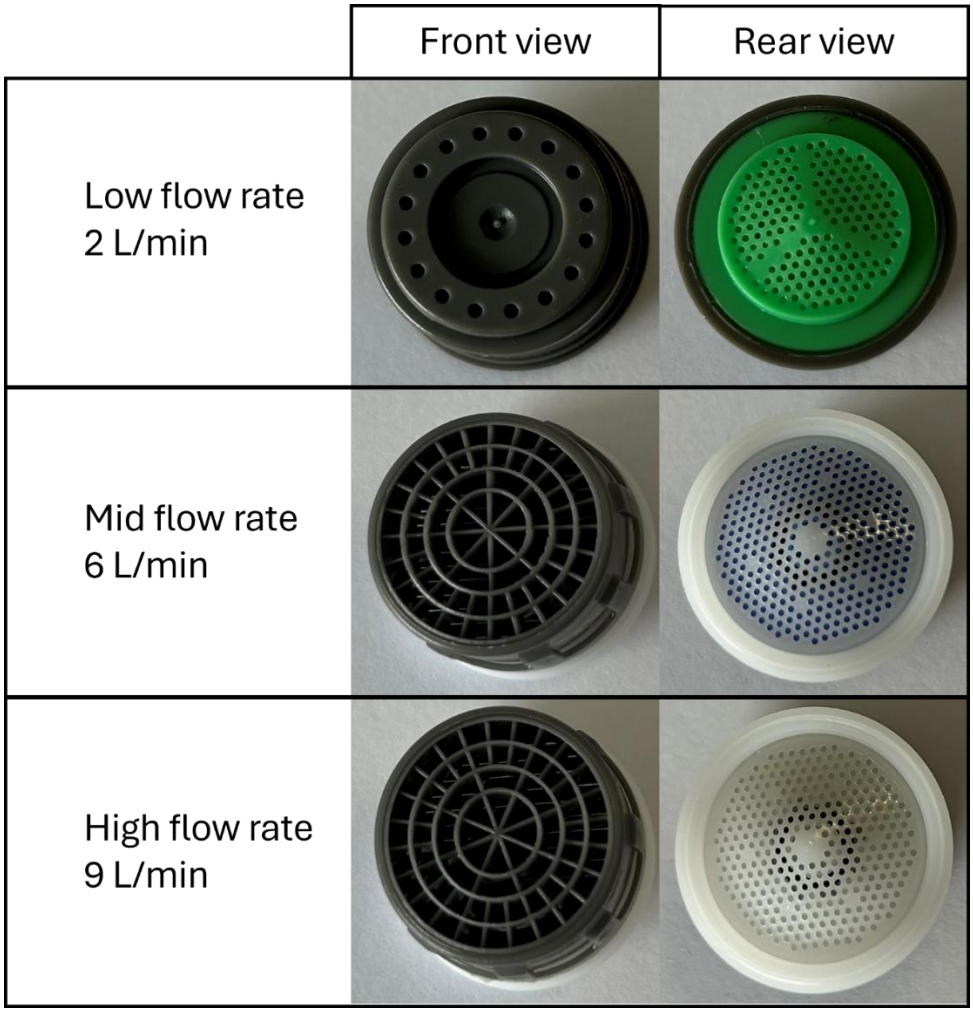

Figure 7.3 - Images of tap flow restrictors used in the hand basin experiment. The flow restrictors differ in design and flow rate, affecting water flow and aerosol generation. The front and rear of each flow restrictor is shown to demonstrate the size and arrangement of holes.

7.4.3. Surface contamination from droplets

MacConkey agar settle plates (CM0007B) (ThermoFisher Scientific: Adelaide, Australia) were used to capture droplet dispersion from the model hand basin. A fixed layout of 50 settle plates was used around the model hand basin (Figure 15.1). The hand basin and counter space was thoroughly disinfected with 70% ethanol between each experiment. The lids of the settle plates were removed for the duration of the experiment. Once the sampling event had finished, the settle plate lids were replaced and incubated at 37 +/- 1^{o}C for 24 +/- 1 hours. *S. epidermidis* and *E. coli* colonies on each plated were identified and enumerated based on selective morphology and the total number of colonies were combined, to be reported as total counts per experiment.

7.4.4. Retrograde contamination

To investigate potential back splash from the hand basin drain to the faucet, the presence of *S. epidermidis* (which was used to contaminate the drain) on the flow restrictor was ascertained. Briefly, following the experiment the flow restrictor was removed and placed in a sterile 50 mL tube containing 10 mL of sterile PBS, followed by 5 min of vortexing (SEM® Vor Mix). One hundred microliters of the mix was plated on MacConkey agar and incubated at 37 +/- 1^{o}C for 24 +/- 1 hours. *S. epidermidis* and *E. coli* colonies were identified and enumerated based on selective morphology.

7.4.5. Statistical analysis

All statistical analyses were performed using SPSS (Version 28.0.1.1). Data were expressed as mean ± standard deviation (SD). Normality of data was assessed using the Shapiro-Wilk test. If data were not normally distributed, log transformation was applied. Comparisons between groups were made using one and two-way ANOVAs and Kruskal-Wallis tests. Tukey's post hoc tests were conducted when significant differences were detected. Statistical significance was set at $p < 0.05$. Adjustments for multiple comparisons were made using Bonferroni correction.

7.5. Results

7.6. Retrograde contamination from the drain to the flow restrictor

Flow restrictors were removed after each experiment and assessed for the presence of *S. epidermidis*, indicating retrograde contamination from the drain to the flow restrictor and faucet. All restrictors showed retrograde contamination, with a significant difference seen between flow rates (p=0.038) (Figure 7.4). As the flow rate increased from 2 L to 9 L/min there was a significant decrease in contamination (p=0.033). However, there was no

significant difference between 2 L and 6 L/min (p=0.423) or between 6 L and 9 L/min (p=0.853).

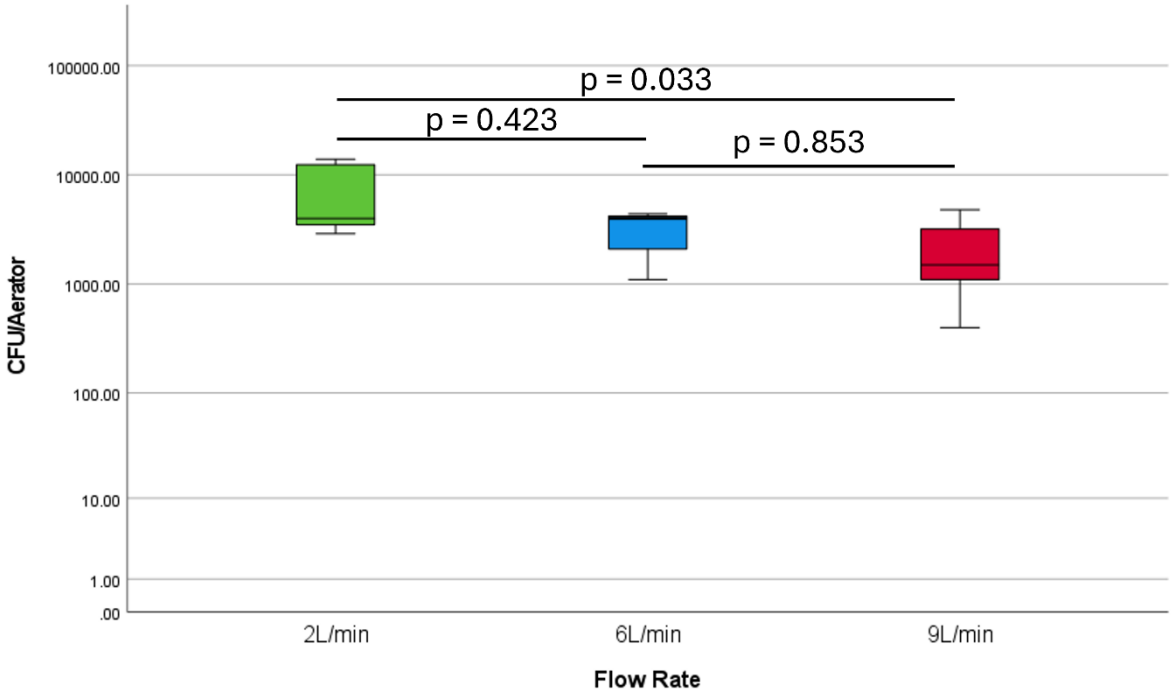

Figure 7.4 - Mean *S. epidermidis* colony forming units (CFU) detected on tap flow restrictors (n = 3 per treatment). This resulted from retrograde contamination from the spiked hand basin drain performed at different flow rates (green, 2 L/min; blue, 6 L/min; and red, 9 L/min). Statistically significant differences between flow rates considered at p = <0.05.

7.6.1. Respirable bioaerosols

All three flow restrictors generated bioaerosols that were of respirable size (0.6 µm to 10 µm) from both the source water and the contaminated drain. The number of respirable bioaerosols generated from the source water or from the drain with the different restrictor flow rates is shown in Figure 7.5. From the source water, there is a slight trend that as flow rate increased there was a decrease in collected aerosols, however, this relationship was not statistically significant (p=0.629) (Figure 7.5). Similarly, as the flow rate increased from 2 L to 9 L/min, there was a decrease in aerosol production from the drain. However, unlike the source water experiments this trend was statistically significant (p=0.021).

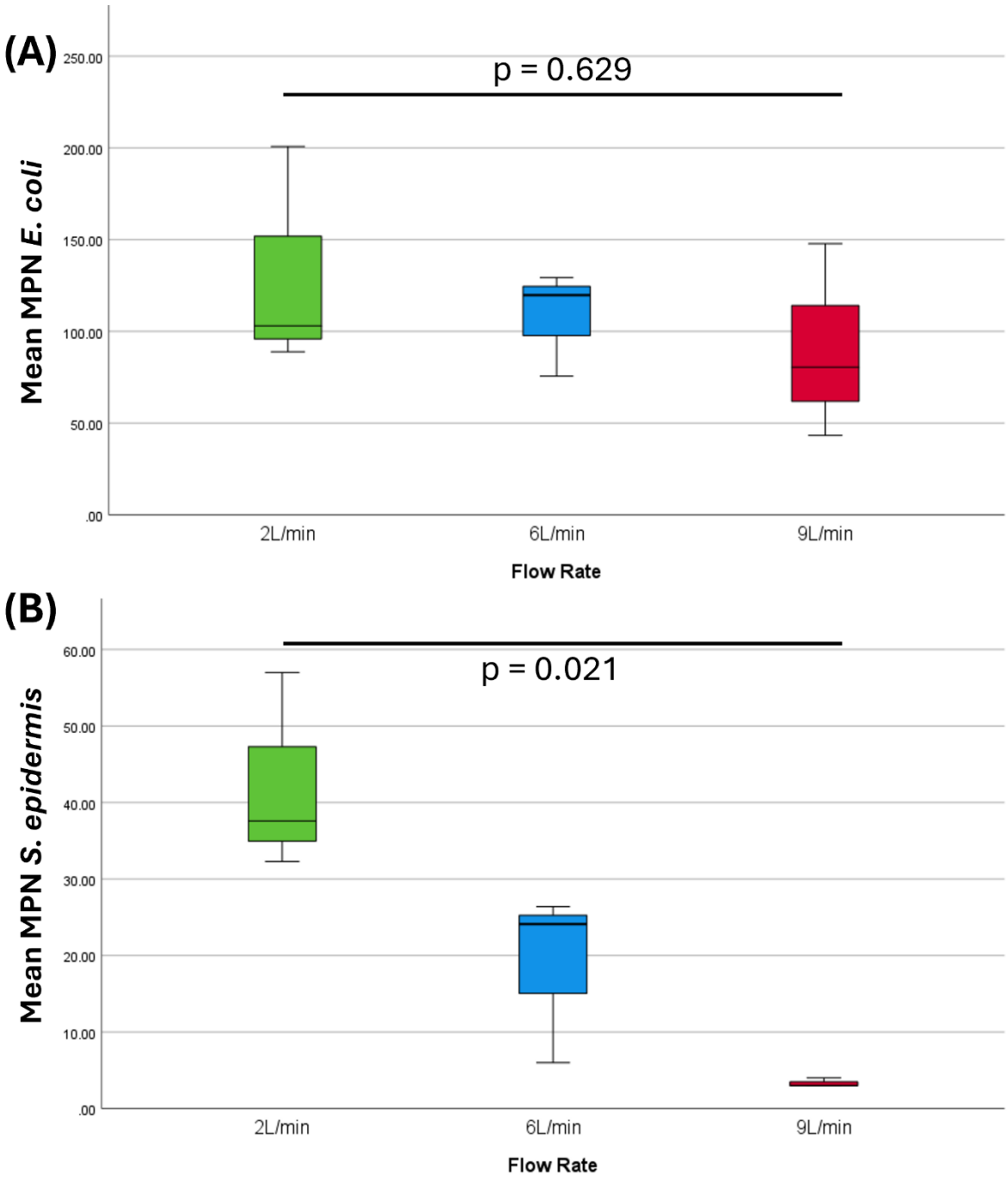

Figure 7.5 - Mean number of positive hole-corrected colonies (MPN) generated from (A) source water spiked with *E. coli* and (B) drain spiked with *S. epidermidis* at different flow rates (n = 3 per flow rate) (green, 2 L/min; blue, 6 L/min; and red, 9 L/min) using a model hand basin. Concentrations measured using 6-stage Anderson Cascade Impactor. Statistically significant differences between flow rates considered at p = <0.05.

Furthermore, there was no significant difference (p= 0.255) between flow rates when comparing the size distribution of the bioaerosols from the source water (Figure 7.6A). For all flow rates, the largest number of bioaerosols were collected at the third stage (3.3-4.7 µm), and all flow rates generated bioaerosols collected at stages 5 and 6 (2.1-0.65 µm diameter).

When the drain was the source of contamination, there was a significant difference between flow rates and the size distribution of the bioaerosols produced (Figure 7.6B). Specifically, there were significantly more aerosols collected in stage 4 of the impactor (2.1-3.3 µm) at 2 L/min than 9 L/min (p=0.009). At 2 L and 6L/min, the highest number of aerosols were collected at the fourth stage (2.1-3.3 µm) whereas at 9 L/min the highest number of aerosols were collected at stage 5 (1.1-2.1 µm).

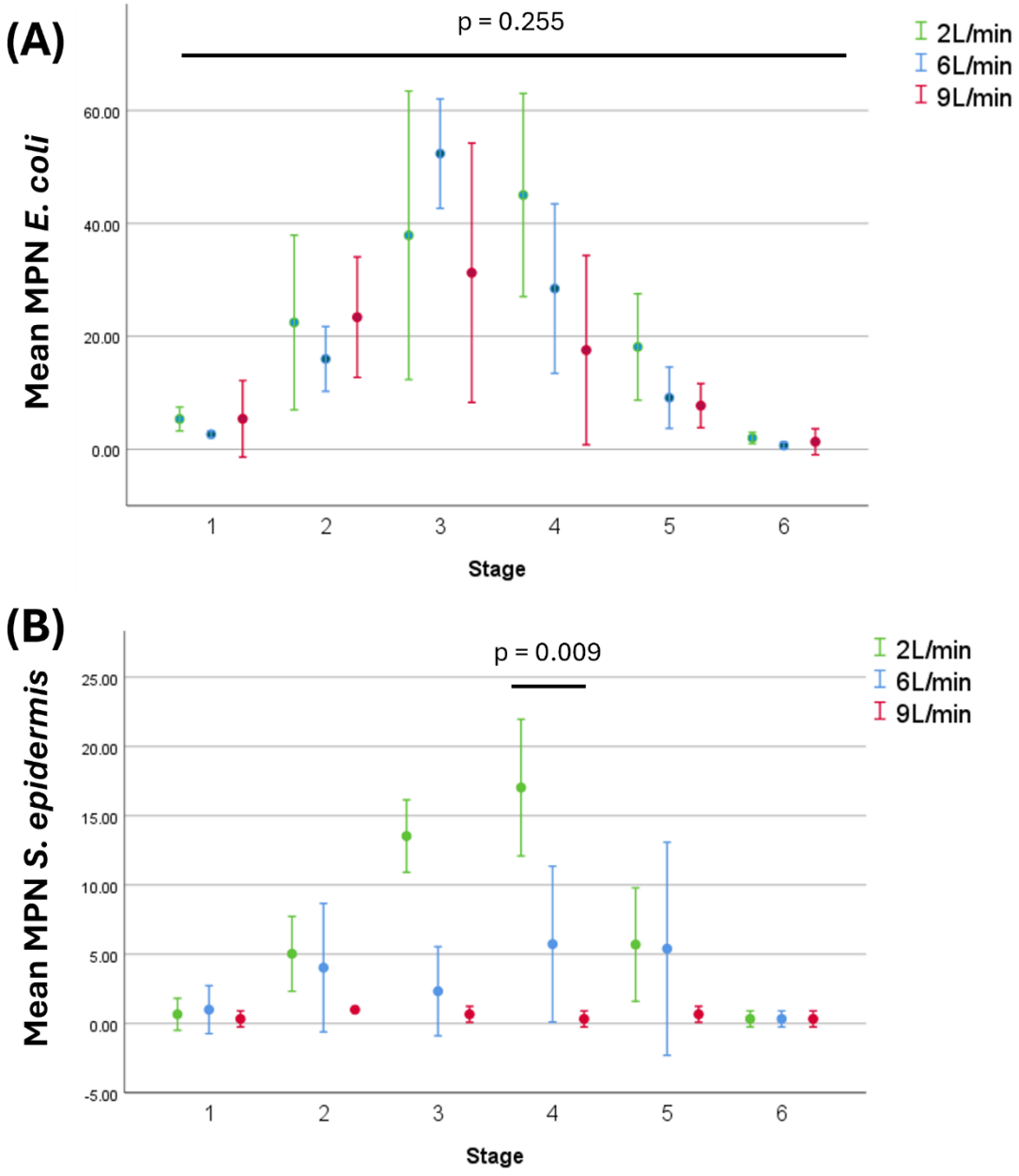

Figure 7.6 - Mean number of positive hole-corrected colonies (MPN) collected at each stage of a 6-stage Anderson Cascade Impactor generated from (A) source water spike with *E. coli* and (B) drain spiked with *S. epidermidis* at different flow rates (n = 3 per flow rate) (green, 2 L/min; blue, 6 L/min; and red, 9 L/min) using a model hand basin. Particles collected at each stage: Stage 1 (>7.0 µm), Stage 2 (4.7–7.0 µm), Stage 3 (3.3–4.7 µm), Stage 4 (2.1–3.3 µm), Stage 5 (1.1–2.1 µm), and Stage 6 (0.65–1.1 µm). Statistically significant differences between flow rates considered at p = <0.05.

7.6.2. Droplet dispersion detection

When comparing larger droplet dispersion from the source water detected by surrounding settle plates, there was also no significant difference between flow rates (p=0.561) (Figure 7.7A). Unlike aerosols collected by Anderson cascade impaction, there was no relationship between flow rate and droplet dispersion. Conversely, when comparing droplet dispersion from the contaminated drains, there was a significant difference between flow rates (p=0.044). As observed with the Anderson cascade impactor, as flow rate increased from 2 L to 9 L/min there was a significant decrease in droplet dispersion onto settle plates (p=0.014) (Figure 7.7B).

There was no significant difference in the direction of droplet dispersion detected on settle plates at 9 L/min from both the source water and the drain (p=0.139 and 0.055, respectively) (Figure 7.8A and B). At 2 L/min there was also no significant difference in dispersion direction from the drain (Figure 7.8B). However, at 2 L/min there were significantly more droplets from the source water dispersed to the back of the basin than to the sides (p=0.047) (Figure 7.8A). When considering droplets from both the source water and drain at 6 L/min, there were significantly more droplets dispersed to the rear of the hand basin than to the front (p=0.041 and 0.015, respectively) (Figure 7.8A and B).

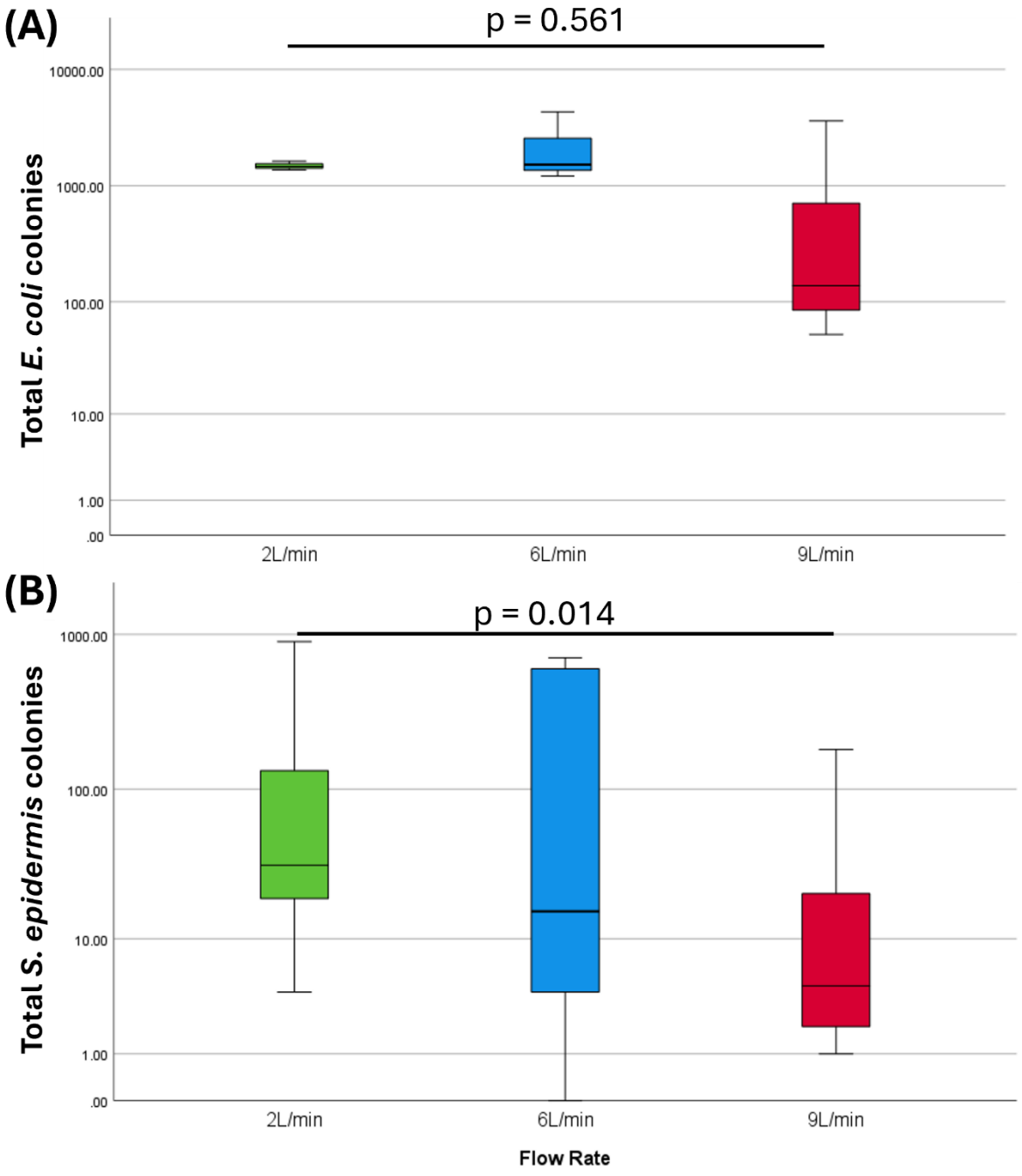

Figure 7.7 - Total number of colonies generated from (A) source water spiked with *E. coli* and from (B) drain spiked with *S. epidermidis* detected on MacConkey settle plates at different flow rates (n = 3 per flow rate) (green, 2 L/min; blue, 6 L/min; and red, 9 L/min) using a model hand basin. Statistically significant differences between flow rates considered at p = <0.05.

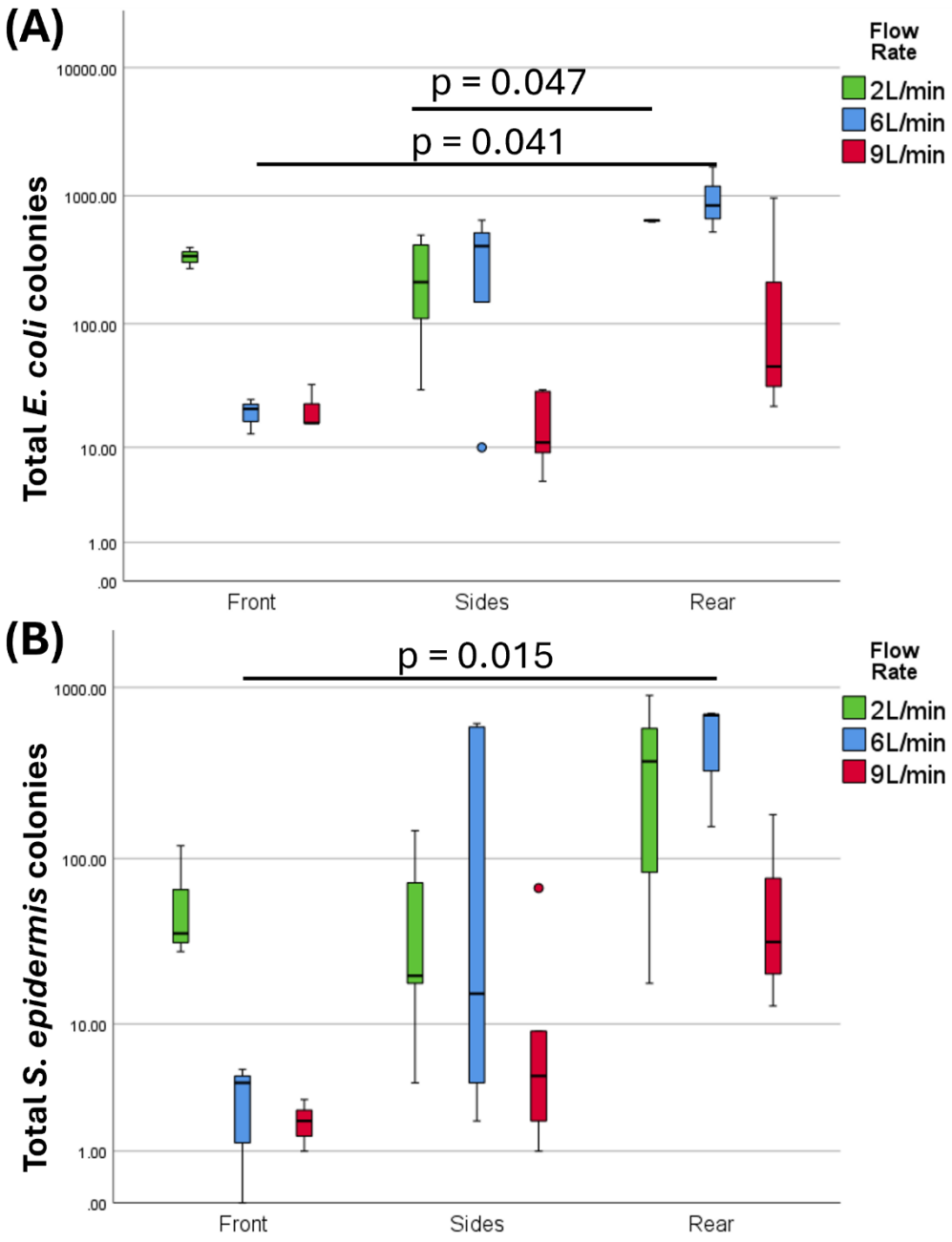

Figure 7.8 - Total number of colonies generated from (A) source water spiked with *E. coli* and (B) drain spiked with *S. epidermidis* detected on MacConkey settle plates to the front, sides and rear of the handwash basin model at different flow rates (n = 3 per flow rate) (green, 2 L/min; blue, 6 L/min; and red, 9 L/min). Statistically significant differences between flow rates considered at p = <0.05.

7.7. Discussion

This study investigated the influence of hand basin design and hydraulic flow on the generation of bioaerosols. Three flow rate restrictors were evaluated in a controlled experimental established to determine the production of bioaerosols generated directly from the source water as well as indirectly through splashback from microbial contamination of the drain. This study showed that all the tested flow restrictors generated bioaerosols that predominantly were 3.3-4.7 µm in diameter which are the size of aerosols that would reach the trachea and primary bronchi regions of the lung (Andersen, 1958; Thomas, 2013) (Figure 7.5). All flow rates restrictors also generated smaller bioaerosols 2.1-0.65 µm diameter (Andersen, 1958) (Figure 7.5) that would be able to reach the deepest regions of the lung such as the terminal bronchioles, alveolar ducts and alveoli (Thomas, 2013). This demonstrates a potential human health risk, if the aerosols produced were contaminated with containing opportunistic premise plumbing pathogens such as *Legionella pneumophila* and *Pseudomonas aeruginosa,* the generated bioaerosols would be small enough to be inhaled into the respiratory system resulting in disease. In immunocompromised individuals this could cause severe and potentially fatal pneumonia like infections (Falkinham et al., 2015; Pourchez et al., 2017).

Previous research that used impaction and/or impingement for aerosol collection placed these devices significantly closer to the faucet (Benoit et al., 2021). However, such experimental designs have been criticised for lacking resolution to distinguish between aerosols (<5 um) and larger droplets (Kotay et al., 2019). By placing the Anderson Cascade Impactor 1500 mm above the ground, the present study demonstrated that aerosols from the source water and the hand basin drain can reach the head height of the user at clinically relevant sizes. Dispersion of larger droplets to surrounding surfaces was captured by settle plates around the model hand basin (Figure 15.1). The settle plates were defined as being at the front (plates 11-16, 36-43; Figure 15.1), to the sides (plates 7-10, 17-20, 29-35, 44-49; Figure 15.1) and to the rear (plates 1-6, 21-28, 50; Figure 15.1) of the hand basin to understand the direction of dispersion and were placed up to 180 mm away from the drain. Previous studies have demonstrated that *P. aeruginosa* was detected up to 15 cm from a hand basin when it was contaminated (Doring et al., 1993). Similarly, Kotay et al. (2019) demonstrated that a green fluorescent tagged-*E. coli* that was spiked into a model hand basin was only dispersed during the faucet flushing and not at subsequent time points, indicating that the dispersion was due to larger droplets quickly settling to the surrounding surfaces rather than finer aerosols. In the present study, droplets from the source water and the hand basin drain were detected in all directions, with the highest dispersion from the source water seen to the rear of the handwashing basin at 9 L/min and from the drain seen

to the rear of the handwashing basin at 6 L/min (Figure 7.8). Outbreaks of multidrug resistant *P. aeruginosa* and carbapenem-resistant Enterobacterales in HAIs have been linked with contaminated handwashing sinks (Hota et al., 2009; Jung et al., 2020). Investigations of these outbreaks identified the water flow directly hitting the contaminated drain resulting in splashing to surrounding surfaces such as medication and sterile dressing preparation areas. These findings highlight the critical role of water flow dynamics in facilitating the dispersion of contaminated droplets from hand basin drains, underscoring the need for considered building design and cleaning interventions to minimize the risk of pathogen transmission in high-risk settings.

Results from this study showed that the lowest number of aerosols and droplets were produced at 9 L/min (Figure 7.5 and Figure 7.8). However, when considering aerosols and droplets originating from the source water, there was no significant difference between the flow rates. This is consistent with previous research that has found inconclusive correlations between flow rates are aerosol production (Benoit et al., 2021; Ehrlich et al., 1970; Fusch et al., 2015; Kotay et al., 2019; Lv et al., 2019; Park et al., 2013; Sebastian Schulz-Stübner et al., 2021; Takajo et al., 2020). The relationship between faucet design, height, and bowl design may also explain the variability in previous studies. Taller faucets positioned further from the drain could increase splash and droplet formation, while shallower or more rounded basin designs may affect how water flows and collects, influencing the generation of aerosols. Additionally, the design of the faucet flow restrictor could either enhance or limit aerosol production, depending on how the water is dispersed upon exit. These factors likely contribute to the inconsistent findings in prior studies, where varying combinations of these elements have been used.

Currently the flow rates recommended for use in high-risk settings such as healthcare, clean rooms and manufacturing facilities vary considerably, and may be chosen for water conservation, usage or gentler flow to manage scolding. For example, the National Health Service Water Management and Efficiency Standard states that hand basin faucets typically use 9 L/min and that flow regulators and restrictors may be fitted where appropriate (National Health Service England, 2013b). Conversely, Ireland's National Health Sustainability Office has released best practice guides for healthcare facilities which recommends lower flow rates of 2 – 4 L/min for washroom taps to reduce water consumption and cost (National Health Sustainability Office, 2020). These results underscore the need for further research to optimize product design and flow rate combinations that minimize aerosolization and associated infection risks. Future studies should explore a wider variety of basin and faucet designs, including variations in flow restrictor configurations and drain

placements. Testing these design variables across different flow rates would provide a more comprehensive understanding of their collective impact on aerosol dynamics and retrograde contamination.

The design of faucet flow restrictors plays a critical role in bioaerosol production, particularly in settings where flow restrictors are used. One hypothesis that could explain the difference in bioaerosol production between restrictor flow rates is the variation in water flow patterns generated by the different restrictors. For example, the 2 L/min flow restrictor used in the present study was of a 'multiple laminar flow' design which is commonly used when the flow rate is too low to produce as aerated or laminar stream. As flow rate decreases, the increased water pressure and shear force onto the basin drain can exacerbate the dispersion of droplets, further amplifying the potential for environmental contamination and infection spread (Kotay et al., 2019). To avoid the dispersal of bacteria from the drain, alternative basin designs such as offset drains have been proposed. For example, the United Kingdom and Germany recommend offset drains for high-risk areas (Commission for Hospital Hygiene and Infection Prevention, 2022; National Health Service England, 2013a) . However, the implementation of this change remains inconsistent with little evidence to demonstrate the changes efficacy at reducing infection transmission (Benoit et al., 2021; Fusch et al., 2015; Knoester et al., 2014; Sebastian Schulz-Stübner et al., 2021).

These aerosols and droplets can also inadvertently contribute to retrograde contamination from the drain to the faucet (Sebastian Schulz-Stübner et al., 2021). This research showed that the lowest flow rate (2 L/min) significantly increased aerosol production and subsequent retrograde contamination from the contaminated drain to the faucet (Figure 7.4). Previous studies have shown that biofouling of low flow restrictors resulted in a significant increase in the aerosols containing bacteria generated compared to initial testing (Benoit et al., 2021). Furthermore, after deep mechanical cleaning the number of aerosols generated still exceeded the initial values (Benoit et al., 2021). The reduced water flow and shear forces along with increased surface area due to complex design both contribute to their risk of becoming a reservoir for biofilm. However, one factor that was not investigated in this study is the role of biofilm. A liquid suspension of indicator bacteria was applied to the hand basin drain to simulate contamination; however, it is unknown how representative this indicator was of bacterial sink biofilms. The methodology developed in this study can be used to explore the aerosolization of biofilms on drain surfaces and other design combinations such as offset drains and laminar flow restrictors.

7.8. Conclusions

In this study, the use of a model hand basin system showed that lower flow rates significantly increased the aerosols generated from contaminated drains and resulted in greater retrograde contamination from the drain to the faucet. While product design modifications such as altering flow restrictor design, drain placement, and flow rate can influence aerosol dynamics, the optimal combination of these factors remains uncertain. This highlights the need for future research and collaboration between design engineers, microbiologists, and infection control practitioners. Such interdisciplinary efforts are essential to inform the optimal design that meets functional needs while preventing bioaerosol production, ultimately protecting public health.

7.9. Statements

Author contributions: C.H., K.E.R., M.H.B., H.W. conceived and designed the experiments. C.H. performed the experiments, analysed the data and drafted the manuscript. H.W., K.E.R. and M.H.B. corrected and contributed to the manuscript. All authors approved the final manuscript.

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Data availability: Data will be made available upon request.

8. EVALUATING PLUMBING MATERIALS FOR ANTIMICROBIL ACITIVITY AND PUBLIC HEALTH IMPLICATIONS

In this chapter, the antimicrobial activity of different plumbing materials, specifically brass and stainless steel, against OPPPs under static conditions was investigated. This chapter addresses Objective 6 and contains a published manuscript that used bioreactors to simulate stagnant conditions typically found within drinking water plumbing systems. These findings underscore the need for evidence-based decisions about plumbing materials and their potential role in infection control and biofilm management within drinking water plumbing systems.

CITATION: Comparison of the antimicrobial activity of brass versus stainless steel against opportunistic premise plumbing pathogens

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8.1. Abstract

Lead contamination is a significant public health issue, as it can cause severe health impacts. Highly publicized instances of elevated lead levels has resulted regulatory changes to prohibit the use of brass materials in potable water in favor of low-lead alternatives, such as stainless steel. However, there are limited studies investigating their effect on opportunistic premise plumbing pathogens (OPPPs). This study used model plumbing bioreactors, made from either brass or stainless-steel, to examine the effects of pipe material and stagnation time on OPPPs, biofilm formation and lead leaching. The bioreactors were filled with water spiked with OPPPs, *Acinetobacter baumannii, Pseudomonas aeruginosa, Mycobacterium avium* complex and *Acanthamoeba polyphaga.* Each OPPP responded differently to pipe material and stagnation time. Overall, brass had greater antimicrobial activity compared to stainless steel. Lead levels exceeding World Health Organization water quality guidelines were found in both brass and stainless-steel bioreactors after 10 weeks stagnation, despite stainless steel be marketed as a low lead alternative. Material analysis found lead deposits on the surface of stainless steel, likely from post-manufacturing contamination. These findings demonstrate the need for future research characterizing the influence of plumbing material on microbial contamination under a range of conditions prior to regulatory changes.

8.2. Synopsis

The study shows that both brass and stainless steel pipes can leach harmful lead with differing impacts microbial growth, demonstrating the need for future research that considered both the microbial and chemical consequences to changes in plumbing material.

8.3. Introduction

Worldwide lead is a common metal contaminant of potable water (Zietz et al., 2010). Its prevalence is due to leaching from plumbing materials (D. Q. Ng et al., 2016). Consumption of lead has been linked to severe health conditions such as developmental neurotoxicity, kidney damage and interference in calcium metabolism for bone formation, with infants, fetuses and pregnant women being most susceptible (Weizsaecker, 2003; Zietz et al., 2010). The World Health Organization has released a guideline value for lead in potable water of 10 ppb, which has been adopted by Japan, Ireland, Australia and the European Union (World Health Organization, 2017).

Highly publicized instances of non-compliance with lead guidelines, such as the ongoing Flint Michigan water crisis that began in 2014, have resulted in widespread public concern

and outrage regarding the safety of municipal water (Molino et al., 2019; Ruckart et al., 2019; Victoria Department of Health and Human Service, 2018). Such instances of elevated lead concentrations are predominantly caused by older potable water distribution systems that still contain lead service lines or corrosion of brass and copper fittings at the outlet (D. Q. Ng et al., 2016; Renner, 2009, 2010). Prolonged water stagnation has also been shown to exacerbate the dissolution of metals, such as lead and copper, from the plumbing materials into the water (Ghoochani et al., 2022). Drinking fountains and tank water supplies in 14 regional and metropolitan New South Wales (NSW) schools were contaminated with elevated lead and copper levels. NSW Health stated that lead materials in the tank water catchment areas were to blame for the contamination, resulting in installation of filtration systems and first flush devices in all affected schools (NSW Health, 2021). Much of the existing literature has focused on the impact of short term stagnation (hours to days) on water quality, whereas extended periods (weeks to months) seen during school holidays and COVID-19 lockdown related closures, have received less attention (Dion-Fortier et al., 2009; Lautenschlager et al., 2010; Lytle et al., 2000; Tian et al., 2021).

Legislators have introduced tighter regulations surrounding the allowable level of lead in the manufacture of materials such as brass and copper including products such as valves, backflow preventors, mixers and water dispensers. For example, the US Environmental Protection Agency announced in 2014 that ‘lead free’ materials may not have more than a weighted average of 0.25% lead in wetted surface, a change that has also been adopted by the Australian Building Codes Board as of 2025 (Australian Building Codes Board, 2021; U.S. Environmental Protection Agency, 2020b). Although these changes have been broadly accepted by the plumbing industry and broader community as a logical response, concerns have been raised regarding the potential unintended impacts on the prevalence of opportunistic premise plumbing pathogens (OPPPs) (Molino et al., 2019).

Microbial contamination of potable water infrastructure is an emerging public health issue, particularly in healthcare settings (Amos et al., 2015; Hayward et al., 2020; Kanamori et al., 2016; Kohlenberg et al., 2010; Stjärne Aspelund et al., 2016). OPPPs have been described as one of the most significant and overlooked source of healthcare associated infections (Anaissie et al., 2002; S. Schulz-Stübner et al., 2021). These OPPPs, such as *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, non-tuberculous mycobacterium, *Legionella pneumophila* and *Aeromonas hydrophila*, are an arbitrary group of opportunistic pathogens that have adapted to survive within premise plumbing environments. OPPPs can form biofilms in water pipelines and on point of use devices such as faucets, drains and shower heads (Falkinham, 2016; Falkinham, 2015; Falkinham et al., 2015; Feazel et al., 2009;

Williams et al., 2013). OPPPs are also able to persist and replicate within free living amoeba (FLA) hosts, providing protection from disinfection strategies(Nisar et al., 2022).
Several studies have demonstrated that brass has antimicrobial properties against OPPPs (Dauvergne et al., 2021). Conversely, there is little understanding of how changing these fixtures may impact the ability of OPPPs to adhere to low lead alternatives (Kimbell et al., 2020). An evidence-based approach should be the foundation for all public policy development, particularly for those that have the potential to significantly impact public health. This study investigated the influence of plumbing material on biofilm formation and growth of OPPPs under stagnant conditions simulating those found within premise plumbing. A biofilm reactor model experiment was developed to compare Australian certified brass and stainless-steel plumbing materials on lead leaching, microbial water quality, and biofilm formation over two periods of extended stagnation. One of the challenges with quantifying microbial contamination in potable water is that traditional culturing techniques may underestimate the pathogenic load of a sample as the culturable population of cells is only a proportion of viable cells. Injured or stressed OPPPs may convert to a viable but non culturable state in response to environmental stressors, however they remain metabolically active and potentially infective (Cateau et al., 2011; Rafik Dey et al., 2019; Kim et al., 2009). To overcome these challenges, a range of pathogen quantification methods including traditional culture, molecular and viability flow cytometry were used.

8.4. Materials and Methods

8.4.1. Biofilm reactor

Biofilm reactors were designed to evaluate biofilm formation and water quality found in stagnant premise plumbing systems. The design of the model plumbing bioreactors is shown in Figure 8.1. Each bioreactor was a closed system with three sampling sections of externally threaded pipe (A, B and C) (50 mm L x 15 mm internal diameter) joined with connectors and sealed with endcaps, all components of the bioreactors to be made of the same material sourced from the same brand. All materials were purchased from Australian plumbing supply stores and were manufactured to Australian Standard 3688: Water supply and gas systems Metallic fittings and end connections (Standards Australia, 2016). All stainless steel materials were 316 grade. Two different brands of brass and two different brands of stainless steel were tested; the brands of brass fittings were not the same as stainless steel fittings. All brass and stainless-steel components used in this study were cleaned by submersion in 5 mg/L chlorine solution for 12 h. Previous research indicates that short term exposure to low levels of chlorine does not result in corrosion or lead leaching significantly higher than what would be expected in distribution systems (Costa et al., 2023; Maas et al., 2007).

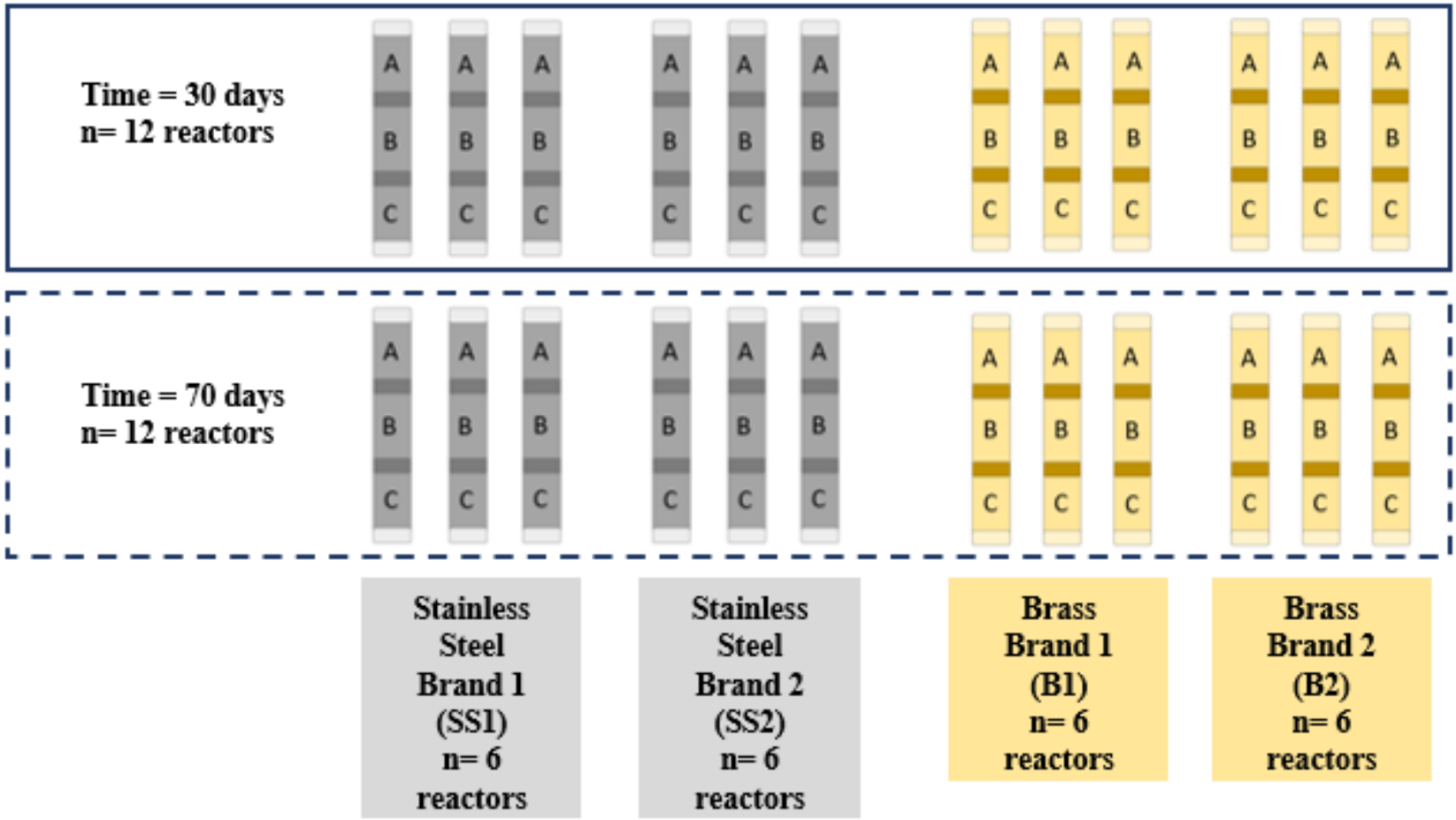

Figure 8.1 - Schematic representation of assembled model biofilm reactors.

Biofilm reactors made entirely from material sourced from Australian plumbing supply stores and were manufactured to AS 3688 Water supply and gas systems specifications. Grey = stainless steel, yellow = brass. Two different brands of brass (B1 and B2) and two different brands of stainless steel (SS1 and SS2) were used to construct a total of 24 bioreactors (n=6 of each brand). Each biofilm reactor was a closed system comprised of three pipe segments (A, B and C), joined with connectors and sealed with endcaps. Twelve reactors (n=3 of each brand) were destructively sampled after 30 days and 70 days.

8.4.2. Source water spiked with opportunistic premise plumbing pathogens

Potable water was collected from one large building distribution system and used as source water. Prior to spiking with targeted pathogens, residual chlorine was quenched with sodium thiosulfate at a final concentration of 1 mg/L. No corrosion inhibitors were added to the source water.

The feed water was then spiked with target OPPPs *A. baumannii* (ATCC 17978)*, P. aeruginosa* (PAO1)*, M. avium* complex (MAC) (clinical isolate provided by SA Pathology, Adelaide, Australia) and *Acanthamoeba polyphaga* (ATCC 30461)*.*

Overnight cultures of *A. baumannii* and *P. aeruginosa* in tryptone soya broth were prepared and adjusted to an OD 600 nm of 1 (1 x10^9 CFU/mL) in sterile water. Feed water was subsequently inoculated with *A. baumannii* and *P. aeruginosa* suspension to a final concentration of 1.53 x 10^5 and 9.93 x 10^4 CFU/mL, respectively to simulate bacterial load seen in environmental settings (Josephson et al., 1997a; Kobayashi et al., 2014; Mombini et al., 2019a). After sufficient growth of *M. avium* complex on R2A agar, all colonies were transferred to a sterile centrifuge tube (15 mL) containing 5 mL of sterile water and homogenized on a vortex mixer (SEM® Vor·Mix) for one min. The turbidity of the suspension was adjusted to an OD 600 nm of 0.1 (1.5 x 10^8 CFU/mL) (Sattar et al., 2018; Thomson et al., 2008). Feed water was inoculated with *M. avium* complex suspension to a final concentration of 1.5x10^4 GU/mL as confirmed through flow cytometry counts using the method described below to simulate bacterial load seen in environmental settings (Briancesco et al., 2014; Donohue et al., 2019; Tichenor et al., 2012b). *A. polyphaga* cells in PYG broth were harvested and resuspended in 5 mL 1X Page's saline and 0.136 g KH_2PO_4 per liter distilled water. Feed water was inoculated with *A. polyphaga* suspension to a final concentration of 8.4x10^2 cells/mL. Once spiked, 25 mL feed water was aliquoted into each bioreactor. Sealed, bioreactors were then placed on their side at 25^{o}C to allow stagnation until sampling.

8.4.3. Sampling procedures

After 30 days stagnation, three of each brand of bioreactors (SS1, SS2, B1 and B2) were sampled whilst the remaining bioreactors remained stagnant at 25^{o}C until the second sampling point of 70 days (Figure 8.1& Table 8.1). The stagnation periods were chosen to represent scenarios encountered by residents returning from vacations and school closure periods (Eurydice, 2022). Stagnant water samples were collected in individual sterile centrifuge tubes for chemical and microbial water quality testing. Biofilms were removed for microbial testing by placing each pipe section (A, B and C) (Figure 8.1) pipe in a sterile 50 mL centrifuge tube with 25 mL sterile water, followed by 5 mins of shaking (Griffin Flash Shaker), vortexing (SEM® Vor·Mix) and sonication (CooperVision® 895 Ultrasonic Cleaner) causing biofilms formed on the internal pipe surface to detach into the sterile water. Each pipe segment was stored at -80^{o}C for material surface characterization.

Table 8.1 - Number of samples taken for plumbing material brand per sampling event*

Sampling event	Sample type	Material [brand number]				Total
		Brass [B1]	Brass [B2]	Stainless Steel [SS1]	Stainless Steel [SS2]	
30 days	Water	3	3	3	3	12
	Biofilm from pipe section	9	9	9	9	36
70 days	Water	3	3	3	3	12
	Biofilm from pipe section	9	9	9	9	36
	Total	24	24	24	24	96

* Two brands of brass (B1 and B2), and two brands of stainless steel (SS1 and SS2) were compared. Three bioreactors from each brand (n=12 total) were destructively sampled at each sampling event, generating three water and nine biofilm samples per brand.

8.4.4. Chemical analysis

Water samples were tested for pH changes over time (HACH PocketPro^{+} Multi2). Water samples were analyzed for total lead, in triplicate, on a Perkin Elmer Nexion 350D ICP-MS.

Prior to measurement, 10 mL water samples were acidified with 0.1 mL of 0.5% HNO_3 and stored at 5°C until analysis.

8.4.5. Microbial testing

8.4.5.1. Selective culture

Water and biofilm samples were analyzed by HPC using the standard method (AS/NZS, 2007). Stagnant water and resuspended biofilm samples were analyzed for the target pathogens *A. baumannii* and *P. aeruginosa* using previously described culture techniques (Ajao et al., 2011; International Organization for Standardization, 2018).

8.4.6. Flow cytometry viability cell sorting and qPCR assay development

8.4.6.1. Flow cytometry viability cell sorting assay

A cell viability kit for microbial viability (Becton Dickinson, Franklin Lakes, NJ, USA) was used to stain and quantify live, injured and dead bacterial cell populations (Nisar et al., 2023a). Analysis and cell sorting were performed on a FACSAria Fusion flow cytometer (Becton Dickinson) under sterile conditions. Thiozole orange (TO) and propidium iodide (PI) fluorescence plots were used to discern alive, injured, and dead bacterial cells. A mixture of untreated target pathogen cells, and heat killed cells (75°C for 10 min) were used as controls to define gates and distinguish between alive and dead cell populations respectively (Nisar et al., 2023a). Approximately 10^2 to 10^6 cells were sorted from each of the alive, injured, and dead populations for further characterization immediately after sampling.

8.4.6.2. Quantitative polymerase chain reaction

DNA was extracted for quantitative polymerase chain reaction (qPCR) analysis from 1 mL of the stagnant water, resuspended biofilm sample, and from each of the bacterial populations (injured and alive) sorted by flow cytometry (VFC+qPCR) using the BIO-RAD Aquadien™ DNA extraction and purification kit following the manufacturer's instructions (Bio-Rad Laboratories, Inc., Sydney, NSW, Australia).

qPCR for *A. baumannii*, *P. aeruginosa*, MAC and *A. polyphaga* was performed as previously described (Lee et al., 2011; McConnell Michael et al., 2012; Park et al., 2000; Qvarnstrom et al., 2006) (Table 16.1).

8.4.7. Surface characterization

The pipe was sectioned with a saw along the length of the pipe and in cross section for analysis by scanning electron microscopy. The samples were mounted on carbon tape, no coating was required for conductivity. An FEI Inspect F50 with an Energy Dispersive

Analysis by X-ray (EDAX) Ocane Pro EDS detector. Images were obtained using both the secondary electron (SE) mode and the back scattered electron (BSE) mode, to detect topographical changes with high resolution. All images were taken with an accelerating voltage of 30 kV, spot size of 7, and a working distance of 10 mm. EDAX was used to analyze and quantify the chemical composition of the pipes. The energy dispersive spectroscopy (EDS) spectra were analyzed using EDAX TEAM version 4.4 software.

8.4.8. Statistical analysis

Statistical analyses of lead levels, microbial culture, qPCR and VFC+qPCR calculated copies/mL of each target organism at the different sampling points and bioreactor material were conducted using IBM SPSS Statistics for Windows, version 25 (IBM Corp., Armonk, N.Y., USA). Comparisons of average lead ppb, CFU/mL, CFU/cm^2, copies/mL and copies/cm^2 were performed using a Wilcoxon signed rank test. Statistical significance was accepted at $p<0.05$.

8.5. Results

Figure 8.2 shows the mean concentration of *A. baumannii, P. aeruginosa, M. avium* complex (MAC) and total bacteria in the water and biofilm samples collected from the bioreactors at the two different time points. *A. baumannii, P. aeruginosa,* MAC concentrations were quantified using selective culture, VFC+qPCR, and qPCR. In 19/48 measurements, stagnant water (GU or CFU/mL) and biofilm (GU or CFU/cm^2) in stainless steel bioreactors presented a significantly ($p<0.05$) higher total concentration of the target pathogens *A. baumannii, P. aeruginosa,* MAC (measured using direct qPCR, alive VFC+qPCR and culture) and indicator heterotrophic bacteria was observed, compared with brass bioreactors. In 16 instances no significant difference between the materials was observed. There were only two instances/measurements where the target number of alive GU/mL was significantly ($p<0.05$) greater in the brass bioreactors than stainless steel. There was no significant difference ($p>0.05$) in *A. polyphaga* GU/mL or cm^2 measured using qPCR between stainless steel or brass bioreactors. These data indicate that stainless steel plumbing materials support the growth of OPPPs to a greater extent compared with brass.

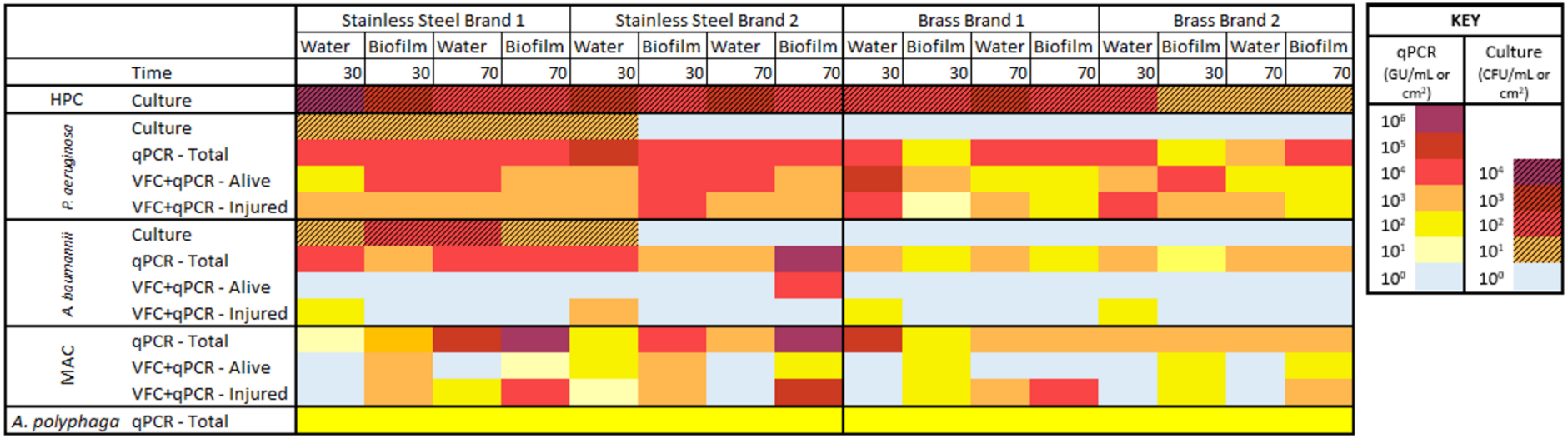

Figure 8.2 - Heat map diagram showing mean concentrations of target pathogens in stagnant water and biofilm from brass and stainless-steel bioreactors after 30 days and 70 days stagnation. Three bioreactors of each brand were destructively sampled after 30 days and 70 days stagnation generating three water samples and nine biofilm samples per brand. Each sample was tested for heterotrophic bacteria (HPC) and spiked pathogens (*A. baumannii*, *P. aeruginosa*, *M. avium* complex (MAC) and *A. polyphaga* using selective culture, qPCR and viability flow cytometry + qPCR (VFC+qPCR). *A. polyphaga* and MAC culture was not performed. Heat map key at the right with colors representing different $\log_{10}$ bacterial cell concentrations. qPCR and VFC+qPCR data expressed as GU/mL or cm^2. Culture data are expressed as CFU/mL or cm^2.

8.5.1. *Acinetobacter baumannii*

The mean concentrations of *A. baumannii,* measured using culture, qPCR, and VFC+qPCR (alive and injured cell fractions), in water and biofilm samples collected from the bioreactors after 30- and 70-days stagnation are shown in Figure 8.3. Culturable concentrations of *A. baumannii* in biofilm (CFU/cm^2) samples were significantly higher in stainless steel bioreactors after 30 days (p=0.027) and 70 days (p=0.0109) stagnation when compared to brass bioreactors. There was also a significant difference in culturable concentrations of *A. baumannii* between brands of stainless steel after 30 and 70 days stagnation. No significant difference was observed between the concentration of *A. baumannii* in stagnant water from either material type after 70 days. Conversely, biofilms that formed on stainless steel plumbing materials had significantly more alive (VFC+qPCR) copies/cm^2 after 70 days stagnation when compared with brass plumbing materials.

8.5.2. *Pseudomonas aeruginosa*

The concentrations of *P. aeruginosa,* measured using culture, qPCR, and VFC+qPCR (alive and injured cell fractions), in water and biofilm samples collected from the bioreactors after 30 days and 70-days stagnation are shown in Figure 8.4. After 30 days the stainless-steel bioreactors had significantly more culturable *P. aeruginosa* in both the stagnant water (p=0.043) and biofilm (p=0.046) samples when compared with the brass bioreactors. After 70 days stagnation, significantly more total (qPCR), alive (VFC+qPCR) and culturable *P. aeruginosa* were observed in the stagnant water from stainless steel bioreactors.

Alive *P. aeruginosa* cells (VFC+qPCR) in stainless steel biofilms increased significantly (p=0.037) between 30 and 70-days stagnation, suggesting growth during this period of stagnation. Whereas there was a decrease in alive cells/cm^2 in brass bioreactors. Significant differences between stainless steel brands were seen in the number of alive *P. aeruginos*a in both the stagnant water (p=0.028) and biofilm (p=0.001). Conversely, significant differences between brass brands were seen with the number of injured *P. aeruginosa* in both the stagnant water (p=0.039) and biofilm (p=0.003).

P. aeruginosa was the only target bacterial pathogen to remain ‘alive’ (VFC+qPCR) in the stagnant water after both 30 days and 70 days. A significantly higher number of total (p=0.002) and alive (p=0.002) *P. aeruginosa* were observed in stagnant water from stainless steel compared to brass bioreactors after 70 days stagnation. Injured (VFC+qPCR) *P. aeruginosa* in stagnant water (p=0.002) and alive (VFC+qPCR) *P. aeruginosa* in the biofilm (p=0.001) after 30 days stagnation were the only samples in which the number of bacteria in the brass bioreactors was significantly higher than stainless steel bioreactors. However, no significant difference was observed in injured *P. aeruginosa* in stagnant water (p=0.616), whereas stainless steel bioreactors had significantly more alive *P. aeruginosa* in biofilm after 70 days (p=0.001).

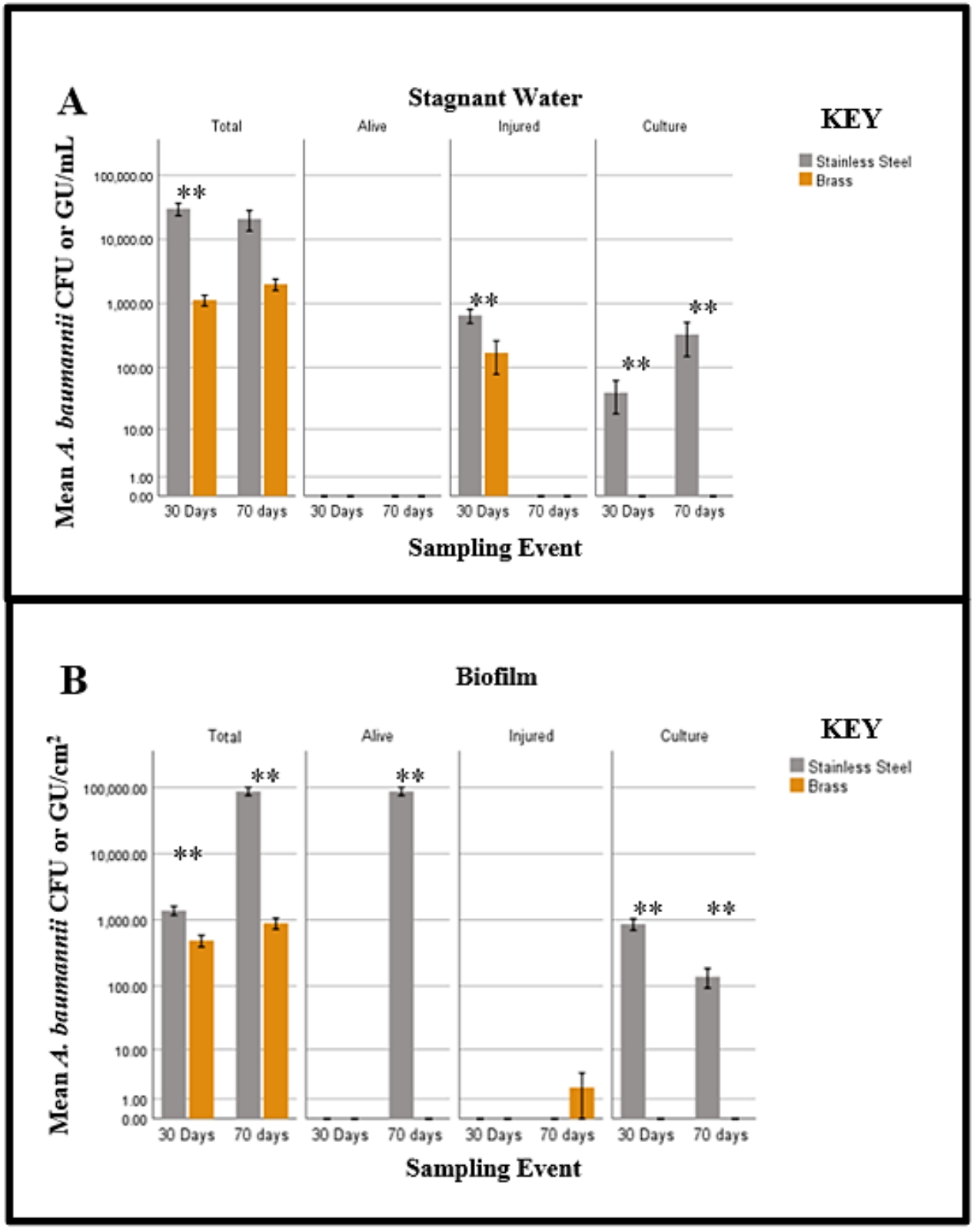

Figure 8.3 - Average concentration of *A. baumannii* CFU or GU in (A) stagnant water (n = 6) and (B) biofilm (n = 18) after 30 days and 70 days stagnation from brass or stainless-steel bioreactors (combining both brands of each material). Concentrations measured using qPCR (total), flow cytometry + qPCR (alive and injured) and selective culture +/- SE. Statistically significant differences between materials (P<0.05) are denoted with a star. Key at the right with stainless steel in grey and brass in orange.

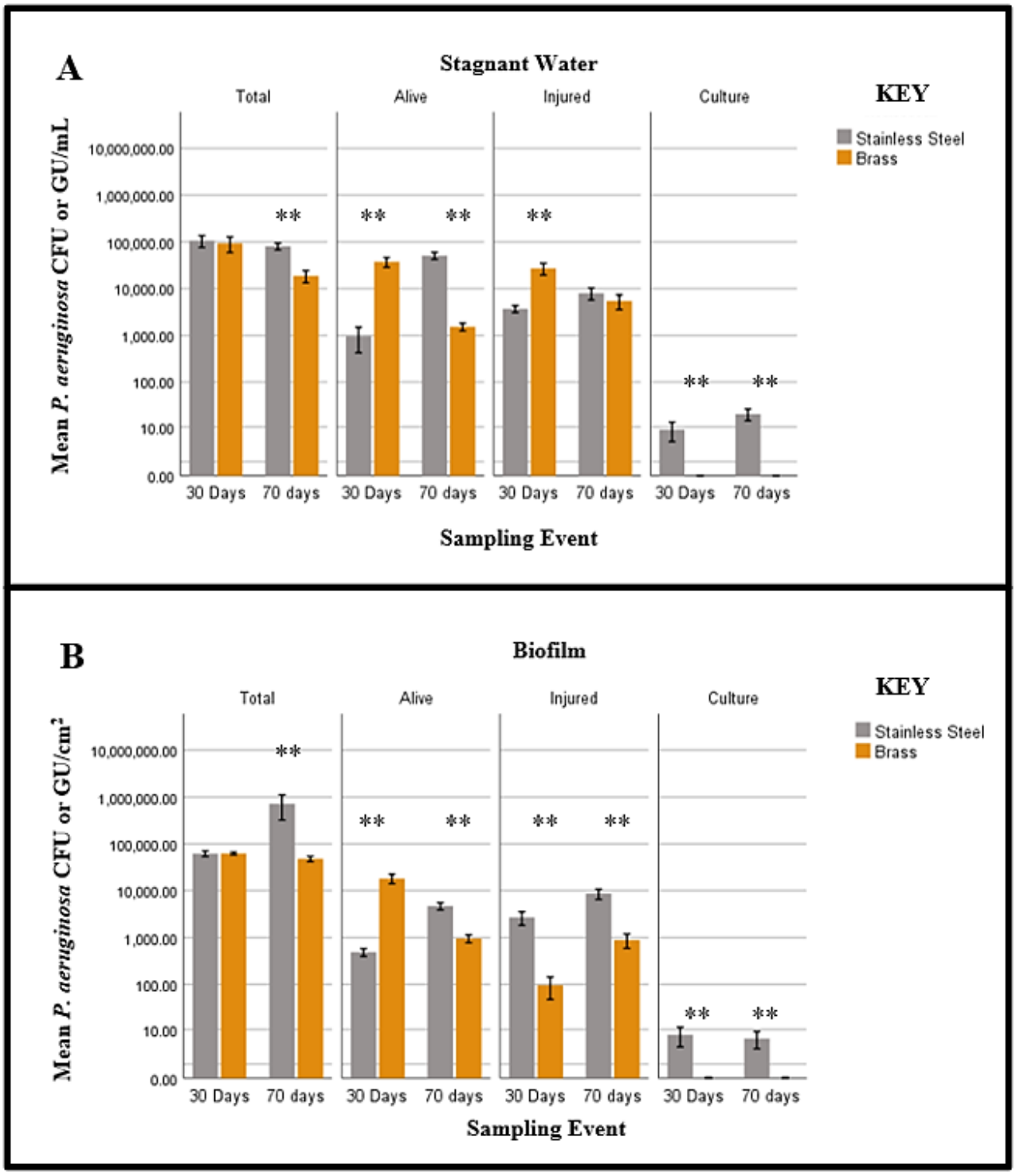

Figure 8.4 - Average concentration of *P. aeruginosa* CFU or GU in (A) stagnant water (n = 6) and (B) biofilm (n= 18) after 30 days and 70 days stagnation from brass or stainless-steel bioreactors (combining both brands of each material). Concentrations measured using qPCR (total), flow cytometry + qPCR (alive and injured) and selective culture +/- SE. Statistically significant differences between materials (P<0.05) denoted with a star. Key at the right with stainless steel in grey and brass in orange.

8.5.3. *Mycobacterium avium complex*

The concentrations of MAC measured using, qPCR and VFC+qPCR (alive and injured cell fractions), in water and biofilm samples collected from the bioreactors after 30- and 70-days stagnation are shown in Figure 8.5. No significant ($p>0.05$) differences in total, alive or injured (VFC+qPCR) MAC copies/mL were observed in any of the bioreactors between samples following 30 days and 70-days stagnation. Conversely, biofilms that formed on stainless steel plumbing materials consistently had significantly more total (qPCR) ($p=0.001$) and injured (VFC+qPCR) ($p=0.01$) MAC copies/cm^2 after 30 days and 70 days stagnation when compared with brass plumbing materials. Significant differences in total MAC stagnant water were seen between different brands of brass. After 30 days stagnation, a significant ($p=0.038$) difference was observed in total (qPCR) MAC copies/mL between the two brass brands.

8.5.4. *Acanthamoeba polyphaga*

A. polyphaga concentrations were measured using qPCR. No statistically significant difference ($p>0.05$) in total qPCR copies/mL of water or copies/cm^2 of biofilm was observed between materials at either sampling event (Figure 8.6).

8.5.5. Heterotrophic plate count

The concentrations of heterotrophic bacteria measured using culture, in water and biofilm samples collected from the bioreactors after 30- and 70-days stagnation are shown in Figure 8.7. After 30 days, the stainless-steel bioreactors had statistically significantly more heterotrophic bacteria in both stagnant water ($p=0.002$) and biofilm ($p=0.001$) compared to brass bioreactors. After 70 days, the concentration of heterotrophic bacteria in biofilm in stainless steel bioreactors remained significantly higher ($p=0.001$), however, there was no significant difference ($p=0.459$) in the stagnant water between materials.

After 30 days stagnation, a significant difference in heterotrophic bacteria culture was observed between stainless steel brands in both the stagnant water and biofilm, and in the stagnant water between brass bioreactors. After 70 days stagnation, a statistically significant difference in HPC culture from stagnant water and biofilm was observed between different brands of both stainless steel and brass

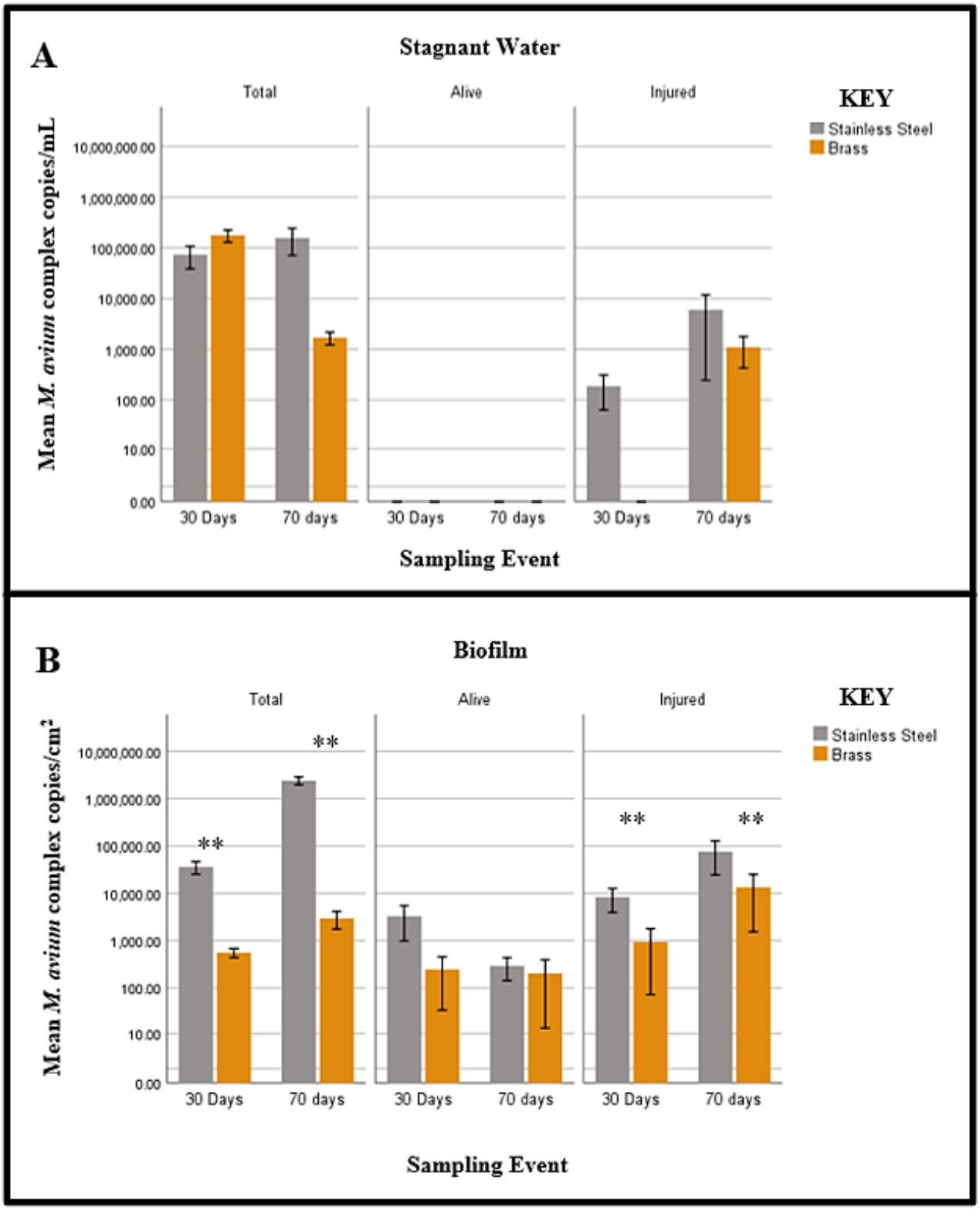

Figure 8.5 - Average concentration of *M. avium* complex copies in (A) stagnant water (n = 6) and (B) biofilm (n= 18) after 30 days and 70 days stagnation from brass or stainless-steel bioreactors (combining both brands of each material). Concentrations measured using qPCR (total) and flow cytometry + qPCR (alive and injured) +/- SE. Statistically significant differences between materials (P<0.05) denoted with a star. Key at the right with stainless steel in grey and brass in orange.

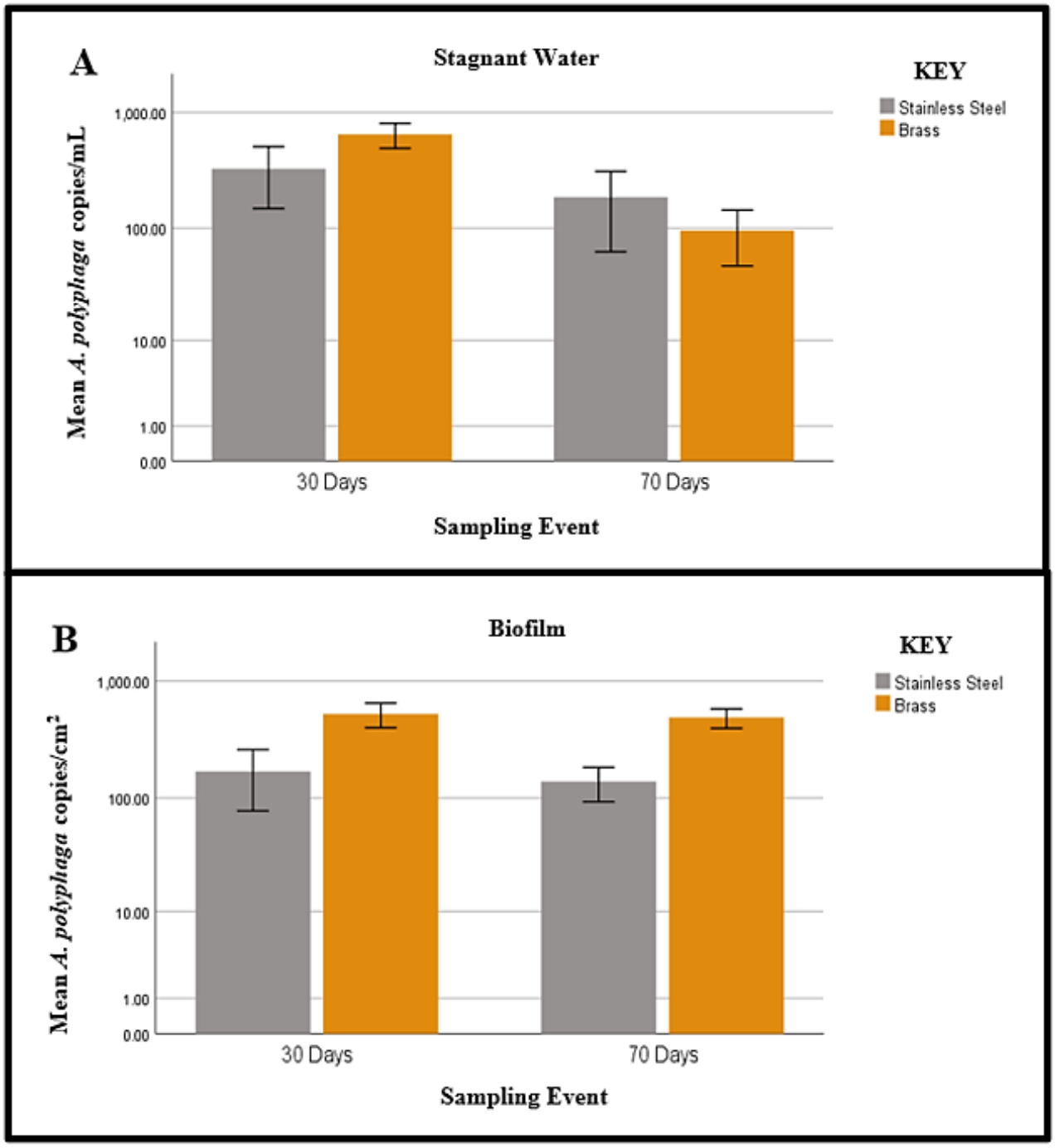

Figure 8.6 - Concentration of *Acanthamoeba polyphaga* GU in (A) stagnant water (n = 6) and (B) biofilm (n= 18) after 30 days and 70 days stagnation measured using qPCR (total) +/- SE. Key at the right with stainless steel in grey and brass in orange

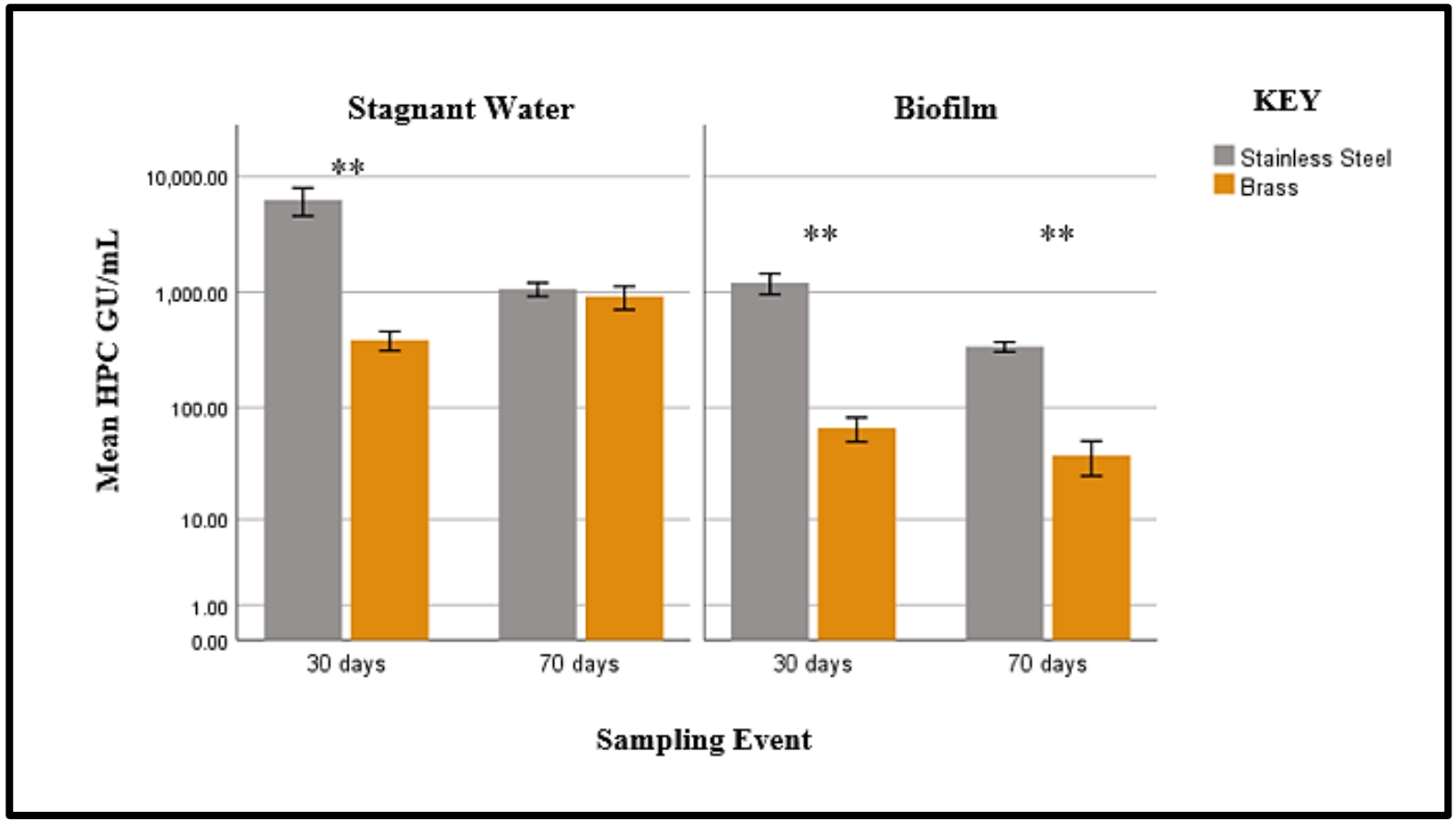

Figure 8.7 - Concentration of Heterotrophic bacteria CFU in (A) stagnant water (n= 6) and (B) biofilm (n=18) after 30 days and 70 days stagnation measured using selective culture. Statistically significant differences between materials (P<0.05) denoted with a star. Key at the right with stainless steel in grey and brass in orange.

8.5.6. Lead leaching

The initial lead concentration of the seed water was found to be below the limit of quantification (0.5 ppb) (Figure 8.8).

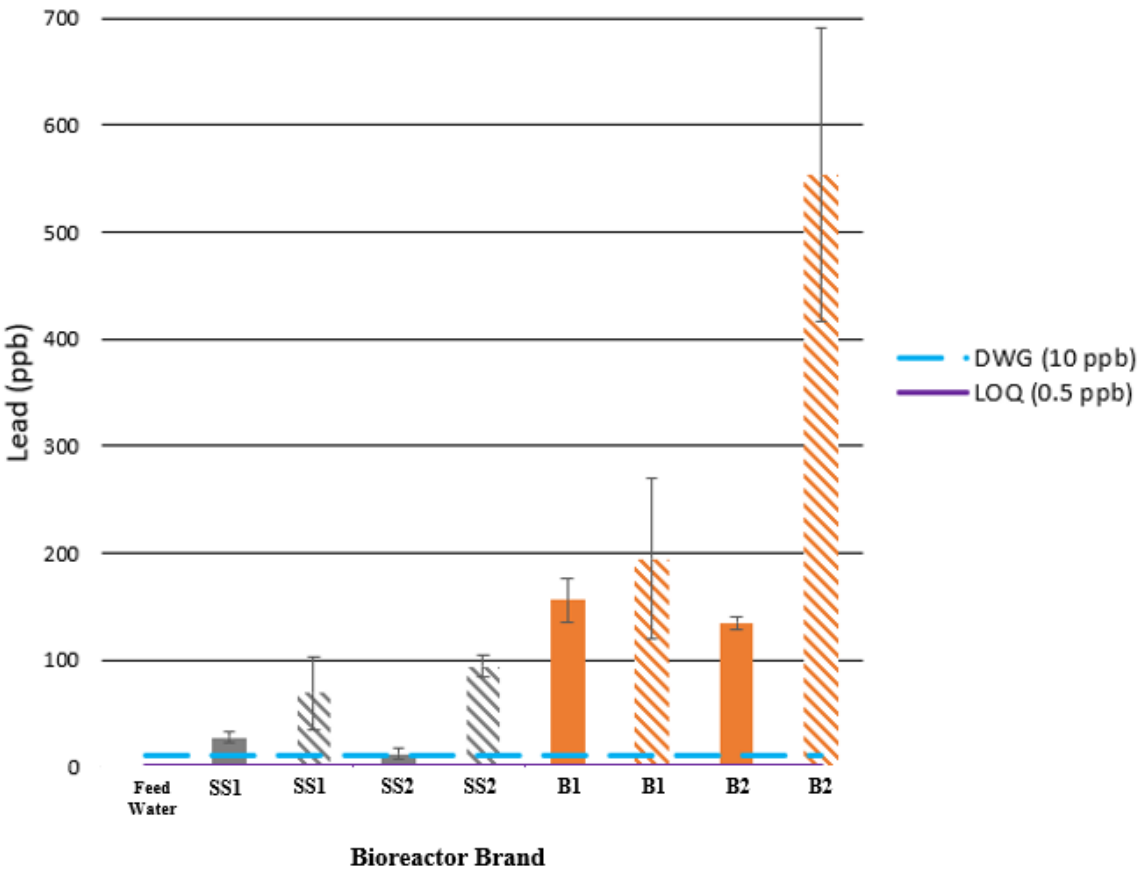

Figure 8.8 - Total lead (ppb) from bioreactors after extended periods of stagnation. Stainless steel brands (SS1 and SS2) shown in grey and brass brands (B1 and B2) shown in orange. Thirty (solid bar) and 70 days (dashed bar) stagnation. World Health Organization drinking water guideline (10 ppb) shown in dashed blue line, ICP-MS limit of quantification (0.5 ppb) shown in solid purple line.

Both brands of brass plumbing materials resulted in stagnant water exceeding the amount listed in the lead drinking water guidelines (DWG). Brass brand 1 (B1) had the highest concentration after 30 days stagnation at 181 +/- 47 ppb. Brass brand 2 (B2) had the highest increase of lead concentration between sampling points with a 312% increase from133 +/- 38 to 553 +/- 236 ppb.

Stagnant water in both brands of stainless steel also exceeded 10 ppb after both 30- and 70-days stagnation. Stainless steel brand 1 (SS1) had the highest lead concentration after 30 days at 43 +/- 27 ppb; however, stainless steel brand 2 (SS2) had a higher lead contamination after 70 days (94 +/- 10 ppb).

8.5.7. Surface characterization

Prior to stagnation, brass plumbing materials showed significant levels of Pb in the matrix of the material. An example of Pb as contaminant particle on the sample surface of brass brand 1 (B1) is shown in Figure 8.9A and B. The unused B1 sample at S1 (blue) contained 5.4 % of Pb, Figure 8.9B and Table 16.2. The B1 plumbing material exhibited traces of Pb as contaminant particles on the sample surface from both the unused and 70 days stagnation samples. The 70 days stagnation samples are shown in Figure 8.9 C and D. At S2 (red) bright particle can be seen indicating a high atomic number material that was confirmed as Pb using EDS. The contaminant particles are at 9.9% Pb and 8.5 % Pb (Table 16.2).

Stainless steel plumbing materials also showed contamination with Pb as surface contaminants. For example, a piece of unused stainless-steel brand 1 (SS1) sample contained traces of Pb (0.5 %) in the form of contaminant particles deposited on the material surface, as shown in Figure 8.10A. However, no Pb was found on SS1 materials after 70 days stagnation. No detectable concentration of Pb was found using EDAX, which has a detection limit of a few ppm, on either the unused material or after 70 days stagnation as shown in Figure 8.10 (S2). If Pb exists on the either brand of stainless-steel samples, it would be below the limit of detection.

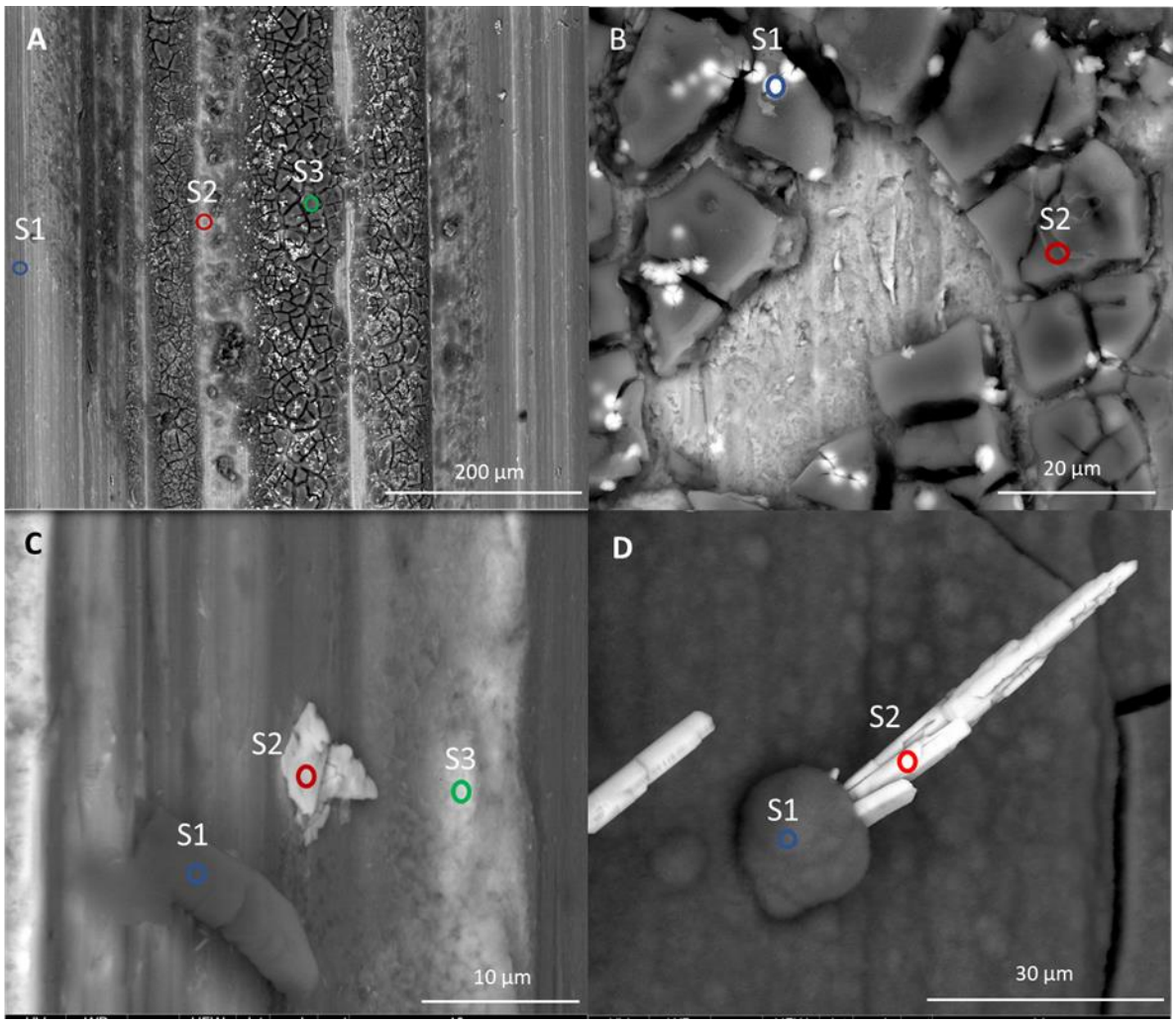

Figure 8.9 - Backscattered electron micrographs of brass brand 1 (B1) plumbing sample.

(A) Unused B1 material with bright regions representing Pb contamination throughout the sample thread. (B) Unused B1 material showing Pb in the brass grain structure. (C) after 70 days stagnation B1 material showing contaminant Pb particles at spot 2 (S2) and impregnated material at spot 3 (S3). (D) 70 days stagnation brass sample with a rod shaped Pb particle

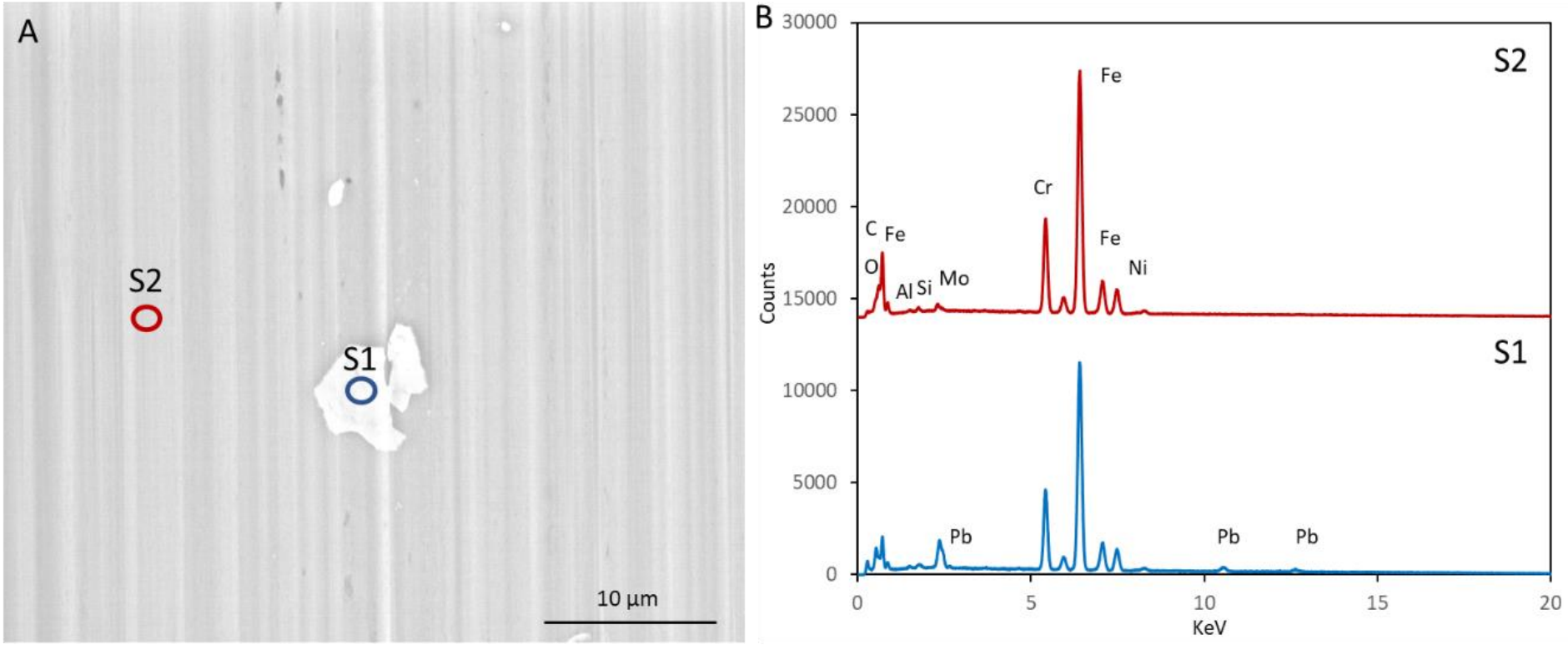

Figure 8.10 - Surface characterization of unused stainless-steel brand 1 plumbing material. (A) Backscattered electron micrograph of unused stainless-steel brand 1 plumbing material. The blue (S1) and red circles (S2) showing the position of spot EDAX analysis (B) EDAX spectra collected from unused samples at S1 (blue spectra) showing small concentration of Pb and S2 (red spectra) with no Pb.

8.6. Discussion

This study used model plumbing bioreactor systems to demonstrate that prolonged periods of stagnation result in significantly higher concentrations of lead leaching in brass potable plumbing systems compared with stainless steel. However, conversely the concentrations of OPPPs, *A. baumannii, P. aeruginosa* and MAC, were predominately higher in stainless steel compared with the brass bioreactors.

8.6.1. Lead

Product testing requirements typically use short leaching times using neutral pH solutions, which do not accurately reflect stagnation conditions seen in premise plumbing environments (Lei et al., 2018). Previous research has suggested that lead leaching from brass reaches a 'peak' after 24 hours and reaches an equilibrium state thereafter (Tam et al., 2009). However, lead levels in stagnant water from bioreactors constructed of both brands of brass significantly exceeded 10 ppb. Lead leaching also increased significantly over time in both brands of brass up to 194 ± 75 (B1) and 553 ± 237 ppb (B2) (Figure 8.8). Despite being marketed as a 'low lead' alternative, stagnant water in both brands of stainless steel plumbing materials exceeded current WHO, US EPA and Australian drinking water guidelines after 30 days stagnation and levels further increased after 70 days stagnation (enHealth, 2018; U.S. Environmental Protection Agency, 2020b; World Health Organization, 2017). Factors such as decreasing pH, changing water treatment, pipe age and galvanic corrosion have been shown to influence lead leaching under periods of stagnation (Cartier et al., 2012; Lei et al., 2018; Montagnino et al., 2022). Long term leaching of lead from brass fittings is due to dezincification from high chlorine concentration exposure or high flow rates. However, in this study, there was no significant change in stagnant water pH over time in either brass or stainless-steel bioreactors and residual chlorine was quenched prior to the experiment (Table 16.4).

The processes used to manufacture plumbing materials have a significant effect on lead contamination of water. SEM and EDAX analysis showed lead particles on the surface and throughout the brass plumbing materials. This was expected as lead is used as an additive in the manufacturing process to improve malleability of the material (Cartier et al., 2012). Conversely, analysis of stainless-steel materials found lead from particles on the material surface rather than embedded within the material itself (Figure 8.10). This is suspected to be a result of unintended impurities from the manufacturing process and postproduction threading (Lei et al., 2018). The Water Mark Certification Scheme is a standard that all manufacturers of tapware need to obtain for products to be sold legally in Australia (Australian Building Codes Board, 2023). However, consumers and contractors may still source non-compliant plumbing materials that have been manufactured overseas.

8.6.2. Microbial enumeration techniques

This study used both culture and molecular based microbial detection methods to overcome limitations associated with either technique. Traditional culture-dependent methods have long been considered the 'gold standard' for microbial water quality monitoring (Deshmukh et al., 2016). Microbial indicators, such as HPC, are often used to provide an indication of the potential pathogenic bacterial load of a water sample (International Organization for Standardization, 2014; Wen et al., 2020). However, many OPPPs such as MAC are more resistant to disinfectant treatment than indicator bacteria and may not follow the same trends (Nisar et al., 2020a; Richards et al., 2018). Additionally, OPPPs may transition to a dormant or viable but not culturable state that would result in a false negative result or an underestimation of the true bacterial load (Dwidjosiswojo et al., 2011a; Whiley et al., 2016). In this study, water in both plumbing materials always exceeded WHO (100 CFU/mL) and CDC (500 CFU/mL) HPC recommendations with stainless steel bioreactors having significantly more HPC than brass in 75% of instances (Centers for Disease Control and Prevention, 2003; World Health Organization, 2017). Despite water in brass bioreactors exceeding these guidelines, no culturable *P. aeruginosa* or *A. baumannii* were recovered from water or biofilm samples at either time points. Conversely, *A. baumannii* and *P. aeruginosa* cultured from water and biofilm taken from stainless steel bioreactors followed the same trend as the HPC. These data suggest that culture dependent methods and the use of microbial indicators do not consistently represent the pathogenic bacterial load of a water sample.

The molecular technique qPCR is frequently used in environmental surveillance due to rapid turnaround time and sensitivity (Botes et al., 2013). qPCR will simultaneously amplify and quantify a target DNA sequence, irrespective of cell viability. Although this technique can detect viable but not culturable bacteria (VBNC) cells that traditional culture typically overlooks, the main limitation is potential overestimation due to the amplification of dead cells and false positive results (Botes et al., 2013; Whiley et al., 2016).

Flow cytometry has also been applied to characterize the total VBNC bacterial cell population in environmental water samples. However, this technique is not species specific unless individual fluorogenic antibodies are used (Füchslin et al., 2010). To overcome these limitations and bridge the gap between qPCR over estimation and culture underestimation, a flow cytometry and qPCR assay (VFC+qPCR) that has been developed to quantify and characterize the viability of OPPPs such as *L. pneumophila* was used (Nisar et al., 2023a). In the present study, VFC+qPCR was able to successfully quantify alive and injured *P. aeruginosa* cells. This is advantageous over traditional culture methods that underestimate the total concentration.

8.6.3. *Acinetobacter baumannii*

Water and biofilm from stainless steel bioreactors had significantly more culturable and injured *A. baumannii* than brass bioreactors. Furthermore, alive (VFC+qPCR) *A. baumannii* cells were only

found in biofilm formed on stainless steel plumbing materials after 70 days stagnation. *A. baumannii* is able to persist in the harsh engineered water system environment due to biofilm formation (Harding et al., 2018). Published literature has demonstrated that when *A. baumannii* cells are injured under prolonged high stress conditions, they become dormant as a form of passive defense (Barth Jr et al., 2013; Dekic et al., 2019). Tolerance to copper ions has been shown when exposed to sub-inhibitory concentrations, with *A. baumannii* in biofilms being more resistant to copper than planktonic cells over time (Williams et al., 2016). Future research is needed to investigate the virulence and public health significance of VBNC *A. baumannii* (Thummeepak et al., 2020).

In this study, if one was to only measure the total *A. baumannii* copies/cm^2 these data would suggest that biofilm formed on stainless steel plumbing materials poses a significantly greater risk to public health than the *A. baumannii* biofilm formed on brass plumbing materials after 30 days stagnation. However, no alive or injured *A. baumannii* were detected in biofilm samples isolated from stainless steel after 30 days stagnation demonstrating that assessing only total qPCR copies/cm^2 is an inappropriate measurement for public health risk. After 30 days, no alive *A. baumannii* was detected by VFC+qPCR regardless of plumbing material in stagnant water; however, stagnant water and biofilms from stainless steel bioreactors were positive for *A. baumannii* using selective culture. However, alive *A. baumannii* were detected via VFC+qPCR in stainless steel biofilms after 70 days stagnation. This discrepancy could be due to the water and biofilm samples going through numerous preparation steps prior to VFC+qPCR analysis (See 8.4.3), which could potential result in the generation of additional dead cells and is a possible limitation with this study. This could have resulted in a reduction of the alive and injured population below the limit of detection which has previously been reported as 10^2 GU/L (Nisar et al., 2023a). Additional concentration steps such as membrane filtration and/or centrifugation of a larger sample size could be considered for future studies to identify pathogens at low concentrations (Safford et al., 2019). This will help determine whether the *A. baumannii* alive VFC+qPCR fractions fell below the limit of detection, or if the culture positive samples were indicative of culturable alive or injured *A. baumannii*.

8.6.4. *Pseudomonas aeruginosa*

P. aeruginosa has long been a model organism for the formation of biofilm in premise plumbing due to its emergence as a multidrug resistant clinical threat and persistence under environmental stress (E. Bédard et al., 2016). *Pseudomonas* spp. are considered the building blocks of plumbing biofilm as they produce proteins and polysaccharides that increase elasticity and cross linking within the biofilm matrix which increases protection against harsh environmental conditions (Liu et al., 2016). Although the ions released by the brass material may have greater antimicrobial activity towards *A. baumannii*, these dead and injured cells can settle onto the pipe surface and act as attachment sites for other persisting species such as *P. aeruginosa* to colonize. Alive *P.*

aeruginosa in water and injured *P. aeruginosa* in biofilm were the only two instances/measurements that brass bioreactors had statistically significantly ($p<0.05$) more copies when compared with stainless steel bioreactors. Longer term studies with more frequent sampling points are required to determine if this 'injured' population is simply transitioning to becoming dead cells, or if this population is maintained overtime in response to copper ion release. *P. aeruginosa* can enter a VBNC state in response to copper ions released by brass materials and can recover their culturability and pathogenicity when this stress is removed (E. Bédard et al., 2016; Dwidjosiswojo et al., 2011a). Although materials such as brass and copper may not completely disrupt the viability of *P. aeruginosa*, it has been considered preferable over materials that promote growth (Dwidjosiswojo et al., 2011a). The *P. aeruginosa* total qPCR copies/mL also suggests that stagnant water and biofilm in stainless steel materials after 70 days stagnation poses a significantly greater public health risk than brass materials. These injured VBNC *P. aeruginosa* are also still able to form biofilm on surfaces and transition to a mucoidal phenotype that results in reduced susceptibility to host defense mechanisms and increased drug resistance (Jeanvoine et al., 2019). Although stainless steel biofilms contained significantly more injured *P. aeruginosa* copies/cm^2 at both time points, both stainless steel and brass showed significant increases in injured *P. aeruginosa* in biofilm over time. Further research is required to investigate the pathogenicity of injured *P. aeruginosa* and to develop more comprehensive risk assessment models.

8.6.5. *Mycobacterium avium complex*

In this study, the highest number of alive and injured MAC cells were recovered from biofilm rather than in the water, with significantly more injured copies/cm^2 evident in stainless steel biofilm than brass with no significant difference between materials in the alive copies/mL. Prolonged stagnation resulted in statistically significant ($p<0.05$) increases in total (qPCR) and injured (VFC+qPCR) copies/cm^2.These data are consistent with previous research suggesting MAC is more frequently found on pipe surfaces compared to bulk water (Falkinham, 2018; Hamilton et al., 2017; Torvinen et al., 2004). Concerningly, aggregate forms of M. avium species have been found to be more virulent than planktonic form (Lopes Leivas Leite, 2015). MAC cells have relatively high resistance to copper ions than other *Mycobacterium* spp. and OPPPs, resulting in brass and copper piping acting as a selective environment for MAC biofilm adherence (Dwidjosiswojo et al., 2011a, 2011b; Falkinham, 2018; Pianetti et al., 2008).

8.6.6. *Acanthamoeba polyphaga*

A defining characteristic of OPPPs is their resistance to, and growth within phagocytic FLA (Falkinham, 2015). Rather than being killed by FLA, the OPPPs are engulfed and the FLA act as a shield, protecting the OPPP from disinfectants and conferring increased virulence (Ashbolt, 2015). There was no significant difference in levels of *A. polyphaga* in stagnant water and biofilm between materials, indicating that plumbing material did not impact *A. polyphaga* growth. FLA are

notoriously resistant to chemical and physical stresses, particularly when forming cysts (Thomas et al., 2010). FLA in growing in potable water benefit from 'grazing' on biofilms as food sources. However, the proliferation of FLA within complex biofilms, particularly under chemical stress requires further investigation (Thomas et al., 2010). *A. polyphaga* persistence may have impacted the detection and viability of the other target OPPPs introduced to the bioreactors. Target OPPPs that remained within *A. polyphaga* at the time of testing may have been missed by the VFC+qPCR and selective culture techniques used. Intracellular multiplication of *A. baumannii*, *P. aeruginosa* and MAC within *A. polyphaga* has been observed in premise plumbing (E. Bédard et al., 2016; Cateau et al., 2011; Steinert et al., 1998). FLA play a vital role in biofilm formation and maturation, as they can become early colonizers of surface biofilms by feeding on dead bacterial cells and provide structure for subsequent bacterial attachment (Thomas et al., 2010). Concerningly, amoeba grown MAC and *P. aeruginosa* have displayed enhanced virulence in animal models and reduced susceptibility to frequently used antibiotics such as clarithromycin and azithromycin (Cirillo et al., 1997; Leong et al., 2022; Miltner et al., 2000).

8.6.7. Recommendations and future considerations

The influence of plumbing materials and water system maintenance on water quality has been the focus of increasing research (Lee et al., 2021; Morvay et al., 2011; Nisar et al., 2020b). Adverse health implications from lead exposure in drinking water are typically associated with long term chronic exposure (Centers for Disease Control and Prevention, 2021). Brass plumbing materials resulted in the highest lead leaching in the present study at both sampling points. Concerningly, despite being marketed as an appropriate low lead alternative, stainless steel plumbing materials also caused lead leaching above public health guidelines. Additionally, there was significant differences in the quantity of OPPPs between brands of the same material despite being manufactured to the same standards. The manufacturing consistency and potential sources of contamination of these alternative materials needs to be investigated before a widespread change in policy is introduced. The present study investigated biofilm formation and lead leaching from brand new plumbing products at a consistent temperature without addition of more disinfectants and corrosions inhibitors not already present in the source water. The long-term relationships between biofilm maturation, corrosion rate and lead leaching along with additional relevant elements such as copper and chromium ions and residual disinfection within a dynamic system remain unclear and requires further investigation.

OPPP persistence and risk in this study was also influenced by material type and stagnation time. However, unlike lead exposure, a once off exposure to an infectious dose of an OPPP via inhalation, ingestion or aspiration could result in a public health consequence through someone developing an infection (Collier et al., 2021). Where comparable data are available, it suggests that the overall burden of disease from OPPPs is likely far greater than that from lead from potable water, with water contributing a relatively minor fraction of the overall lead body burden. It is

therefore critical to understand how different plumbing materials contribute to both lead contamination and the proliferation of OPPPs, with an evidenced based approach essential to any regulatory intervention that will guide materials available to the plumbing industry.

Although this study was a laboratory model system, steps were taken in method development to ensure conditions simulated real world premise plumbing scenarios. Municipal drinking water was used in this system and was spiked with concentrations of OPPPs that are comparable to those found in the environment and to simulate the mutualistic relationship these pathogens have when present in biofilms (Briancesco et al., 2010; Josephson et al., 1997b; Masaka et al., 2021; Ojima et al., 2002a; Rhodes et al., 2014; Tichenor et al., 2012a). Previous research characterizing the bacterial communities found in potable water and biofilms has found positive correlations between *Pseudomonas* and *Acinetobacter,* and no significant correlations between these genera and *Mycobacterium* (Hayward et al., 2024; Nisar et al., 2023b; Thomas et al., 2010). It has also previously been shown that *Acanthamoeba* and other FLA in drinking water harbour intracellular *M. avium* complex, *P. aeruginosa* and/or *A. baumannii* (Thomas et al., 2010). This study focused on brass and stainless steel as a 'low lead' alternative. However, premise plumbing systems are comprised of many types of materials including galvanized steel, cement, cast iron, copper and polyvinyl chloride materials (Cullom et al., 2020). The methodology developed in the present study can be used to explore the antimicrobial activity of other materials. Future research is needed to compare a wider range of material types and include additional sampling time points to provide a more detailed profile of lead leaching and corrosion over time.

The present study showed that control of both lead contamination and OPPPs cannot be addressed by pipe material choice alone. However, extended stagnation resulted in lead concentrations and OPPP persistence that is considered a risk to public health. Increased stagnation and low water demand, due to routine school holidays and COVID-19 related lockdowns, raised concerns about increased lead leaching from brass tapware and OPPP proliferation (Liang et al., 2021; Salehi et al., 2021). Longer term studies have demonstrated elevated levels can persist for as long as 5 months (D.-Q. Ng et al., 2016). Prolonged stagnation can also result in the accumulation of nutrients, corrosion products that promote the growth and dissemination of OPPPs (Ling et al., 2018; W. J. Rhoads et al., 2016). Therefore, the consistent flushing of stagnant water can be used as an effective strategy to reduce both water quality risks if implemented appropriately. The US EPA and enHealth recommended flushing outlets for 30 seconds when first used after periods of stagnation to minimize lead exposure (enHealth, 2018; Katner et al., 2018). Reducing temporal and permanent dead leg stagnation is also recommended to building owners, facility managers and plumbing in drinking water guidelines to control OPPPs (enHealth, 2015; National Health and Medical Research Council, 2011; Nisar et al., 2020b; World Health Organization, 2017). Flushing plumbing outlets brings in fresh municipal drinking water with higher residual disinfectant and reduces contact time with pipe surface to minimize biofilm

establishment (Ling et al., 2018; Nisar et al., 2020b). Further controlled studies such as the present model, and broader premise plumbing case studies are required to establish appropriate control strategies that can minimize multiple water related public health threats.

8.7. Conclusions

In this study, the use of a model plumbing system showed that stainless steel plumbing materials support the growth of OPPPs to a greater extent compared with brass. Lead leaching into the stagnant water in both these bioreactors exceeded drinking water guidelines for lead contamination; however, higher lead concentrations were seen in the brass bioreactor compared with the stainless steel. Further research is needed to explore the influence of plumbing materials on water quality under a range of variable environmental conditions before changes are made to current regulations.

8.8. Acknowledgments

C.H., K.E.R., M.H.B., R.B., H.W. conceived and designed the experiments. C.H. performed the experiments. G.B. and C.H. conducted flow cytometry + qPCR assay. S.H. conducted scanning electron microscopy with X-ray photoelectron spectroscopy analysis. C.H., S.H., G.B. and H.W. drafted and edited the manuscript. K.E.R., M.H.B., R.B., P.J.M and J.H. corrected and contributed to the manuscript. All authors approved the final manuscript. The research was funded by Enware Australia Pty Ltd through contract research. The funders had no role in the experimental design, collection or analyses of data.

8.9. Statements and declarations

8.9.1. Financial Disclosure Statement

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8.9.2. Supporting information

This material is available free of charge via the internet at <a href='http://pubs.acs.org.xn--ivg/'>http://pubs.acs.org.

Tables describing the qPCR conditions including primer and probe sequences, assay conditions and references along with atomic percentages of elements from brass and stainless steel plumbing

materials measured using Energy Dispersive Analysis by X-ray (EDAX) Ocane Pro EDS detector, and water quality parameters collected from stagnant water samples.

9. DISCUSSION

OPPPs, also referred to as drinking water associated pathogens, are waterborne pathogens that can pose a significant health risk to vulnerable populations (Falkinham, 2015; Proctor et al., 2022). These pathogens share characteristics that enable them to persist and grow in drinking water plumbing systems (Falkinham, 2015). Despite these pathogens gaining increased attention, particularly due to the rise of antimicrobial resistance, the incidence of infections associated with them remains high (Centers for Disease Control and Prevention, 2019a, 2020b; Collier et al., 2021; Jasen M. Kunz, 2024; World Health Organization, 2024). This trend emphasises the need for improved water management strategies to control OPPPs in drinking water plumbing systems. It is essential that these strategies are evidence based, and risk assessments are conducted to understand how these different design and management strategies may impact the public health risk.

The research presented in this thesis explores the interactions OPPP prevalence, antimicrobial resistance, interactions with protozoan hosts and the effects of abiotic factors including building type, product design and plumbing material on the growth and proliferation of these OPPPs. This research was conducted in four phases:

1. In the **first phase**, a systematic literature review was conducted to identify pathogens present in drinking water and water related devices such as showers, hand washing basins and baths in residential properties (CITATION: The presence of opportunistic premise plumbing pathogens in residential buildings: A systematic review, Page 5). It was identified that:
    a. Residential buildings have increasingly been identified as reservoirs for OPPPs such as *Legionella pneumophila*, *Pseudomonas aeruginosa*, and *Mycobacterium avium*, in addition to pathogens not typically considered waterborne such as *Staphylococcus aureus* and *Enterobacter* spp. which all pose health risks to immunocompromised individuals and the elderly;
    b. Factors such as plumbing material, building age, temporal stagnation, and water heater type all influence OPPP growth and persistence. Stagnation was found to lower disinfectant efficacy and create an environment conducive to biofilm formation;
    c. Unlike hospitals, residential properties lacked routine water treatment and monitoring. There is a need for guidelines specifically tailored to address OPPP risks in residential environments;

d. Current research and regulatory guidelines focus largely on public and commercial water systems, with limited attention to residential drinking water plumbing systems. Addressing this gap is critical for developing effective OPPP management strategies in healthcare at home environments.

2. The **second phase** of this study consisted of three parts. In the first part, drinking water and biofilm samples were collected from hospitals and residential water systems and screened for *P. aeruginosa*, *S. aureus*, *Legionella* spp., *L. pneumophila*, *A. baumannii* and protozoan hosts *Acanthamoeba* spp. and *Vermamoeba vermiformis* using molecular based methods (CITATION: Microbial risks associated with drinking water and plumbing biofilms: Prevalence of opportunistic premise plumbing pathogens in healthcare and residential settings, Page 52). In the second part, a subset of these samples were screened for AMR *P. aeruginosa*, *A. baumannii* and *S. aureus*, informed by a narrative review investigating the role of hospital water as the source of antimicrobial resistant infections (CITATION: Drinking water plumbing systems are a hot spot for antimicrobial resistant pathogens, Page 84). In the third part, the microbial community composition of biofilm samples from hospital and residential handwashing basin faucets and drains were compared (CITATION: Handwashing basins and healthcare associated infections: Bacterial diversity in biofilms on faucets and drains, Page 102). It was identified that:
    a. Australian hospital and residential drinking water systems were frequently colonised by *P. aeruginosa* (41%), *S. aureus* (26%), *Legionella* spp. (26%), *L. pneumophila* (24%) and *A. baumannii* (14%);
    b. Free living amoeba *V. vermiformis* (46%) and *Acanthamoeba* spp. (25%), were frequently detected, with *Acanthamoeba* spp. demonstrating a significant positive correlation with all bacterial OPPPs;
    c. The detection of *S. aureus*, not typically considered waterborne, highlights the need to reconsider drinking water plumbing biofilms as a reservoir for unexpected health risks;
    d. Overall, results indicated a statistically higher prevalence of OPPPs in residential properties and biofilms. However, building characteristics, including stagnation, hot water system type, and building age, showed inconsistent impacts on individual OPPP prevalence;
    e. Drain biofilms were the most common reservoir for AMR *A. baumannii, S. aureus* and *P. aeruginosa*;
    f. Using culture, 10% of samples were positive for *P. aeruginosa*, 8% for *A. baumannii* and 7% for *S. aureus*. Of these culture isolates, 29% of *P. aeruginosa* and 28% of *A. baumannii* culture isolates were carbapenem resistant, and 54% of *S. aureus* isolates were identified as MRSA.

3. In the **third phase** of this study, a model handwashing basin was built to determine the role of flow rate on aerosol production from source water and contaminated drains (CITATION: The impact of water flow rates on bioaerosol production from handwashing basins, Page 129). It was identified that:
    a. Lower flow rates led to significantly a higher generation of aerosols from the contaminated drain (p=0.021) and increased retrograde contamination from the drain to the faucet compared to higher flow rates (p=0.033);
    b. Modifying design elements, such as aerator design and flow rates, could potentially reduce infection risks, but the optimal configuration remains unclear.
4. The **fourth phase** of this study investigated the influence of plumbing material on biofilm formation, growth of OPPPs and lead leaching under stagnant conditions simulating those found within premise plumbing in response to legislative changes limiting the allowable level of lead in the manufacture of materials such as brass and copper with the promotion of 'lead free alternatives' such as stainless steel (CITATION: Comparison of the antimicrobial activity of brass versus stainless steel against opportunistic premise plumbing pathogens, Page 148). This phase identified that:
    a. Each OPPP responded differently to pipe material and stagnation time;
    b. Overall, brass had greater antimicrobial activity compared to that of stainless steel;
    c. Both brass and stainless steel leached lead into the stagnant water at levels exceeding World Health Organisation (WHO) water quality guidelines.

9.1. Persistence and growth of opportunistic premise plumbing pathogens in drinking water plumbing systems

The growth and persistence of OPPPs in drinking water plumbing systems is influenced by multiple biotic and abiotic factors, including existing microbial water quality, subinhibitory residual disinfection, stagnation and building infrastructure design (Gomez-Alvarez et al., 2023; Hayward et al., 2024; Logan-Jackson et al., 2023; Nisar et al., 2020b). Furthermore, the risk of OPPP transmission is influenced by product design, flow rate and the users vulnerability (Benoit et al., 2021; Ehrlich et al., 1970; Fusch et al., 2015; Kotay et al., 2019; Lv et al., 2019; Park et al., 2013; Sebastian Schulz-Stübner et al., 2021; Takajo et al., 2020). Many current international infection control and prevention guidelines recognize contaminated water sources as potential reservoir for HAIs. However, these guidelines note that risk factors such as facility design, point-of-use filters, and water temperature, require further study (Centers for Disease Control and Prevention, 2024a; National Health and Medical Research Council, 2011, 2019; World Health Organization, 2022). Based on the findings from this research, it is suggested that these standard guidelines need to use a dynamic multi-barrier approach to control OPPP persistence in drinking water and water related devices and minimise the risk of disease transmission to vulnerable populations.

Throughout this thesis, the following three aspects were consistently identified as areas that need to be addressed to deliver this multi-barrier solution:

- Expand the understanding of OPPP diversity
- Structural and design considerations
- Enhanced surveillance of OPPPs

9.1.1. Expand the understanding of opportunistic premise plumbing pathogen diversity

Over the past few decades, the threat of waterborne illness has shifted from enteric pathogens to OPPPs such as *P. aeruginosa*, *L. pneumophila*, and MAC (Collier et al., 2021). OPPPs are traditionally considered to be waterborne bacteria that originate from the source water and are therefore ubiquitous throughout the drinking water distribution system (Falkinham et al., 2015). This perspective has informed water treatment approaches to primarily target these waterborne pathogens, neglecting potential threats from alternative sources. As evidenced throughout this thesis, the scope of OPPPs and the resultant drinking water treatment approaches, must be broadened to include pathogens not typically considered waterborne, such as those originating from the end user or from surrounding environmental surfaces.

The literature review, conducted in Chapter 1, identified gaps in the current understanding of OPPPs, highlighting that pathogens such as *Enterobacter* spp., *K. pneumoniae*, *A. baumannii*, and *S. aureus* are clinically significant, however reports of their presence in drinking water plumbing systems was infrequent (Hayward et al., 2022b). Detection was often limited to chronic infection case studies rather apart of routine surveillance, unlike *L. pneumophila*, which is regularly monitored and reported (Abera et al., 2014; Antony et al., 2016; Barna et al., 2016a; Buttery et al., 1998; Chapuis et al., 2016; Cox et al., 1998; Decraene et al., 2018; Dijk et al., 2002; Donohue et al., 2019; French et al., 2004; Katz et al., 2015; Sexton et al., 2006; Ziwa et al., 2019). This reporting inconsistency was further complicated by inconsistent detection methods, that make it difficult to accurately understand the role of drinking water and water related devices in diverse HAI infection transmission (Deshmukh et al., 2016; Nisar et al., 2023a). In this thesis, both molecular and culture-based surveillance was conducted (Chapters 4 & 5), and demonstrated the widespread prevalence of these diverse pathogens in both hospital and residential drinking water plumbing systems. Quantitative PCR demonstrated a high prevalence of *P. aeruginosa* (41%), *S. aureus* (26%), *Legionella* spp. (26%), *L. pneumophila* (24%), and *A. baumannii* (14%). Additionally, free-living amoebae such as *V. vermiformis* (46%) and *Acanthamoeba* spp. (25%) were frequently detected. *Acanthamoeba* spp. showed significant positive correlations with all target bacterial OPPPs, demonstrating complex interactions within these microbial communities. Conversely,

selective culture revealed that 10% of samples were positive for *P. aeruginosa*, 8% for *A. baumannii* and 7% for *S. aureus* (Chapter 5).

Further molecular characterization of biofilms from handwashing basins collected from both residential and hospital settings (Chapter 6) demonstrated diverse microbial communities, including high abundances of potentially corrosive, biofilm-forming, and pathogenic genera. The frequent detection of potentially AMR species such as *P. aeruginosa*, *A. baumannii* and *S. aureus* in Chapters 4 – 6 led to further analysis investigating the prevalence of key AMR threats identified by the WHO and CDC (Centers for Disease Control and Prevention, 2019a; World Health Organization, 2024). This analysis found that 29% of *P. aeruginosa* and 28% of *A. baumannii* isolates were carbapenem-resistant, while 54% of *S. aureus* isolates were identified as MRSA. Additionally, *P. aeruginosa* carbapenem resistance genes, such as $bla_{\text{NDM-1}}$, $bla_{\text{OXA-48}}$, $bla_{\text{KPC-2}}$, and bla_{VIM}, were detected in biofilm samples that were otherwise negative for *P. aeruginosa*. This finding suggests that biofilms in drinking water plumbing systems may serve as a reservoir for extracellular DNA.

The findings of **phases 1 and 2** challenge the traditional perspective of OPPPs and emphasizes the need to expand water treatment and surveillance strategies to address the diverse pathogens present in drinking water plumbing systems. Current water treatment strategies focus primarily on pathogens originating from source water through disinfection and maintenance of residual disinfectant levels through to the POU. However, these approaches may not be appropriate to address pathogens introduced at the POU or those that persist in biofilms on plumbing fixtures.

9.1.2. Structural and design considerations

The implications of building age and type, stagnation, fixture design and drinking water system maintenance on the persistence of OPPPs and associated risk of HAI transmission are not well understood (Hayward et al., 2022b; Logan-Jackson et al., 2023; Nisar et al., 2020b; Nisar et al., 2023b). These factors interact in complex ways, impacting the microbial ecology of drinking water plumbing systems and the resultant public health risks.

Traditional water treatment protocols, building maintenance practices, and infection control guidelines to address the risk of OPPP are typically tailored to “high risk” environments like hospitals (Centers for Disease Control and Prevention, 2003; Cervia et al., 2008; Decker et al., 2014; World Health Organization, 2018). In these settings, there is greater control over building infrastructure design, as well as an ability to implement additional water disinfection and control strategies, such as heat shock and hyperchlorination (Decker et al., 2014). These practices aim to minimize the risks associated with OPPPs, with an emphasis on maintaining water quality standards to protect vulnerable populations.

However, this thesis highlights that residential properties present an emerging risk for the growth of OPPPs, particularly in light of the promotion of healthcare-at-home services (Chapter 4). Unlike hospitals, residential properties often lack standardized guidelines for product and infrastructure design. This means that many homes may not be equipped with appropriate plumbing infrastructure suitable for controlling microbial growth.

9.1.2.1. Building design and infrastructure

For example, water management protocols have emphasized minimizing periods of low water demand and stagnation, as these conditions can increase pathogen growth and biofilm formation (Chen et al., 2020; Lautenschlager et al., 2010; Ley et al., 2020; Lipphaus et al., 2014; Nisar et al., 2020b). Removal of long-term stagnation areas such as dead legs in hospitals has been highlighted in current WHO, SA Health and enHealth *Legionella* management guidelines (enHealth, 2015; World Health Organization, 2017). However, in residential buildings, water also stagnates temporarily in water storage tanks, piping, and in water outlets (i.e., shower heads, tap faucets, etc.) for a few hours to weeks (Salehi et al., 2020; Zlatanović et al., 2017). In this thesis, the impact of temporary stagnation on OPPP growth and biofilm formation was examined through real-world drinking water plumbing samples surveillance (Chapter 4) and a laboratory-scale plumbing model (Chapter 8). In Chapter 4, results revealed that the prevalence of *Acanthamoeba* spp., *V. vermiformis*, *P. aeruginosa*, and *S. aureus* increased significantly as outlet usage decreased. Conversely, *A. baumannii* prevalence was higher in areas with increased outlet use, likely due to frequent end-point contamination. Although minimizing stagnation by encouraging regular outlet use may reduce pathogen growth in stagnant drinking water, this intervention could inadvertently increase end-point contamination rates. This may result in seeding biofilms with a broader range of pathogens, complicating microbial risk control efforts. In Chapter 8, the results of the laboratory scale bioreactor model demonstrated that plumbing material influenced each OPPPs response to periods of stagnation. For example, alive *P. aeruginosa* cells (VFC+qPCR) in stainless steel biofilms increased significantly ($p=0.037$) between 30 and 70-days stagnation, demonstrating growth during temporal stagnation. However, there was a decrease in alive *P. aeruginosa* cells/cm^2 in brass bioreactors over time. Conversely, there was no significant ($p>0.05$) differences in total, alive or injured (VFC+qPCR) MAC copies/mL observed in any of the bioreactors following 30 days and 70-days stagnation.

The results of both the real-world surveillance and laboratory scale bioreactor model study conducted in **phases 2 and 4** demonstrated that reducing periods of stagnation is an effective management strategy for controlling the growth of some OPPPs. However, this strategy should be complemented by additional approaches to address those pathogens that are not influenced by stagnation.

9.1.2.2. Influence of product design and flow rate

Product design and flow rates are factors that can influence the growth and transmission of OPPPs. Using an evidence-based approach to decision making in these areas can significantly reduce public health risks. However, past approaches to drinking water plumbing system design has often lacked robust evidence, resulting in unintended outcomes such as failing to address causes of contamination, increase microbial growth, or the introduction of new health risks. This oversight highlights the need for future innovations to be informed by multidisciplinary research to ensure that product and infrastructure modifications effectively reduce OPPP risks without creating additional hazards.

This research demonstrates that handwashing basins may play a pivotal role in the persistence and transmission of OPPPs. While handwashing is a cornerstone of infection prevention, the basin itself may paradoxically act as a reservoir and dissemination point for pathogens. Basin design, material, and operation, such as water flow rates and drainage systems, can significantly influence microbial growth and biofilm formation (Hayward et al., 2022a; Hayward et al., 2024; Hota et al., 2009; Jung et al., 2020; Kanamori et al., 2016; Sharma et al., 2021). Additionally, interactions between product design and usage patterns can increase risks. Droplets and aerosols generated during handwashing can serve as potential pathways for pathogen transmission (Benoit et al., 2021; Kotay et al., 2019).

This connection between handwashing basins and OPPP transmission has been made primarily from extensive outbreak investigations (Hayward et al., 2022a; Hayward et al., 2024; Hota et al., 2009; Jung et al., 2020; Kanamori et al., 2016; Sharma et al., 2021). Typically, these studies have detected the causative pathogen in the handwashing basin, and hypothesized the spread of the pathogen was from splashing to the user or the surrounding surface. As a result, the identified handwashing basin is disinfected or removed (Tracy et al., 2020). However, these measures are often not effective as they do not address the original causes of contamination or mechanisms of transmission (Volling et al., 2021). In response, several health authorities have updated water management and infection control guidelines to address aerosol and droplet dispersion from handwashing basins including specifying drain placement and flow rate (National Health Service England, 2013b; National Health Sustainability Office, 2020). Flow rates recommended for handwashing basins in high-risk settings vary widely depending on priorities such as water conservation, user experience, and safety concerns such as scalding. However, these changes are often based on limited evidence regarding their effectiveness in reducing infection transmission risk. This thesis has demonstrated that handwashing basins are able to aerosolize bacteria from the source water and drain. Lower flow rates increased aerosol and droplet dispersion from drains compared to higher flow rates. This is likely due to the increased water pressure hitting the drain, which increases aerosolization. These findings suggest that while the intention behind design

modifications may be to improve safety, some changes may inadvertently increase the risk of OPPP transmission, emphasising the need for robust evidence to guide design and policy decisions.

9.1.3. Enhanced surveillance of opportunistic premise plumbing pathogens

Surveillance and data reporting are critical components in controlling waterborne AMR HAIs. Surveillance systems are necessary to identify outbreaks, assess risks, and implement appropriate interventions to prevent future outbreaks (Collier et al., 2021; Hayward et al., 2022b). However, this surveillance approach must be informed by the risk factors identified in this thesis, particularly those relevant to residential properties.

A comprehensive surveillance framework must address both water and biofilms formed on drinking water related devices. This thesis highlights that biofilms can harbor pathogens not traditionally considered waterborne, such as *S. aureus*. Testing strategies must move beyond traditional targets such as *Legionella* spp., to include a broader spectrum of potential pathogens. To do this, methods must include molecular techniques alongside culture-based approaches and acknowledge the limitations of relying solely on metrics such as HPCs. Water utilities and building managers must recognize that while HPC can provide general insights into microbial drinking water quality, this approach won't capture complexity and diversity of biofilm-associated microbial communities.

However, using any testing approach, whether culture-based or molecular, to continuously monitor all high-risk areas is not logistically or fiscally feasible. Instead, a proactive approach should be adopted. Surveillance frameworks should focus on identifying high-risk buildings and outlets through the integration of operational data, such as water temperature, flow rates, and stagnation. In hospitals, this approach can be integrated into existing infrastructure and regular facility assessments. To support decision-making, hospitals could use a digital twin of their drinking water systems to create a virtual replica that integrates real-time data from sensors and predictive models (Li et al., 2024). This approach would enable dynamic risk assessments and simulate the effects of potential interventions (Li et al., 2024). This would identify high-risk outlets, such as rarely used faucets or drains near patient care areas, which can then undergo targeted testing and interventions.

Implementing this approach to residential settings would be challenging, as these buildings typically lack centralized monitoring systems and consistent maintenance schedules. Vulnerable individuals receiving healthcare at home require tailored approaches. Infection control teams can implement simple but effective measures, such as identifying infrequently used outlets, advise on regular flushing to prevent temporal stagnation, and maintain appropriate water temperatures.

Low-cost fixture modifications like replacing faucet aerators can reduce biofilm development and minimise aerosolization risks without requiring extensive plumbing infrastructure overhauls.

These strategies allow for efficient allocation of resources while maintaining a high standard of infection control and drinking water maintenance. By combining real-time data analysis with targeted testing, it is possible to proactively manage risks and respond effectively to emerging threats in high-risk environments.

9.2. Implications for infection control and prevention guidelines and areas for improvement

Current water treatment methods were developed in the mid-20th century to target enteric waterborne pathogens such as *E. coli*, *Salmonella*, and *V. cholerae*, when the predominant waterborne infections threats were cholera and typhoid fever (Armstrong et al., 1999; Collier et al., 2021). At the time, the implementation of chlorination and filtration strategies, paired with behavioural changes such as regular handwashing and improved infrastructure was revolutionary. This multidisciplinary approach involved public health professional, engineers, microbiologists, and policymakers. As a result, rates of enteric illness decreased significantly, leading to vastly improved health outcomes (Control et al., 1999).

However, today's primary waterborne risks have evolved. The emerging threats are infections caused by AMR pathogens such as *S. aureus*, *A. baumannii*, and *P. aeruginosa;* organisms that thrive in biofilms within drinking water plumbing systems rather than as planktonic cells in the water. To address these new risks effectively, we must learn from the success of mid-20th-century interventions, which were grounded in multidisciplinary collaboration. The challenges presented by AMR pathogens require similar collaboration between building managers, plumbers, infection control professionals, and water utilities. This collaborative approach should focus on designing drinking water plumbing systems that minimize biofilm development, monitoring AMR pathogen prevalence, and implementing tailored evidence-based interventions. Protecting vulnerable individuals, particularly in residential settings where healthcare delivery is increasing, requires a proactive approach. For example, strategies like optimizing water flow rates, improving cleaning protocols, and redesigning high-risk fixtures can be implemented. By adopting a collaborative perspective that combines expertise from multiple fields, we can develop resilient drinking water systems that are capable of addressing modern health threats to safeguard public health.

10. CONCLUSIONS

OPPPs are a significant public health concern. In particular, the rise of AMR OPPPs has resulted in limited treatment options and has complicated infection control efforts**.** This research was the first in Australia to investigate the prevalence of OPPPs in drinking water and biofilm samples from healthcare and residential drinking water plumbing systems and to determine what factors may influence their growth and transmission. Valuable insight was gained into the diversity of the pathogens present in the drinking water plumbing environment, which established that current water treatment and infection control approaches are overlooking a significant public health risk**.** Notably, it identified the presence of non-waterborne bacteria and key AMR threats such as MRSA in plumbing systems.

This thesis advocates for the integration a dynamic multi-barrier approach into water treatment and infection control protocols that addresses the complex factors that influence OPPP growth and transmission. Hospitals, with centralized monitoring systems and supplementary water treatment protocols, can more readily integrate these changes. However, in residential settings, where healthcare-associated infection risks are increasing, these measures are harder to incorporate. Vulnerable individuals receiving care at home require tailored strategies, including effective cleaning, preventing temporal stagnation, and adjusting flow rates through simple solutions like replacing faucet restrictors.

A collaborative approach between microbiologists, engineers, public health professionals, and policymakers is essential to ensure these tailored water management strategies effectively address the diverse microbial risks, infrastructure design, and public health outcomes. By incorporating the findings of this thesis, future water management strategies can better protect public health and address the evolving risks posed by AMR pathogens.

11. APPENDIX -1

Supplementary data for the manuscript entitled CITATION: The presence of opportunistic premise plumbing pathogens in residential buildings: A systematic review (Chapter 1)

Table 11.1 Summary of reports and studies identifying opportunistic premise pluming pathogens in residential drinking water systems.

Study Site	Reservoir	Pathogen [a]	Prevalence [b]	Detection Method [c]	Antimicrobial Characteristics [d]	Country [e]	Year [f]	Reference
House	Tap Well	*Acinetobacter haemolyticus* *Aeromonas hydrophila* *Pseudomonas aeruginosa*	Water: *P. aeruginosa*: 11.36% well water (166.7 CFU/mL) *A. haemolyticus*: 11.1% tap water (333.3 CFU/mL) *A. hydrophila* 6.82% tap water (333.3 CFU/mL)	Culture	N/A	Saudi Arabia	2014	(Abdel Haleem et al., 2016)
House	Tap	*P. aeruginosa*	Water: 7.14%*	Culture	Disc diffusion: 100% AMC^R 100% AMP^R 100% CHL^R	Ethiopia	2013	(Abera et al., 2014)

					100% TET^{R} 100% SXT^{R} 50% CRO^{R} 40% GEN^{R}			
House Hotel University	Sink	*P. aeruginosa*	<u>Biofilm:</u> 26.1% *	Culture	N/A	Nigeria	2013[f]	(Abubakar et al., 2013)
House	Hot water system Shower	*Legionella pneumophila*	<u>Water:</u> 23.3% hot water system * 7.5% tap water * 6% shower water *	Culture	Disc diffusion: 100% CIP^{S} 100% RIF^{S} 89.2% AZM^{R} 71.4% MXF^{R} 64.3% CLR^{R} 35.7% TGC^{R} 32.1% ERY^{R} 21.4% CRO^{R}	Iraq	2019[f]	(Adday et al., 2019)

					17.8% DOX^{R} 14.2% LVX^{R}			
House Hotel	Cooling tower	*L. pneumophila*	Water: 22.5% *	Culture	N/A	Iran	2015	(Ahmadrajabi et al., 2016)
House	Water heater Tap Shower	*Legionella* spp.	Water: 37% hot water heaters * 15% showers * 12% taps *	Culture	N/A	Canada	1991[f]	(Alary et al., 1991)
House	Tap Water heater Shower	*L. pneumophila*	Biofilm: 11.9% shower heads * Water: 17.7% hot water heaters * 19.4% taps * 23% shower heads *	Culture and Immunofluorescence	N/A	Canada*	1989	(Alary et al., 1992)
Drinking water distribution system	Water	*Aeromonas* spp.	Water:	Culture	100% AMP^{R} 100% PMB^{R}	India	1999[f]	(Alavandi et al., 1999)

			32.4% *		77.8% CEF^{R} 83.3% ERY^{R}			
House	Water storage tank	*Aeromonas* spp. *Pseudomonas* spp.	Biofilm: *Aeromonas* spp.: 77.5% * *Pseudomonas* spp.: 97.5% *	Culture	N/A	Oman	2011[f]	(Al-Bahry et al., 2011)
House	Tap	*P. aeruginosa*	Water: 23.3% *	Culture	N/A	United Arab Emirates	2015	(Ali et al., 2019)
House	Shower Bath	*Mycobacterium avium* subsp. *hominissuis*	Biofilm: 33.3% of bath inlet * 4% drain Water: 5.4% of shower *	Culture	N/A	Japan	2019	(Arikawa et al., 2019)
House	Hot water system Tap	*L. pneumophila*	Water: (1–10^{4} CFU/L) 32% hot water	Culture	N/A	USA	1982	(Arnow et al., 1985)

			<u>Biofilm:</u> 100% tap *					
House	Water	*Mycobacterium* spp.	<u>Water:</u> NTM: 82% house water* MAC: 22% house water (1–10^3 CFU/500mL) *M. avium*: 5% house water* *Mycobacterium intracellulare*: 13% house water *	Culture	N/A	USA	1995	(Aronson et al., 1999)
House	Water	*Aeromonas spp.* *Legionella spp.* *Pseudomonas fluorescence*	Not specified	Culture	N/A	South Africa	1995[f]	(Augoustinos et al., 1995)
House	Tap	*A. baumannii*	Not specified	qPCR	N/A	Cameroon	2014	(Bae et al., 2019)
House	Tap	*Acinetobacter* spp.	<u>Water:</u>	Culture	N/A	Pakistan	1991[f]	(Baqai et al., 1991)

		Aeromonas spp. *P. aeruginosa*	*Aeromonas* spp.: 12% * *Acinetobacter* spp.: 1–8% * *P. aeruginosa*: 6–40% *					
House Accommodation sites	Hot water system Shower Tap	*Legionella* spp.	<u>Water:</u> 71.4% accommodation buildings (>1000 CFU/L) 61.5% house (central hot water supplies 50 CFU/L) 7.7% house (individual hot water supplies 50-5300 CFU/L)	Culture	N/A	Hungary	2013	(Barna et al., 2016b)
House	Tap	*Legionella* spp.	<u>Water:</u> 10% * <u>Biofilm:</u> 4% *	Culture and molecular	N/A	New Zealand	2000[f]	(Bates et al., 2000)
House	Shower	*Legionella* spp.	<u>Water:</u>	Culture	N/A	Italy	2002	(Borella et al., 2004)

	Tap	*Pseudomonas* spp.	*Pseudomonas* spp.: 38.4% (1 to 6.4×10^4 CFU/100mL) *Legionella* spp.: 22.6% (25 to 8.7×10^4 CFU/L)					
House	Water	*Pseudomonas* spp.	<u>Water:</u> 20.8% (6–1100 CFU/ 250 mL)	Culture	N/A	Cyprus	2015[f]	(Botsaris et al., 2015)
House	Bath Shower	*Legionella* spp.	<u>Water:</u> *Legionella* spp.: 72.5% (5–5625 CFU/L) *L. pneumophila*: 59.5% (3.5.5–990 CFU/L)	Culture	N/A	Morocco	2013	(Boudouaya et al., 2017)
House	Water	NTM	<u>Water:</u> 60% (300 CFU/L)	Culture	N/A	Italy	2010[f]	(Briancesco et al., 2010)
House School	Water	NTM	<u>Water:</u> 65% houses ($1–3\times10^2$ CFU/L)	Culture	N/A	Italy	2014[f]	(Briancesco et al., 2014)

			100% water meter (5×10^2 CFU/L) 100% schools (1.6×10^2–6×10^2 CFU/L) 100% swimming pools (2.9×10^1 −3.1×10^4 CFU/L) <u>Biofilm:</u> 100% house (shower floor) (1×10^2–1.2×10^5 CFU/cm^2) 67% public building (shower floor) 5–3.7×10^3 CFU/cm^2) 50% pool edge (4.5 CFU/cm^2)				
House	Tap Shower	*Legionella* spp.	<u>Water:</u> 32.3% negative by all methods *	Culture and molecular	N/A	Germany*	2002[f]	(Buchbinder et al., 2002)

			2.9% positive by all methods * 41.2% positive by PCR only * 23.5% positive by FISH and PCR *					
House	Water	*Legionella* spp.	<u>Water:</u> Residential water linked to 28 clinical cases *	Culture	N/A	Germany	2016-2019	(Buchholz et al., 2020)
House	Tap	*Mycobacterium xenopei*	<u>Water:</u> 6% *	Culture	N/A	United Kingdom	1970[f]	(Bullin et al., 1970)
House	Shower	*L. pneumophila*	<u>Water:</u> 19.6% *(5×10^{1}–26.6×10^{3} CFU/mL) <u>Biofilm:</u> 6.5% (2.9×10^{3}–28.6×10^{3} CFU/mL)	Culture	N/A	Turkey	2009[f]	(Burak et al., 2011)
House	Water	*Aeromonas* spp.	<u>Water:</u>	Culture	N/A	Australia	1984[f]	(Burke et al., 1984)

			33.3% *					
House	Hot water tank Tap Shower	*Legionella* spp.	Water: *Legionella* spp.: 1.5% * *L. pneumophila*: 11.5% *	Culture	N/A	USA	2016	(Byrne et al., 2018)
House	Tap	*Helicobacter pylori*	Water: 12.2% *	Molecular	N/A	Peru	2017	(Castillo et al., 2019)
House	Tap	*A. hydrophila* *P. aeruginosa*	Water: *A. hydrophila*: 6.6% without POU filter (10 CFU/500mL) 10.5% with POU filter (29.5 CFU/500mL) *P. aeruginosa*: 16.6% without POU (15 CFU/500mL) 33.3% with POU filter (102 CFU/500mL)	Culture	N/A	USA	1998	(Chaidez et al., 2004)

House	Rainwater	*P. aeruginosa*	Water: 28% (1–100 CFU/100mL)	Culture	N/A	Mexico	1999[f]	(Chaidez et al., 1999)
House	Water	*P. aeruginosa*	Water: 15% (1–975 CFU/100mL)	Culture	N/A	Mexico	2004	(Chaidez et al., 2008)
House	Tap	*A. hydrophila* *P. aeruginosa*	Water: *P. aeruginosa*: 100% sites * *A. hydrophila*: 33% sites *	Molecular	N/A	India	2013	(Chandra et al., 2016)
House	Tap	*Methylobacterium* spp.	Not specified	Culture	N/A	Norway	2019[f]	(Charnock et al., 2019)
House	Tap	*L. pneumophila*	Water: Residential water linked to 1 clinical case *	Culture	N/A	China*	2002[f]	(Chen et al., 2002)
House	Tap	*P. aeruginosa*	Not specified	Culture	N/A	India	2012	(Chouhan et al., 2014)
House	Water	*Legionella* spp.	Water: 8% patient houses (1.3×10^2 to 2.7×10^4 CFU/L)	Culture and molecular	N/A	Spain	2000	(Codony et al., 2002)

			19% control houses (2.3 × 10^2 to 5.5 × 10^4 CFU/L)					
DWDS	Municipal water	*L. pneumophila*	<u>Water:</u> 25% of pre flush* 50% flushed (1–2100 CFU/25·2mL)	Culture	N/A	USA	2011	(Cohn et al., 2015)
Building	Tap	*L. pneumophila*	<u>Water:</u> 2.4% (2×10^2 to 3x10^4 CFU/L)	Culture	N/A	England	1986	(Colbourne et al., 1986)
House	Shower	*Legionella* spp.	<u>Water:</u> 8.1% (4.0 × 10^1−1.3 × 10^4 CFU/L) <u>Biofilm:</u> 1.1% (5.4x10^2 CFU/swab)	Culture and molecular	N/A	United Kingdom	2017[f]	(Collins et al., 2017)
House	Shower	*L. pneumophila*	<u>Water:</u> continuously detected for 2.5 yrs (380–600 CFU/L)	Culture	N/A	United Kingdom	2005	(Cooper et al., 2008)

House	Water	*Legionella* spp.	Water: 77.5% *	Molecular	N/A	USA	2016	(Dai et al., 2019)
House	Shower head	*Mycobacterium* spp.	Biofilm: 100%*	Molecular	N/A	Singapore	2020[f]	(De Sotto et al., 2020)
House	Water	*P. aeruginosa*	Water: 89.5% (4–130 CFU/100mL)	Culture	N/A	Mexico*	2001[f]	(de Victorica et al., 2001)
House	Shower Tap Drain	*Stenotrophomonas maltophila*	Biofilm: Tap: 36.3%* Drain: 72%*	Culture	N/A	United Kingdom	1996	(Denton et al., 1998)
House	Water	*L. pneumophila*	Water: Hot water tank: 33% (1–100 CFU/mL) Bath: 4% (<50 CFU/mL) Biofilm: 40.9%*	Culture	N/A	Canada	1984	(Dewailly et al., 1991)

DWDS	Municipal water	*Mycobacterium mucogenicum*	Water: Incoming municipal water linked to one clinical case*	Culture	N/A	Canada*	2017[f]	(Dhruve et al., 2017)
House	Water	*Legionella* spp. *P. aeruginosa*	Water: *Legionella* spp.: 30% (200–2650 CFU/L^{-1}) *P. aeruginosa*: 50% (<1 CFU/100mL)	Culture	N/A	Italy*	2015[f]	(Donati et al., 2015)
House	Tap	*L. pneumophila*	Water: 28.6% (>1×10^4 CFU/L)	Culture and molecular	N/A	USA	2010	(Donohue et al., 2014)
House Commercial building	Tap	*L. pneumophila* *M. avium*	Water: *L. pneumophila*: 38% (3188 CE/L) *M. avium*: 42% (2006 CE/L)	Culture and molecular	N/A	USA	2014	(Donohue et al., 2019)
House	Water	NTM	Water: 11.2% *	Culture	N/A	Greece	2013	(Dovriki et al., 2016)

House	Tap Shower Hot water tank	*L. pneumophila*	<u>Water:</u> 33% houses Residential water linked to 14% of clinical cases *	Culture	N/A	Canada	2012[f]	(Dufresne et al., 2012)
House	Water	*P. aeruginosa*	<u>Water:</u> Bathroom linked to one clinical case *	Molecular	N/A	Japan*	2013[f]	(Eguchi et al., 2013)
House	Water	*L. pneumophila*	<u>Water:</u> Residential water linked to 2 clinical cases (10–100 CFU/mL)	Culture	N/A	Turkey*	2016[f]	(Erdoğan et al., 2016)
House	Spa Garden hose	*Legionella* spp.	<u>Water:</u> Spa and garden hose linked to 3 clinical isolates *	Culture	N/A	Netherlands	2009	(Euser et al., 2010)
House	Tap Shower	*Methylobacterium* spp. *M. avium*	<u>Biofilm:</u> *Methylobacterium* spp.: 46%(>10 CFU/mL)	Culture	N/A	USA	2016[f]	(Falkinham et al., 2016)

			M. avium: 27% (>1 CFU/mL)					
House	Shower	*M. avium*	<u>Biofilm and water:</u> Residential shower linked to 1 clinical isolate (2–240 CFU/mL^{-1})	Culture	N/A	USA	2002	(Falkinham Iii et al., 2008)
House	Tap Shower	NTM	<u>Biofilm:</u> 28% * <u>Water:</u> 24% *	Culture	N/A	USA	2011[f]	(Falkinham Iii, 2011)
House	Water	*A. baumannii* *P. aeruginosa*	<u>Water:</u> *A. baumannii*: 80% * *P. aeruginosa*: 85% *	Culture	N/A	Pakistan	2004	(Farooqui et al., 2009)
House	Water	*Mycobacterium* spp.	<u>Water:</u> 81.9% (10^2–10^3 CFU/L)	Culture	N/A	Germany	1991[f]	(Fischeder et al., 1991)
House	Tap	*L. pneumophila*	<u>Water:</u> 6% *	Culture	N/A	USA	2016	(Garner et al., 2019)
House	Washing machine	*Methylobacterium* spp.	Not specified	Culture	N/A	USA Switzerland	2010[f]	(Gattlen et al., 2010)

		Pseudomonas spp. *Stenotrophomonas* spp.				Germany South Korea		
House	Shower	Mycobacterium spp.	Biofilm: 13.5% *	Molecular	N/A	USA	2016	(Gebert et al., 2018)
House	Spa Shower Tap	*P. aeruginosa*	Water: 18.2% (100–500 CFU/250mL)	Culture	N/A	Italy*	2009	(Germinario et al., 2012)
House	Tap Shower	MAC	Water: NTM: 93% houses MAC: 21% houses (1->10^3 CFU/500^{-1}mL)	Culture	N/A	USA	1994[f]	(Glover et al., 1994)
Drinking water distribution system	Municipal water	*L. pneumophila* *Mycobacterium* spp.	Biofilm and Water: *Mycobacterium* spp.: 66% buildings * *L. pneumophila*: 83% buildings *	Molecular	N/A	Canada	2018	(Gora et al., 2020)

House	Tap	NTM	<u>Water:</u> 100% *	Molecular	N/A	USA	2016	(Haig et al., 2018)
House	Tap	NTM *P. aeruginosa* *S. maltophila*	<u>Biofilm & water:</u> NTM: 100% * *P. aeruginosa:* 100% * *S. maltophila:* 100% *	Molecular	N/A	USA	2016	(Haig et al., 2020)
House	Shower	NTM	<u>Water:</u> Shower water linked to 1 clinical case *	Culture	N/A	USA*	2011[f]	(Hankwitz et al., 2011)
House	Shower	*Legionella* spp.	<u>Water:</u> *Legionella* spp.: 73.5% (7603 copies/mL) *L. pneumophila*: 63.2% (4295 copies/mL)	Molecular	N/A	Australia	2018	(Hayes-Phillips et al., 2019)
House	Tap Shower Drain	NTM	<u>Biofilm:</u> Showerhead: 69%* Kitchen: 59% *	Molecular	N/A	USA	2013	(Honda et al., 2016)

			Bathroom: 67% *					
House	Shower Tap	*L. pneumophila*	<u>Water:</u> Residential water linked to 2 clinical cases (6×10^3– 2.3×10^4 CFU/L)	Culture	N/A	Israel*	2003[f]	(Huerta et al., 2003)
House	Tap	*Methylobacterium* spp.	<u>Water:</u> 12% *	Molecular	N/A	USA	2015[f]	(Hull et al., 2015)
House	Tap	*Helicobacter* spp.	<u>Water:</u> 12% *	Molecular	N/A	Sweden	1998[f]	(Hultén et al., 1998)
House	Shower	*P. aeruginosa*	<u>Water:</u> Shower water linked to 1 clinical case *	Culture	N/A	Israel	1986	(Huminer et al., 1989)
House	Shower	*M. avium*	<u>Water:</u> 11% *	Culture	N/A	USA	2016	(Iakhiaeva et al., 2016)
House	Drain Bath Shower	*Mycobacterium* spp.	<u>Biofilm:</u> 96.5% drains ($<10^1$ – 10^7 cells/cm^2)	Molecular	N/A	Japan	2014[f]	(Ichijo et al., 2014)

			44.4% baths (<10^1 – 10^7 cells/cm^2) 48.7% kitchen drains (<10^1 – 10^7 cells/cm^2) 13% inner shower head (<10^1 – 10^5 cells/cm^2) 2.5% outer showerhead (<10^1 – 10^2 cells/cm^2)					
House	Water	*Legionella* spp. *Mycobacterium* spp.	<u>Water:</u> *Legionella* spp.: 72% * *Mycobacterium* spp.: 67% *	Molecular	N/A	USA	2020[f]	(T. S. Isaac et al., 2020)
House	Bath	*L. pneumophila*	<u>Water:</u> Bath water linked to 1 clinical case *	Culture	N/A	Japan*	2012[f]	(Ishimaru et al., 2012)
House	Rainwater	*Pseudomonas* spp.	<u>Water:</u> Dry season: 9% * Wet season: 91% *	Culture	N/A	Bangladesh	2009	(Islam et al., 2011)

House	Tap Shower	*L. pneumophila*	Water: Tap: 1 isolate *	Molecular	N/A	China	2014	(Jiang et al., 2020)
House	Hot water heater	*L. pneumophila*	Water: Electric heater: 30% * Oil/gas heater: 6% *	Culture	N/A	Canada	1985[f]	(Joly, 1985)
House	Tap	*L. pneumophila*	Water: 1.9% *	Culture	N/A	England	1987[f]	(Jones et al., 1988)
House	Sink	*P. aeruginosa*	Biofilm: 19% (1×10^2– 1.5×10^5 CFU/swab)	Culture	N/A	USA	1997[f]	(Josephson et al., 1997b)
House	Tap	*A. hydrophila*	Water: Contaminated well water linked to 1 recurrent clinical case *	Culture	N/A	USA	2015	(Katz et al., 2015)
House	Shower	*Mycobacterium xenopi*	Biofilm: 95.4% * Water: 90.9% *	Culture	N/A	Czech Republic	1990	(Kaustova et al., 1993)

			Water					
House	Rainwater	*L. pneumophila* *M. avium*	*L. pneumophila*: 8.7% (2.9 log_{10} genomic targets/L) *M. avium*: 30% (3.9 log_{10} genomic targets/L)	Molecular	N/A	USA	2013	(T. Kim et al., 2016)
House	Tap	NTM	Water: 33.9% *	Culture	N/A	Czech Republic	2013[f]	(Klanicova et al., 2013)
Public bath House	Bath	*Legionella* spp. *Mycobacterium* spp.	Water: Public bath: *Legionella* spp.: 3% (<10-1×10^4 CFU/100mL) NTM: 0.6% * House bath: *Legionella* spp: 60.5% (<10-1×10^4 CFU/100mL) NTM: 21% *	Culture	N/A	Japan	2009-2011	(Kobayashi et al., 2014)
House	Tap	*P. aeruginosa*	Water:	Culture	N/A	Germany*	2001	(Kohnen et al., 2005)

			12% *					
House	Tap Water storage	*Aeromonas* spp.	<u>Water:</u> Tap: 6% * Water storage: 12% *	Culture	Disc diffusion: 55% R AMP^{R} 48% R ERY^{R} 41% R AMC^{R} 28% R CAZ^{R} 27% R FOX^{R} 26% R CRO^{R} 26% CTX^{R} 22% R PIP^{R} 14% R SXT^{R} 12% R TET^{R} 11% R ATM^{R} 8% R MEM^{R} 6% R IPM^{R} 2% R NAL^{R}	Turkey	2005	(Koksal et al., 2007)

					1% R CIP^{R} 1% R TOB^{R} 1% R GEN^{R}			
House	Water	*Legionella* spp.	<u>Water</u>: 47.8% (15 – 370 CFU/100 mL) <u>Biofilm:</u> 18.9%*	Culture	N/A	Japan	2014	(Kuroki et al., 2017)
House	Tap Shower Ice dispenser	*M. avium*	<u>Biofilm:</u> 56.8% kitchen sink tap * 41.7% bathroom taps * 37.1% shower heads * 37.9% shower pipes * 14.2% ice dispensers *	Culture	N/A	USA	2012	(Lande et al., 2019)
House	Shower Tap	*L. pneumophila*	<u>Water:</u> Water heater linked to 1 clinical case (10^{9} CFU/L)	Culture	N/A	USA	2001[f]	(Laverdière et al., 2001)

	Water heater							
House	Tap	*Mycobacterium canariasense*	Water: 47.4% *	Culture	N/A	Spain	2014	(Lecuona et al., 2016)
House	Hot water system	*Legionella* spp. *P. aeruginosa*	Water: *Legionella* spp.: 30.5% (25–9.75×10^4 CFU/L^{-1}) *P. aeruginosa*: 7.1% (4–8.2×10^3 CFU/100mL)	Culture	N/A	Italy	2005	(Leoni et al., 2005)
House	Water	*L. pneumophila*	Water: Residential water linked to 1 clinical case (10^8 CFU/L)	Culture	N/A	Netherlands*	1993	(Leverstein van Hall et al., 1994)
House	Tap	*Aeromonas* spp.	Water: *Aeromonas* spp.: 4% houses *	Culture	N/A	Canada	1992	(Levesque et al., 1994)

House	Shower	*Legionella* spp. *Mycobacterium* spp.	Biofilm: *Legionella* spp.: 12.5% * *Mycobacterium* spp.: 6.25% * Water: *Legionella* spp.: 23% * *Mycobacterium* spp.: 10.4% *	Molecular	N/A	Switzerland	2011	(Lienard et al., 2017)
House	Water purifier	*P. aeruginosa*	Not reported	Molecular	N/A	Germany*	2020[f]	(Lin et al., 2020)
House	Tap	*Aeromonas* spp. *Mycobacterium* spp.	Biofilm and Water: Aeromonas spp.: 90% * *Mycobacterium* spp.: 60% *	Culture and molecular	N/A	Netherlands	2017[f]	(Liu et al., 2017)
House	Sink U-bend	*Pseudomonas* spp. *S. maltophila* *A. hydrophila*	Biofilm: *Pseudomonas putida*: 20.9% * *P. aeruginosa*: 18.7% *	Molecular	VITEK-2: *P. aeruginosa* (1) isolate MDR	Germany	2018	(Lucassen et al., 2019)

		A. baumannii	*A. hydrophila*: 12.1% * *S. maltophila*: 11% * *A. baumannii*: 2.2% *		*S. maltophila* (3) isolates MDR BAC^{R} >640 µg/mL			
House	Shower Tap	*Legionella* spp.	Water: 65% (1–4000 CFU/mL)	Culture	N/A	Germany	1993[f]	(Lück et al., 1993)
House	Water	*Legionella* spp.	Water: Residential water linked to 1 clinical (1–2×10^{4} CFU/L^{-1})	Culture	N/A	Germany	2004	(Lück et al., 2008)
DWDS	Biofilm	*Mycobacterium* spp.	Biofilm: 100%*	Molecular	N/A	Sweden	2011	(Lührig et al., 2015)
House	Water	*L. pneumophila* *Acinetobacter* spp. *Pseudomonas* spp.	Water: 5%*	Molecular	*P. aeruginosa* aph(3')-I	China	2019[f]	(Ma et al., 2019)
House	Water	*L. pneumophila*	Not specified	Culture	N/A	Canada	2021[f]	(MacMartin et al., 2021)

House	Tap	*A. hydrophila*	<u>Biofilm:</u> 24% *	Culture	VITEK-2: TIC[R] CAZ[R] ATM[R] MIN[I]	Iraq	2019[f]	(Maki, 2019)
House	Water	*L. pneumophila*	<u>Water:</u> 15.9% *	Molecular	N/A	USA	2020[f]	(Mapili et al., 2020)
House	Tap	*L. pneumophila* *M. avium*	Not specified	Culture	N/A	USA	2007	(Marciano-Cabral et al., 2010)
House	Bath Shower	*M. avium*	<u>Water:</u> Residential water linked to 1 clinical case *	Culture	N/A	Canada	2005[f]	(Marras et al., 2005)
DWDS	Municipal water	*Mycobacterium* spp.	<u>Water:</u> 70% *	Culture	N/A	Australia	2008	(Marshall et al., 2011)
House	Shower Water heater	*L. pneumophila*	<u>Water:</u> 30% hot water tanks *	Culture	N/A	Italy	2000	(Martinelli et al., 2000)

			6.2% shower *					
House Commercial building	Tap	*P. aeruginosa*	<u>Water:</u> 40% (1–75 CFU/100mL)	Culture	Disc diffusion: 54.5% MDR	India	2003	(Mathias et al., 2007)
House	Hot water system	*Legionella* spp.	<u>Water:</u> 12% (0 to 1×10^5 CFU/100mL)	Culture	N/A	Germany	2003	(Mathys et al., 2008)
House	Drain	*P. aeruginosa*	<u>Biofilm:</u> 100% (<4–9.52 $\log_{10}$ CFU)	Culture and molecular	N/A	United Kingdom	2003[f]	(McBain et al., 2003b)
House	Water	*Aeromonas* spp.	<u>Water:</u> 3.7% houses (5 CFU/mL)	Culture	N/A	Japan	2011	(Miyagi et al., 2017)
House	Water	*Mycobacterium leprae*	<u>Water:</u> 24.2% *	Molecular	N/A	India	2016[f]	(Mohanty et al., 2016)
House	Tap	*P. aeruginosa*	<u>Water:</u> 100% (10^5–10^9 CFU)	Culture	Disc diffusion: 13 isolates MDR	Iran	2019[f]	(Mombini et al., 2019b)

House	Tap Shower Bath	*Legionella* spp.	Water: 36.7% (>10^2 CFU/L)	Culture	N/A	Italy	2005	(Montagna et al., 2006)
House Commercial building Hotel	Tap	*Legionella* spp.	Water: 19.8% total buildings *	Culture	N/A	USA	2002	(Moore et al., 2006)
House	Tap	*L. pneumophila*	Water: Residential water linked to 1 clinical case (3×10^4 CFU/L)	Culture	N/A	Israel	2012	(Moran-Gilad et al., 2012)
House	Well	*P. aeruginosa* *Acinetobacter* spp.	Water: *P. aeruginosa*: 17 urban * 19 rural * *Acinetobacter* spp.: 5 urban *	Culture	Disc diffusion: *P. aeruginosa*: 2.8% R PIP^R 5.6% R CIP^R 5.6% R NET^R *Acinetobacter* spp.:	India	2009	(Mukhopadhyay et al., 2012)

					62.5% R AMP^{R} 37.5% R AMX^{R} 50% R CFZ^{R} 37.5% R SEF^{R}			
House	Water	*H. pylori*	<u>Water:</u> 53.3% *	Culture	N/A	India	2013[f]	(Mulchandani et al., 2013)
Apartment	Water	*Legionella* spp.	<u>Water:</u> 42.9% (10^{2}–10^{4} CFU/L)	Culture	N/A	Italy	2009	(Napoli et al., 2010)
House	Tap Shower Drain	MAC	<u>Biofilm:</u> 5.4% shower * 4% bath drain * <u>Water:</u> 6.5% shower water * 6.25% bath water *	Culture	N/A	Japan*	2007[f]	(Nishiuchi et al., 2007)
House	Water	*Aeromonas* spp.	<u>Water:</u> 20% *	Culture	N/A	South Africa	2006	(Obi et al., 2007a)

House	Water	*Aeromonas* spp.	Not specified	Culture	Disc diffusion: CHL^{R} PEN^{R} AMP^{R} CLOXA^{R}	South Africa	2006	(Obi et al., 2007b)
House	Drain Shower Tap	*P. aeruginosa*	Not specified	Culture	N/A	Japan	1999	(Ojima et al., 2002a)
House	Drain Tap Shower	*P. aeruginosa*	<u>Biofilm:</u> (>1 CFU/10 cm^{2}) Kitchen tap: 7.2% Kitchen sink: 12.9% Drain: 27.1% Bathroom faucet: 2.3% Bath: 1.2%	Culture	N/A	Japan	2002[f]	(Ojima et al., 2002b)
House	Water	*Legionella* spp.	<u>Water:</u> 25% (10^{3} cells/mL)	Culture	N/A	Germany*	1996[f]	(Okpara et al., 1996)

House	Tap	*Pseudomonas* spp.	<u>Water:</u> Rain water: 25%* Well water: 75% *	Culture	Disc diffusion: 100% R AMP^{R} 100%R COL^{R} 75% R GEN^{R} 100% R STR^{R} 100% R TET^{R} 50% R COT^{R}	Nigeria	2011	(Oluyege et al., 2011)
House	Tap	*Acinetobacter* spp. *P. aeruginosa*	Not specified	Culture	N/A	Nigeria	2008[f]	(Omezuruike et al., 2008)
House	Tap	*Pseudomonas* spp.	Not specified	Culture	N/A	Australia Germany India Malaysia Saudi Arabia South Africa England	2009	(Oxford et al., 2013)

						USA		
House	Tap	*Mycobacterium* spp.	Water: 39.42% *	Culture and molecular	N/A	India	2000	(Parashar et al., 2009)
House	Shower	*L. pneumophila*	Water: Residential shower linked to 1 clinical case *	Molecular	N/A	Italy	1985	(Pastoris et al., 1986)
House	Tap	*L. pneumophila*	Water: Residential water linked to 3 clinical cases *	N/A	N/A	Italy*	1986	(Pastoris et al., 1988)
House	Tap	*Pseudomonas* spp. *S. maltophila*	Not specified	Culture	N/A	Canada*	1988	(Payment, 1989)
House	Shower	*Legionella* spp.	Water: 16.1% shower * 1 boiler tank *	Culture and molecular	N/A	Brazil	2005	(Pellizari et al., 1995)
House	Tap	NTM	Water: 16% *	Culture	N/A	Mexico	2009	(Perez-Martinez et al., 2013)

House	Water storage	*Pseudomonas* spp. *L. pneumophila*	Water: *L. pneumophila*: 13.3% (100–800 CFU/L) *Pseudomonas* spp.: 86.6% (7–1000 CFU/100mL)	Culture	N/A	United Kingdom	2016	(Peter et al., 2018)
House	Tap	*Mycobacterium* spp.	Water: 25% (4–1600 CFU/L)	Culture	N/A	Germany	1992	(Peters et al., 1995)
House	Tap	*P. aeruginosa*	Water: 12% (2–100 CFU/100mL)	Culture	N/A	Cyprus	2013	(Pieri et al., 2014)
House	Water	*Legionella* spp.	Water: 24.1% (3.75–415.5 CFU/mL)	Culture	N/A	USA	2019[f]	(Pierre et al., 2019)
House DWDS	Tap	*Aeromonas* spp.	Not specified	Culture	N/A	Canada	1997[f]	(Prevost et al., 1997)
House	Drain	*P. aeruginosa*	Biofilm: 28% *	Molecular	N/A	USA	2012	(Purdy-Gibson et al., 2015)

House	Dishwasher	*Pseudomonas* spp. Acinetobacter spp.	Not specified	Molecular	N/A	Slovenia	2018[f]	(Raghupathi et al., 2018)
Hotel Retirement home	Water	*Legionella* spp.	<u>Water:</u> 66.6% of seasonal facilities (450 CFU/L^{-1})	Culture	N/A	Croatia	2009	(Rakić et al., 2011)
House	Tap	*Legionella* spp.	<u>Water:</u> 20% (250–1000 CFU/L)	Culture	N/A	Croatia	2009	(Rakić et al., 2012)
House	Hot water	*L. pneumophila*	<u>Water:</u> 12.7% (500–13,000 CFU/L)	Culture	N/A	Croatia	2011	(Rakić et al., 2013)
Accommodation site	Water	*Legionella* spp.	<u>Water:</u> 27.3% *	Culture	N/A	Croatia	2012	(Rakic et al., 2017)
House	Water	*Methylobacterium* spp.	Not specified	Molecular	N/A	United Kingdom	2013[f]	(Ramalingam et al., 2013)
House	Tap Drain	*P. aeruginosa*	<u>Water:</u> 71.6% *	Culture	N/A	Germany	2004[f]	(Regnath et al., 2004)

House	Shower Tap Drain Ice dispenser	*Pseudomonas* spp.	Biofilm: 48.6% *	Culture	N/A	USA	2007	(Remold et al., 2011)
House	Water heater	*Legionella* spp.	Water: 6.6% houses *	Culture	N/A	USA	2016	(Rhoads et al., 2020)
House	Shower	*M. avium*	Biofilm: 93% (10^2–10^{10} CE/L)	Molecular	N/A	United Kingdom	2011	(Rhodes et al., 2014)
House	Tap	*Legionella* spp. *Mycobacterium* spp. *Helicobacter* spp.	Water: *Legionella* spp.: 21% * *Mycobacterium* spp.: 35.1% * *Helicobacter* spp.: 7% *	Culture and molecular	N/A	USA	2018[f]	(Richards et al., 2018)
House	Water	NTM	Water: 12% *	N/A	N/A	USA and Finland	2015[f]	(Ristola et al., 2015)
House	Tap	*L. pneumophila*	Water:	Culture	N/A	Korea*	2016	(Ryu et al., 2017)

			Residential water linked to 1 clinical case (2.9–7.2×10^4 CFU/L)					
House	Water storage	*Pseudomonas* spp. *S. maltophila* *Acinetobacter lwoffii* *A. hydrophila*	<u>Water:</u> *P. aeruginosa* 6% * *P. fluorescens* 6.75%* *P. luteola* 0.7%* *P. stutzeri* 2.2%* *S. maltophila* 1.5%* *A. lwoffii* 7.5%* *A. hydrophila* 0.7%*	Culture	Broth microdilution: 80.6% MDR	South Africa	2012	(Samie et al., 2012)
House DWDS	Shower Tap Municipal water Cooling tower	*Legionella* spp.	<u>Water:</u> 30% * <u>Biofilm:</u> 56% *	Culture	N/A	USA*	1992[f]	(Sanden et al., 1992)

House	Shower	*L. pneumophila*	<u>Water:</u> Shower water linked to 1 clinical case (1.95×10^4 CFU/L)	Culture	N/A	Switzerland	1999	(Sax et al., 2002)
House	Tap	*Legionella* spp.	<u>Water:</u> Residential: 52% (10^2–10^5 CFU/L)	Culture	N/A	Italy	2008	(Scaturro et al., 2015)
House	Shower drain	*S. maltophila* *P. aeruginosa* *Acinetobacter* spp. *Aeromonas* spp.	<u>Biofilm:</u> *S. maltophila*: 27.9%* *P. aeruginosa*: 9.3%* *Acinetobacter* spp.: 1.6%* *Aeromonas* spp.: 3.9%*	Culture	*Bla* CMY-2, *bla* ACT/MIR and *bla* OXA-48	Germany	2019	(Schages et al., 2020)
House	Tap Drain	*P. aeruginosa*	<u>Biofilm:</u> Bath drain: 12.4%* Bath tap: 14.3%* Shower drain: 17.6%*	Culture	N/A	Belgium	2005	(Schelstraete et al., 2008)

			Showerhead: 0%* Kitchen drain: 5%* Kitchen tap: 4.5%*					
House	Water	*P. aeruginosa*	<u>Water:</u> House: 2.13%*	Culture	Disc diffusion: 13.2% MDR	Italy	2015	(Schiavano et al., 2017)
House	Shower	*Mycobacterium* spp.	<u>Biofilm:</u> 78.5% (15 to 5.6x10^6 CFU/cm^2)	Culture	N/A	Germany	1992	(Schulze-Röbbecke et al., 1992)
House	Tap	*Legionella* spp.	<u>Water:</u> 89.5% (5.45 CFU/mL)	Culture	N/A	Germany	1999[f]	(Schulze-Robbecke et al., 1999)
House	Shower	*L. pneumophila*	<u>Water:</u> Shower linked to 1 clinical case (2.54 CFU/mL)	Culture	N/A	USA	2018	(Schumacher et al., 2020)
House	Drain Tap	*Pseudomonas* spp. *A. hydrophila*	<u>Biofilm:</u> Drain: 2.2%*	Culture	N/A	United Kingdom	1982[f]	(Scott et al., 1982)

			Tap: 10.9%* Water: 8.1%*					
House	Drain Tap	*Pseudomonas* spp.	<u>Biofilm:</u> Kitchen sink: 24%* Kitchen drain: 40%* Bathroom sink: 21%* Bath: 47%*	Culture	N/A	USA	2006	(Scott et al., 2009)
House	Ice cube	*Pseudomonas* spp. *Acinetobacter* spp.	Not specified	Culture	N/A	Italy	2017	(Settanni et al., 2017)
House	Tap Shower	*Legionella* spp.	<u>Water:</u> 74%*	Culture	N/A	USA	2009	(Silk et al., 2013)
House	Shower Sprinkler	*L. pneumophila*	<u>Water:</u> 10% (3.0×10^2 – 8.0×10^6 CFU/L)	Culture	N/A	New Zealand	2006	(Simmons et al., 2008)
House	Tap	*L. pneumophila*	<u>Water:</u> 20% (1.0×10^4 – 2.1×10^5 CFU/L)	Culture	N/A	Finland*	1999	(Skogberg et al., 2002)

House	Water	*A. hydrophila*	Water: 64.28%*	Culture	N/A	Saudi Arabia	1985	(Slade et al., 1986)
House	Water	*M. xenopi*	Water: 45.5% of patients' houses 29.4% of neighbours' houses*	Culture	N/A	Prague	1993[f]	(Slosarek et al., 1993)
DWDS	Water	*Mycobacterium* spp.	Water: 38.7%*	Culture	N/A	Czech Republic	1994[f]	(Slosarek et al., 1994)
House	Tap Drain	*Pseudomonas* spp.	Biofilm: Tap: 1.2%* Sink: 2%*	Culture	N/A	Scotland*	1991	(Speirs et al., 1995)
House	Tap Water storage	*Legionella* spp.	Water: 5% *	Culture	N/A	Canada	1992	(Stephens, 1992)
House	Tap	*Legionella* spp. *P. aeruginosa*	Water:	Culture	N/A	Poland	2007-2010	(Stojek et al., 2011)

		Acinetobacter spp. *Aeromonas* spp.	*Legionella* spp.: 77.5% (<200 CFU/mL) *P. aeruginosa*: 19.8%* *Acinetobacter* spp.: 13.5%* *Aeromonas* spp.: 16.2%*					
House	Tap Shower	*Legionella* spp.	<u>Water:</u> Hot water tank linked to 1 clinical case (400–2000 CFU/mL)	Culture	N/A	USA*	1987[f]	(Stout et al., 1987)
House	Water	*Legionella* spp.	<u>Water:</u> Municipal water linked to 8 clinical cases (1×10^4 – 6×10^5 CFU/L)	Culture	N/A	USA	1992[f]	(Stout et al., 1992a)
House	Tap Shower Hot water tank	*L. pneumophila*	<u>Water:</u> 6.4%*	Culture	N/A	USA	1992[f]	(Stout et al., 1992b)

House	Tap Shower	*L. pneumophila*	Water: 6.2%*	Culture	N/A	USA	1992	(Straus et al., 1996)
House	Bath	*M. avium*	Water: Bath water linked to 1 clinical case*	Culture and molecular	N/A	Japan*	2001	(Takahara et al., 2002)
House	Shower Tap	NTM	Water: Residential water linked to 35% of clinical cases*	Culture	N/A	Australia	2013[f]	(Thomson et al., 2013)
House	Shower Tap	NTM	Water: 40% (27 to 1.7×10^4 CFU/mL)	Molecular	N/A	USA	2011	(Tichenor et al., 2012a)
House	Water storage	*Pseudomonas* spp. *Mycobacterium* spp. *Methylobacterium* spp.	Not specified	Culture	N/A	Lebanon	2004[f]	(Tokajian et al., 2004)
House	Bath	*L. pneumophila*	Water: Bath water linked to 1 clinical case*	Culture	N/A	Japan	2018[f]	(Tomari et al., 2018)

House	Building inlet	*Legionella* spp.	Water: 23% (2×10^2 to 4.8×10^4 CFU/L)	Culture	N/A	Italy	2017	(Totaro et al., 2017)
House	Water	*Legionella* spp.	Water: Hot water: 40% (2×10^2 to 7.6×10^5 CFU/L) Cold water: 12% (1×10^2 and 1.2×10^4 CFU/L)	Culture	N/A	Italy	2019	(Totaro et al., 2020)
House	Tap Shower	*Mycobacterium* spp.	Water: Case residence: Bathroom tap: 23%* Kitchen tap: 23%* Shower aerosol: 18%* Control residence: Bathroom tap: 11%* Kitchen tap: 14%* Shower aerosol: 6%*	Culture	N/A	USA	2011	(Tzou et al., 2020)

House	Tap	*Pseudomonas* spp. *Acinetobacter* spp.	Water: *Pseudomonas* spp.: 2.2%* *Acinetobacter* spp.: 4.4%*	Molecular	N/A	Belgium	2013	(Van Assche et al., 2019)
House	Tap Shower	NTM	Water: 65%*	Culture	N/A	Netherlands	2010[f]	(Van Ingen et al., 2010)
House	Tap	*Acinetobacter* spp.	Not specified	Culture	N/A	Portugal*	2009	(Vaz-Moreira et al., 2013)
House	Water	*A. hydrophila*	Water: 27%*	N/A	N/A	Philippines	2013	(Ventura et al., 2015)
House	Shower Tap	*Legionella* spp.	Water: 20.5%*	Culture	N/A	Netherlands	2003	(Verhoef et al., 2004)
House	Tap Shower	*Mycobacterium* spp. *Legionella* spp. *P. aeruginosa*	Water: *Mycobacterium* spp. Cold water: 95.4% (1–500 CFU/500mL)	Culture	N/A	Germany	2007	(Von Baum et al., 2010)

			Warm water: 15.4% 1–1000 CFU/500mL) *Legionella* spp.: 9.2% (50–5000 CFU/500mL) *P. aeruginosa*: 10.8% (5–2500 CFU/500mL)					
House	Tap	*M. avium*	<u>Water:</u> 12.5%*	Culture	N/A	USA	2002[f]	(von Reyn et al., 2002)
House	Shower	*Methylobacterium* spp.	Not specified	Culture	N/A	USA*	2012[f]	(Vornhagen et al., 2013)
House	Tap Shower	*Mycobacterium* spp.	<u>Water:</u> *M. chimaera*: 73%* MAC: 19.5%*	Culture	N/A	USA	2013[f]	(Wallace Jr et al., 2013)
House	Hot water system	*Legionella* spp.	<u>Water: (</u><250->10^4 CFU/L) Culture Hot water: 6.5%	Culture and molecular	N/A	France	2006	(Wallet et al., 2016)

			Mixed water: 5.6% IFA Hot water: 41% Mixed water: 52%					
House	Bath	*M. avium*	<u>Water:</u> Bath water linked to 1 clinical case*	Culture	RIF^R STR^R EMB^R	Japan*	2000	(Watando et al., 2001)
House DWDS	Tap	*H. pylori*	<u>Water:</u> 15%*	Culture and molecular	N/A	England	2004[f]	(Watson et al., 2004)
House	Tap Drain	*P. aeruginosa*	<u>Biofilm:</u> Sink: 6.1%* Tap: 4.7%*	Culture	N/A	England*	1972[f]	(Whitby et al., 1972)
House	Water	*Legionella* spp. *Mycobacterium* spp.	<u>Water:</u> *Legionella* spp: 86.7%* *Mycobacterium* spp.: 68.1%*	Culture and molecular	N/A	USA	2020[f]	(Xue et al., 2020)

House	Tap Shower	MAC	Water: *Mycobacterium* spp.: 17%* MAC: 2%*	Culture	N/A	USA	1992[f]	(Yajko et al., 1995)
House	Tap	*L. pneumophila*	Water: Residential water linked to 1 clinical case (500–4.5×10^4 CFU/L)	Culture	N/A	Australia	2003	(Young et al., 2005)
House	Tap	*Legionella* spp. *Mycobacterium* spp. *Pseudomonas* spp.	Not specified	Molecular	N/A	China	2021[f]	(Zhang et al., 2021)
House	Water meter	*Mycobacterium* spp. *Pseudomonas* spp.	Biofilm: *Mycobacterium* spp.: 93%* *Pseudomonas* spp.: 100%*	Molecular	N/A	China	2014	(Zhu et al., 2019)
Dormitory	Tap	*Legionella* spp.	Water: 50% (5-68 CFU/L)	Culture	N/A	Germany	1999	(Zietz et al., 2001)

		S. maltophila	Biofilm:		Broth microdilution:			
House	Dishwasher	*P. aeruginosa*	*S. maltophila*: 33%*	Culture	57% R CTX^R	Slovenia*	2019[f]	(Zupančič et al., 2019)
			P. aeruginosa: 20%*		70% R CAZ^R			

a Abbreviations*: Mycobacterium avium* complex, MAC; Non-tuberculous mycobacteria, NTM .

b Abbreviations: Fluorescence in situ hybridization, FISH; Immunofluorescence assay, IFA; Polymerase chain reaction, PCR; Point of use, POU; Colony forming unit, CFU. Where the concentration of pathogen was not specified in the article, it was denoted with an asterisk (*).

c Abbreviations: Quantitative polymerase chain reaction, qPCR; drinking water distribution systems (DWDS) .

d Abbreviations: Amoxicillin- clavulanic acid, AMC; Ampicillin, AMP; Amoxicillin, AMX; Aztreonam, ATM; Azithromycin, AZM; Ceftazidime, CAZ; Cephalothin, CEF; Cefazolin, CFZ; Chloramphenicol, CHL; Ciprofloxacin, CIP; Cloxacillin, CLOXA; Clarithromycin, CLR; Colistin, COL; Cotrimoxazole, COT; Ceftriaxone, CRO; Cefotaxime, CTX; Doxycycline, DOX; Erythromycin, ERY; Cefoxitin, FOX; Gentamicin, GEN; Imipenem, IPM; Levofloxacin, LVX; Multidrug resistant, MDR; Meropenem, MEM; Minimum inhibitory concentration, MIC; Minocycline, MIN; Moxifloxacin, MXF; Nalidixic acid, NAL; Netilmicin, NET; Penicillin, PEN; Piperacillin, PIP; Polymyxin B, PMB; Resistant, R; Rifampin, RIF; Streptomycin, STR; Trimethoprim-sulfamethoxazole, SXT; Tetracycline, TET; Tigecycline, TGC; Ticarcillin, TIC; Tobramycin, TOB; BioMerieux identification and antibiotic susceptibility testing instrument, VITEK-2; Not applicable, N/A; R, Antimicrobial resistance; S, Antimicrobial sensitivity; I, Intermediate antimicrobial resistance.

e In countries where the study location was not specified in the article, it was assumed that the country of origin was denoted by the country of the authors.

f Where the year of study was not specified in the article, it was assumed that the year of research was denoted by the year of publication.

12. APPENDIX – 2

Supplementary data for the manuscript entitled CITATION: Microbial risks associated with drinking water and plumbing biofilms: Prevalence of opportunistic premise plumbing pathogens in healthcare and residential settings(Chapter 4)

Table 12.1 – Residential sample abiotic factors

Sample ID	Building Type◊	Sample site	Sample type§	Collector □	Water heating System	Hot water storage	Building age (years)	Plumbing system age (years)	Usage
R1	H	Handbasin	BF	CH	Did not know	Did not know	N/A	N/A	N/A
R10	H	Handbasin	BF	CH	Did not know	Did not know	N/A	N/A	N/A
R100	H	Handbasin	BF	CH	Electric	Did not know	More than 20	More than 20	2 to 10/day
R101	H	Handbasin	BF	CH	Electric	Did not know	More than 20	More than 20	2 to 10/day
R102	A	Handbasin	BF	CH	Gas	Did not know	Less than 5	Less than 5	1/day
R103	A	Handbasin	BF	CH	Gas	Did not know	Less than 5	Less than 5	1/day

R104	H	Handbasin	BF	CH	Electric	No	More than 20	More than 20	2/day
R105	H	Handbasin	BF	CH	Electric	No	More than 20	More than 20	2/day
R106	H	Showerdrain	BF	CH	Electric	Yes	More than 20	More than 20	2 to 10/day
R107	H	Showerhead	BF	CH	Electric	Yes	More than 20	More than 20	2 to 10/day
R108	H	Bath Faucet	BF	CH	Electric	No	More than 20	More than 20	Less than 1/month
R109	H	Bath Drain	BF	CH	Electric	No	More than 20	More than 20	Less than 1/month
R11	H	Kitchen	BF	CH	Did not know	Did not know	N/A	N/A	N/A
R12	H	Showerhead	BF	CH	Did not know	Did not know	N/A	N/A	N/A
R13	H	Handbasin	BF	CH	Did not know	Did not know	N/A	N/A	N/A
R14	H	Kitchen	BF	CH	Did not know	Did not know	N/A	N/A	N/A

R15	H	Handbasin	BF	CH	Did not know	Did not know	N/A	N/A	N/A
R16	H	Handbasin	BF	CH	Did not know	Did not know	N/A	N/A	N/A
R17	H	Handbasin	BF	CH	Did not know	Did not know	N/A	N/A	N/A
R18	H	Handbasin	BF	CH	Did not know	Did not know	More than 20	N/A	More than 10/day
R19	University housing	Handbasin	BF	CH	Did not know	Did not know	More than 20	N/A	More than 10/day
R2	H	Kitchen	BF	CH	Did not know	Did not know	N/A	N/A	N/A
R20	H	Handbasin	BF	CH	Electric	No	More than 20	More than 20	More than 10/day
R21	H	Handbasin	BF	CH	Electric	No	More than 20	More than 20	More than 10/day
R22	H	Showerhead	BF	CH	Did not know	Did not know	More than 20	10 to 14	2/day
R23	H	Handbasin	BF	CH	Did not know	Did not know	More than 20	More than 20	2 to 10/day

R24	H	Showerhead	BF	CH	Gas	Did not know	N/A	N/A	2 to 10/day
R25	H	Showerdrain	BF	CH	Gas	Did not know	N/A	N/A	2 to 10/day
R26	H	Handbasin	BF	CH	Gas	No	More than 20	More than 20	2 to 10/day
R27	H	Handbasin	BF	CH	Gas	No	More than 20	More than 20	2 to 10/day
R28	H	Handbasin	BF	CH	Gas	No	More than 20	Less than 5	More than 10/day
R29	H	Handbasin	BF	CH	Gas	No	More than 20	Less than 5	More than 10/day
R3	H	Kitchen	BF	CH	Did not know	Did not know	N/A	N/A	N/A
R30	N/A	Handbasin	BF	CH	Did not know	Did not know	Less than 5	Less than 5	More than 10/day
R31	N/A	Handbasin	BF	CH	Did not know	Did not know	Less than 5	Less than 5	More than 10/day

R32	H	Showerhead	BF	CH	Electric	Did not know	More than 20	More than 20	2 to 10/day
R34	A	Handbasin	BF	CH	Did not know	Did not know	More than 20	10 to 14	2 to 10/day
R35	A	Handbasin	BF	CH	Did not know	Did not know	More than 20	10 to 14	2 to 10/day
R36	A	Handbasin	BF	CH	Gas	Did not know	Less than 5	Less than 5	2 to 10/day
R37	A	Handbasin	BF	CH	Gas	Did not know	Less than 5	Less than 5	2 to 10/day
R38	N/A	Kitchen	BF	CH	Gas	Did not know	More than 20	Less than 5	More than 10/day
R39	N/A	Kitchen	BF	CH	Gas	No	More than 20	Less than 5	More than 10/day
R4	H	Showerhead	BF	CH	Did not know	Did not know	N/A	N/A	N/A
R40	N/A	Handbasin	BF	CH	Did not know	Did not know	5 to 9	5 to 9	2 to 10/day
R41	N/A	Handbasin	BF	CH	Did not know	Did not know	5 to 9	5 to 9	2 to 10/day

R44	H	Kitchen	BF	CH	Gas	Yes	More than 20	10 to 14	2 to 10/day
R45	H	Kitchen	BF	CH	Gas	Yes	More than 20	10 to 14	2 to 10/day
R46	H	Handbasin	BF	CH	Gas	No	Less than 5	Less than 5	2 to 10/day
R47	H	Handbasin	BF	CH	Gas	No	Less than 5	Less than 5	2 to 10/day
R5	H	Handbasin	BF	CH	Did not know	Did not know	N/A	N/A	N/A
R50	H	Handbasin	BF	CH	Gas	Yes	5 to 9	5 to 9	2 to 10/day
R51	H	Kitchen	BF	CH	Gas	Yes	5 to 9	5 to 9	2 to 10/day
R6	H	Handbasin	BF	CH	Did not know	Did not know	N/A	N/A	N/A
R60	H	Handbasin	BF	CH	Gas	Did not know	N/A	N/A	More than 10/day
R61	H	Handbasin	BF	CH	Gas	Did not know	N/A	N/A	More than 10/day

R68	H	Kitchen	BF	CH	Gas	No	Less than 5	Less than 5	2 to 10/day
R69	H	Kitchen	BF	CH	Gas	No	Less than 5	Less than 5	2 to 10/day
R7	H	Handbasin	BF	CH	Did not know	Did not know	N/A	N/A	N/A
R70	H	Handbasin	BF	CH	Gas	No	Less than 5	Less than 5	2 to 10/day
R71	H	Handbasin	BF	CH	Gas	No	Less than 5	Less than 5	2 to 10/day
R72	H	Handbasin	BF	CH	Gas	No	Less than 5	Less than 5	2 to 10/day
R73	H	Handbasin	BF	CH	Gas	No	Less than 5	Less than 5	2 to 10/day
R74	N/A	Handbasin	BF	CH	Did not know	Did not know	More than 20	10 to 14	More than 10/day
R75	N/A	Handbasin	BF	CH	Did not know	Did not know	More than 20	10 to 14	More than 10/day

R76	H	Handbasin	BF	CH	Electric	Yes	More than 20	More than 20	2 to 10/day
R77	H	Handbasin	BF	CH	Electric	Yes	More than 20	More than 20	2 to 10/day
R78	H	Showerhead	BF	CH	Gas	No	More than 20	More than 20	2/day
R79	H	Shower drain	BF	CH	Gas	No	More than 20	More than 20	2/day
R8	H	Handbasin	BF	CH	Did not know	Did not know	N/A	N/A	N/A
R80	H	Handbasin	BF	CH	Electric	Yes	More than 20	More than 20	More than 10/day
R81	H	Handbasin	BF	CH	Electric	Yes	More than 20	More than 20	More than 10/day
R84	H	Handbasin	BF	CH	Electric	No	More than 20	More than 20	2/day
R85	H	Handbasin	BF	CH	Electric	No	More than 20	More than 20	2/day

R86	H	Handbasin	BF	CH	Electric	No	More than 20	More than 20	2/day
R87	H	Handbasin	BF	CH	Electric	No	More than 20	More than 20	2/day
R88	H	Bath Drain	BF	CH	Electric	Yes	More than 20	More than 20	1/fortnight
R89	H	Bath Faucet	BF	CH	Electric	Yes	More than 20	More than 20	1/fortnight
R9	H	Handbasin	BF	CH	Did not know	Did not know	N/A	N/A	N/A
R90	H	Bath Drain	BF	CH	Electric	Did not know	More than 20	More than 20	2 to 10/day
R91	H	Bath Faucet	BF	CH	Electric	Did not know	More than 20	More than 20	2 to 10/day
R94	H	Showerhead	BF	CH	Electric	Yes	More than 20	More than 20	2 to 10/day
R95	H	Showerdrain	BF	CH	Electric	Yes	More than 20	More than 20	2 to 10/day

R96	H	Bath Faucet	BF	CH	Electric	Yes	More than 20	More than 20	1/day
R97	H	Bath Drain	BF	CH	Electric	Yes	More than 20	More than 20	1/day
R98	H	Handbasin	BF	CH	Electric	Yes	More than 20	More than 20	More than 10/day
R99	H	Handbasin	BF	CH	Electric	Yes	More than 20	More than 20	More than 10/day
58S1	N/A	Shower	W	MAN	Gas	No	More than 20	5 to 9	2 to 10/day
58S2	N/A	Shower	W	MAN	Gas	No	More than 20	5 to 9	2 to 10/day
60T	N/A	Tap faucet	BF	MAN	Gas	No	More than 20	5 to 9	More than 10/day
68S1	N/A	Shower	W	MAN	Gas	No	More than 20	5 to 9	2 to 10/day
68S2	N/A	Shower	W	MAN	Gas	No	More than 20	5 to 9	2 to 10/day

71S1	N/A	Shower	W	MAN	Gas	No	More than 20	5 to 9	2 to 10/day
71S2	N/A	Shower	W	MAN	Gas	No	More than 20	5 to 9	2 to 10/day
71T1	N/A	Tap faucet	BF	MAN	Gas	No	More than 20	5 to 9	More than 10/day
71T2	N/A	Tap faucet	BF	MAN	Gas	No	More than 20	5 to 9	More than 10/day
74S1	N/A	Shower	W	MAN	Gas	No	More than 20	5 to 9	2 to 10/day
74S2	N/A	Shower	W	MAN	Gas	No	More than 20	5 to 9	2 to 10/day
74T	N/A	Tap faucet	BF	MAN	Gas	No	More than 20	5 to 9	More than 10/day
75S1	N/A	Shower	W	MAN	Gas	No	More than 20	5 to 9	2 to 10/day
75S2	N/A	Shower	W	MAN	Gas	No	More than 20	5 to 9	2 to 10/day

AE01	N/A	Shower	W	MAN	Electric	No	N/A	Less than 5	2 to 10/day
AWDS	N/A	Shower	W	MAN	Did not know	Did not know	N/A	N/A	N/A
AWUS	N/A	Shower	W	MAN	Did not know	Did not know	N/A	N/A	N/A
CH01	H	Shower	W	MAN	Gas	No	More than 20	More than 20	2/day
CH02	H	Shower	W	MAN	Gas	No	More than 20	More than 20	1/day
CT01	N/A	Shower	W	MAN	Did not know	Did not know	N/A	N/A	N/A
EF01	N/A	Shower	W	MAN	Gas	No	More than 20	Less than 5	2/day
EK01	N/A	Shower	W	MAN	Gas	No	More than 20	N/A	2 to 10/day
EK03	N/A	Shower	W	MAN	Gas	No	Less than 5	Less than 5	2/day
EK04	N/A	Shower	W	MAN	Gas	No	Less than 5	Less than 5	Less than 1/month

FL2-1	A	Shower	W	MAN	Gas	No	More than 20	Less than 5	2 to 10/day
FL2-2	A	Shower	W	MAN	Gas	No	More than 20	Less than 5	2 to 10/day
FL2-3	A	Shower	W	MAN	Gas	No	More than 20	Less than 5	2 to 10/day
FL2-4	A	Shower	W	MAN	Gas	No	More than 20	Less than 5	2 to 10/day
FL2-5	A	Shower	W	MAN	Gas	No	More than 20	Less than 5	2 to 10/day
FL2-6	A	Shower	W	MAN	Gas	No	More than 20	Less than 5	2 to 10/day
FL3-1	A	Shower	W	MAN	Gas	No	More than 20	5 to 9	2 to 10/day
FL3-2	A	Shower	W	MAN	Gas	No	More than 20	5 to 9	2 to 10/day
FL3-3	A	Shower	W	MAN	Ga	No	More than 20	5 to 9	2 to 10/day

FL3-4	A	Shower	W	MAN	Gas	No	More than 20	5 to 9	2 to 10/day
HW01	H	Shower	W	MAN	Gas	No	More than 20	Less than 5	2/day
HW02	H	Shower	W	MAN	Gas	No	More than 20	Less than 5	1/week
HW03	H	Shower	W	MAN	Gas	No	Less than 5	Less than 5	Less than 1/month
HW04	H	Shower	W	MAN	Gas	No	Less than 5	Less than 5	Less than 1/month
HW05	H	Shower	W	MAN	Gas	No	Less than 5	Less than 5	2/day
JW01	H	Shower	W	MAN	Gas	No	More than 20	5 to 9	1/week
JW02	H	Shower	W	MAN	Gas	No	More than 20	5 to 9	2/day
KRDS	H	Shower	W	MAN	Electric	Yes	More than 20	10 to 14	1/week

KRUS	H	Shower	W	MAN	Electric	Yes	More than 20	10 to 14	1/day
KS01	N/A	Shower	W	MAN	Gas	No	More than 20	Less than 5	1/day
KS02	N/A	Shower	W	MAN	Gas	No	More than 20	Less than 5	1/day
KS03	N/A	Shower	W	MAN	Gas	No	More than 20	Less than 5	1/day
KS04	N/A	Shower	W	MAN	Gas	No	More than 20	Less than 5	Less than 1/month
MW01	N/A	Shower	W	MAN	Solar	Yes	More than 20	Less than 5	2/day
NK01	N/A	Shower	W	MAN	Solar	Yes	More than 20	Less than 5	2/day
NM01	N/A	Shower	W	MAN	Did not know	Did not know	More than 20	More than 20	2 to 10/day
NS01	N/A	Shower	W	MAN	Electric	Yes	N/A	N/A	1/day

P1	N/A	Tap fauc	BF	MAN	Gas	No	More than 20	10 to 14	N/A
P2	N/A	Tap fauc	BF	MAN	Gas	No	More than 20	10 to 14	N/A
P3	N/A	Tap fauc	BF	MAN	Gas	No	More than 20	10 to 14	N/A
P4	N/A	Tap fauc	BF	MAN	Gas	No	More than 20	10 to 14	N/A
PT01	N/A	Shower	W	MAN	Gas	No	More than 20	More than 20	1/day
PT04	N/A	Shower	W	MAN	Electric	Yes	More than 20	Less than 5	1/day
PT05	N/A	Shower	W	MAN	Electric	Yes	More than 20	Less than 5	Less than 1/month
PT06	N/A	Shower	W	MAN	Electric	Yes	More than 20	Less than 5	1/day
RJ01	N/A	Shower	W	MAN	Gas	No	5 to 9	5 to 9	2 to 10/day

RJ02	N/A	Shower	W	MAN	Gas	No	5 to 9	5 to 9	Less than 1/month
SB01	N/A	Shower	W	MAN	Electric	Yes	5 to 9	5 to 9	1/day
SB02	N/A	Shower	W	MAN	Electric	Yes	5 to 9	5 to 9	1/day
SB03	N/A	Shower	W	MAN	Electric	Yes	5 to 9	5 to 9	1/day
T0	N/A	Tap faucet	BF	MAN	Gas	No	More than 20	10 to 14	More than 10/day
TD01	N/A	Shower	W	MAN	Gas	No	More than 20	10 to 14	2 to 10/day
TD02	N/A	Shower	W	MAN	Gas	No	More than 20	10 to 14	1/day
TK01	N/A	Shower	W	MAN	Gas	Did not know	N/A	Less than 5	2 to 10/day

N/A = Data not collected

◊ = H: house; A: apartment

§ = BF: biofilm; W: water

□ = CH: Claire Hayward; MAN: Muhammad Atif Nisar

Table 12.2 – Hospital abiotic factors

Sample ID	Sample Site	Sample Type	One month prior to sampling (flow count)
1	Basin	Biofilm	125
2	Basin	Biofilm	88
3	Basin	Biofilm	163
4	Basin	Biofilm	147
5	Basin	Biofilm	206
6	Shower	Biofilm	55
7	Basin	Biofilm	88
8	Shower	Biofilm	152
9	Basin	Biofilm	104
10	Shower	Biofilm	7
11	Shower	Biofilm	242
12	Basin	Biofilm	161
13	Shower	Biofilm	37
14	Basin	Biofilm	79
15	Shower	Biofilm	39
16	Basin	Biofilm	44
17	Basin	Biofilm	27
18	Shower	Biofilm	247
19	Basin	Biofilm	359
20	Shower	Biofilm	183
21	Basin	Biofilm	235

22	Basin	Biofilm	422
23	Basin	Water	163
24	Basin	Water	35
25	Basin	Water	125
26	Shower	Water	125
27	Basin	Water	183
28	Basin	Water	147
29	Shower	Water	242
30	Shower	Water	383
31	Basin	Water	88
32	Basin	Water	44
33	Shower	Water	55
34	Basin	Water	206
35	Shower	Water	152
36	Basin	Water	62
37	Shower	Water	202
38	Basin	Water	235
39	Shower	Water	7
40	Basin	Water	79
41	Shower	Water	37
42	Basin	Water	27
43	WC	Water	267
44	WC	Water	N/A

45	Shower	Water	39
46	Basin	Water	104
47	Shower	Water	422
48	Shower	Water	242
49	Basin	Water	247
50	WC	Water	64359
51	Basin	Water	161
52	Shower	Water	359
61	Basin	Water	N/A
62	Basin	Water	N/A
63	Basin	Water	N/A
65	Shower	Water	N/A
66	Basin	Water	N/A
67	Basin	Water	N/A
68	Basin	Water	N/A
70	Basin	Water	N/A
71	Basin	Water	N/A
72	Tap	Biofilm	N/A
73	Drain	Biofilm	N/A
74	Tap	Biofilm	N/A
75	Faucet	Biofilm	N/A
76	Drain	Biofilm	N/A
77	Tap	Biofilm	N/A

78	Faucet	Biofilm	N/A
79	Drain	Biofilm	N/A
80	Tap	Biofilm	N/A
81	Faucet	Biofilm	N/A
82	Drain	Biofilm	N/A
83	Faucet	Biofilm	N/A
84	Drain	Biofilm	N/A
85	Tap	Biofilm	N/A
86	Faucet	Biofilm	N/A
87	Drain	Biofilm	N/A
88	Tap	Biofilm	N/A
89	Faucet	Biofilm	N/A
90	Drain	Biofilm	N/A
10B	Faucet	Biofilm	N/A
11B	Faucet	Biofilm	N/A
12B	TMV	Biofilm	N/A
13B	TMV	Biofilm	N/A
14B	TMV	Biofilm	280
1B	Faucet	Biofilm	2
2B	Faucet	Biofilm	2
3B	Faucet	Biofilm	2
4B	TMV	Biofilm	109
5B	TMV	Biofilm	109

6B	Faucet	Biofilm	96
7B	Faucet	Biofilm	96
8B	TMV	Biofilm	N/A
9B	TMV	Biofilm	N/A
B63R20	Basin	Water	94
HBR12T76	Basin	Water	421
HBR12T76	Basin	Water	477
HBR12T76	Basin	Water	531
R13B	Basin	Water	175
R13B	Basin	Water	46
R13B	Basin	Water	123
R13S	Shower	Water	437
R15B	Basin	Water	72
R15B	Basin	Water	477
R15B	Basin	Water	207
R15B	Basin	Water	221
R15S	Shower	Water	330
R15S	Shower	Water	418
R15S	Shower	Water	416
R15S	Shower	Water	796
R17B	Basin	Water	148
R17S	Shower	Water	796
R19B	Basin	Water	307

R19B	Basin	Water	253
R19B	Basin	Water	4
R19B	Basin	Water	109
R19S	Shower	Water	210
R19S	Shower	Water	325
R19S	Shower	Water	59
R19S	Shower	Water	141
R20B	Basin	Water	325
R20B	Basin	Water	273
R20B	Basin	Water	192
R20S	Shower	Water	18
R20S	Shower	Water	64
R20S	Shower	Water	57
R20S	Shower	Water	54
R21B	Basin	Water	N/A
R21B	Basin	Water	102
R21B	Basin	Water	357
R21B	Basin	Water	N/A
R21S	Shower	Water	2
R21S	Shower	Water	313
R21S	Shower	Water	10
R21S	Shower	Water	136
R22B	Basin	Water	267

R22B	Basin	Water	188
R22B	Basin	Water	152
R22B	Basin	Water	363
R22S	Shower	Water	192
R22S	Shower	Water	251
R22S	Shower	Water	135
R22S	Shower	Water	324
R23B	Basin	Water	206
R23S	Shower	Water	N/A
R24B	Basin	Water	290
R24B	Basin	Water	358
R24B	Basin	Water	71
R24B	Basin	Water	131
R24S	Shower	Water	429
R24S	Shower	Water	390
R24S	Shower	Water	163
R24S	Shower	Water	147
R25B	Basin	Water	883
R25B	Basin	Water	355
R25B	Basin	Water	176
R25B	Basin	Water	242
R25S	Shower	Water	889
R25S	Shower	Water	168

R25S	Shower	Water	85
R25S	Shower	Water	361
R27B	Basin	Water	N/A
R27S	Shower	Water	N/A
R28S	Shower	Water	N/A
R29B	Basin	Water	148
R29B	Basin	Water	171
R29B	Basin	Water	288
R29B	Basin	Water	257
R29S	Shower	Water	423
R29S	Shower	Water	437
R29S	Shower	Water	413
R29S	Shower	Water	127
R31B	Basin	Water	269
R31B	Basin	Water	286
R31B	Basin	Water	538
R31B	Basin	Water	139
R31S	Shower	Water	304
R31S	Shower	Water	462
R31S	Shower	Water	244
R31S	Shower	Water	359
R32B	Basin	Water	217
R32B	Basin	Water	258

R32B	Basin	Water	400
R32B0T1	Basin	Water	96
R32B0T2	Basin	Water	96
R32S	Shower	Water	102
R32S	Shower	Water	144
R32S	Shower	Water	148
R32S0T1	Shower	Water	109
R32S0T2	Shower	Water	109
R33B	Basin	Water	414
R33B	Basin	Water	229
R33B	Basin	Water	46
R33B	Basin	Water	334
R33S	Shower	Water	N/A
R33S	Shower	Water	295
R33S	Shower	Water	361
R33S	Shower	Water	N/A
R34B	Basin	Water	N/A
R34B	Basin	Water	N/A
R34B	Basin	Water	59
R34S	Shower	Water	132
R34S	Shower	Water	103
R34S	Shower	Water	363
S1	Shower	Biofilm	388

S10	Shower	Biofilm	363
S2	Shower	Biofilm	388
S22	TMV	Biofilm	530
S23	Shower	Biofilm	210
S24	Basin	Biofilm	307
S25	TMV	Biofilm	54
S26	Basin	Biofilm	139
S27	Shower	Biofilm	390
S28	TMV	Biofilm	147
S29	Basin	Biofilm	123
S3	Faucet	Biofilm	123
S30	Basin	Biofilm	206
S31	TMV	Biofilm	530
S32	Basin	Biofilm	363
S33	TMV	Biofilm	54
S34	Shower	Biofilm	144
S35	Shower	Biofilm	359
S36	TMV	Biofilm	147
S37	Faucet	Biofilm	450
S38	Basin	Biofilm	147
S4	Shower	Biofilm	64
S5	Shower	Biofilm	64
S6	Faucet	Biofilm	400

S7	Shower	Biofilm	148
S8	Faucet	Biofilm	59
S9	Shower	Biofilm	363
STB15	Basin	Water	N/A
STB15	Basin	Water	530
STB15	Basin	Water	N/A
VWCT69	Basin	Water	147
VWCT69	Basin	Water	269
VWCT69	Basin	Water	192
VWCT69	Basin	Water	416
W1	Basin	Water	N/A
W11	Basin	Biofilm	450
W12	Basin	Biofilm	450
W13	Basin	Biofilm	520
W14	Basin	Biofilm	520
W15	Basin	Biofilm	147
W2	Basin	Water	N/A
W3	Basin	Water	N/A

N/A = data not collected

Table 12.3 - Minimum and maximum microbial concentrations present in the positive water and biofilm samples.

Target pathogen	Minimum Concentration	Maximum concentration
Water (GU/L)		
Vermamoeba vermiformis	2.7×10^2	7.47×10^7
Acanthamoeba spp.	1.40×10^2	2.33×10^6
Pseudomonas aeruginosa	1.08×10^3	1.3×10^7
Staphylococcus aureus	4.73×10^3	3.27×10^9
Legionella spp.	1×10^2	2.8×10^6
Legionella pneumophila	4.0×10^1	3.5×10^5
Acinetobacter baumannii	2.67×10^2	2.4×10^3
Biofilm (GU/swab)		
Vermamoeba vermiformis	1.2×10^2	3.45×10^8
Acanthamoeba spp.	1.16×10^2	3.63×10^8
Pseudomonas aeruginosa	1.36×10^3	1.67×10^{10}
Staphylococcus aureus	1.5×10^2	2.62×10^8
Legionella spp.	1.3×10^1	7.7×10^4
Legionella pneumophila	5.0×10^1	1.12×10^6
Acinetobacter baumannii	1.36×10^2	3.33×10^5

13. APPENDIX – 3

Supplementary data for the manuscript entitled "CITATION: Drinking water plumbing systems are a hot spot for antimicrobial resistant pathogens (Chapter 5)

Table 13.1 - Sequences of oligos and fluorogenic probes, and reaction conditions used for pathogen identification qPCR assays

	Sequence and Fluorescence signal (5' – 3')	Assay Conditions	Reference
Acinetobacter baumannii ompA gene			
Forward Primer	TCTTGGTGGTCACTTGAAGC	Hold: 95°C/5 min 45 cycles: 95°C/15 s, 60°C/30 s and 72°C/20 s	(McConnell Michael et al., 2012)
Reverse Primer	ACTCTTGTGGTTGTGGAGCA		
Probe	6FAM-AAGTTGCTCCAGTTGAACCAACTCCA-Iowa Black FQ		
Pseudomonas aeruginosa gyrB gene			
Forward Primer	GGCGTGGGTGTGGAAGTC	Hold: 95°C/5 min 45 cycles: 95°C/15 s, 60°C/30 s and 72°C/20 s	(Lee et al., 2011)
Reverse Primer	TGGTGGCGATCTTGAACTTCTT		
Probe	6FAM-TGCAGTGGAACGACA- Iowa Black FQ		
Staphylococcus aureus nuc gene			
Forward Primer	TGCCTTTACAGATAGCATGCCA	Hold: 95°C/5 min 45 cycles: 95°C/15 s, 60°C/30 s and 72°C/20 s	(Galia et al., 2019)
Reverse Primer	CCAGTAACGTCGAACGAATGAATGA		
Probe	6FAM-TCATTTCACGCAAACTGTTGGCC-Iowa Black FQ		

Table 13.2 - Antibiotics tested, including respective classes, names, and disc contents used for antimicrobial susceptibility testing.

Species	Class	Antibiotic	Disc content
Acinetobacter baumannii	Penicillins	Piperacillin	100 µG
	β-lactam combination agents	Piperacillin-tazobactam	100/10 µG
	Cephems	Ceftazidime	30 µG
		Cefepime	30 µG
	Carbapenems	Doripenem	10 µG
		Imipenem	10 µG
		Meropenem	10 µG
	Aminoglycosides	Gentamicin	10 µG
		Tobramycin	10 µG
	Fluoroquinolones	Ciprofloxacin	5 µG
		Levofloxacin	5 µG
	Folate pathway antagonist	Trimethoprim-sulfamethoxazole	1.25/23.75 µG
Pseudomonas aeruginosa	Penicillins	Piperacillin	100 µG
	β-lactam combination agents	Piperacillin-tazobactam	100/10 µG
		Ceftazidime-avibactam	30/20 µG
	Cephems	Ceftazidime	30 µG
		Cefepime	30 µG
	Carbapenems	Doripenem	10 µG

		Imipenem	10 µG
		Meropenem	10 µG
	Aminoglycosides	Gentamicin	10 µG
		Tobramycin	10 µG
	Fluoroquinolones	Ciprofloxacin	5 µG
		Levofloxacin	5 µG
Staphylococcus aureus	Penicillinase-labile penicillins	Penicillin	10 units
	Penicillinase-stable penicillins	Cefoxitin	30 µG
	Aminoglycosides	Gentamicin	10 µG
		Tobramycin	10 µG
	Fluoroquinolones	Ciprofloxacin	5 µG
		Levofloxacin	5 µG
	Folate pathway antagonist	Trimethoprim-sulfamethoxazole	1.25/23.75 µG

Table 13.3 - Sequences of oligos and fluorogenic probes, and reaction conditions used for carbapenem resistance gene qPCR assays

	Sequence and Fluorescence signal (5' – 3')	Assay Conditions	Reference
***bla*$_{NDM-1}$**			
Forward primer	GGGCAGTCGCTTCCAACGGT	30 cycles: 95°C/45 s, 53°C/ 30 s. and 72°C/30 s Melt: 65-95°C, 0.5°C increments at 5 s/step	(Gondal et al., 2024)
Reverse primer	GTAGTGCTCAGTGTCGGCAT		
***bla*$_{OXA-48}$**			
Forward primer	GCGTGGTTAAGGATGAACAC	30 cycles: 95°C/45 s, 52°C/ 30 s. and 72°C/30 s Melt: 65-95°C, 0.5°C increments at 5 s/step	(Gondal et al., 2024)
Reverse primer	CATCAAGTTCAACCCAACCG		
***bla*$_{KPC-2}$**			
Forward primer	GCTACACCTAGCTCCACCTTC	30 cycles: 95°C/45 s, 55°C/ 30 s. and 72°C/30 s Melt: 65-95°C, 0.5°C increments at 5 s/step	(Gondal et al., 2024)
Reverse primer	ACAGTGGTTGGTAATCCATGC		

***bla*$_{VIM}$**			
Forward primer	GATGGTGTTTGGTCGCATA	30 cycles: 95^{o}C/45 s, 52^{o}C/ 30 s. and 72^{o}C/30 s Melt: 65-95^{o}C, 0.5^{o}C increments at 5 s/step	(Gondal et al., 2024)
Reverse primer	CGAATGCGCAGCACCAG		

14. APPENDIX – 4

Supplementary data for the manuscript entitled CITATION: Handwashing basins and healthcare associated infections: Bacterial diversity in biofilms on faucets and drains (Chapter 6)

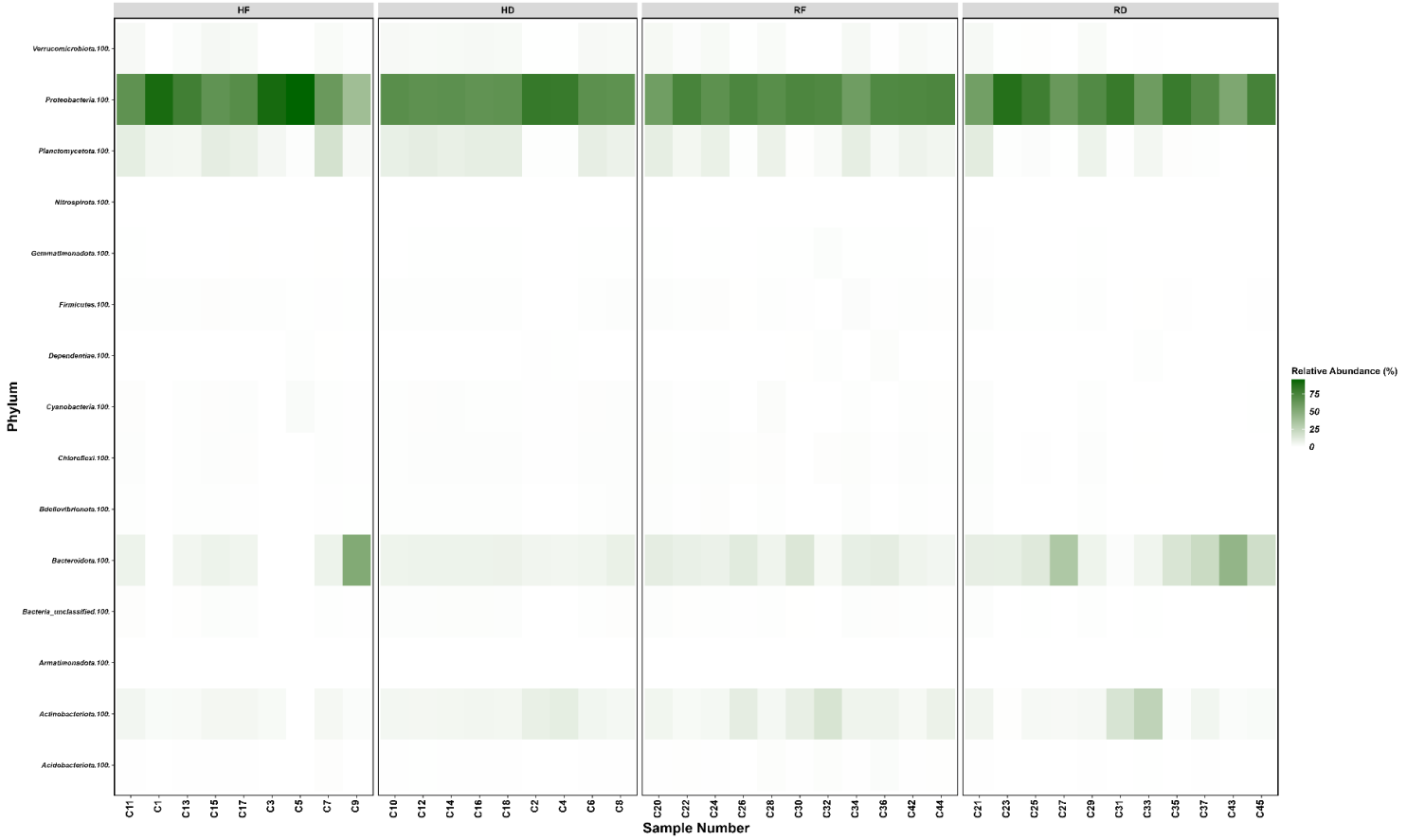

Figure 14.1 - Heatmap of abundant bacterial phyla present in hospital and residential handbasin biofilms. Biofilm samples are indicated along the X-axis and divided by sampling factor (HF = hospital faucet; HD, hospital drain; RF = residential drain and RD = residential drain). Bacterial phyla are indicated along the Y-axis. The relative abundance of each phyla is indicated by colors ranging from white (low abundance) to dark green (high abundance). Numbers included after the genus name denotes sequence resolution

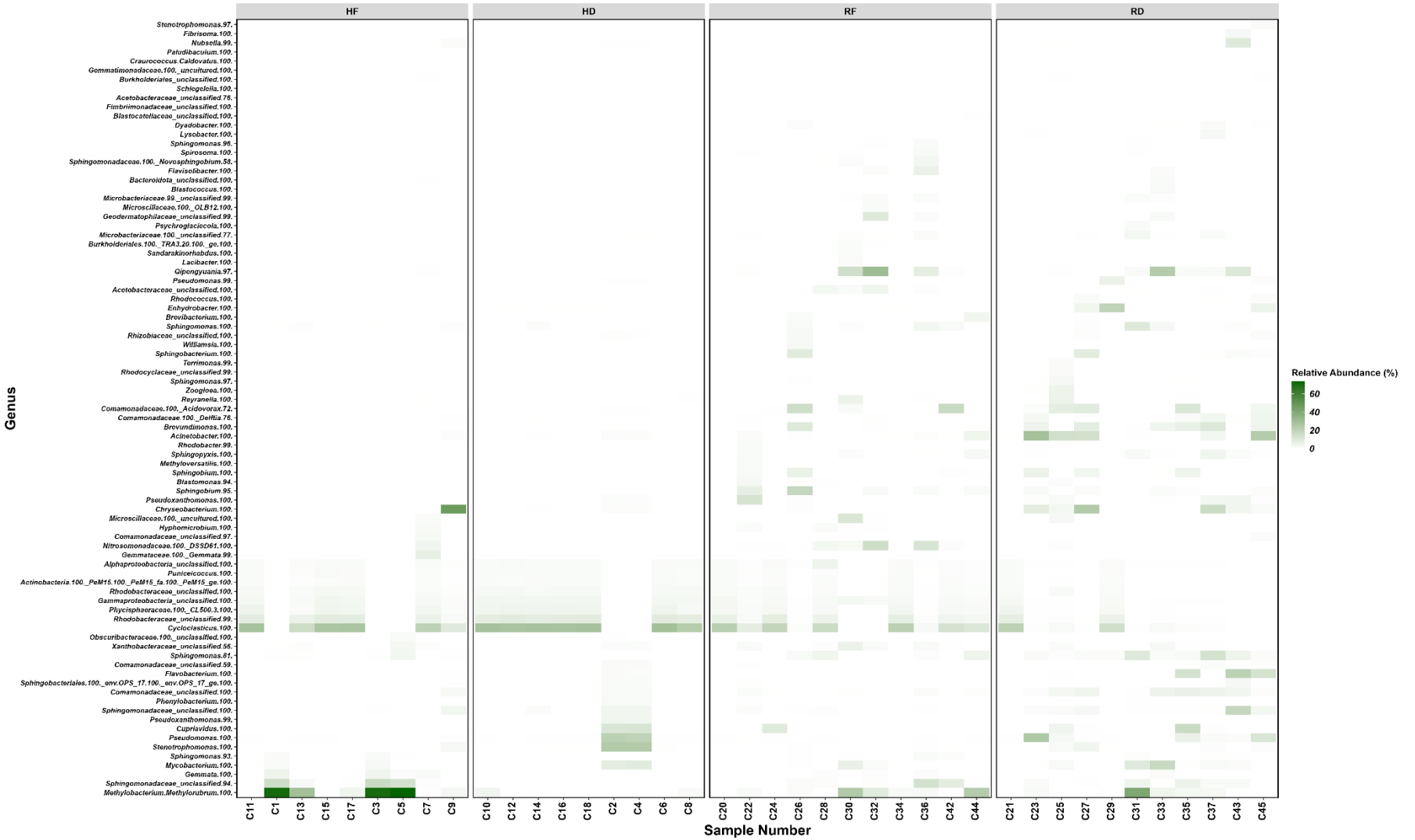

Figure 14.2 - Heatmap of abundant bacterial genera present in hospital and residential handbasin biofilms. Biofilm samples are indicated along the X-axis and divided by sampling factor (HF = hospital faucet; HD, hospital drain; RF = residential drain and RD = residential drain). Bacterial genera are indicated along the Y-axis. The relative abundance of each genera is indicated by colors ranging from white (low abundance) to dark green (high abundance). Numbers included after the genus name denotes sequence resolution

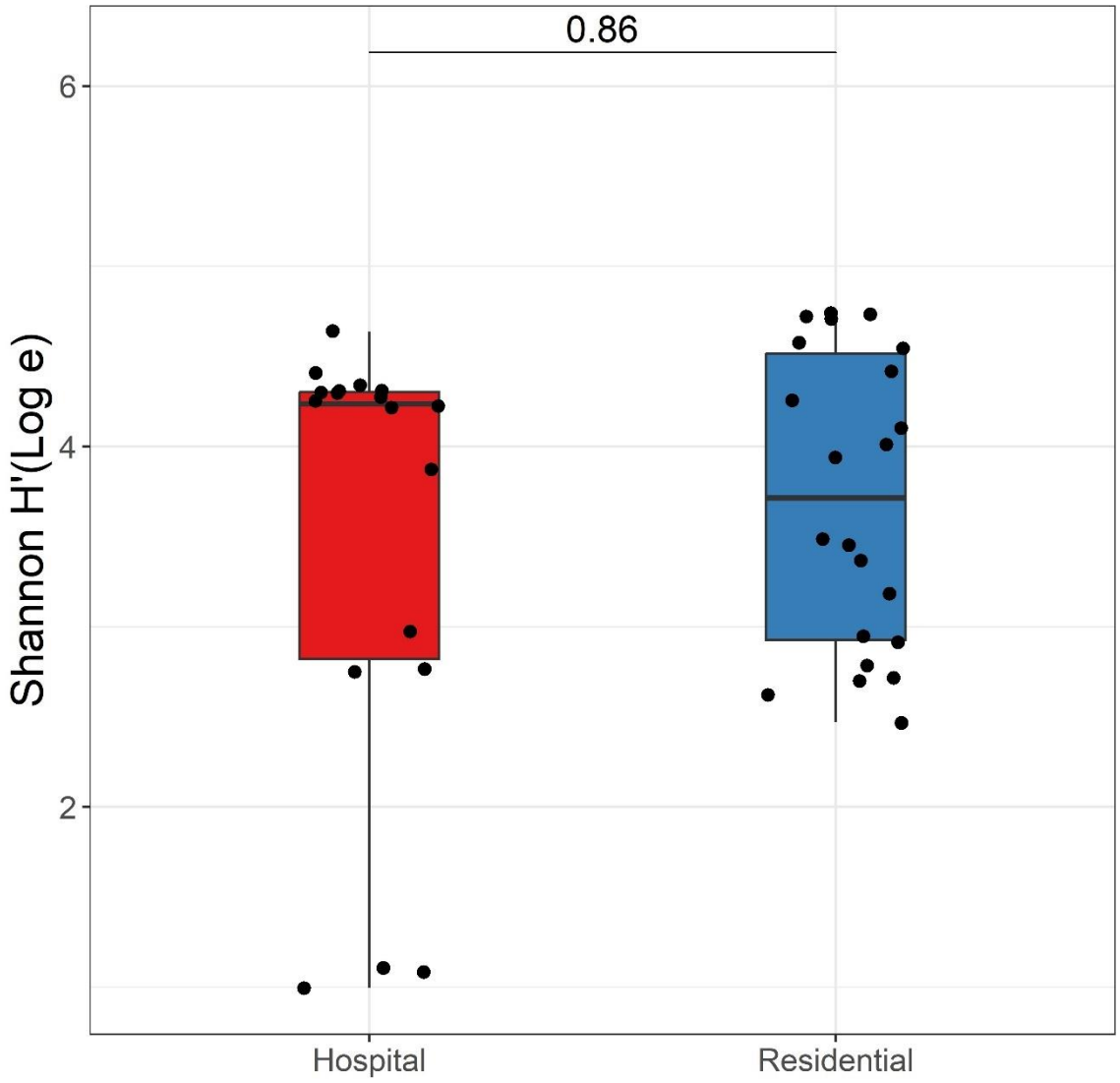

Figure 14.3 - Alpha diversity indices (Shannon Index) of bacterial communities at genus level affected by building type

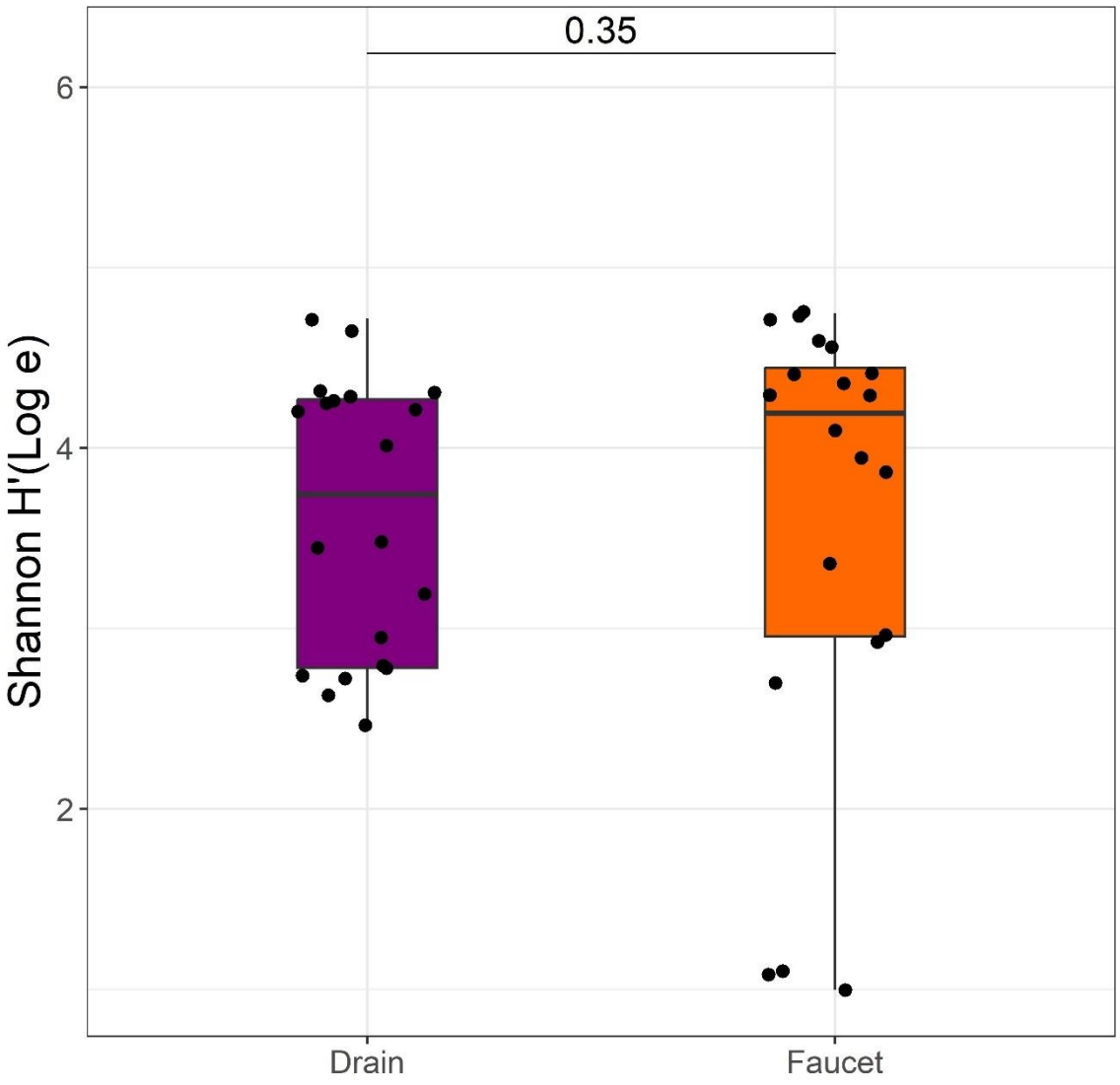

Figure 14.4 - Alpha diversity indices (Shannon Index) of bacterial communities at genus level affected by sampling site

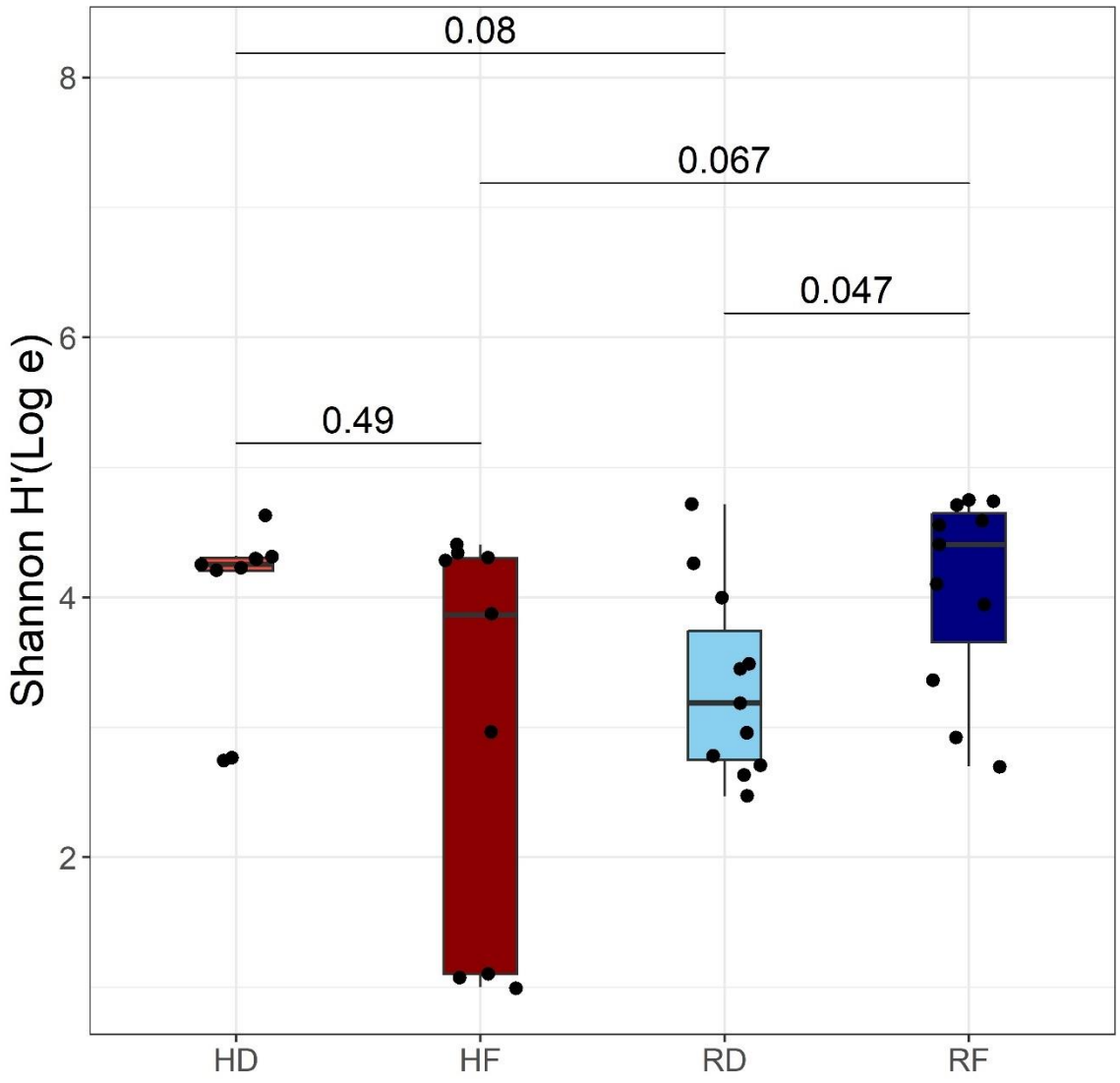

Figure 14.5 - Alpha diversity indices (Shannon Index) of bacterial communities at genus level affected by building type and site hospital faucet

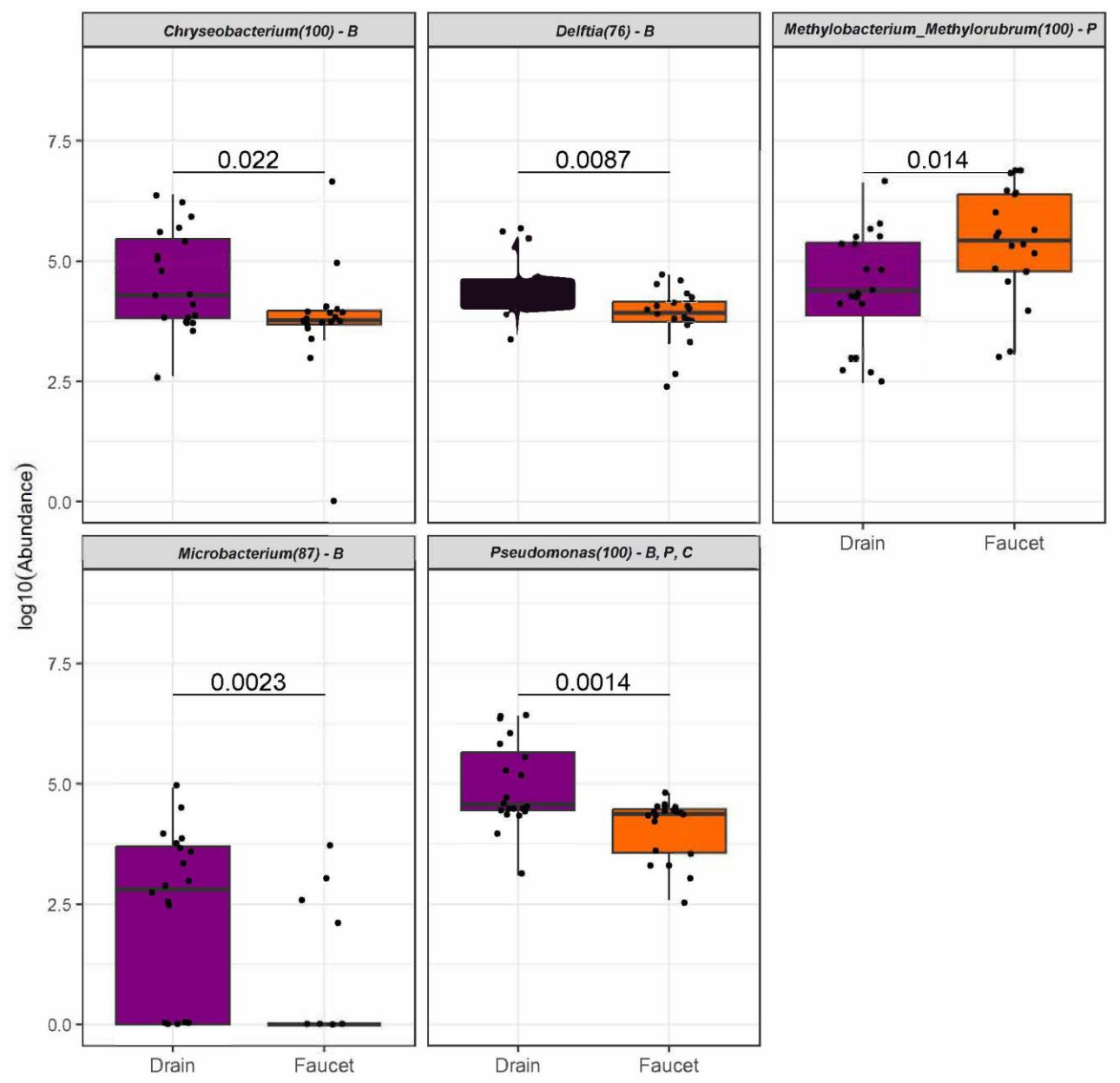

Figure 14.6 - Relative abundance (Log^{10} transformed) of pathogenic, corrosive and biofilm forming bacterial genera (n=5) that differed significantly between faucets (orange) and drains (purple). B = Biofilm forming, P = pathogenic and C = corrosive genera.

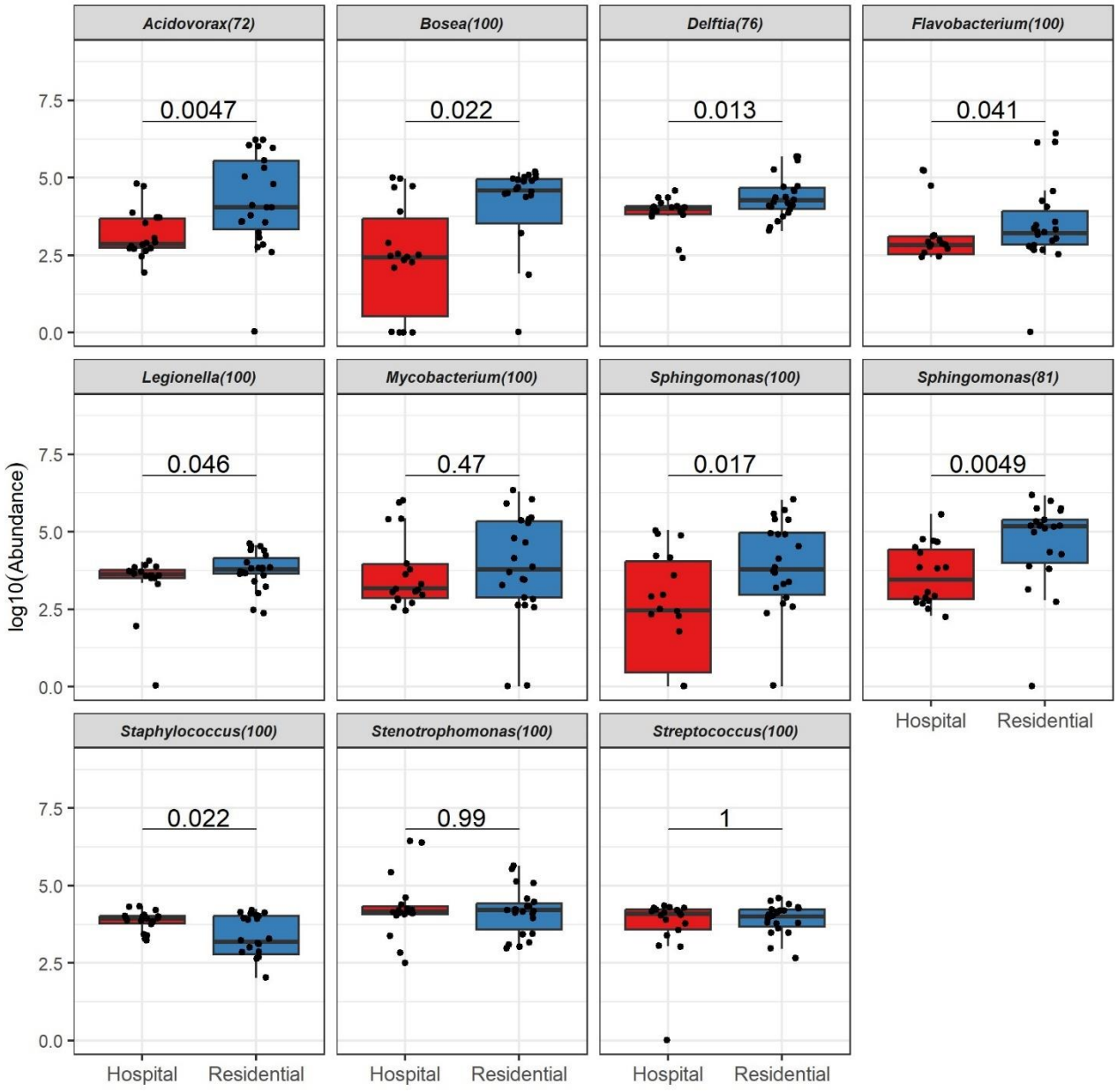

Figure 14.7 - Relative abundance (Log^{10} transformed) of pathogenic, corrosive and biofilm forming bacterial genera (n=8) that differed significantly between hospital (red) and residential (blue) buildings

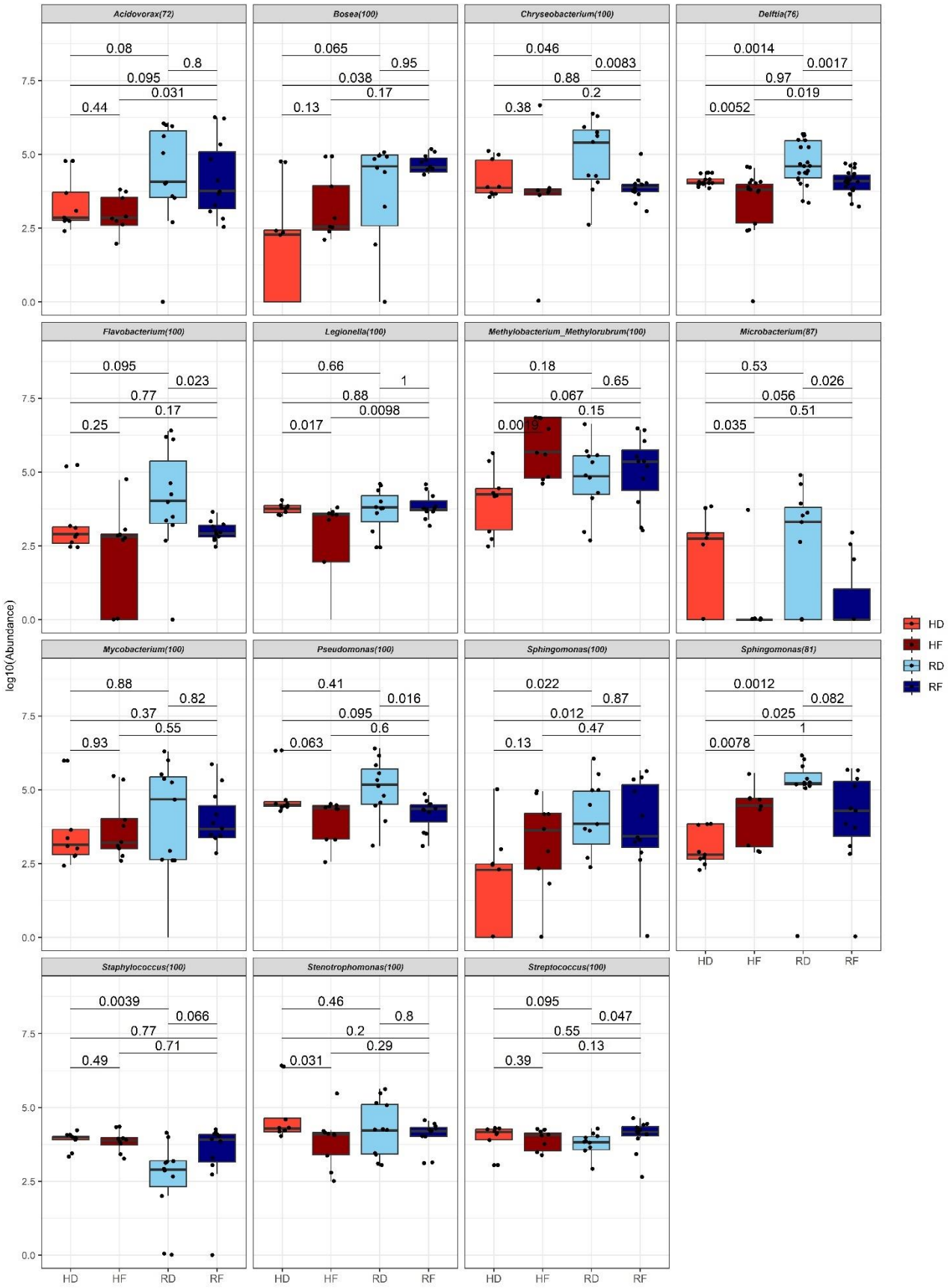

Figure 14.8 - Relative abundance (Log[10] transformed) of pathogenic, corrosive and biofilm forming bacterial genera (n=8) that differed significantly between sampling factors

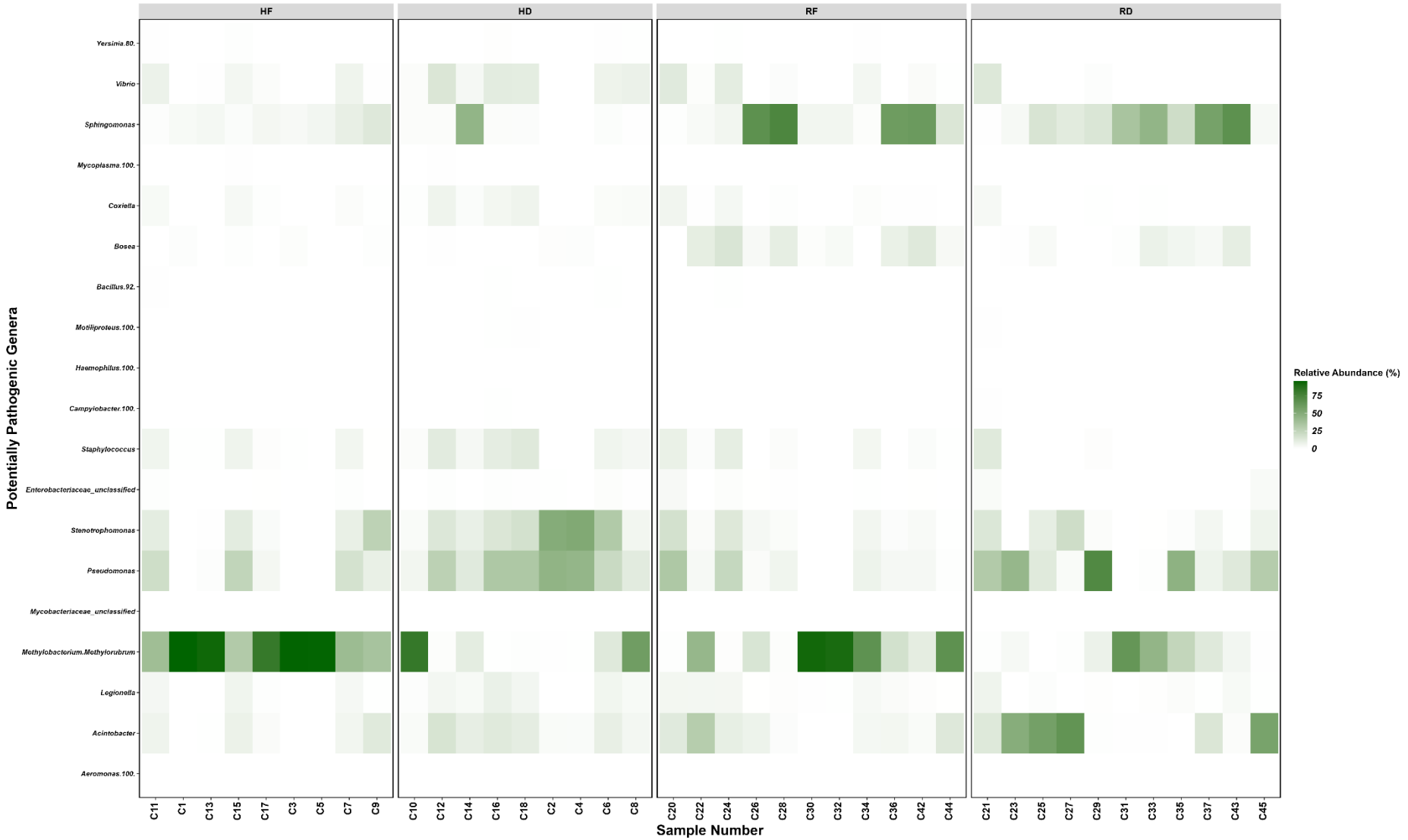

Figure 14.9 - Heatmap of abundant potentially pathogenic genera present in hospital and residential handbasin biofilms. Biofilm samples are indicated along the X-axis and divided by sampling factor (HF = hospital faucet; HD, hospital drain; RF = residential drain and RD = residential drain). Bacterial genera are indicated along the Y-axis. The relative abundance of each genera is indicated by colors ranging from white (low abundance) to dark green (high abundance). Numbers included after the genus name denotes sequence resolution

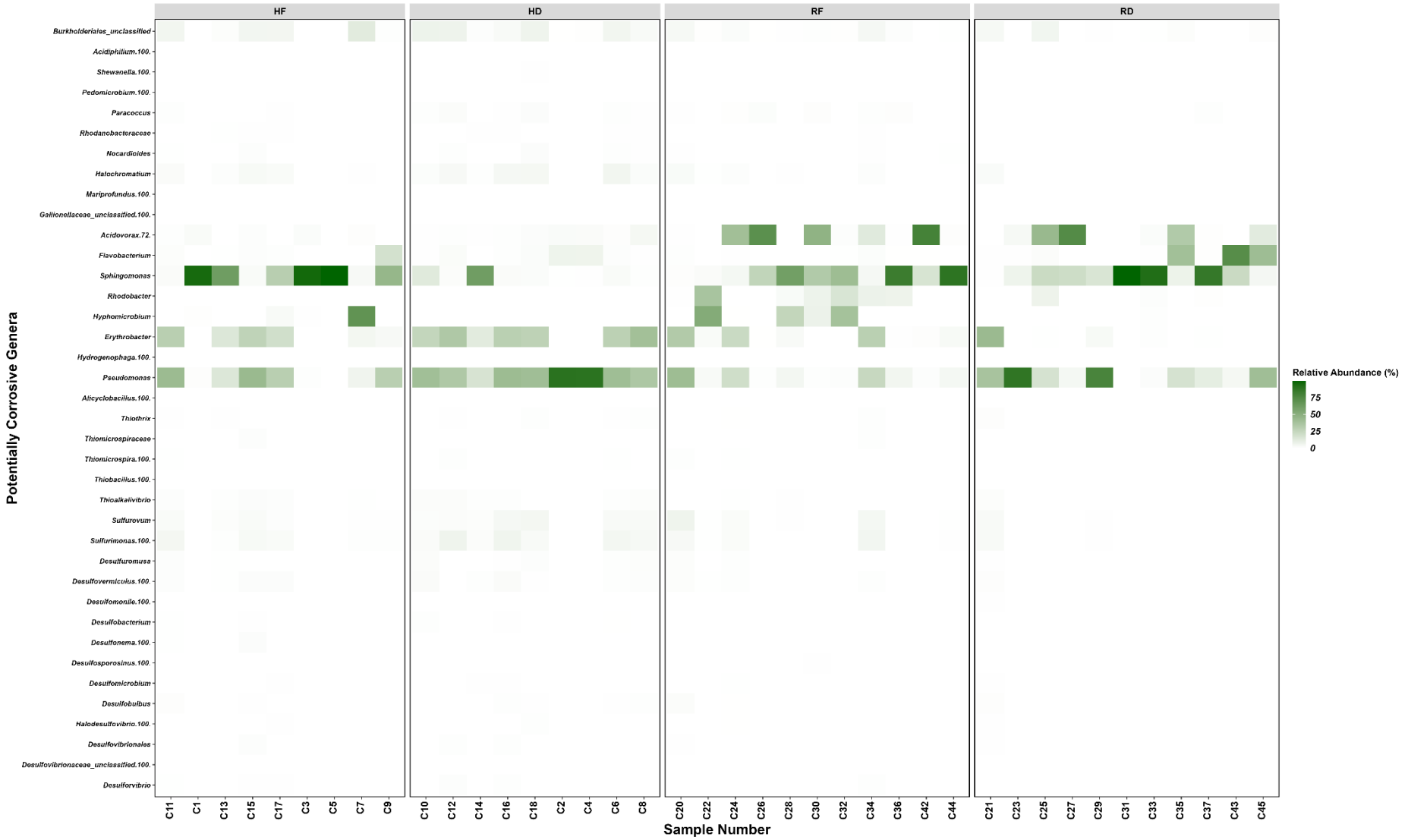

Figure 14.10 - Heatmap of abundant potentially corrosive bacterial genera present in hospital and residential handbasin biofilms. Biofilm samples are indicated along the X-axis and divided by sampling factor (HF = hospital faucet; HD, hospital drain; RF = residential drain and RD = residential drain). Bacterial genera are indicated along the Y-axis. The relative abundance of each genera is indicated by colors ranging from white (low abundance) to dark green (high abundance). Numbers included after the genus name denotes sequence resolution

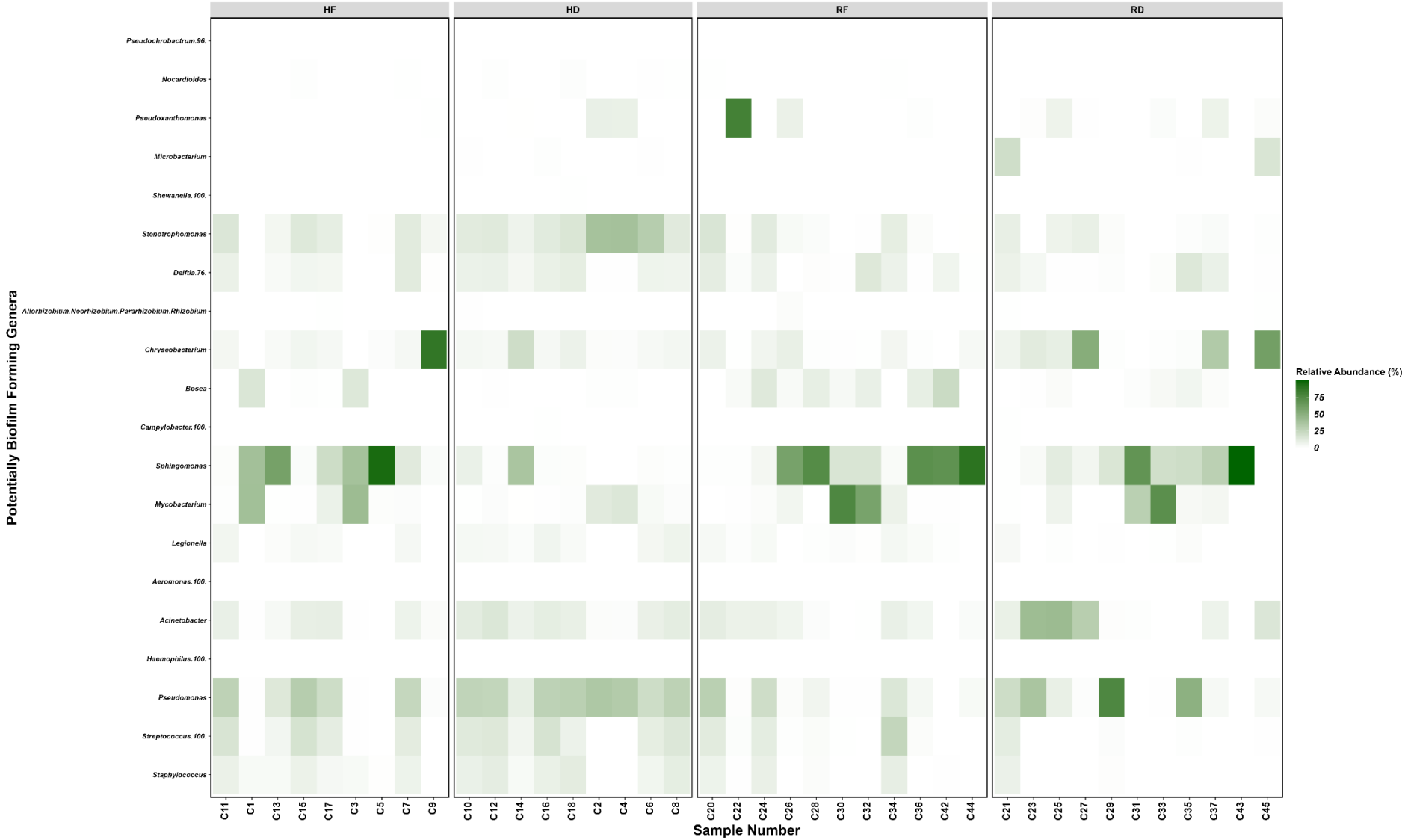

Figure 14.11 - Heatmap of abundant potentially biofilm forming bacerial genera present in hospital and residential handbasin biofilms. Biofilm samples are indicated along the X-axis and divided by sampling factor (HF = hospital faucet; HD, hospital drain; RF = residential drain and RD = residential drain). Bacterial genera are indicated along the Y-axis. The relative abundance of each genera is indicated by colors ranging from white (low abundance) to dark green (high abundance). Numbers included after the genus name denotes sequence

Text 14.1 - Spearmans correlation analysis (ρ) of potentially pathogenic genera identified in handwashing basin biofilm samples

Legionella showed a significant ($p<0.05$) positive correlations with *Pseudomonas*, *Stenotrophomonas*, *Staphylococcus*, *Coxiella*, *Vibrio* and *Yersinia* ($\rho=0.527$, 0.548, 0.731, 0.842, 0.803 and 0.512 respectively). *Stenotrophomonas* showed significant ($p<0.05$) positive correlation with *Pseudomonas, Enterobacteriaceae, Coxiella* and *Vibrio* ($\rho= 0.671$, 0.580, 0.512, 0.557, respectively). *Staphylococcus* showed a significant ($p<0.05$) positive correlation with *Coxiella, Vibrio, Yersinia* and Enterobacteriaceae ($\rho= 0.877$, 0.863, 0.505 and 0.593, respectively). Finally, *Coxiella* was positively correlated ($p<0.05$) with *Vibrio* and *Yersinia* ($\rho= 0.939$ and 0.531, respectively). This positive correlation shows that these potentially pathogenic genera co-occur in handwashing basins biofilms. *Methylobacterium-Methylorubrum* showed a significant ($p<0.05$) negative correlation with *Acinetobacter, Pseudomonas* and *Stenotrophomonas* ($\rho=-0.533$, -0.591 and -0.534 respectively). *Stenotrophomonas* was also negatively correlated ($p<0.05$) with *Sphingomonas* ($\rho=-0.502$).

Table 14.2 - Potentially corrosive bacterial genera identified in handwashing basin biofilms.

Potentially corrosive genera (Hernandez-Santana et al., 2022; Kushkevych et al., 2021; Li et al., 2018; Piazza et al., 2019; Rana et al., 2020; Singh et al., 2018)	
Sulphate-reducing bacteria	*Desulfovibrio* *Desulfobulbus* *Desulfomicrobium* *Desulfosporosinus* *Desulfonema* *Desulfobacterium* *Desulfomonile* *Desulfuromusa* *Desulfovermiculus* *Sulfurimonas* *Sulfurovum*
Sulfur-oxidising bacteria	*Thioalkalivibrio* *Thiobacillus* *Thiomicrospira* *Thiothrix* *Alicyclobacillus*
Manganese-oxidising bacteria	*Pseudomonas* *Hydrogenophaga* *Erythrobacter* *Hyphomicrobium* *Rhodobacter* *Sphingomonas* *Flavobacterium* *Acidovorax*
Iron-oxidising bacteria	*Gallionella* *Thiobacillus*

	Mariprofundus *Chromatium* *Nocardioides* *Rhodanobacter* *Acidovorax* *Paracoccus* *Pseudogulbenkiania* *Gallionellaceae* *Pedomicrobium*
Iron-reducing bacteria	*Shewanella* *Acidiphilium*
Bacteria secreting organic acids	*Pseudomonas* *Burkholderia* *Pinus*

Text 14.3 - Spearmans correlation analysis (ρ) of potentially corrosive genera identified in handwashing basin biofilm samples

Spearman's correlation analysis (ρ) was used to study the co-occurrence of these potentially corrosive genera (Figure 14.9). *Desulforvibrio* showed a positive correlation ($p<0.05$) with *Thioalkalivibrio*, *Erythrobacter, Halochromatium* and Rhodanobacteraceae (ρ= 0.578, 0.580, 0.594 and 0.647, respectively). *Desulfobulbus* positively correlated ($p<0.05$) to *Desulfovibrionales, Halodesulfovibrio, Desulfomonile, Desulfovermiculus, Desulfuromusa, Sulfurimonas, Sulfurovum* and *Thioalkalivibrio* (ρ= 0.509, 0.538, 0.581, 0.725, 0.755, 0.693, 0.767, 0.582, 0.727 and 0.521, respectively). *Desulfomicrobium* was positively correlated ($p<0.05$) to *Desulfomonile, Desulfovermiculus* and *Thioalkalivibrio* (ρ= 0.505, 0.531 and 0.515, respectively). *Desulfosporosinus* was positively correlated ($p<0.05$) to Gallionellaceae (ρ=0.715). *Desulfobacterium* was positively correlated ($p<0.05$) to *Desulfuromusa, Thioalkalivibrio, Halochromatium* and *Rhodanobacteraceae* (ρ= 0.552, 0.629, 0.531 and 0.571, respectively). *Desulfovermiculus* was positively correlated ($p<0.05$) with *Desulfobacterium, Desulfuromusa, Sulfurimonas, Thioalkalivibrio, Erythrobacter, Halochromatium*, Rhodanobacteraceae and Burkholderiales (ρ= 0.601, 0.664, 0.831, 0.744, 0.829, 0.838, 0.664 and 0.508, respectively). *Desulfuromusa* was positively correlated ($p<0.05$) to *Halodesulfovibrio, Thiomicrospira, Erythrobacter* and *Halochromatium* (ρ= 0.553, 0.536, 0.660 and 0.639 respectively). *Sulfurimonas* was positively correlated ($p<0.05$) with *Desulforvibrio*, Desulfovibrionales, *Desulfobacterium, Desulfomonile, Desulfuromusa, Sulfurovum, Thioalkalivibrio, Thiothrix, Pseudomonas, Erythrobacter, Halochromatium, Nocardioides*, Rhodanobacteraceae and *Burkholderiales* (ρ= 0.642, 0.536, 0.529, 0.617, 0.962, 0.760, 0.537, 0.522, 0.964, 0.939, 0.538, 0.632 and 0.568 respectively). *Sulfurovum* was positively correlated ($p<0.05$) with *Desulfovibrio*, Desulfovibrionales, *Desulfobacterium, Desulfovermiculus, Desulfuromusa, Thioalkalivibrio, Thiothrix, Erythrobacter, Halochromatium, Nocardioideas*, Rhodanobacteraceae and *Burkholderiales* (ρ= 0.559, 0.533, 0.505, 0.864, 0.680, 0.705, 0.523, 0.958, 0.944, 0.552, 0.595 and 0.591, respectively). *Thioalkalivibrio* was positively correlated ($p<0.05$) with *Erythrobacter, Halochromatium* and Rhodanobacteraceae (ρ= 0.762, 0.765 and 0.582, respectively) and negatively correlated with *Rhodobacter* (ρ= -0.508). *Erythrobacter* was positively correlated ($p<0.05$) with *Desulgovibrionales, Thiothrix, Pseudomonas, Halochromatium, Nocardioides,* Rhodanobacteraceae and *Burkholderiales* (ρ= 0.564, 0.545, 0.517, 0.949, 0.515, 0.572 and 0.552, respectively). *Halochromatium* was positively correlated ($p<0.05$) with Desulfovibrionales, *Pseudomonas, Nocardioides*, Rhodanobacteraceae and *Burkholderiales* (ρ= 0.532, 0.504, 0.549, 0.557 and 0.602, respectively). *Sphingomonas* was negatively correlated ($p<0.05$) with *Pseudomonas, Flavobacterium* and *Burkholderiales* (ρ= -0.750, -0.563 and -0.525, respectively).

Table 14.4 - Potential strong biofilm forming bacterial genera identified in handwashing basin biofilms.

Potential strong biofilm forming genera (Khatoon et al., 2018; Maes et al., 2019; Mahapatra et al., 2015)		
Staphylococcus	*Legionella*	*Chryseobacterium*
Streptococcus	*Mycobacterium*	*Pseudochrobactrum*
Pseudomonas	*Sphingomonas*	*Delftia*
Acinetobacter	*Campylobacter*	*Stenotrophomonas*
Aeromonas	*Bosea*	*Shewanella*
Microbacterium	*Pseudoxanthomonas*	*Nocardioides*
Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium		

Text 14.5 - Spearmans correlation analysis (ρ) of potentially strong biofilm forming genera identified in handwashing basin biofilm samples.

Staphylococcus was positively correlated ($p<0.05$) with *Streptococcus*, *Stenotrophomonas* and *Nocardioides* ($\rho= 0.869$, 0.547 and 0.569, respectively) and negatively correlated ($p<0.05$) with *Pseudoxanthomonas* ($\rho= -0.634$). *Streptococcus* was positively correlated ($p<0.05$) with *Delftia*, *Stenotrophomonas* and *Nocardioides* ($\rho= 0.535$, 0.546 and 0.532, respectively). *Pseudomonas* was positively correlated ($p<0.05$) with *Delftia* and *Stenotrophomonas* ($\rho=0.537$ and 0.682 respectively). *Acinetobacter* was positively correlated ($p<0.05$) with *Chryseobacterium* and *Stenotrophomonas* ($\rho= 0.614$ and 0.516, respectively), and negatively correlated with *Sphingomonas* ($\rho= -0.534$). *Legionella* was positively correlated ($p<0.05$) with *Staphylococcus, Streptococcus, Pseudomonas, Delftia* and *Stenotrophomonas* ($\rho= 0.709$, 0.844, 0.530, 0.715 and 0.648 respectively). *Sphingomonas* was negatively correlated ($p<0.05$) with *Stenotrophomonas* ($\rho= -0.647$).

15. APPENDIX – 5

Supplementary data for the manuscript entitled CITATION: The impact of water flow rates on bioaerosol production from handwashing basins (Chapter 7)

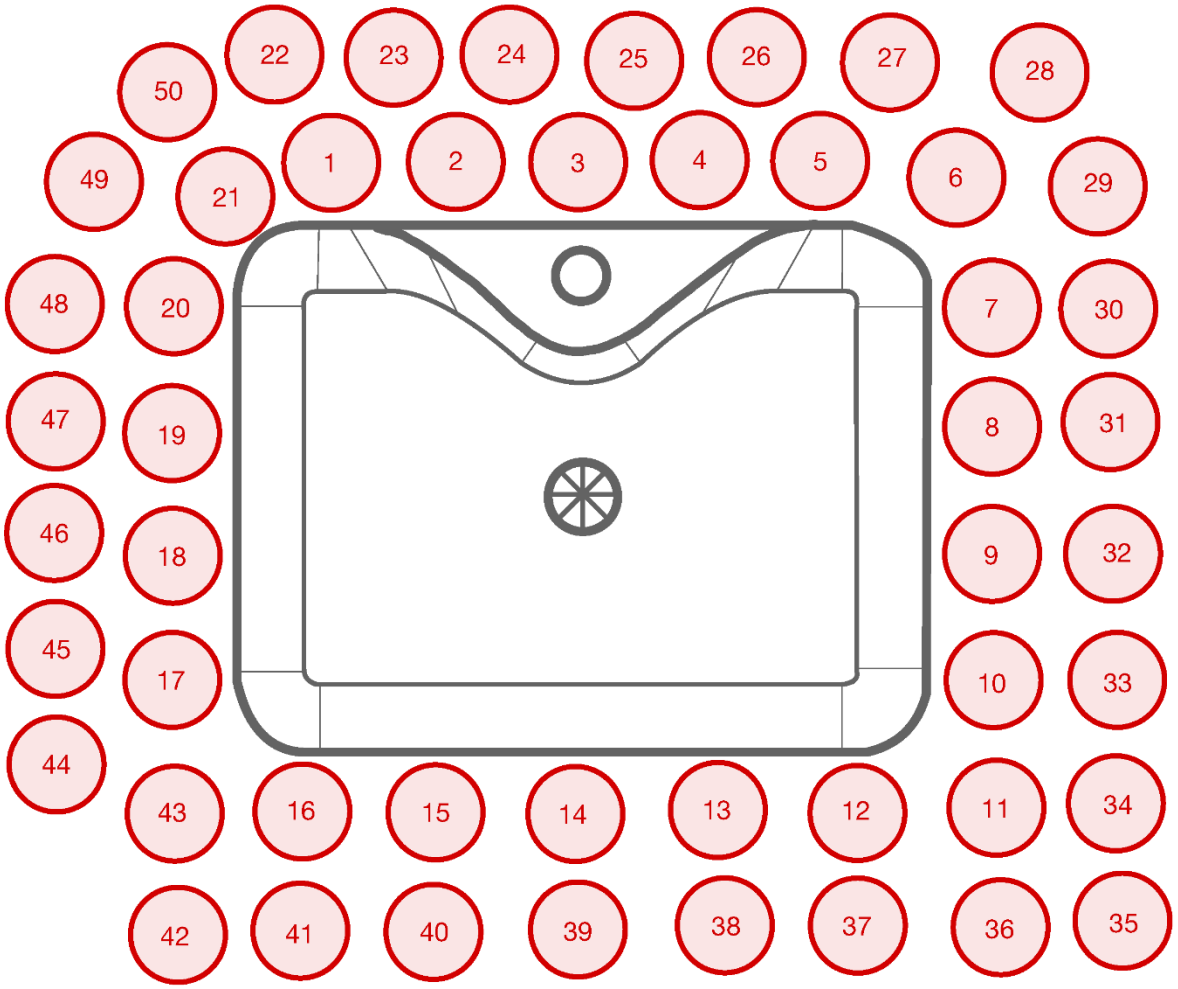

Figure 15.1 - Graphical representation depicting the layout of settle plates around the hand basin

16. APPENDIX – 6

Supplementary data for the manuscript entitled CITATION: Comparison of the antimicrobial activity of brass versus stainless steel against opportunistic premise plumbing pathogens (Chapter 8)

Table 16.1 - qPCR conditions

Name	Sequence and Fluorescence signal (5' – 3')	Assay Conditions	Reference
***Acinetobacter baumannii ompA* gene**			
Forward Primer	TCTTGGTGGTCACTTGAAGC	Hold: 95°C/5 mins 45 cycles: 95°C/15 sec, 60°C/30 sec and 72°C/20 sec	(McConnell Michael et al., 2012)
Reverse Primer	ACTCTTGTGGTTGTGGAGCA		
Probe	6FAM-AAGTTGCTCCAGTTGAACCAACTCCA-Iowa Black FQ		
***Pseudomonas aeruginosa gyrB* gene**			
Forward Primer	GGCGTGGGTGTGGAAGTC	Hold: 95°C/5 mins 45 cycles: 95°C/15 sec, 60°C/30 sec and 72°C/20 sec	(Lee et al., 2011)
Reverse Primer	TGGTGGCGATCTTGAACTTCTT		
Probe	6FAM-TGCAGTGGAACGACA- Iowa Black FQ		
***Acanthamoeba* 18s rDNA gene**			
Forward Primer	CCCAGATCGTTT ACCGTGAA	Hold: 95°C/5 mins 45 cycles: 95°C/15 sec, 60°C/30 sec	(Qvarnstrom et al., 2006)
Reverse Primer	TAAATATTAATG CCCCCAACTATCC		
Probe	CTGCCACCGAATACATT AGCATGG		

		and 72°C/20 sec	
***Mycobacterium avium* complex**			
Forward Primer	CCCTGAGACAACACTCGGTC	Hold: 95°C/5 mins 45 cycles: 94°C/15 sec, 50°C/30 sec and 72°C/20 sec	(Park et al., 2000)
Reverse Primer	ATTACACATTTCGATGAACGC		
Probe	SYTO9 fluorescent dye		

Table 16.2 - Atomic percentages of elements in brass brand 1 (B1) plumbing sample unused and after 70 days stagnation.

Brass	Unused B1 - A*			Unused B1 - B*		70 Days B1 - C*			70 Days B1 - D*	
Element	**S1 (%)**	**S2 (%)**	**S3 (%)**	**S1 (%)**	**S2 (%)**	**S1 (%)**	**S2 (%)**	**S3 (%)**	**S1 (%)**	**S2 (%)**
C	30.5	35.8	49.1	44.2	44.4	65.7	0.0	21.0	23.0	28.2
O	12.9	46.2	30.3	29.5	38.7	19.2	58.1	40.7	56.1	56.6
Ni	0.9	1.7	0.2	0.2	4.8	5.6	0.0	4.0	0.2	0.0
Cu	41.7	11.0	9.5	14.1	6.9	4.4	1.2	18.3	10.1	3.9
Mg	2.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Al	0.6	0.1	10.0	0.2	1.1	0.6	0.0	3.0	0.0	0.0
Si	0.0	0.0	2.1	0.0	0.4	0.2	0.0	1.3	0.0	0.0
Pb	0.9	0.4	0.2	5.4	0.2	0.1	9.9	0.2	0.2	8.5
Sn	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0
Fe	0.4	0.3	5.4	0.8	0.3	1.0	0.0	0.5	0.0	0.1
Zn	10.3	4.3	4.9	5.4	3.1	2.3	30.9	10.7	10.4	3.1
Ca	0.0	0.1	0.0	0.1	0.0	0.6	0.0	0.0	0.0	0.0
K	0.0	0.0	0.0	0.0	0.0	0.5	0.0	0.0	0.0	0.0

* Data collected from spots labelled S1, S2 and S3 on electron micrographs A, B, C and D in Figure 9.

Table 16.3 - Lead leaching (ppb) from stagnant water in stainless steel (SS1=brand 1; SS2=brand2) and brass (B1=brand 1; B2=brand 2) bioreactors after 30 days and 70 days stagnation.

	Stainless Steel		Brass	
30 Days Stagnation	SS1	SS2	B1	B2
Replicate 1	31.25775	7.927	141.488	138.5413
Replicate 2	23.86227	15.53532	169.6542	130.0707
Replicate 3	73.98564	69.75554	234.3163	68.57132

	Stainless Steel		Brass	
70 Days Stagnation	SS1	SS2	B1	B2
Replicate 1	40.615	92.112	256.352	354.017
Replicate 2	106.337	105.519	217.12	753.196
Replicate 3	59.573	84.681	110.56	331.456

Table 16.4 - pH of stagnant water in stainless steel (SS1=brand 1; SS2=brand2) and brass (B1=brand 1; B2=brand 2) bioreactors after 30 days and 70 days stagnation

	Stainless Steel		Brass	
30 Days Stagnation	SS1	SS2	B1	B2
1	7	7.1	6.89	7.1
2	7.02	7.11	6.9	7
3	6.98	5.71	7.1	6.7

	Stainless Steel		Brass	
70 Days Stagnation	SS1	SS2	B1	B2
1	7.03	7.05	6.86	6.94
2	7	7.03	6.75	7.1
3	7.1	6.83	6.97	6.93

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