Examining biofilm formation and copper susceptibility testing methods in *Pseudomonas aeruginosa* sink drain isolates to study the role of the GI-7 genomic island

By

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A thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

In partial fulfillment of the requirements of the degree of

Master of Science

Department of Medical Microbiology and Infectious Diseases

University of Manitoba

Winnipeg, Manitoba

Abstract

Copper is an antimicrobial metal used in sink drains that is becoming ineffective due to copper tolerance of species such as *Pseudomonas aeruginosa* enhanced by the presence of the genomic island GI-7. Studies of copper tolerance associated with GI-7 are hindered by the lack of accurate planktonic and biofilm testing methods to determine copper susceptibility, and a lack of knowledge regarding the prevalence of this sequence amongst Canadian *Pseudomonas* isolates. In chapter 3, we developed a new deep well biofilm device that provided increased surface area for biofilm biomass formation. This method can be used to screen antimicrobial susceptibility in a 96-well high throughput format. The increased surface area allowed for greater biofilm biomass per mm^2 as compared to the standard device and revealed plastic preferences in biofilm biomass formation by *P. aeruginosa* and *Escherichia coli.* Both devices produced different minimum biofilm eradication (MBEC) values for benzalkonium chloride and bleach, yet both were suitable for biofilm cultivation. In chapter 4, we examined 2467 genome sequenced *Pseudomonas* isolates collected from a 2017-2019 Ontario hospital intensive care unit study. Isolates from patients and hospital rooms with regular and copper sink drains were collected and used to explore if GI-7 presence was associated with copper sink drains. This analysis revealed that GI-7 is widely spread across clinical and environmental isolates of certain multi-locus sequence types, but statistical analyses did not show significant associations between GI-7 and copper sink drains due limited sampling from copper sink drains. Some correlation trends amongst GI-7 isolates were noted and discussed. Finally, phenotypic characterization of *P. aeruginosa* GI-7 copper tolerance through planktonic and biofilm culturing methods was examined with various *P. aeruginosa* strains. Copper salt addition to media caused lethal acidification that inhibited both planktonic and biofilm culture growth, regardless of GI-7 presence. To overcome this, we modified a previously used copper water exposure method to eliminate the effects of copper acidity and verify copper tolerance differences between susceptible PAO1 and PA-8 strains with and without GI-7. Overall, our findings offer improved techniques for studying copper tolerance and exploring the role of GI-7 in Canadian hospital *Pseudomonas* isolates.

Acknowledgements

I would like to begin by expressing my gratitude to my supervisors, Drs. Denice Bay and George Golding. Your support, encouragement, intelligence, and scientific creativity were monumental in shaping and completing this project over the last 3 years. I'd also like to thank the members of my committee— Drs. Michael Mulvey, George Zhanel, and Ayush Kumar, for their guidance and expertise throughout all stages of my project. I would like to give additional thanks to Dr. Mulvey for passionately leading the copper sink drain study, and Dr. Zhanel for enhancing my visual, written, and spoken scientific communication skills with his thoughtful and constructive feedback during the course MMIC 7160.

To Andrew Clarey, Jamie Parsons, Mindy McCarville, Dr. Katy Garant, Alexandra Fenton, and the entire SuperNOVA at Dalhousie University team. From high school and through my undergraduate and graduate degrees, your passion for science education inspired me to become the researcher and scientific communicator I am today, which I am forever grateful for.

Moving to a new province for graduate school was scary, but I was quickly reassured everything would be okay with the help of friends made through the department. Jeremiah Yarmie, Domenica DeLuca, Derek Tan, Toby Le, Megan Allardice, Mona Mahmoudi, Bay Lab members Branden Gregorchuk, Carmine Slipski, Kari Green, Kieran Milner, and Shelby Reimer, and so many others— thank you for making me feel at home, listening to complaints, helping me troubleshoot, and always being there for a laugh (or beer) when needed. A special thank you to Kari and Shelby—I truly don't think I would have come to love Winnipeg the way that I do now if it weren't for our adventures.

And to my dear friends and family spread out around the globe: my mom, Kelly Doucet; Grampie, Bill Cameron; aunt, Ashley Cameron; siblings Haley, Thea, Brielle, and Brayden; in-laws, Jo and Ralph; our sweet dogs, Rory and Sulley; the CamFam; and my incredible best friends. Thank you for encouraging me to follow my dreams (even though they took me all the way to Winnipeg) and being there to help me push through many hard, lonely days from near and far away. I love you!

Dedication

To Kelly, David, Wilma, and Bill

My biggest supporters, here and over the rainbow.

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List of Abbreviations

A	Absorbance
AMR	Antimicrobial resistance
ANOVA	Analysis of variance
AST	Antimicrobial susceptibility testing
BCIP	Build in Canada Innovation Program
Bp	Base pair
BZK	Benzalkonium chloride
CDF	Cation diffusion facilitator
CFU	Colony forming units
CLSI	Clinical Laboratory Standards Institute
CV	Crystal violet
DG	Davis-Glucose
DMSO	Dimethyl sulfoxide
EPS	Extra polymeric substances
EUCAST	European Committee on AST
GC	Guanine cytosine
GRDI	Genomics Research and Development Initiative
HAI	Hospital acquired infection
ICU	Intensive care unit
JoVE	Journal of Visualized Experiments
KATsect	K-mer analysis toolkit and sequence coverage estimator tool
L	Litre(s)
LB	Luria-Bertani
LBA	Luria-Bertani agar
M	Molar
MBEC	Minimum biofilm eradication concentration
MBIC	Minimum biofilm inhibition concentration
mg	Milligram(s)

MHB	Cation-adjusted Mueller-Hinton broth
MIC	Minimum inhibitory concentration
mL	Millilitre(s)
MLST	Multilocus sequence type
mM	Millimolar
nm	Nanometer(s)
NML	National Microbiology Laboratory
OD	Optical density
PHAC	Public Health Agency of Canada
ppm	Parts per million
RND	Resistance nodulation-cell-division
ROS	Reactive oxygen species
rpm	Revolutions per minute
USD	United States of America dollars
UV	Ultraviolet
Vis	Visible
w/v	Weight per volume
WGS	Whole-genome sequencing
WHO	World Health Organization
XDR	Extensively drug resistance
µg	Microgram(s)
µL	Microlitre(s)

Contribution of Authors

Overall thesis writing, experiment conduction, figure creation, and data analysis were completed by Ali N. Doucet unless otherwise stated. Denice C. Bay edited this thesis and contributed to Chapter 4 BCIP isolate analysis and figure creation. Carmine J. Slipski contributed to section 1.5.1, section 2.5.4, and Chapter 3 writing, editing, and experiment conduction as part of our Journal of Visualized Experiments (JoVE) 2022 publication (1) which was included in this thesis with permission from JoVE. Michael R. Mulvey and George G. Golding edited the JoVE 2022 publication. David A. Boyd developed the bioinformatic method for GI-7 detection in BCIP isolates.

Chapter 1: Introduction

1.1 Chapter Introduction

The background information to be presented in this chapter of the thesis provides a brief overview of *Pseudomonas aeruginosa* and its role as a human nosocomial pathogen and sink drain colonizer. This chapter will provide background information on *P. aeruginosa*, including its pathogenicity, relevance as an opportunistic pathogen, its presence in hospital sink drains, and antimicrobial resistance (AMR) mechanisms as they pertain to copper and acid tolerance. Additionally, this section will discuss current methods of growing biofilm and testing copper tolerance, and the issues with these methods. Mechanisms of AMR specific to *Pseudomonas* sp. as well as other relevant proteobacteria such as *Escherichia coli* will also be reviewed and outlined in this chapter. A summary of the main thesis aims and hypotheses will end this chapter, where both aims and their findings will be discussed in Chapters 3-4.

1.2 Introduction to *Pseudomonas aeruginosa*

1.2.1 Pathogenicity and clinical relevance as an opportunistic pathogen

P. aeruginosa is a Gram-negative, aerobic, rod-shaped bacterium that was first discovered in 1882 by pharmacist Carle Gessard while examining a blue and green coloured bandage, indicative of the characteristic colours of this species (2–5). *P. aeruginosa* is a ubiquitous species found in soil, seawater, freshwater, plants, humans, and other animals (2, 3, 6). Like other members of its genera, *P. aeruginosa* has an impressive metabolic diversity allowing it to persist in a variety of unconventional conditions, including hot tubs, swimming pools, and implanted medical equipment, even after sterilization with disinfectants (3, 7–9).

P. aeruginosa is well known as an opportunistic pathogen and it often causes infections among the most vulnerable patients, including the elderly, immunocompromised, and those in intensive care units (ICUs) (8, 10). Additionally, *P. aeruginosa* infections target diverse host regions, where these infections can occur broadly, in nearly any part of the body (11). For example, respiratory tract infections by *P. aeruginosa* are common amongst those with compromised lungs, where chronic *P. aeruginosa* infections are the most common causes of morbidity and mortality in cystic fibrosis patients (12). *P. aeruginosa* also causes skin and soft

tissue infections that manifest in several ways, including benign infections that typically resolve on their own, such as folliculitis (13), or cause more rare but serious and life-threatening infections such as necrotizing fasciitis, also known as flesh eating disease (14).

P. aeruginosa can cause community acquired infections, but hospital acquired infections (HAIs), also known as nosocomial infections, are much more common (15). Indeed, *P. aeruginosa* is among the most common causes of nosocomial infections worldwide (16). Additionally, AMR among outbreak strains of *P. aeruginosa* has made nosocomial infections increasingly difficult to treat and prevent, leading to increased morbidity and mortality (16). In 2017, the World Health Organization (WHO) listed carbapenem-resistant *P. aeruginosa* as a critical priority pathogen to guide the research and development of new antibiotics (17). *P. aeruginosa* was included on this list due to its tremendous burden on the health care system, as carbapenem and multi-drug resistant *P. aeruginosa* frequently causes pneumonia and bloodstream infections and has limited antibiotic treatment options remaining (17). Other species listed as critical priority pathogens include carbapenem-resistant *Acinetobacter baumannii* and carbapenem-resistant, 3rd generation cephalosporin-resistant *Enterobacteriaceae* (17). However, this thesis will focus on *P. aeruginosa* based on its ability to form difficult to eradicate biofilms and concerning its prevalence in hospital sink drains.

There are a variety of AMR mechanisms that allow *P. aeruginosa* to persist and survive, but one of the most defining AMR features of *P. aeruginosa* is its ability to produce biofilms. A biofilm is defined as a matrix composed of bacterial cells encased by extracellular polymeric substances (EPS) such as polysaccharides, proteins, extracellular DNA, and lipids that typically forms on surfaces and air-liquid interfaces (18). Besides cells, EPS materials make up approximately 50-90% of the biofilm (3, 8). A diagram summarizing key components of biofilms is shown in Figure 1.1.

Biofilms form readily on abiotic surfaces such as catheters, artificial joints, and ventilation tubes and contribute to persistence on these surfaces (8, 9). Additionally, biofilms aid in *P. aeruginosa* chronic infections, especially in the lungs of cystic fibrosis patients where polymicrobial biofilms containing *P. aeruginosa* form biofilm within the lungs and often cannot be eliminated, even with aggressive antibiotic treatment (9, 19). Chronic biofilm formation is

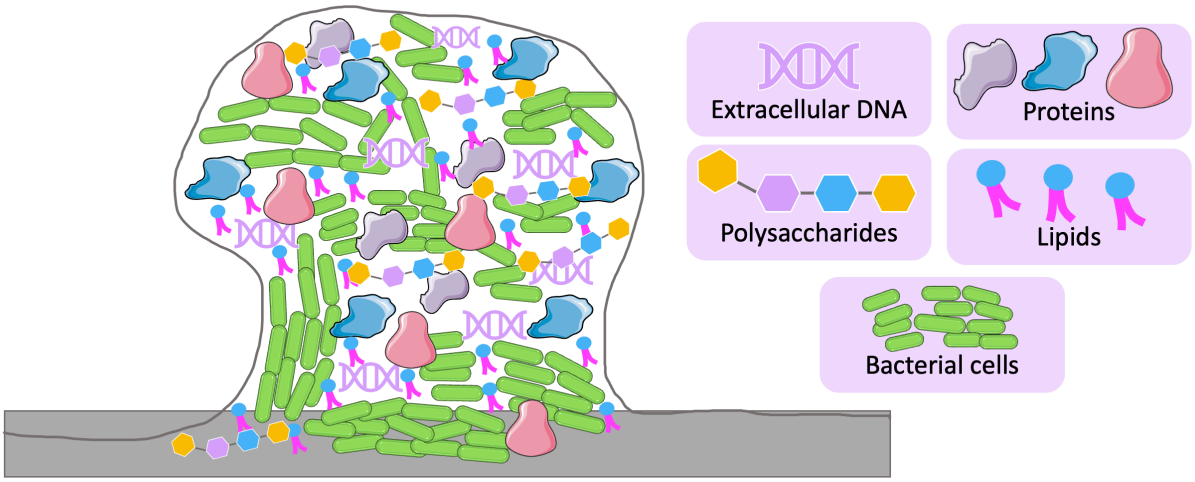

Figure 0.1 A diagram of bacterial biofilm structure and key components of biofilm and EPS. Items shown are not drawn to scale (DNA, proteins, sugars, and lipids) but are shown at sizes to highlight their presence. Key biofilm components include bacterial cells and macromolecules (DNA, proteins, polysaccharides, and lipids).

also of concern in chronic wounds, as biofilms are estimated to be involved in at least 90% of these infections (20).

Biofilms enhance the AMR of many species, including *P. aeruginosa,* in a variety of ways. Firstly, EPS thickness and chemistry prevents most antimicrobials from permeating into and reaching cells within the biofilm. EPS acts as a solute barrier and can also deactivate the antimicrobial through enzymatic reactions, chelation, or adsorption (9, 21, 22). Additionally, biofilms also produce bacterial metabolically inactive persister cells that do not grow, especially when exposed to high concentrations of an antimicrobial, but can survive exposure and seed a new biofilm community (23).

1.2.2 P. aeruginosa as an environmental sink drain contaminant

As mentioned above, *P. aeruginosa* is a ubiquitous microorganism with the ability to persist in harsh environments. This ability is especially concerning in hospitals, where *P. aeruginosa* can easily spread from abiotic hospital surfaces to patients, causing nosocomial infections (24). A variety of surfaces within a hospital can harbour microbes, including common patient care items (blood pressure cuffs, thermometers, stethoscopes), medical equipment (monitors, supply carts), and touch surfaces (bed rails, chairs, light switches, doorknobs) (25). On these types of dry, inanimate surfaces, *P. aeruginosa* can persist between 6 hours (h) and 6 months (24).

While the dry surfaces listed above can serve as bacterial infection sources, persistent reservoirs of *P. aeruginosa* are of increasing concern in aqueous hospital settings. Aqueous environments provide surface-liquid-air interfaces that allow biofilms to form and thrive (18). Specifically, sink drains are among the most prevalent aqueous environments that harbour biofilm communities and allow *P. aeruginosa* to spread widely within the hospital (26). When the sink is turned on, water flows from the tap and can aerosolize bacteria from the biofilms that formed within the sink tap(s), on the sink surface(s), and within sink drain(s), leading to the spread of opportunistic AMR bacteria, including *P. aeruginosa*, to hospital staff and patients (27). A figure outlining bacterial spread from sink drain biofilms to patients is shown in Figure 1.2.

The spread of *P. aeruginosa* from sink drains has been reported as a cause of nosocomial outbreaks worldwide. The outbreak environments are diverse and include critical care units (28),

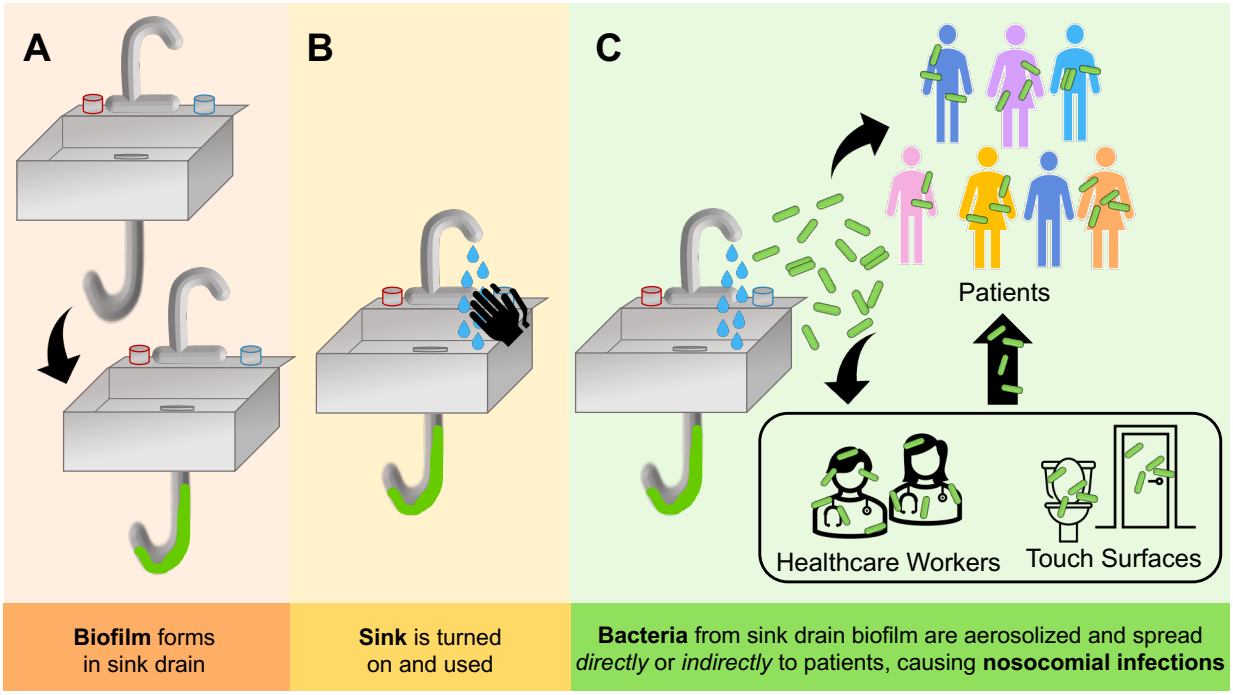

Figure 0.2 A diagram illustrating how bacteria can spread from sink drain to patients. Biofilm forms in the hospital sink drain (A), the biofilm contaminated sink is turned on and used (B), and cells from the biofilm aerosolize, spreading directly to patients or indirectly to patients through healthcare workers or touch surfaces (C).

hematology wards (29), neonatal ICUs (30), nephrology transplant ICUs (31), and even hospital-wide outbreaks caused by contaminated wastewater systems (32). Contaminated hospital sink drains are sometimes removed and replaced to end or prevent *P. aeruginosa* outbreaks; however, the cost of sink replacement and the high potential for recolonization prohibits this method as a long-term, sustainable solution (33). To prevent sink related outbreaks, a variety of other mitigation methods have been trialled. Such methods include ultraviolet (UV) decontamination of sinks (34); sinks that self-disinfect with heat and vibration (27); and sinks have even been completely removed from patient rooms (35). However, one aspect of this thesis focuses on the use of copper pipes within sink drains and their ability to prevent *P. aeruginosa* nosocomial infections.

1.2.3 The Build in Canada Innovation Program (BCIP) Ontario sink drain study

In addition to the research and development of new antibiotics, tackling AMR is expanding to involve the usage of other kinds of antimicrobials, especially in clinical settings, to treat AMR infections and prevent infections before they occur (36). The use of copper to prevent bacterial contamination within healthcare settings has been explored in previous studies, and these studies have examined the usage of copper on touch surfaces (37) as well as in materials for sink drains (38). In this thesis, a specific focus was placed on a study entitled "Effectiveness of a novel sink drain system in reducing sink surface and air contamination around ICU sinks, and gammaproteobacterial infections in ICU patients" which was funded and completed through a BCIP grant held and lead by the principal applicant, Dr. Allison McGeer (Professor, University of Toronto, Canada) (39). The purpose of this study was to determine if the installation of copper alloy sink drains, specifically LISH drainset, Cuverro® splashproof cover, plug, tailpiece, and traps could reduce biofilm formation within sink drains (39). The goal was to ultimately determine if copper could lead to a reduction in bacterial infections caused by *P. aeruginosa* in ICU patients (39). ICU rooms in the study were assessed for their baseline bacterial contamination rates, and pairs of rooms were then matched based on the similarity of sink configuration and baseline contamination (39). Among paired rooms, one sink was randomly assigned as the copper alloy sink drain, while the other had a chrome-coated control drain installed with a similar appearance to the copper alloy sink drain (39).

Over the course of two years (2017-2019), seven ICUs in the greater Toronto area of Ontario, Canada, collected 2467 *P. aeruginosa* isolates from faucets, sink drains, high-touch surfaces, the air, and from patients in these hospital rooms (39). Unfortunately, there was insufficient funding to do further characterization of the *P. aeruginosa* isolates collected in the study. Specifically, the BCIP study was not able to address concerns that *P. aeruginosa* isolates from copper alloy sink drains might develop tolerance to copper due to insufficient isolate collection, which would render the copper alloy sink drains ineffective (39) and may lead to cross-tolerance to other antimicrobials (40). Some of the research completed in this thesis (Chapter 4) explores these aspects based on the small number of samples that were collected from the study. Hence, it is important to understand the antimicrobial actions of copper and its known effects on *P. aeruginosa,* which will be discussed further in the sections below.

1.3 The genomic island GI-7 in *Pseudomonads*

P. aeruginosa has a large genome that is composed of a conserved core genome as well as an accessory genome that is highly variable in composition (41). Genomic islands are genetic elements found within chromosomes that are part of the accessory genome, meaning that these islands are not found in every *P. aeruginosa* strain (41). Genomic islands are clusters of genes that are believed to be acquired by horizontal gene transfer, and thus often have mobility genes, insertion sequences, and direct repeats (41). Additional characteristics of genomic islands include higher-than-average guanine-cytosine (GC) contents, phage-related genes, and DNA insertion-specific loci “hot spots”, such as frequently detected core genome transfer RNA (tRNA) genes (41).

Among these genomic islands, the genomic island “GI-7” is distinguished by its transposable elements (42) and has been found in *P. aeruginosa* isolates (43). The GI-7 genomic island is of growing interest as a marker for copper tolerance due to the presence of genes that encode copper tolerance systems (42–44). In a recent study of a group of seven *P. aeruginosa* ST395 isolates from hospital outbreaks, GI-7 was identified as a cause of copper tolerance (43). This genomic island consists of a 17,000- base pair (bp) region containing six copper transport genes (43). These genes encode for cupredoxins, a copper ABC transporter (CopABC), CopZ, CopA, and Cop B (43). However, a larger, 37,000-bp version of GI-7 was also reported in the *P.*

aeruginosa strain PA-8, which was isolated from a copper pipe in the plumbing system of a French hospital (Besançon, France) and contained 13 copper resistance genes, including those found in ST395, the *czcABC* copper and zinc efflux system, a CopG export protein, the cytochrome C oxidase CopM, and two copper-binding plastocyanins (42, 45). GI-7 was also found in Besançon Epidemic Strain (BES)-4, which was isolated from a sink drain and responsible for a 12-year *P. aeruginosa* outbreak at the University Hospital of Besançon, France (44). Diagrams of GI-7 can be found in Figure 1.3.

1.4 Copper tolerance

Copper is a group 11 heavy metal that is reddish in colour when mined but becomes blue-green when oxidized (46). It serves as an essential element for a variety of cellular processes and is required by many organisms in trace amounts as a co-factor in proteins used during redox reactions (47), but is toxic at eukaryotic intracellular concentrations greater than 10 µM (48). At toxic copper concentrations, this heavy metal has antimicrobial activity and can cause lethal toxicity that leads to bacterial cell death through a variety of mechanisms (49). This section will focus on the antimicrobial mechanisms of copper, copper tolerance mechanisms found within bacterial species, current methods of studying copper tolerance, and the issues/ limitations of these methods.

1.4.1 Antimicrobial action of copper

Heavy metals such as copper are known to cause toxicity within cells when present in low amounts (48). Copper acts as an antimicrobial compound by causing protein dysfunction and the production of reactive oxygen species (ROS), it also alters membrane structure through lipid peroxidation and nutrient uptake (50). While the antimicrobial mechanisms of copper are not fully understood, it is widely known that copper acts in a multitude of ways (51).

Firstly, the oxidation states of copper play a major role in its solubility and toxicity. The transition from Cu^{1+} (I) and Cu^{2+} (II) ions enhances copper's ability to interact with and be recognized by important cellular components involved in active transport, electron transportation, and metabolism (52). Additionally, when Cu^{2+} is reduced to Cu^{1+}, it enhances hydroxyl radical generation as well as catalyzes the formation of other

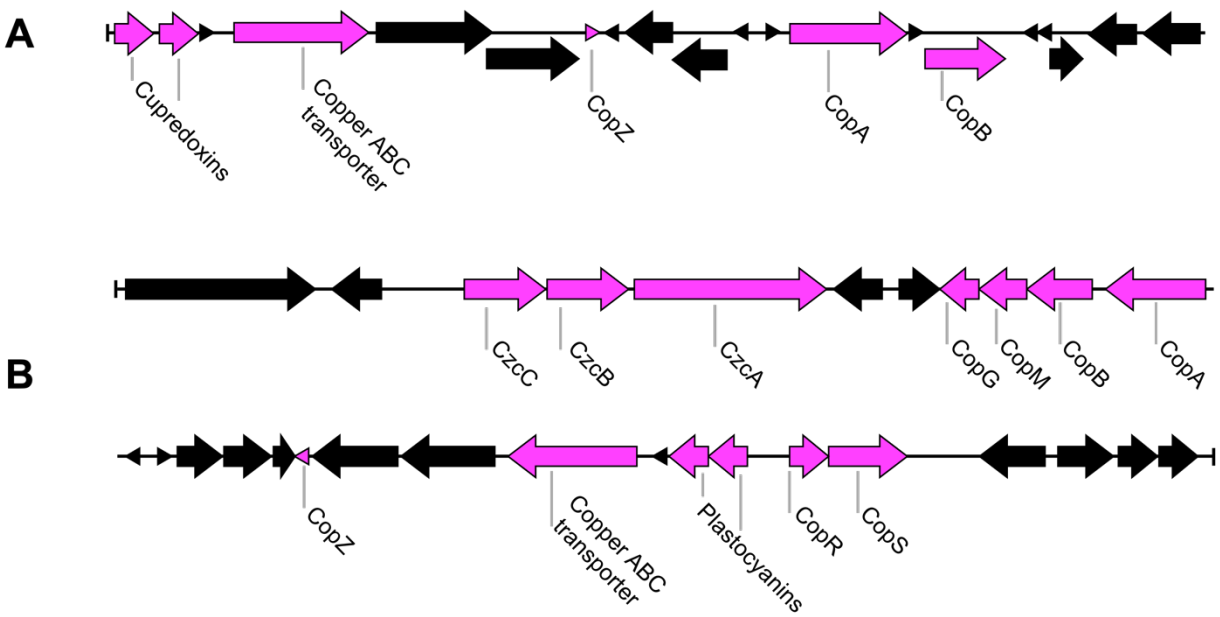

Figure 0.3 Genomic representations of the copper tolerance genomic island GI-7. Genes that are relevant to copper tolerance are highlighted in pink. Panel A represents a ~17,000 bp version of GI-7 first found in the *P. aeruginosa* ST395 DHS01 isolate in Besançon, France and contains six copper tolerance genes (43). Panel B represents a larger ~37,000 bp GI-7 found in *P. aeruginosa* ST308 PA-8 in Besançon, France and contains 13 copper tolerance genes (42).

ROS that cause oxidative damage to DNA, membrane lipids, and proteins (51). It has also been shown in *E. coli* that copper can exert toxic effects by displacing iron from iron-sulfur clusters; essentially, this shows that copper can compete for metal binding sites within the cell, which may interrupt protein function and biosynthetic pathways (53). Lastly, in *E. coli* it was recently shown that under anaerobic conditions, copper causes protein aggregation due to non-specific interactions with cysteine and histidine protein residues (54). While not all antimicrobial mechanisms of copper are understood, it is clear that copper's effects on the cell are broad.

1.4.2 Mechanisms of copper tolerance

It is important to discern the difference between resistance and tolerance, and why we have chosen to use the term copper tolerance over copper resistance. Resistance to an antimicrobial is determined by specific breakpoint concentrations defined by the Clinical Laboratory Standards Institute (CLSI) and European Union Committee on Antimicrobial Susceptibility Testing (EUCAST) by conducting antimicrobial susceptibility tests (AST) with many bacterial species and antimicrobials (55, 56). Each antimicrobial and species combination that is tested has defined breakpoint antibiotic concentration values for "susceptible", "intermediate", and "resistant" (55, 56). Given that CLSI and EUCAST do not define such breakpoints for copper, we have chosen to use the term "copper tolerance" herein. Without established breakpoints, methods of testing copper susceptibility, or copper tolerant reference isolates there is often variation in the defined tolerant copper concentration between experiments, making it difficult to compare studies; despite this, we are still able to detect bacterial copper tolerance mechanisms. Thus, the remainder of this section will describe molecular mechanisms that contribute to bacterial copper tolerance with a focus on efflux-mediated copper tolerance mechanisms due to their abundance and importance.

Efflux pumps are important membrane systems that transport a variety of molecules out of the cellular milieu. These expelled substrates include biocides, drugs, detergents, dyes, and heavy metals such as copper (52). Efflux mechanisms expel metal cations within the cytosol into the periplasmic space and/or out of the cell (49). The overexpression of efflux pumps can lead to resistance to the antimicrobials they extrude from the cell (57), and this includes copper. Copper efflux system genes can be genetically encoded and inherited chromosomally or inherited by

mobile genetic elements (plasmids transposons). A summary of these copper-selective efflux systems is discussed below.

A well-characterized Cu^{1+} efflux pump of *E. coli* is the CopA system; CopA is a soft metal P1-type ATPase (49, 58). The expression of CopA, as well as the multi-copper oxidase CueO, are both regulated by CueR in the *cue* copper efflux system (58). In the *cue* system, cytoplasmic copper induces the action of CueR, which goes on to upregulate CopA and CueO production (57, 58). CopA effluxes Cu^{1+} ions into the periplasm where they are oxidized to Cu^{2+}, a less toxic form of copper, in an oxygen-dependent reaction by CueO (49, 58). The *P. aeruginosa* gene PA3920 is homologous to P-type ATPases and is upregulated under conditions of copper shock and adaptation as published in a study by Teitzel et al. 2006 (49).

The *cus* operon is another chromosomally encoded copper efflux system. The *cusCFBA* is activated by a phosphorylated CusRS two-component regulatory system when extracellular copper concentrations are high (58). The *cusR* gene encodes a response regulator, while *cusS* encodes a histidine kinase (57). CusRS activation results in the production of CusA, a silver/copper resistance nodulation-cell-division (RND) family transporter; CusB, a periplasmic membrane fusion protein; CusC, an outer membrane channel; and CusF, a periplasmic metallochaperone (57, 58). CusCBA allows for the export of Cu^{1+} to the outside of the cell from CusF, the cytoplasm, or periplasm (58). In *P. aeruginosa*, twelve loci were determined to be homologous to RND family members (49). Of these, PA2520 to PA2522, which were upregulated under copper shock and adaptation conditions, encode *czrCBA*, a system important for tolerance to copper and other heavy metals (49).

The final group of transporters involved in chromosomally inherited copper efflux are the cation diffusion facilitator (CDF) transporters (49, 58). In *E. coli,* CDF proteins transport copper and other heavy metals across the inner membrane (49). *P. aeruginosa* encodes PA0397, a CDF transporter that was upregulated in copper adapted *P. aeruginosa* (49). PA0397 is homologous to a CDF gene in *Ralstonia metallidurans*; however, it has been suggested that copper is not large enough to act as a substrate for this protein, although this has not been proven experimentally to date (49).

The plasmid-borne operon *pco* has a role in the resistance to high concentrations of copper in *E. coli* (59). The most well-characterized plasmids that encode *pco* operons is pRJ1004, which was initially isolated from a pig farm in the United Kingdom (60). The *pco* system involves the soluble proteins PcoA, PcoC, and PcoE; copper pumps PcoB and PcoD; and a copper sensing system composed of PcoS and PcoR (59). PcoA is a multicopper oxidase protein with a similar function to chromosomally encoded *cueO* (60). PcoC is a is a soluble periplasmic β-barrel protein that binds both oxidation states of copper at two different sites. This allows the protein to transfer oxidized, reduced, and non-redox copper states between the two binding sites (59). It appears that PcoA catalyzes the oxidation of Cu^{1+} to Cu^{2+} when the ion is bound to PcoC in the periplasm (59). It is then possible that PcoB, an efflux pump protein, exports periplasmic Cu^{2+} (59).

1.4.3 Methods of studying bacterial tolerance to copper

Multiple studies have been conducted that seek to examine the effectiveness of copper metal as an antimicrobial material (copper metal/ore or copper salt) and as a surface coating. In studies testing copper as an antimicrobial surface material, studies have largely focused on experiments that spot pure bacterial broth culture dilutions directly onto copper coupons (60), into copper bowls containing water (61), and have installed copper touch surfaces in hospitals to determine if they are effective at preventing antimicrobial contamination within healthcare settings (62). In all of these studies, after a defined copper exposure period, the viable bacterial load is determined by taking samples from the copper surface and determining the number of colony forming units (CFUs) (60–62). However, the focus of this section will be on planktonic and biofilm copper susceptibility testing, as these methods are used in Chapter 2 of this thesis.

1.4.3.1 Copper susceptibility testing methods for planktonic bacteria

While the studies described above are suitable for studying copper as an antimicrobial surface, they are more qualitative as they do not allow for the quantification of exact concentrations of exposure (63). Thus, in many cases, it is more suitable to study bacterial copper tolerance using more standardizable antimicrobial susceptibility testing (AST) involving broth culture dilution assays with defined and increasing amounts of a dissolved copper salt (typically

copper sulfate or copper chloride). Copper salts are added to growth media and diluted bacterial cultures are allowed to incubate for 12-24 h in the copper amended growth media to determine the lowest concentration of copper at which growth is inhibited, i.e. the MIC. Copper minimum inhibitory concentration (MIC) values using this approach are determined as the lowest concentration of copper that inhibited growth as shown by visual inspection or reading of the optical density at 600 nm (OD_{600nm}) in a UV/ visible (Vis) range spectrophotometer. To date, copper concentration ranges and methods used to establish bacterial copper tolerance vary significantly between experiments, as shown in Table 1.1. Additionally, copper tolerance has been defined quite differently between studies. Copper tolerance has been defined as an organism's ability to survive copper concentrations that are higher than a reference strain (64); in other studies, the microorganism is considered tolerant when it can grow at concentrations of the metal that are higher than what is naturally found in the environment that the bacterium itself was isolated from (42); and finally, copper tolerance has been defined by researchers arbitrarily, based on chosen a set of defined Cu concentrations at which they consider to be a threshold for bacterial species copper tolerance (65). As a result, there is considerable variability in defining precise copper concentrations that define Cu tolerance or resistance values from past studies.

1.4.3.2 Copper susceptibility testing methods for bacterial biofilms

Bacterial biofilms have also been examined for their enhanced tolerance to copper and other metals. Biofilms have been shown to increase *P. aeruginosa* tolerance to copper up to 600-fold (23, 66). Therefore, it is essential that we do not apply conclusions related to metal susceptibility in planktonic culture to the same species growing as a biofilm, as metal tolerance between the two can vary greatly.

The first step of determining biofilm susceptibility to metals is to decide on a method for growing biofilm. Many methods exist for growing biofilm, including growth on the sides of microtiter plate wells, in spinning disk bioreactors/ growth chambers, and tube biofilm flow reactors (67). Most rapid *in vitro* biofilm culturing methods examine biofilm growth as a "static" biofilm culture rather than in more natural "continuous" cultures, where static bacterial growth

Table 0.1 A summary of the methods of previously conducted bacterial planktonic and biofilm copper tolerance studies.

Species	Media type	Copper salt used	Copper conc. or range tested	Exposure Time	Planktonic (P) or biofilm (B)	Inhibition (I) or eradication (E)	Source
P. aeruginosa, E. coli, S. aureus	CSWM	$CuSO_4$	0.03125-8 mM	4 h inhibition, 24 h eradication	P,B	I, E	(68)
P. aeruginosa	MSVP, or MOPSO-buffered saline	Not specified	0.03-2mM (planktonic), 0.015-225 mM (biofilm)	56 h (planktonic), 5 h (biofilm)	P,B	I (planktonic), E (Biofilm)	(66)
P. aeruginosa	Water	$CuSO_4$	150 µg/L	24 h	P	E	(42)
P. aeruginosa, E. coli	LB + Vitamin B1 or MSVP	$CuSO_4 \bullet 5 H_2O$	<0.25-16 mM	2 or 24 h	P,B	I,B	(23)
P. aeruginosa, E. coli, S. aureus	LB + Vitamin B1	$CuSO_4 \bullet 5 H_2O$	2-15 mM	24 h	P,B	I,E (Planktonic); E (Biofilm)	(69)
P. aeruginosa, S. maltophila, A. baumannii	Water	$CuCl_2$	0.1-0.8 mg/mL	1.5-24 h	P	E	(70)

Abbreviations: Conc.=concentration; CSWM= Chemically simulated wound media; MSVP=Minimal salts and vitamins pyruvate; MOPSO= β-Hydroxy-4-morpholinepropanesulfonic acid; LB= Luria-Bertani

in 96 well microplates is monitored in early to late stages of biofilm formation over a short (24-96 h) timeframe in the same growth medium. Static biofilms are finite and have early, mid, and late stages which can affect biofilm physiology and nutrient uptake (71). Continuous biofilm methods (bioreactors and flow chambers) are cumbersome for rapid biofilm analysis as they require biofilm growth assessed over longer timeframes (days to weeks) and cultures grown in chambers that allow continuous flow require replacement of growth media and experiments can only be conducted with fewer replicates (71). The MBEC device (Innovotech Inc; previously known as the Calgary Biofilm Device, and herein known as the standard biofilm device), is another commonly used static biofilm method as it allows high-throughput biofilm testing on lids that possess pegs that insert into wells, allowing lower volumes of culture medium and biofilms to form on a removable peg surface (67, 68). The standard biofilm device fits onto a standard 96 well microtiter plate and consists of a lid with 96 polystyrene pegs upon which biofilm can grow (72). The peg lid devices are advantageous in comparison to other methods for growing biofilm as they allow researchers to conduct high-throughput biofilm assays and the pegs can be detached for use as pre-exposure controls and examination of biofilm by microscopy (67).

After a biofilm has been established in the various methods described above, many of these methods allow for the determination of either a minimal biofilm inhibition concentration (MBICs) or a minimum biofilm eradication concentration (MBEC). An MBIC is defined as the lowest concentration of antimicrobial showing 90% inhibition of biofilm formation compared to the untreated control (73). In MBIC experiments, exposure to metals or other potential antimicrobials would be initiated upon inoculation of the device with bacteria, rather than after biofilm has been established (67). The MBIC is determined by staining the pegs with crystal violet (CV), de-staining in ethanol or acetic acid, and then measuring the absorbance of the solution to approximate biofilm biomass (73). In contrast, an MBEC value is defined as “the lowest concentration of antimicrobial agent that prevents visible growth from occurring in the recovery medium used to collect biofilm cells” (18, p. 1242). Both static and continuous biofilm methods can be used to determine MBIC and MBEC values (74).

1.4.4 Knowledge gaps and limitations of current methods to study bacterial copper tolerance

Many parameters impact bacterial killing by metal ions and metal surfaces. The most notable are inoculation techniques used with the metal, the incubation temperature(s), culture humidity, and final metal concentration(s) (62). Surprisingly, increased copper concentration in an alloy does not necessarily increase antimicrobial activity; some studies have reported that alloys with lower concentrations of copper have greater antibacterial effects. For example, Elguindi *et al*. 2009 reported that a copper alloy containing 88.6% copper showed a more effective bactericidal effect than an alloy containing 99.9% copper (75). While the exact reason for this has not been explored, it is likely that minor metals within the alloy also contribute to the antimicrobial effects of copper (76). Additionally, bacterial susceptibility to copper as a surface material is more effective at higher temperatures (20 °C in comparison to 4 °C) (77) and increased humidity (90% relative humidity vs. 22%) (78). Finally, agents that inhibit the corrosion of metal surfaces can also decrease the antimicrobial effect of the metal by preventing the release of copper ions from surfaces (62, 79). Thus, it is important to report all of these conditions in studies, so that the conditions influencing copper susceptibility results can be accurately replicated.

Studying bacterial copper/heavy metal tolerance is made more difficult due to a variety of conditions that must be controlled for. For example, the type of media chosen for tolerance studies is extremely important as various heavy metals including copper can bind and complex with media additives such as peptones and sugars (65). Binding of metals to growth media components decreases their soluble availability in solution and thus makes metal tolerance concentration value measurements appear to be higher than values for minimal medium or water (65). Researchers have tried to circumvent this by using various minimal media formulations to increase copper bioavailability in solution; however, many bacterial species require complex rich medium for growth, growth in minimal media is often much slower, and copper can precipitate due to phosphates present in minimal media (80). Due to differences in bacterial growth and physiology in rich and minimal media, it can be difficult to accurately detect copper tolerance over and above copper concentrations that carry over in rich medium formulations. It is also challenging to consistently use the same medium when studying different bacterial species that may require specific nutrient supplementation (65). Therefore, the growth

medium used to culture bacteria and the variations in media formulations make it difficult to compare the antimicrobial effects of copper between studies (65).

pH can also affect bacterial growth and metal availability (65). Bacterial species have optimal pH ranges, so an increase or decrease of pH outside of this range can significantly diminish growth (65). The addition of heavy metal ions to an aqueous solution can cause changes that lower the pH out of optimal biological pH ranges (6.5-7.5); for example, the addition of $CuSO_4 \bullet 5H_2O$ to media causes the copper and sulfate ions to dissociate and creates sulfuric acid in solution(65). Alterations of pH outside of the range of pH 5-7 can also change the solubility, oxidation state and bioavailability of the metal ion in solution, causing there to be fewer available free ions for bacterial uptake (65). For example, copper solubility in pure water (pH 5.5) can significantly vary as the most soluble free Cu^{2+} ions form hydroxide ($Cu(OH)_2$) complexes, which increase as pH increases; these copper hydroxides precipitate as Cu^{2+} (II) (81). As solutions become more basic, copper complexes formed with anions (OH^-, NO_3^-, CO_3^{2-}, SO_4^{2-}) precipitate out of solution. In water, the most common multi-anionic copper precipitate is malachite [$Cu^2(OH)_2(CO_3)$], which has a blue-green coloured precipitate (81). More acidic solutions can also reduce the ability of copper ions to bind ligands (65). It is also important to note that Cu^{2+} is the most soluble form of copper ion in aqueous solutions, typically at pH values of 6 and lower; pH 6 is lower than the optimal biological pH of most clinically relevant proteobacteria (*E. coli* and *P. aeruginosa* can grow at pH ranges of 5-8). Therefore, the pH of the growth medium and the solubility of copper ions can impact the bacterial species and metal being studied. Few if any studies discuss adjustments in pH to account for copper ion effects, where experimental and control conditions have the same initial pH and more efforts to report pH changes induced by copper in studies are needed so experiments can be accurately replicated (65).

1.5 The strengths and limitations of current anti-biofilm compound testing methods

The recalcitrant growth properties of bacterial biofilms often make them more resistant to antimicrobial/ heavy metal inhibition and/or eradication (82, 83). Thus, establishing *in vitro* methods to study bacterial biofilm antimicrobial or anti-biofilm compound eradication concentrations is paramount for selecting effective compounds to eradicate chronic infections when they form on medical plastics (e.g., in-dwelling catheters) and medical implants. Due to the

time and effort required to maintain and establish continuous biofilms, static *in vitro* biofilms are the most popular when assessing anti-biofilm compounds and drugs. Static biofilm experiments are easily adapted for high-throughput AST assays in plastic 96-well microtiter plates rather than elaborate flow chamber systems that limit the number of cultures simultaneously tested (84, 85). The simplest static "in-well" biofilm microplate assays use standard polystyrene or vinyl (300 µL capacity) microtiter plates to measure biofilm formation on the sides and bottoms of each well and often as rings at the air-to-liquid surface interface. Bacterial in-well biofilm formation is measured by staining the deposited biomass adhered to the wells after the growth medium liquid is removed and the biofilms are washed (84, 85). These assays are economically popular but often have reproducibility issues due to their inherent design, as deposited biofilms are prone to damage or loss during rinsing for cell recovery and biomass staining procedures (71, 86).

To reduce biofilm losses, standard commercial biofilm devices have improved upon in-well biofilm microplate designs by adding an insertable 96-well pegged polystyrene lid to the standard 96 in-well plate design, referred to as the standard biofilm device. The addition of a pegged lid expands the available surface area in each microplate well, allowing for enhanced surface adherence and biofilm biomass formation (87, 88). Standard biofilm pegged lid devices allow for greater static biofilm recovery, removal, and rinsing for subsequent antimicrobial biofilm susceptibility and eradication testing when pegged lids are inserted into new microtiter plates containing drug or growth condition challenges. Similar to in-well biofilm microplate techniques, the recovered materials from the removed and washed pegged lid devices allows for cell survival testing and biofilm biomass staining, typically involving CV dye formulations (67, 89, 90). Standard biofilm devices are also optimal for screening biofilm antimicrobial susceptibilities. These assays monitor biofilm growth inhibition in two ways: 1) When antimicrobials are added to cells at the start of growth or MBIC values. 2) When established biofilms are formed on the pegs after 24-48 h and then exposed to antimicrobials, it can determine the MBEC value (67, 91).

Similar to in-well biofilm microplate devices, standard biofilm devices have some notable limitations, such as their high cost per device, they are non-autoclavable, and less durable to chemical solvents due to the polystyrene plate materials used in their fabrication. Standard biofilm devices also have a low surface area to peg ratio, which limits the maximum working

volumes in each well to 200 µL. These factors can make standard biofilm devices more challenging to use for studies that demand greater quantities of biofilm in a high-throughput format.

1.6 Acid tolerance

1.6.1 Acidity as an antimicrobial mechanism

Proton concentrations (measured by pH) within a bacterial cell and in its environment are heavily influenced by bacterial physiology during growth, reproduction, and survival (92). In general, most bacteria are neutrophiles that live best at neutral pH of around 7.0; however, the optimal pH range for growth can range from 3-4 pH units, and some species prefer more acidic (acidophiles) or basic (alkaliphiles) conditions (93). *P. aeruginosa*, which is known for its ability to survive a wide range of environmental conditions, is typically found in habitats with a pH range of 4.5-9.5 (94). While acidophiles are less affected by high proton concentrations, for many species, including *P. aeruginosa,* low pH has many antimicrobial effects (92, 93).

Acidic pH effects can be harmful to the function of proteins and other macromolecules (92). At an acidic pH (pH<5), amino acids can become protonated, which causes protein denaturation through increased protonation and charge repulsion (95), as well as protein misfolding caused by changes in salt bridge formation and hydrogen bond formation (96, 97). Additionally, enzymes within the cell generally operate within a specific pH range, and thus a lowering of cytoplasmic pH will decrease enzyme activity and impact the metabolic processes necessary for survival that require such enzymes (95).

Acidic pH values also have antimicrobial effects at the cellular membrane. Proton gradients that exist on either side of a cellular membrane are impermeable to protons by diffusion, which is important for cellular energy production (98). When pH becomes more acidic, uncharged, protonated organic acids can diffuse through the lipid bilayer, disrupting the production of energy by the proton motive force (92, 93). Additionally, at pH extremes, solute transportation across the membrane is altered (93).

1.6.2 Mechanisms of tolerance to acidic conditions

Due to regular exposure to acidic conditions in nearly every environment and within the human body, methods for maintaining pH homeostasis are essential for bacterial survival (92). General homeostasis mechanisms that prevent pH from dropping to unviable levels include increasing reactions that use protons or create basic compounds to neutralize the acidic pH, and ATP-dependent proton efflux (92). However, many bacterial species live in environments in which acid stress is more severe and prolonged. Thus, bacteria may require additional inducible tolerance mechanisms beyond the general maintenance of pH homeostasis (95).

Membranes play a major role in acid tolerance. While the outer membrane of Gram-negative bacteria provides little resistance to proton influx due to the size of porin channels, the inner membrane provides an efficient barrier that prevents proton movement from the periplasm to the cytoplasm (95). As previously mentioned, the periplasm is most susceptible to extreme changes in pH due to the proton permeable outer membrane (95). However, many acid-tolerant species can resist periplasmic damage by low pH through periplasmic chaperones such as HdeA, Skp, DegP, and SurA, which prevent aggregation of unfolded proteins and facilitate configurations that lead to proper refolding (99).

Additionally, membrane modifications can further increase the impermeability of protons. For example, when exposed to acidic conditions outside of the ideal physiologic range (pH 3) for 16 h, *E. coli* strains with increased cyclopropane fatty acids in the cell membrane were shown to be more tolerant to the acidic conditions in comparison to those with lower levels of cyclopropane fatty acids (100). This change in membrane composition is likely beneficial because the presence of cyclopropane fatty acids makes the membrane more rigid and therefore less permeable to protons (100).

Finally, biofilms can play a role in acid tolerance. This is believed to be in part due to the density of cells within a biofilm, as cells in the innermost biofilm layers are less exposed to acidic environmental conditions (101). This has been shown in a study of *P. aeruginosa* disinfection of endoscopes with peracetic acid, as planktonic cultures were eradicated by 20 parts per million (ppm) of peracetic acid, while it took more than 10 times this amount to eradicate 96 h biofilms of the same species (102). Additionally, planktonic cultures supplemented with extracellular DNA, a structural component of *P. aeruginosa* biofilms, have been shown through

transcriptomics to induce the expression of acid tolerance genes such as the *cyoABCDE* terminal oxidase system, which is involved in proton transport under acidic conditions (103).

1.7 Thesis objectives

The original intent of this thesis was to explore the planktonic and biofilm copper tolerance of the *P. aeruginosa* sink drain isolates collected from Ontario hospitals in the BCIP study. However, many limiting factors complicated the pursuit and completion of this objective in a high-throughput AST approach. The first was the lack of reliable biofilm formation methods that could be used to produce sufficient quantities of biofilm for reliable copper testing on materials that were not porous to heavy metals or did not degrade due to copper's acidity. This knowledge gap was addressed in Chapter 3 of this thesis, where I led the development of a new method to explore static biofilm formation using larger 96 well deep well devices composed of polypropylene. This method was recently published (1) and is discussed below in section 1.7.1 and Chapter 3. The second experimental hurdle was resolving the problem of accurately measuring bioavailable copper in conventional rich and minimal growth media or copper added to standing water, to determine the copper susceptibly of *P. aeruginosa* strains. As there are no standards for copper tolerance determination or defined copper salt breakpoints by either the CLSI or EUCAST, Chapter 4 of this thesis explored growth media copper tolerances, copper media acidification, and other methods of copper exposure to reliably determine copper susceptibility by *P. aeruginosa* reference strains. As part of this analysis, two sets of copper tolerant *P. aeruginosa* reference strains with and without GI-7 provided by Dr. Didier Hocquet (Besançon, France) were included as part of this study for use as copper tolerant controls. Section 1.7.2 and Chapter 4 below describe the findings from all the copper susceptibility testing approaches examined using *P. aeruginosa* reference strains. Chapter 4 also shows the results from bioinformatic analysis of whole-genome sequenced (WGS) BCIP *P. aeruginosa* isolates for the presence of the GI-7 region.

1.7.1 Chapter 3 rationale and hypothesis

In this chapter, we describe a new deeper static biofilm pegged-lid 96-well method to grow larger biofilm biomass from bacterial cultures of *E. coli* and *P. aeruginosa* for high-throughput AST. The larger devices were assembled from commercially available polypropylene

semi-skirted 0.5 mL 96-well PCR plates fitted to 96-well polypropylene 1.1 mL microtiter plates. These assembled devices have a maximum working volume of 750 µL when used for growing biofilm (herein known as "deep well biofilm device"). With this method, we show the applications of these devices for growing and characterizing biofilm biomass formed by *E. coli* BW25113 and *P. aeruginosa* PAO1. Methods to determine MBEC values using biofilm eradication assays are also described. These MBEC tests examined the antimicrobial effects of quaternary ammonium compound disinfectant, benzalkonium chloride (BZK) and sodium hypochlorite (bleach), which are both commonly used as anti-biofilm compounds.

We **hypothesized** that larger high-throughput 96 well devices used for static biofilm formation made from chemically durable plastic (polypropylene) may be suitable for increasing biofilm biomass and will be suitable for other downstream biofilm experiments, including MBEC determination of disinfectants.

1.7.2 Chapter 4 rationale and hypothesis

In this chapter, the copper tolerance of presumably copper susceptible *P. aeruginosa* (PAO1) strains and copper tolerant *P. aeruginosa* isolates with (PA-8 and BES-4) and without GI-7 (PA-8 ΔGI-7 and BES-4 ΔGI-7) was explored both genotypically and phenotypically to determine the potential role of GI-7 among BCIP sink drain isolates and their copper tolerance. The genotypic analysis sought to determine if the copper tolerant genomic island GI-7 was present in the genomes of *P. aeruginosa* isolates from the BCIP Ontario sink drain study. After bioinformatically surveying the genome sequenced isolates for the presence of GI-7 and their association to copper sink drains from the BCIP study, a more in-depth phenotypic analysis of *P. aeruginosa* reference isolates with and without GI-7 was conducted. As part of the bioinformatic BCIP genome analysis, associations between isolate strain types and/or where they were isolated from (i.e., hospital rooms containing control or copper sink drains) and the presence and absence of GI-7 sequences were performed. **We hypothesized** that GI-7 would be present at a higher frequency among isolates from copper sink drains and that this GI-7 presence would associate with specific strain types, particularly ST308 and ST395, which have been previously reported to contain GI-7 (42, 43).

As part of our phenotypic analysis of *P. aeruginosa* with and without GI-7, we performed various copper tolerance testing methods. We began by performing copper tolerance methods using an AST approach. With this technique, we sought to determine if commonly tested copper salts, such as $CuSO_4 \bullet 5H_2O$ and $CuCl_2 \bullet 2H_2O$, inhibited *P. aeruginosa* reference isolate and strain growth. As part of this analysis, we also determined if these salts resulted in acidic pH changes in the different growth media tested. We noted an acidification effect that was proportional to copper concentration, i.e., increased concentrations of copper salts also acidify growth media which can be inhibitory to growth (80). Therefore, I examined the copper tolerance of planktonic and biofilm *P. aeruginosa* cultures to $CuSO_4 \bullet 5H_2O$ and acidic media conditions using traditional broth microdilution susceptibility assays. The rationale for these testing methods was that copper with media components can decrease bioavailable copper concentrations, and thus, high concentrations of copper must be added to have the desired antimicrobial effect, but high copper concentrations also acidify media. Therefore, we **hypothesized** that the antimicrobial effects of both copper salts may be distinguishable from pH acidification values that inhibit bacterial growth at particular concentrations.

To distinguish the effects of acidification from copper susceptibility, we also exposed tolerant and susceptible *P. aeruginosa* planktonic cultures to a smaller concentration of $CuSO_4 \bullet 5H_2O$ in water and determined survival after 24 h by counting CFU/mL. This method has been used in previous studies to determine copper susceptibility after prolonged copper exposures, similar to sink drains (42, 44). This method eliminated the acidification effects caused by higher copper concentrations in solution and as required for AST techniques, as only minimal amounts of copper were needed for an antimicrobial effect. This approach also more accurately represented sink drain copper concentration values and conditions as these exposure experiments were performed in sterile water. **We hypothesized** that this method of copper tolerance testing is a more reliable technique than AST for detecting differences in copper susceptibility amongst copper tolerant and susceptible *P. aeruginosa* strains with and without GI-7.

Chapter 2: Materials and Methods

2.1 Chemicals and media used in this study

Cation-adjusted Mueller-Hinton broth (MHB), sodium chloride, copper (II) chloride dihydrate, and BZK were purchased from MilliporeSigma (St. Louis, MO, USA). Tryptone, yeast extract, copper (II) sulfate pentahydrate, hydrochloric acid, glacial acetic acid, and sodium hypochlorite (bleach) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). All other chemicals used in this the studies described below were purchased from VWR/Avantor (Avantor; Radnor, PA, USA) or Tokyo Chemical Industries (TCI; Tokyo, Japan).

2.2 Bacterial strains and growth conditions

2.2.1 P. aeruginosa and E. coli reference strains

All bacterial strains discussed in this section are summarized in Table 2.1. *P. aeruginosa* PAO1 (DSM 6974) was obtained from Dr. Ayush Kumar (Winnipeg, MB, Canada). *P. aeruginosa* Boston 41501 (DSM 1117), ATCC 15442 (DSM 939), ATCC 9027 (DSM 1128), and NCCP 14571 (DSM 102274) were purchased from The Leibniz Institute DSMZ German Collection of Microorganisms and Cell Cultures (Braunschewig, Germany). *Escherichia coli* BW25113 was purchased from the Coli Genetic Stock Centre (104).

P. aeruginosa reference strains PA-8, PA-8 ΔGI-7, BES-4, and BES-4 ΔGI-7 were generously donated by Dr. Didier Hocquet (Besançon, France). Both PA-8 and BES-4 were isolated from copper sink drains at the University Hospital of Besançon (Besançon, France) and showed tolerance to copper when grown under conditions mimicking a sink drain (25 °C in the dark) due to the presence of well-characterized copper tolerance operons within the genomic island GI-7 (42, 105). PA-8 ΔGI-7 and BES-4 ΔGI-7 have deletions of the genomic island GI-7 (constructed by overlapping PCR and recombination events), and this deletion conferred reduced tolerance to copper (42, 105). WGS of isolates was performed by the study group using Illumina NextSeq technology and multi-locus sequence type (MLST) was determined using the WGS data (42, 105).

Table 6.1 *P. aeruginosa* and *E. coli* strains phenotypically characterized in this study.

Name	Strain	GI-7	Source
PAO1	*P. aeruginosa* DSM 6974	-	Dr. Ayush Kumar*
DSM 1117	*P. aeruginosa* Boston 41501	-	DSMZ
DSM 939	*P. aeruginosa* ATCC 15442	-	DSMZ
DSM 1128	*P. aeruginosa* ATCC 9027	-	DSMZ
DSM 102274	*P. aeruginosa* NCCP 14571	-	DSMZ
BW25113	*E. coli BW25113*	-	CGSC
PA-8	*P. aeruginosa* PA-8	Yes	(42)
PA-8 ΔGI-7	*P. aeruginosa* PA-8 *ΔGI-7*	-	(42)
BES-4	*P. aeruginosa* BES-4	Yes	(44)
BES-4 ΔGI-7	*P. aeruginosa* BES-4 ΔGI-7	-	(44)

*University of Manitoba (Winnipeg, MB, Canada)

2.2.2 BCIP P. aeruginosa clinical and environmental isolates

A Build in Canada Innovation Program (BCIP) funded study, led by Dr. Allison McGeer, was conducted across 7 hospitals in the greater Toronto area. This study sought to determine if copper sink drains would reduce *P. aeruginosa* infections in ICU patients. Over the course of 2 years (2017-2019), 2,467 *P. aeruginosa* isolates were collected from sink drains, faucets, rims, surfaces, the air, or patients (via rectal swab) at seven Ontario hospitals (Hospitals A-G) (39). Drains were matched based on similarity of sink design baseline contamination levels, and then randomly assigned to be refitted with a copper sink drain or a control drain. A summary of the isolates and their isolation locations from the BCIP study is provided in Table 2.2.

As part of the Genomics Research and Development Initiative (GRDI) project which sought to further analyze the isolates collected in the BCIP study, isolates were WGS using the NextSeq 550 Illumina platform by the DNA core at the National Microbiology Laboratory (NML; Winnipeg, Manitoba, Canada) (protocol discussed in section 2.2.4), characterized by multi-locus sequence typing (MLST), and underwent AST.

2.2.3 Bacterial growth and cryopreservation conditions

All bacterial strains used in this study were cryopreserved at -80 °C in Luria-Bertani (LB) broth (106) with 8% (v/v) dimethyl sulfoxide (DMSO). Unless otherwise stated, *P. aeruginosa* overnight cultures were grown from cryopreserved stocks at 37°C in LB or MHB with shaking at 250 revolutions per minute (rpm) for 18 h, while *E. coli* overnight cultures were grown in LB with shaking at 170 rpm for 18 h. Alternatively, when plating cultures to count CFU per mL (CFU/mL), strains were grown overnight at 37 °C on Luria-Bertani agar (LBA).

2.2.4 P. aeruginosa strain and clinical and environmental isolate whole-genome sequencing

All genome sequencing performed by the NML involved the use of the following DNA extraction procedures. Total cellular DNA was prepared using Epicentre Masterpure ™ Complete kits (Mandel Scientific, Guelph, Canada) and libraries from these preparations were created with Nextera XT kits (Illumina, San Diego, United States). Paired-end 150 bp indexed reads were

Table 6.2 Summary of the number of clinical and environmental isolates collected from the BCIP Ontario hospital sink drain study from 2017-2019.

	Clinical*	**Environmental^**		**Total isolates**
Study Hospitals		Control sink drain room	Copper sink drain room	
Hospital A	127	356	14	497
Hospital B	195	92	3	290
Hospital C	184	206	12	402
Hospital D	218	92	9	319
Hospital E	212	74	5	291
Hospital F	80	104	18	202
Hospital G	130	306	31	467
Total	1146	1229	92	**2467**

*Clinical isolates obtained by rectal swab

^ All environmental isolates were collected from the air, sink drain, sink faucet, sink rim, or sink surface of hospital rooms with standard or copper sink drains.

generated on an Illumina NextSeq™ platform using the 500/550 v2.5 (300 cycles) preparation kits (Illumina, San Diego, United States).

2.3 Build in Canada Innovation Program (BCIP) sink drain study

2.3.1 Bioinformatic analyses of BCIP sink drain isolates to detect GI-7

Bioinformatic analysis of genome sequenced *P. aeruginosa* BCIP isolates was conducted to detect the presence of the GI-7 genomic island, which is associated with copper tolerance in *P. aeruginosa* (42, 105). This analysis was completed with the assistance of David Boyd using the Public Health Agency of Canada's (PHAC) in-house software pipeline where BCIP genome sequences were assessed using the K-mer Analysis Toolkit (107) and sequence coverage estimator tool (KAT sect). The nucleotide sequence of GI-7 (Appendix A) was used as the query input sequence for KAT sect genome sequence searches. This analysis examines k-mer query coverage in sequenced genomes, where k-mers are defined as substrings of length k contained within a biological nucleotide sequence. Using this program, the K-mer coverage was estimated in all 2,467 *P. aeruginosa* BCIP isolates to verify GI-7 genome presence. GI-7 was determined to be present in any sequenced isolate's genome when its %_non-zero, which is defined as the percentage of the sequence which has a K-mer coverage greater than 1 (108), was >99%. This high 99% cutoff value was selected based on randomly selected manual genome sequence verifications that confirmed the presence of GI-7 presence in all instances. The isolates were sorted based on their multi-locus sequencing type (MLST) and the type of sink they were collected from (*i.e.*, standard pipe vs. copper pipe, or the air within these rooms) to determine if there was any correlation between MLST, GI-7, and presence in copper sink drains.

2.3.2 Statistical analyses of BCIP sink drain isolates to detect GI-7

Statistical analyses were performed using Microsoft 365 Excel v16.60 and GraphPad Prism software v9.3.1. Data averaging from sorted dataset totals and standard deviation calculations were performed using Excel analysis. Two-tailed Student's t-tests and ANOVA calculations were used to assess statistically significant differences between variables, where p-values and particular calculations are indicated in Table footnotes or in specified figure captions in chapters 3-4. Linear correlations to deter r values used a Pearson co-efficient correlation calculation in

Excel where *r*-values are reported in specified Chapter 4.1 section Figures, Tables and text. A power calculation was used to assess the minimum sample sizes needed to avoid type I or type II statistical errors with respect to copper sink drain and regular sink drain isolate sampling at an α= 0.05 at 80% power (109). This Power calculation determined that a minimum of 40 copper and 40 regular sink drain isolate samples were needed from each hospital to avoid type I and II errors; unfortunately, copper sink drain isolate sampling was underpowered at all hospitals in the BCIP study.

2.4 pH testing of copper solutions

Before completing experiments conducted in chapter 4, we first needed to determine the extent of growth media acidification by copper salts. A brief description of the pH testing methods used is described below. Copper solutions at concentrations of 0-32 mM $CuSO_4 \bullet 5H_2O$ or $CuCl_2 \bullet 2H_2O$ were created in LB, MHB, and distilled water (dH_2O) using a 2-fold dilution series. The pH of all solutions was measured three times using the Orion™ DualStar pH/ISE benchtop meter and ROSS Ultra™ pH Electrode by Thermo Fisher Scientific (Waltham, MA, USA) which was first calibrated using Orion™ pH buffers (pH 4.01, 7.0, 10.01). The experiment was repeated three times and averaged across the experiments.

2.5 Susceptibility testing of *P. aeruginosa* to antimicrobials and acidic pH conditions

2.5.1 Determining growth media pH before and after growth in copper or acidic pH media

For experiments described in Chapter 4, MHB growth media required pH adjustments to properly compare the acidic effects caused by copper sulfate addition. To complete these experiments, MHB was amended with 0-8 mg/mL $CuSO_4 \bullet 5H_2O$ (2-fold dilutions) or pH adjusted to final values of 3.5, 4.5, 5.5, 6.5, 7.0 or 7.5 using 1.0 M HCl. The pH of copper supplemented and pH-adjusted media was measured using a ROSS Ultra™ pH Electrode and then filter sterilized using a 0.2 µm nylon syringe filter (Thermo Fisher Scientific, Waltham, MA, USA). Overnight cultures of *P. aeruginosa* strains grown in MHB from cryopreserved stocks were standardized to an optical density at 600 nm (OD_{600nm}) of 0.5 units in a Thermo Scientific Multiskan® Spectrum UV/Vis Spectrophotometer and diluted to a final concentration of 10^{-4} in 10 mL MHB (control),

$CuSO_4 \bullet 5H_2O$ supplemented MHB, or pH adjusted MHB. *P. aeruginosa* cultures were incubated overnight, and cells were pelleted in the Beckman Coulter (Brea, California, USA) Avanti J-E Centrifuge (5,000 x g, 30 min) prior to measuring the pH of spent media using the procedure describe above.

2.5.2 Planktonic broth microdilution susceptibility testing of copper and pH

In Chapter 4, modified antimicrobial susceptibility testing methods were used to determine the copper and acid tolerance of each *P. aeruginosa* reference strain. Briefly, overnight cultures of each *P. aeruginosa* strain were grown in MHB from cryopreserved stocks were standardized to OD_{600nm} 0.5 units in MHB. Standardized cultures were inoculated in 96 well microtitre plates at final diluted concentration of 10^{-4}. Each well of the plate contained filter sterilized MHB amended with a 2-fold dilution series of $CuSO_4 \bullet 5H_2O$ (0-8 mg/mL/0-32 mM); the final well volume in the plate was 200 µl/well. For pH-adjusted acid susceptibility testing, diluted standardized cultures were inoculated into microtitre plates containing filter sterilized MHB adjusted to the same pH as each tested $CuSO_4 \bullet 5H_2O$ concentration ± 0.2 pH units at pH values of 3.5, 4.5, 5.5, 6.5, 7.0, and 7.5 and a final well volume of 200 µl/well. These pH values matched the tested copper concentrations of 0, 0.5, 1, 2, 4, and 8 mg/mL, respectively.

All inoculated microtitre plates for copper/ pH susceptibility testing were incubated at 37°C overnight with shaking at 150 rpm. The minimum inhibitory concentration (MIC) of a specified condition (pH and or copper) was calculated based on the lowest concentration of $CuSO_4 \bullet 5H_2O$, or the most basic pH value, that was indistinguishable in OD_{600nm} value from its matching negative growth control well based on UV/Vis spectrophotometer readings (Thermo Scientific Multiskan® Spectrum UV/Vis Spectrophotometer). A total of 6 biological replicates were tested for each strain and OD_{600nm} values were averaged for the biological replicates.

2.5.3 Planktonic copper exposure mimicking sink drain conditions

In Chapter 4, a copper exposure in water testing method was selected to determine each *P. aeruginosa* reference strain's copper tolerance under more physiologically relevant sink drain exposure conditions and this method was adapted from Jeanvoine *et al*. 2019 (42). Briefly, this

method used mid-logarithmic phase (OD_{600nm} ~0.5 units) cultures inoculated from overnight cultures of *P. aeruginosa* that were diluted 1/200 in MHB. 10 mL of mid-log cells were pelleted (5,000 x g, 30 min) and standardized to OD_{600nm} 0.5 units in sterile dH_2O with and without 600 µg/L (0.0006 mg/mL) $CuSO_4 \bullet 5H_2O$. To determine each strain's viability over time during copper exposure testing, a sample of standardized cultures in water without $CuSO_4 \bullet 5H_2O$ was diluted 10-fold (10^{-3}-10^{-5}) in LB and dilutions were plated on LBA to determine CFU/mL before copper exposure. Control and copper exposed *P. aeruginosa* in water were incubated for 24 h at room temperature (~25°C) in the dark with shaking at 250 rpm. After 24 h of copper exposure, cultures were diluted 10-fold (10^{-3}-10^{-5}) in LB, where 100 µl of each dilution was plated onto LBA. Agar plates were incubated at 37°C overnight. The following day, bacterial colonies were counted on each plate to determine CFU/mL. Three biological replicates were tested for each isolate.

2.5.4 Biofilm standard vs deep well MBEC determination of BZK and bleach

In Chapter 3, a comparison of static high-throughput 96 well/ pegged device biofilm growth and formation methods were tested between standard MBEC devices sold by Innovotech Inc. (Catalogue number: 91112) and self-assembled 96 well PCR-plates inserted into deep well microplates. The detailed protocol published in the Journal of Visualized Experiments (JoVE) can be found in our recent manuscript (1). A summary of the methods used for comparing *P. aeruginosa* PAO1 and *E. coli* BW25113 biofilm formation on both devices is described below, and a schematic overview of the experiment is shown in Figure 2.1. *P. aeruginosa* and *E. coli* were selected as reference strains to compare the strengths and limitations of each biofilm formation device.

Overnight cultures of 3 biological replicates of *P. aeruginosa* and *E. coli* grown from cryopreserved stock in LB were standardized to $OD_{600\ nm}$= 1.0 in a UV/Vis spectrophotometer (Thermo Scientific Multiskan® Spectrum), diluted to a final in-plate dilution of 10^{-4}, and grown in LB as 24 h biofilms on the standard and deep well biofilm devices. The plate layout for biofilm growth is shown in Figure 2.2A.

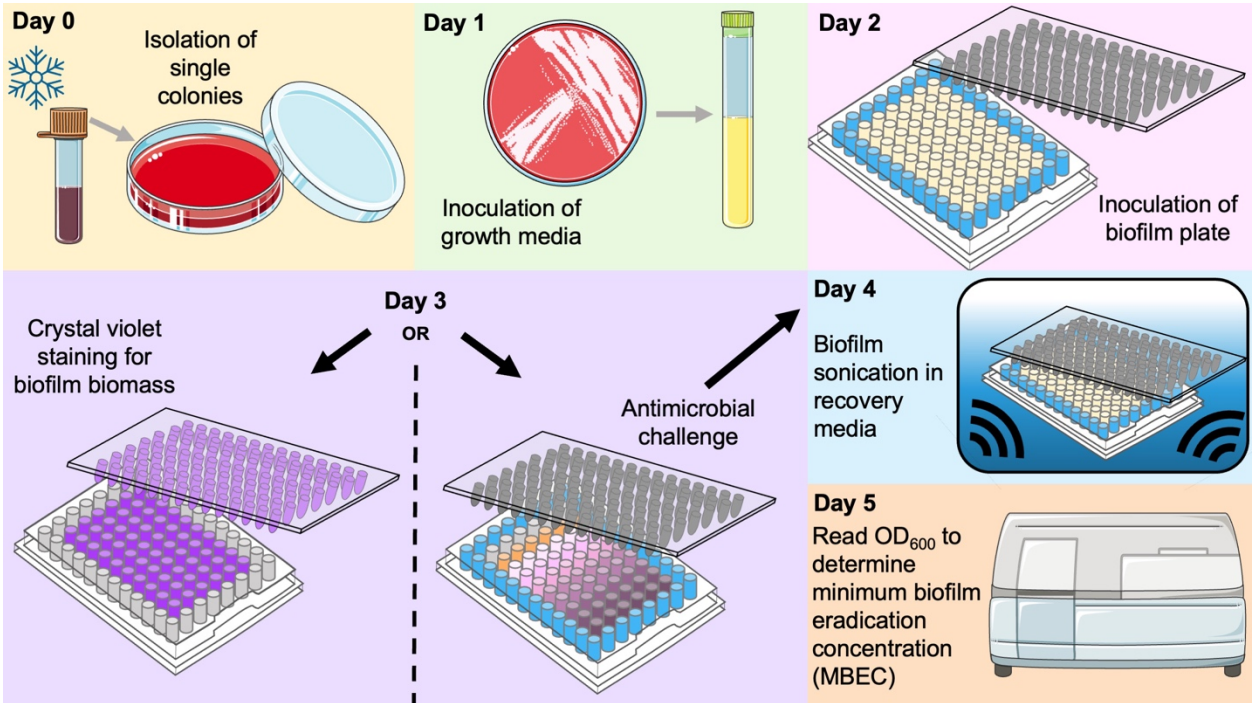

Figure 6.1 Schematic overview of deep well biofilm device experiments. Day 0 isolation of single colonies from cryopreserved stock; Day 1 inoculation of growth media with single colonies; Day 2 inoculation of biofilm plate; Day 3 CV staining or antimicrobial challenge; Day 4 biofilm sonication into recovery media; and Day 5 reading OD_{600nm} of recovery plate to determine MBEC values. Some images in the figure were provided by Servier Medical Art (smart.servier.com). This figure was originally published in JoVE (1) and is reproduced here with journal and authorship permission.

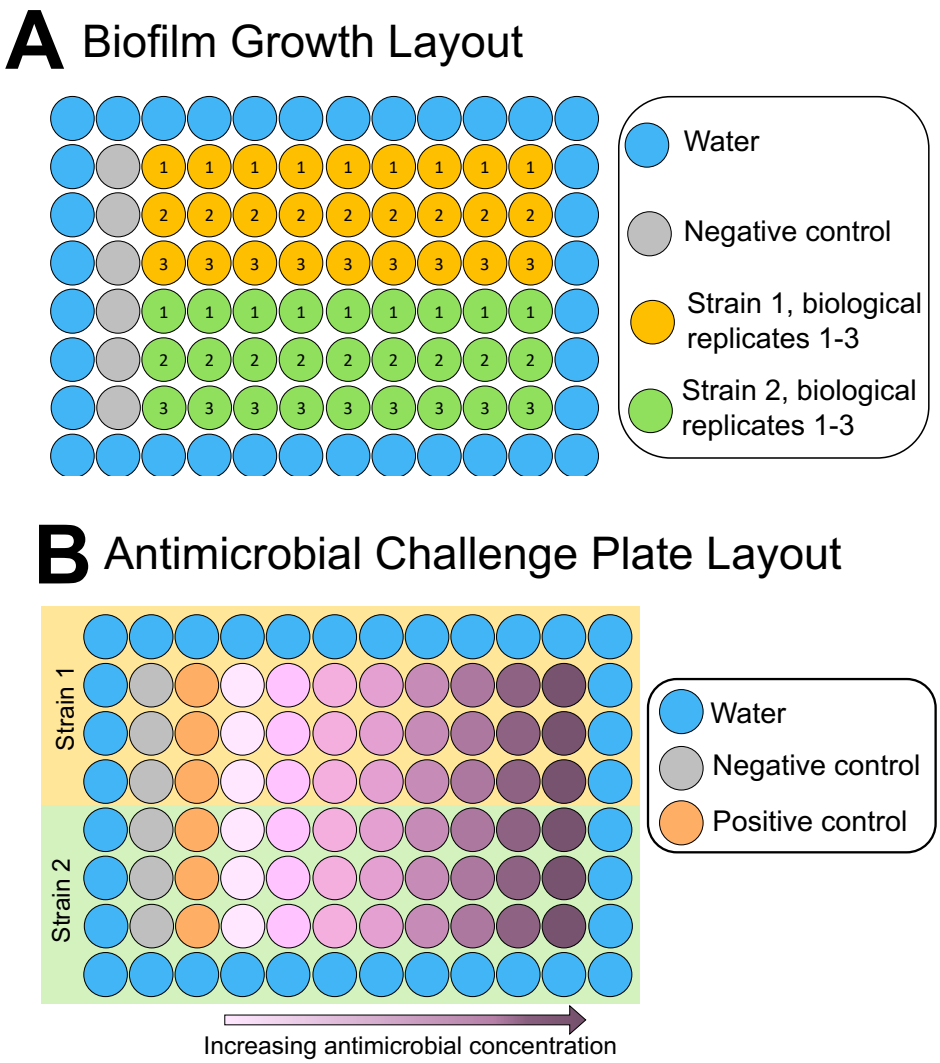

Figure 6.2 Example plate layouts shown for various steps of the deep well biofilm device protocol. A) The final plate setup for the initial 24 h biofilm growth using Day 1 bacterial cultures from two bacterial strains (*E. coli* and *P. aeruginosa*), each with three biological replicates. Negative control wells (grey) are used as sterility controls (no bacteria added). **B)** Plate setup for antimicrobial challenge of biofilms. Darkening colours indicate increasing antimicrobial concentration. Negative control are wells with no bacteria added (Grey) and positive controls are biofilms with no antimicrobial exposure (orange). Biofilm peg lids taken from panel A are transferred to this deep well plate for antimicrobial exposure. This figure was originally published in JoVE (1) and is reproduced here with journal and authorship permission.

Crystal violet (CV) staining was used to compare biofilm biomass formation on each of the devices. 24 h biofilms were rinsed in microtiter plates containing phosphate buffered saline (PBS), stained for 5 min in 0.1% (w/v) CV in dH_2O, rinsed again in PBS, dried for 5 min, and destained in 30% (v/v) glacial acetic acid in water. The absorbance at 550 nm (A_{550nm}) of the destaining solution was read in a UV/Vis spectrophotometer (Thermo Scientific Multiskan® Spectrum).

We also sought to determine the minimum biofilm eradication concentration (MBEC) of two common disinfectants, benzalkonium chloride (BZK) and sodium hypochlorite (bleach) of *P. aeruginosa* and *E. coli* when grown on each device. 24 h biofilms were rinsed in sterile PBS and exposed to a 2-fold dilution of BZK (0-40960 µg/mL) or bleach (0-2.5% (v/v)) in LB for 24 h (Figure 2.2B). Biofilm eradication was determined by sonicating biofilms for 30 min into a new standard or deep well microtitre plate containing recovery media and incubating overnight. The following day the OD_{600nm} was read in a UV/Vis spectrophotometer (Thermo Scientific Multiskan® Spectrum) and the MBEC value was determined as the lowest antimicrobial concentration with no growth.

2.5.5 Copper and acidic pH biofilm inhibition

In Chapter 4, biofilm copper and acidic pH inhibition testing experiments were set up in the same manner as described in 2.4.2. However, these copper and pH susceptibility assays were modified by inserting sterile MBEC Assay® Biofilm Inoculator (Innovotech, Edmonton, AB, Canada) pegged lids into the inoculated 96 well microtiter plates to allow for biofilm growth. The MBEC device biofilms were grown for 24 h at 37°C with shaking at 150 rpm.

To determine the $CuSO_4 \bullet 5H_2O$ concentration or pH that inhibited growth, biofilms grown on the pegs were stained following the protocol described in 2.4.4. Minimum biofilm inhibition concentration (MBIC) was determined by the lowest concentration of $CuSO_4 \bullet 5H_2O$ or the most basic pH that inhibited biofilm formation on the pegs as determined by the $A_{550\ nm}$.

2.6 Transcriptomics of copper and acid exposed *P. aeruginosa*

Although an analysis of the *P. aeruginosa* reference strains was prepared during this thesis its analysis is not included in the thesis to meet graduation timeline requirements. These analyses

will be included in publications derived from the work conducted in Chapter 4. Overnight cultures of PAO1, PA-8, and PA-8 ΔGI-7 were diluted 1/100 in MHB and grown to early-mid logarithmic phase (OD_{600nm}= ~0.4). 10 mL mid-log cultures were centrifuged to pellet cells (5,000 x g, 30 min) and resuspended in 10 mL MHB (control), MHB amended with 2 mg/mL $CuSO_4 \bullet 5H_2O$ (copper exposed), or MHB adjusted to pH 5.5 (pH exposed). After 2 h exposure at 37 °C with shaking at 250 rpm, 2 mL were pelleted (14,000 x g, 1 min) resuspended in 500 µl RNAlater (Thermo Fisher Scientific, Waltham, MA, USA) and frozen at -20 °C.

RNA extraction from the collected samples above was completed using the RiboPure™-Bacteria Kit by Thermo Fisher with DNAse I treatment. Briefly, samples were lysed in RNAWiz with zirconia beads for 10 min, RNA was extracted with 0.2 volumes of chloroform, and the aqueous layer was treated with 0.5 volumes of ethanol before washing and purification of RNA from the aqueous phase using a spin column. RNA was eluted in two 40 µl volumes of warmed (95-100 °C) elution buffer and then treated with DNAse I following the kit manufacturer's recommended instructions.

RNA purity was determined by measuring the 260/280 nm and 260/230 nm ratios using the NanoDrop™ One/OneC Microvolume UV-Vis Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and RNA concentration was determined using the Qubit™ 2.0 Fluorometer and RNA Broad Range Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). RNA samples were sent to LC Sciences (Houston, TX, USA) for total RNA sequencing and analysis. Reference sequences of PAO1, PA-8, and PA-8 ΔGI-7 used for analysis were sequenced at the NML (Winnipeg, MB; refer to section 2.2.4 above for details as the same extraction and sequencing conditions were used).

Chapter 3: Generation of Greater Bacterial Biofilm Biomass Using PCR-Plate Deep Well Microplate Devices

3.1 Chapter Aim

This study aimed to provide a method for conducting larger volume, high-throughput (96-well), static biofilm device measurements using deep well biofilm devices. The rationale for this aim was to improve upon the yields of biofilm biomass generated by conventional high-throughput static biofilm testing methods so this approach could be used to screen the BCIP study *Pseudomonas* spp. environmental isolates in greater amounts and using a more affordable biofilm culturing device. Here, we compared a self-assembled semi-skirted PCR plate inserted into a deep well microplate, known as the deep well device, to a commonly used standard biofilm pegged lid device to examine their capabilities to form static biofilms. CV-stained biomass and MBEC assays were used to assess biofilm formation by both devices. The results and discussion presented below have been peer-reviewed and published in a recent 2022 JoVE article (DOI: 10.3791/63069; (1)).

3.2 Results

3.2.1 CV staining of MBEC and deep well biofilm device biofilms shows increased biofilm formation on the deep well device and species-specific plastic preference

To compare biofilm formation by different species on each device, we assessed biofilm biomasses formed by *E. coli* BW25113 and *P. aeruginosa* PAO1 using the biofilm CV staining protocol (67). Although CV staining is generally reported as A_{550nm} values, due to the differences in growth media volumes of each device and their available surface areas we converted CV stain A_{550nm} values to molar CV concentrations in solution. Molar CV concentrations accounted for volume differences of each device and allowed a comparison of CV concentrations representing biomasses recovered from each device. The results showed that both species produced significantly more biomass on deep well biofilm devices (2.1 fold *E. coli*; 4.1 fold *P. aeruginosa*) compared to the standard biofilm device (Figure 3.1 A-B). This outcome was expected given the larger surface area of the deep well PCR peg (320.4 mm^2) as compared to the standard device

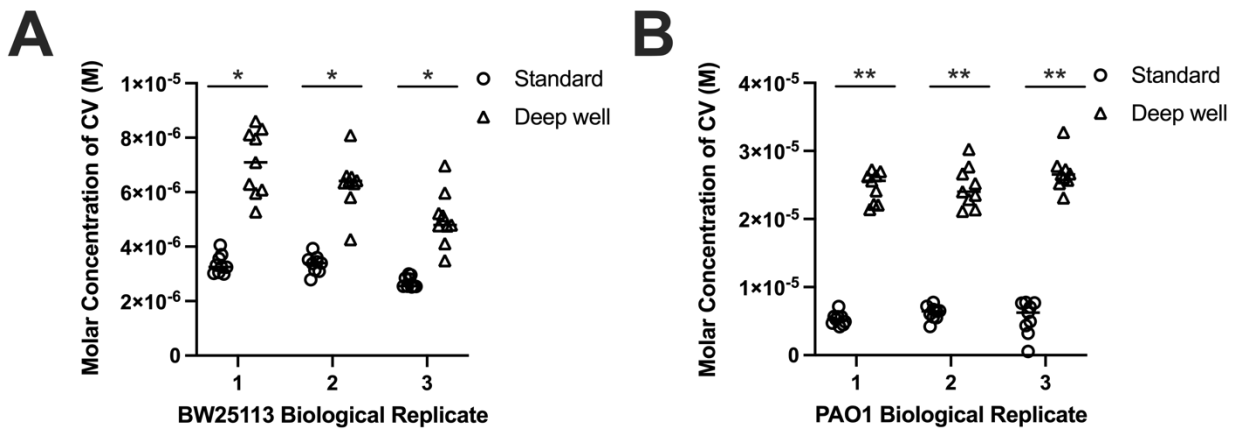

Figure 7.1 CV staining of biofilm biomass formed on pegged lids. The molar concentration (M) of CV-stained *E. coli* BW25113. (A) and *P. aeruginosa* PAO1 (B) biofilm biomass recovered from standard (circles) and deep well (triangles) devices. CV staining was measured by reading the absorbance of CV at 550 nm (A_{550nm}), and deep well A_{550nm} readings were adjusted by a factor of 3.809 (800 µL/ 210 µL) to account for the volume differences between the devices. CV molar concentration (M) was determined by the Beer-Lambert law (CV Concentration =$A_{550nm}/\varepsilon l$); where the pathlength (l) of 210 µL in the 96 well flat bottom microtitre plate was determined to be 0.56 cm and the extinction coefficient (ε) of CV in water at A_{550nm} of 251500 $cm^{-1}M^{-1}$ [39]. Results represent 9 technical replicates from three biological replicates of each strain grown as a biofilm and each biological replicate's median value is shown as a horizontal bar in each plot. Statistically significant differences in biomass between the devices was determined between biological replicate median values using a two-tailed paired t-test (*E. coli* p= 0.008-0.018 (*); *P. aeruginosa* p= 0.002-0.009 (**)) shown as bars with asterisks. This figure was originally published in JoVE (1) and is reproduced here with journal and authorship permission.

peg surface area (108.9 mm^2). This finding is also consistent with the increased volumes (750 µL) used in the deep well biofilm device as compared to the standard device wells (200 µL). Hence, deep well devices increased biofilm biomass accumulation on PCR tube pegs as compared to smaller pegs with standard biofilm devices.

Both biofilm devices formed reproducible biofilms when we compared biologically replicated biofilms formed by *E. coli* and *P. aeruginosa* (Figure 3.1 A-B). Despite observing greater variability in technical replicate CV-stained M A_{550nm} values for either strain grown on the deep well device, there were no statistically significant differences when we compared the median CV M values for each species' biological replicates on the deep well or standard devices using pairwise two-way Analysis of variance (ANOVA) or Student's T-tests (both $p > 0.05$). This finding shows that biofilm formation by deep well and standard devices form reproducible biofilms. However, the CV-staining results also showed that when we accounted for peg surface area differences on each device, the biofilm biomass formation by *E. coli* and *P. aeruginosa* showed statistically significant differences in biomass accumulation (Table 3.1). Calculation of mean CV-stained biomass (M) per peg surface area in mm^2 (CV M/mm^2) for *E. coli* showed that biofilm formation was 1.5-fold lower on polypropylene deep well devices when compared to the standard biofilm device (Table 3.1). However, the opposite result was obtained for *P. aeruginosa*, which demonstrated 1.4-fold higher CV M/mm^2 on polypropylene deep well devices when compared to standard biofilm devices (Table 3.1). Despite the observed species-specific differences in biofilm biomass accumulation with each device, the deep well device still demonstrated greater overall (2-4-fold increases) biofilm biomass formation by each species (Figure 3.1).

3.2.2 Bleach MBEC values decrease for biofilms grown on deep well biofilm devices while BZK shows variation in eradication on both devices

To determine the AST applications of the deep well biofilm device, we compared two commonly used disinfectants, BZK and bleach, for their biofilm eradicating potential. Both chemicals are commonly used to prevent (BZK) and/or eradicate (bleach) bacterial biofilms in clinical and industrial applications (110–112). Each compound was added in increasing 2-fold concentrations to biofilms formed by *E. coli* and *P. aeruginosa* on the standard and deep well

biofilm devices (111, 112) (Figures 1, 2B). The lowest concentration of BZK or bleach that resulted in OD_{600nm} values that were indistinguishable from negative control wells were defined as the MBEC value. Treatment of biofilms formed on standard biofilm devices with bleach resulted in the same bleach MBEC values of 0.625% for *E. coli* and *P. aeruginosa* (Table 3.1, Figure 3.2 A-B). Bleach MBEC values determined for *E. coli* and *P. aeruginosa* biofilm formed on deep well devices showed 2-4 fold lower MBEC values by both species (*E. coli*; 0.156%; *P. aeruginosa* 0.313%) when compared to standard devices (Table 3.1). A 2-fold difference in bleach MBEC values between species was noted for the deep well device, where *P. aeruginosa* required a 2-fold higher concentration of bleach to eradicate biofilms as compared to *E. coli* (Figure 3.2 A-B, Table 3.1). The 2-fold bleach MBEC difference between *P. aeruginosa* and *E. coli* in deep well device appears to inversely correlate with 1.5-fold increased CV-stained biomass formation by *P. aeruginosa* as compared to *E. coli* (Figure 3.1 A-B). The larger amount of biomass formed by *P. aeruginosa* on deep well device pegs may also explain why a higher bleach concentration was required to eradicate *P. aeruginosa* biofilms when compared to *E. coli* on deep wells (Figure 3.1 A-B, Table 3.1). Hence, deep well device biofilm eradication assays show that both species were susceptible to bleach but at lower (2-4-fold) bleach concentrations as compared to standard devices. This inversely correlates with the 3-fold greater peg surface area and volumes of the deep well device PCR plate pegs. This suggests that for bleach exposure, greater biomass surface area may lower the bleach concentrations necessary for biofilm eradication.

BZK biofilm eradication testing showed greater variability in recovered growth (OD_{600nm} values) and MBEC values by each species when compared to bleach results (Figure 3.2 C-D). This variability is not unexpected based on previous studies showing that BZK was more effective at preventing biofilm formation than eradicating well-established biofilms (113–115). Using the standard biofilm device, only *P. aeruginosa* biofilms treated with BZK showed a consistent MBEC value (2560 µg/mL) that was 8-fold lower than the MBEC value (20480 µg/mL) obtained for this strain in the deep well device (Table 3.1, Figure 3.2 C). These results may reflect differences in the amounts of *P. aeruginosa* biomass formed on deep well pegged surfaces and plastic

Table 7.1 A summary of *E. coli* BW25113 and *P. aeruginosa* PAO1 mean CV-stained biomass M/ mm^2 values and biofilm eradication MBEC values for BZK and bleach on various devices.

Strain tested	BZK MBEC (µg/mL)		Bleach MBEC (% v/v)	
	Deep well Device	Standard Device	Deep well Device	Standard Device
E. coli BW25113	2560-40960	320-10240	0.156	0.625
P. aeruginosa PAO1	20480	2560	0.313	0.625
Strain tested	**Deep well Device Mean CV M*/ mm^2 ± SD**	**Standard Device Mean CV M*/ mm^2 ± SD**	***p*-value****	**Fold change (Deep well/ Standard)**
E. coli BW25113	1.99×10^{-8} ± 3.25 x10-9	3.03×10^{-8} ± 3.31 x 10-9	0.0176	-1.52
P. aeruginosa PAO1	8.01×10^{-8} ± 9.42×10^{-9}	5.72×10^{-8} ± 9.42×10^{-9}	0.034	1.4

*Mean CV M/mm2 values calculated using median biological replicate CV M values (Figure 3.1).
**p-values determined using Two-tailed Student's T-test.
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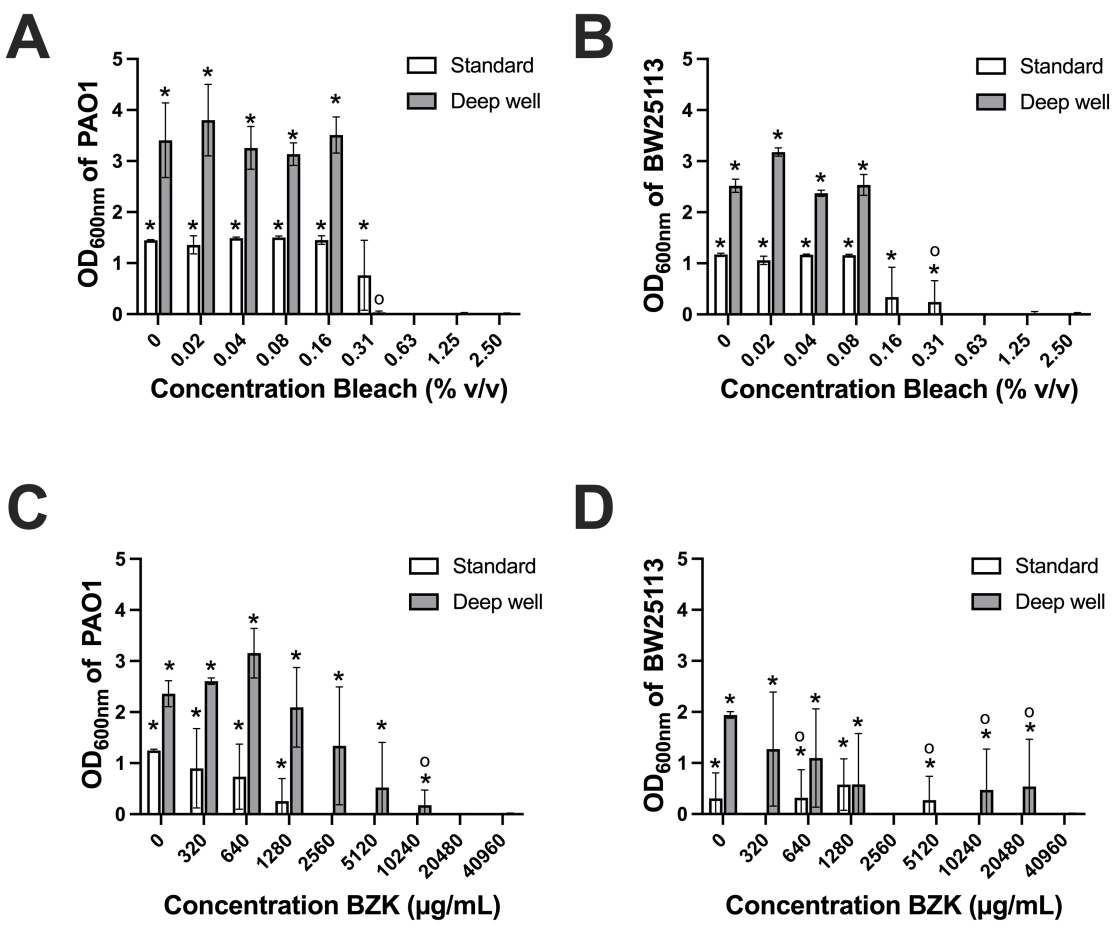

Figure 7.2 Determination of antimicrobial MBEC concentrations. Antimicrobial MBEC concentrations for bleach (A,B) and BZK (C,D) for *P. aeruginosa* PAO1 (A, C) and *E. coli* BW25113 (B,D) when grown on standard (white bars) and deep well (grey bars) biofilm pegged devices. Results were determined by reading of the OD_{600nm} of biofilm recovered after sonication into recovery media and overnight incubation. Deep well OD_{600nm} was adjusted by a factor of 3.75 (750 µL /210 µL) to account for volume differences between the devices. Results are representative of three biological replicates and error bars show the standard deviation between replicates at each antimicrobial concentration[39]. Asterisks (*) above each bar plot indicate statistically significant differences in OD_{600nm} values from blank controls with p-values < 0.05 using two-tailed Student's T-test calculations. Circle symbols (o) above each bar plot indicate measurements where only 1 or 2 replicates had statistically significant OD_{600nm} values from blank controls were defined as the MBEC value. This figure was originally published in JoVE (1) and is reproduced here with journal and authorship permission.

compositions when compared to the standard device pegs. BZK eradication of biofilms formed by *E. coli* on both the deep well and standard devices were equally poor, resulting in a broad range of BZK MBEC values from 2560-40960 µg/mL for the deep well device and 320-10240 µg/mL on the standard device (Figure 3.2 D). For *E. coli*, this variability was explained by the occasional instance where 1-2 replicate wells showed low but statistically significant growth in recovery media, that increased error and reduced BZK MBEC determination accuracy with either device (Figure 3.2 D, Table 3.1). This variability highlights the ineffectiveness of using BZK in eradicating *E. coli* biofilms as previously noted in studies (113–115). In contrast *P. aeruginosa* biofilms could be reliably eradicated by BZK at a defined concentration with both devices, but with an 8-fold BZK concentration difference (Figure 3.2 C). In summary, BZK eradication MBEC values of biofilms formed by *P. aeruginosa* show distinct MBEC values with each device, however, both devices are equally poor in distinguishing *E. coli* precise BZK eradication phenotypes. Hence, the deep well biofilm device method described herein is similarly effective for forming reproducible biofilms when compared to a standard biofilm device.

3.3 Discussion and Conclusions

This study describes methods for using a larger volume 96-well high throughput static biofilm growth device involving a polypropylene deep well microplate fitted with a semi-skirted PCR-plate lid for biofilm formation (deep well biofilm device; Table of Materials available in JoVE supplementary materials (1)). We compared biofilms generated with this device to a commercially available polystyrene standard biofilm device (Table of Materials available in JoVE supplementary materials (1)). The deep well device method uses the same methodological steps and solutions as the standard device (67) at adjusted volumes modified for the deep well devices, making this device ideal for large-scale biofilm formation and experimental analysis. The growth of *E. coli* BW25113 and *P. aeruginosa* PAO1, two Gram-negative species that are known to form biofilms, were examined for their biomass formation and disinfectant (BZK/bleach) MBEC values on both devices. Comparison of CV-stained biomass formed on pegs from each device showed that both *E. coli* and *P. aeruginosa* formed biofilms with higher biomass on the deep well biofilm device when compared to the standard device (Figure 3.1 A-B). Increased biofilm biomass

reflected the larger surface area of deep well pegs when compared to the standard device peg surface area. When we accounted for differences in peg surface areas (mm^2) with both devices, differences in biomass formation were noted, where *E. coli* formed significantly greater CV biomass per mm^2 on polystyrene standard device pegs than with the deep well device's PCR-tube pegs (Table 3.1). *P. aeruginosa* formed greater CV-stained biomass/ peg mm^2 as compared to standard devices (Table 3.1). These findings may highlight species-specific differences in biofilm biomass formation on the different devices.

It should be noted that bleach eradication of *E. coli* and *P. aeruginosa* biofilms on deep well devices occurred at 2-4-fold lower concentrations than standard device (Figure 3.2A-B, Table 3.1). The differences in bleach MBEC values identified from each device are likely impacted by differences in device peg shapes (deep well "pegs" are tapered tubes and standard pegs are cylindrical), plastic composition differences (polypropylene versus polystyrene), and volume differences (750 µL versus 200 µL). For example, *P. aeruginosa* had greater CV M biomass/ mm^2 on deep well device pegs when compared to standard devices, but *E. coli* had less biomass on deep well pegs (Figure 3.1). This suggests that disinfectant concentrations required for biofilm eradication may be impacted by the biomass formed by each species as well as the available surface area. Additionally, differences in device peg shape may influence various growth conditions for particular species. In our study, *P. aeruginosa* may form greater biomass on deep well pegs due to their greater surface area and aeration, as this species is an obligate aerobe in contrast to *E. coli*, which is a facultative anaerobe. To date, we have not identified any published studies that have directly compared *E. coli* and *P. aeruginosa* biofilm formation together on both polypropylene (116–118) and polystyrene (119, 120) materials. However, reports of robust *E. coli* and *P. aeruginosa* biofilm formation have been noted in independent studies examining either polypropylene or polystyrene materials. With respect to Pseudomonads, many *Pseudomonas* spp. can use plastics such as polypropylene as potential carbon sources (118). Hence, the availability of this polypropylene deep well biofilm device is a useful advance in static biofilm studies. Polypropylene is chemically more durable than polystyrene and is a clinically relevant material, as it is frequently used in medical implants, sutures, and meshes for hernia or pelvic surgeries (121, 122).

Although biofilm biomass was formed by both devices, the deep well device had slightly higher variability in biomass based on the CV staining method and OD_{600nm} biofilm eradication MBEC values for bleach and BZK. This may be explained by 3 main factors: 1) Deep well devices have greater peg surface area than standard devices that were angled as compared to standard device pegs. 2) Both species tested may have differing abilities to adhere to polypropylene and polystyrene materials of each device. 3) The volumes of growth media used in each device (750 µL deep well, 200 µL standard) and spacing between the inserted peg to the well side walls differs. These issues are not a problem if only one type of device is used for all biofilm experiments, however, if both devices are selected then the comparisons we conducted herein should be performed to identify differences (123, 124). Due to the differences in plastic material used in each device, the CV-stained biofilm biomass and MBEC values should not be directly compared between different devices. However, if methods and experiments are conducted on the same device (deep well or standard), the results obtained for species and antimicrobials tested are comparable.

This protocol shows that self-assembled deep well PCR-plate devices are larger volume biofilm device for measuring biofilm formation and eradication that is also cost-effective. From a cost perspective, standard biofilm devices with a 96 well pegged lids range retail at $29-36 US dollars (USD) per device (Table of Materials available in JoVE supplementary materials (1)). Polystyrene standard biofilm devices are not autoclavable and are less tolerant to solvents/acids due to its plastic chemical properties. The self-assembled polypropylene deep well plates described herein, fitted with a separate semi-skirted 96 well PCR-plate (Table of Materials available in JoVE supplementary materials (1)) cost a total of $14 USD per assembled device, which is half the standard biofilm device cost. It should be noted that prices may vary depending on region, distributor, and availability, and our costs after institutional discounts worked out to $9 USD/deep well device (prices as of Oct 2021). These self-assembled deep well polypropylene PCR-plate devices have the added advantage of being autoclavable for sterilization and offer 2-4 times more biofilm biomass than standard devices.

In conclusion, this protocol and the representative findings from biofilm growth comparisons of the deep well and standard biofilm devices show that both devices are capable

of cultivating bacterial biofilms, but deep well devices form 2-4 times more biofilm. The deep well biofilm device offers a viable and affordable alternative for larger volume high-throughput biofilm formation experiments such as drug susceptibility screening studies. This technique may generate biofilms useful for downstream '-omics' extractions (proteomic, metabolomic, transcriptomic) or experimental assays (enzymatic, fluorescent) that may require larger quantities of biofilm materials for analyses. The deep well biofilm device is recommended for labs that would like to study biofilms in a high-throughput 96-well assay using lower-cost, larger volume, chemically durable plastic materials that are clinically relevant.

Chapter 4: Genomic and Phenotypic Exploration of Copper Tolerance and Copper Susceptibility Testing Methods in Pseudomonads

4.1 Introduction and Aims

P. aeruginosa is a ubiquitous bacterial pathogen that is one of the most common causes of nosocomial infections worldwide (16). Infections by *P. aeruginosa* are of increasing concern due to its intrinsic and acquired resistance to antibiotics, particularly last resort beta-lactam therapies such as carbapenems (17). For this reason, the WHO has listed carbapenem-resistant *P. aeruginosa* as a critical priority pathogen for the development of new antibiotics (17). In addition to developing new antibiotics to treat *P. aeruginosa* infections, we must utilize alternative antimicrobial compounds to prevent or reduce *P. aeruginosa* colonization on surfaces within healthcare settings (36). This chapter focuses on the use of copper as an antimicrobial metal, since copper alloys and copper coatings are frequently used on touch surfaces as well as within sink drains to prevent bacterial contamination due to its reported antimicrobial actions (38, 76). Sink drains are a major reservoir for bacterial colonization within hospitals because they provide ideal conditions for biofilm growth, and numerous *P. aeruginosa* nosocomial outbreaks have been reported due to contaminated sink drains (18, 27). Copper sink drains have been suggested as a method of preventing sink drain *P. aeruginosa* outbreaks and consequently reducing nosocomial infections from these environmental sources. As copper gains popularity with more frequent usage on materials, there is growing concern that *P. aeruginosa* will develop tolerance to copper. There are many copper tolerance mechanisms identified in *P. aeruginosa* as well as other clinically relevant Gram-negative species, suggesting that copper tolerance is important to monitor. One of the most concerning is the spread of a genomic island known as GI-7 among *Pseudomonas* isolates (45). As reviewed in chapter 1, GI-7 is a concerning mobile genetic element that encodes for many known copper tolerance genes. It is also a growing environmental and clinical concern due to its increasing detection in hospital *Pseudomonas* isolates (45).

To study copper tolerance associated with GI-7 in *P. aeruginosa,* it is important to detect copper tolerance phenotypically and genotypically. This is important to ensure associations between copper tolerance phenotypes and genotypes are consistently related to copper

susceptibility and exposure testing methods in use to detect and predict copper tolerance from genome sequences. It is also important to measure accurate levels of copper in solutions to relate copper exposure concentrations to antimicrobial effects and limit confounding factors related to copper use. These confounding variables include its known acidification effects and propensity to form phosphate precipitates which change bioavailable copper in solutions (65, 125). Thus, the focus of this chapter was to survey *P. aeruginosa* sink drain isolates collected from various hospital ICUs in Ontario, Canada for their copper tolerance GI-7 genotypes and then characterize GI-7 phenotypic contributions in greater depth. Due to the limitations in reliable copper susceptibility testing methods which have been explored in this chapter, this study was needed to determine the effect of acidification of growth media by copper salts during planktonic and biofilm *P. aeruginosa* strain growth. This involved copper susceptibility testing of various *P. aeruginosa* reference strains with and without the copper tolerance genomic island GI-7. These studies also examine copper tolerance in *P. aeruginosa* reference strains where a previously published method of copper susceptibility testing (42) was modified in an effort to eliminate the issue of copper acidification at growth inhibitory concentrations in complex growth media.

4.2 Results

4.2.1 Genome surveillance of BCIP Pseudomonas isolates for the copper tolerance genomic island GI-7

To determine the frequency of GI-7 presence among the clinical and environmental *Pseudomonas* spp. isolates collected from the BCIP study, a bioinformatic survey was conducted on the 2467 genome sequenced *Pseudomonas* isolates collected from seven different Ontario hospitals between 2017-2019 (Table 2.2). A total of 2452 *P. aeruginosa* and 15 non-*aeruginosa* species were collected, where 1146 isolates were clinical samples collected from patients via rectal swab, and 1321 isolates were from environmental sources, including air, faucets, sink drains, sink rims, and the sink surface. A visual breakdown of all *Pseudomonas* isolate amounts based on their hospital sampling location is summarized in Figure 4.1A. This chart shows that several hospital environmental sampling locations had few or no samples taken at certain sites, specifically for isolates collected from sampled room sink rims, faucets, and surfaces. Since many hospitals lacked isolates collected from these locations, we chose to exclude environmental sink

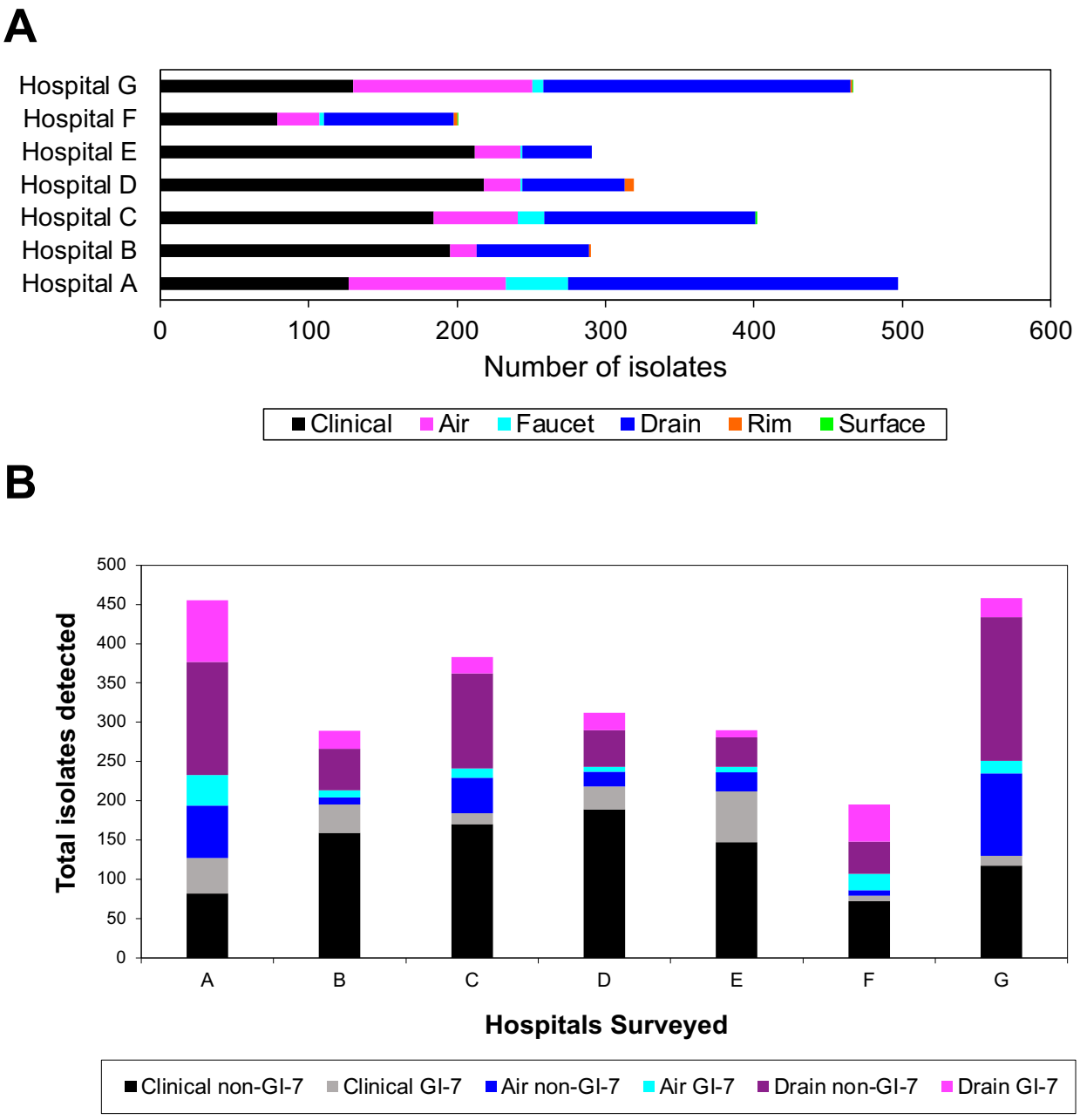

Figure 8.1 BCIP isolates organized by sampling location and presence or absence of GI-7. A) Total number of *Pseudomonas* isolates collected within BCIP study hospitals, organized by hospital (A-G) and isolation location. B) Total number of *Pseudomonas* isolates from various locations within BCIP study hospitals, organized by isolation location and the presence or absence of GI-7. GI-7 detection was done using the tool KATsect, and the presence of GI-7 was indicated by a KAT score >99%.

rims, faucets and surfaces from further bioinformatic genomic analysis, resulting in only drain and air environmental isolates (n=1237) for final analysis (Figure 4.1).

To identify the extent of genomic island GI-7 presence in the sequenced genomes of clinical and environmental (air and drain) BCIP *Pseudomonas* isolates the tool KATsect was used to identify GI-7 sequence presence. KATsect is a program belonging to the KAT program and was used to generate a pairwise comparison KAT score based on the previously identified GI-7 sequence to each assembled sequenced genome; the higher the KAT score the greater the pairwise k-mer identity (107). A KAT score of >99% was selected as the cut-off value to confidently detect the presence of a full length GI-7 sequence (Figure 4.1B). This value was selected based on randomly selected genome sequence verification to detect full length GI-7 sequence coverage in assembled isolate genomes with a previously published GI-7 sequence (Appendix A). Overall, each sampled BCIP hospital location (clinical, air and sink drain) contained a higher proportion of *P. aeruginosa* isolates without GI-7 than isolates with GI-7 (mean % GI-7 detection values of 17.8% clinical, 34.7% air, and 28.0% drain; Figure 4.1B, Table 4.1). The only exception was identified for Hospital F, where environmental air and drain sample locations had more GI-7 isolates than non-GI-7 isolates, with 75% (21/28) of air isolates and 53.4% (47/88) of drain isolates containing GI-7 (Figure 4.1B; Table 4.1). Additionally, sampled locations from Hospital B showed an equal number of GI-7 and non-GI-7 isolates in air samples (9 GI-7, 9 non-GI-7) (Figure 4.1B).

Statistical analysis using Student's t-test showed that there was no significant difference between % GI-7 detection in clinical isolates and both environmental isolate locations (Table 4.1). It is important to note that the standard deviation values in total % GI-7 detection among the clinical, air, and drain sampled groups were quite large, indicating that GI-7 detection is extremely variable between hospitals. Thus, these results may not accurately reflect the difference in GI-7 detection between isolation locations and more isolate samples may have helped reduce this variation. Overall, these results show that among surveyed Ontario BCIP study hospitals, the average % GI-7 sequence detection among *Pseudomonas* isolate genomes is within 20-35% and similar across clinical, air and sink drain sampled hospital locations.

Table 8.1 The percentage and number of BCIP isolates with a detectable GI-7 sequence, separated by hospital and sampling location.

	% GI-7 detection			Total number of samples with GI-7		
Hospital	**Clinical**	**Air**	**Drain**	**Clinical**	**Air**	**Drain**
A	35.43	36.79	35.14	45	39	78
B	18.46	50.00	30.26	36	9	23
C	7.61	21.05	14.79	14	12	21
D	13.30	24.00	31.88	29	6	22
E	30.66	22.58	19.15	65	7	9
F	8.86	75.00	53.41	7	21	47
G	10.00	13.22	11.59	13	16	24
Mean Hospitals	**17.76**	**34.66**	**28.03**			
Stdev	11.12	21.49	14.37			
	Student's t-tests	**Clinical vs. Air** 0.0976	**Clinical vs. Drain** 0.1621			

Mean hospital isolate % GI-7 detection in each isolation location (clinical, air, and drain) is listed as well as its standard deviation (Stdev).

Two-tailed Student's t-tests compared the %GI-7 detection in environmental isolates (sink or drain) to clinical isolates.

GI-7 detection was done using the tool KATsect, and the presence of GI-7 was indicated by a KAT score >99%.

To assess whether GI-7 detection in *Pseudomonas* isolates increased in hospital rooms where copper sink drains were used, the environmental isolates were separated and analyzed according to hospital rooms containing a regular drain (denoted as "regular") or copper sink drain (denoted as "copper") and the percentage of isolates with detectable GI-7 was determined (Table 4.2; Figure 4.2). Due to differences in BCIP room sampling frequency, it was noted that the number of isolates collected in rooms with copper sink replacements was under sampled as compared to rooms with regular sinks (Table 4.2) where only 84 environmental *Pseudomonas* isolates (from both air and drain samples) were collected from rooms with a copper sink drain as compared to the 1153 air and sink drain isolates sampled from rooms with pre-existing "regular" sink drains. The limited proportion of isolates from copper sink drain rooms in comparison to isolates sampled from regular sink drain rooms prevented more robust multivariate statistical analysis and significance determinations; however, our findings based on available data are discussed below.

To determine if there were any correlations between isolates with GI-7 collected from regular or copper sink drains among air and drain sampled hospital rooms, a Pearson correlation coefficient calculation was used (often referred to as Pearson's *r* value). Positive *r* values ranging from 0.75-1.0 typically indicate a reliable linear correlation between variables, whereas negative *r* values from -0.75 to -1.0 indicate an inverse relationship between compared samples (as one sample increases the other decreases) and r values of zero have no linear relationship. Pearson's *r* values comparing % GI-7 detection in air and drain samples from regular or copper sink drain rooms showed high positive correlations (+0.88 and +0.74, respectively) (Table 4.2). This finding indicates that as the % detection of GI-7 in a regular sink drain room increases, the air sampled from the same room as the sink drain also increases; the same is true of copper sink drain room air and drain % GI-7 detection among isolates (Table 4.2). These results are expected since we know that bacteria growing within sink drains can be aerosolized and spread into the air and surrounding environment. Thus, if isolates from a sink drain had a higher % GI-7 detection, we would expect that air samples would also have an increased % GI-7 detection due to bacterial

Table 8.2 The percentage of BCIP environmental isolates in each hospital with detectable GI-7 and the total number of samples surveyed, organized by type of sample and sink drain.

	% GI-7 Detection				Total number of samples surveyed			
	Air		Drain		Air		Drain	
Hospital	**Regular**	**Copper**	**Regular**	**Copper**	**Regular**	**Copper**	**Regular**	**Copper**
A	39.00	0.00	35.81	14.29	100	6	215	7
B	50.00	0.00	28.77	66.67	18	0	73	3
C	19.23	40.00	13.97	33.33	52	5	136	6
D	25.00	0.00	32.31	25.00	24	1	65	4
E	23.33	0.00	18.60	25.00	30	1	43	4
F	68.42	88.89	48.75	100.00	19	9	80	8
G	11.82	27.27	9.57	31.58	110	11	188	19
Average	33.83	22.31	26.83	42.27	353	33	800	51
Average Excluding 0	33.83	52.05	26.83	42.27				
Correlation Coefficients	Air- Reg vs. Cu	0.47	Drain- Reg vs. Cu	0.57				
	Reg- Air vs. Drain	0.88	Cu- Air vs. Drain	0.74				
Student's t-test	Air- Reg vs Cu	0.45	Drain- Reg vs Cu	0.25				
	Reg- Air vs. Drain	0.46	Cu- Air vs. Drain	0.27				

Pearson's correlation coefficients were calculated to assess any linear correlations between isolate location (air or drain) and the type of sink drain (regular or copper).
Student's t-tests assessed if statistically significant differences between the % GI-7 values of each hospital's sampled isolate locations (air or drain) and type of sink drain (regular or copper) occurred; p-values of <0.05 were considered significant.

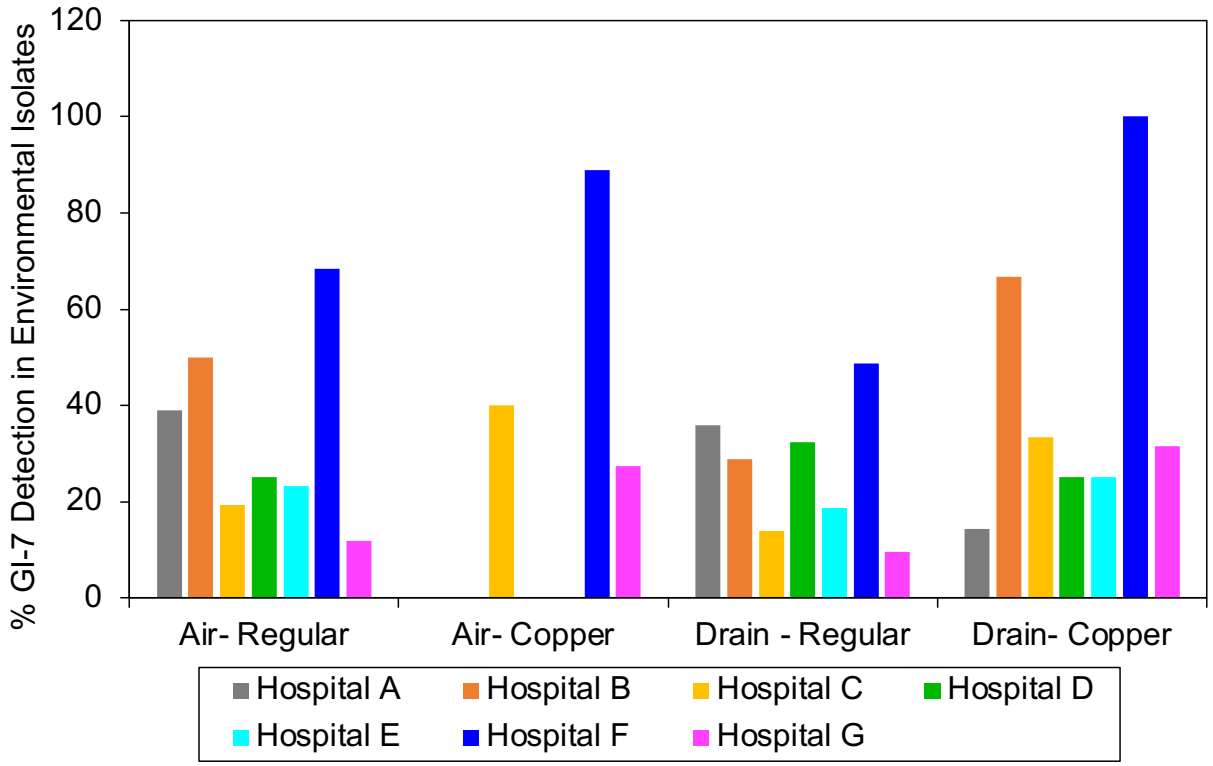

Figure 8.2 A summary plot of the percentage of BCIP environmental isolates in each hospital with detectable GI-7, separated by type of sample and sink drain. The samples were isolated from the air or sink drains of hospital rooms with regular (control) or copper sink drains.

spread between these two areas. It was also important to note that statistical analysis using a Student's t-test indicated that there was no significant difference between % GI-7 detection values from isolates sampled from copper or regular air and drains ($p>0.05$) (Table 4.2).

To test the hypothesis that copper sink drains should increase the frequency of isolates with detectable GI-7, Pearson correlations between % GI-7 detection in regular and copper air isolates, or regular and copper drain isolates, were examined. We hypothesized that this would result in negative correlation values, since the detection of GI-7 in copper sink drain room isolates should be higher than % GI-7 detected isolates in regular sink drain room isolates if GI-7 mediated copper tolerance enhanced fitness in copper sink drains. If *Pseudomonas* isolates from copper sink drain rooms are exposed to copper, they may be more likely to have selected for the copper tolerant GI-7 genomic island, whereas isolates from regular sink drain rooms are less likely to have been exposed to copper and would not require this AMR mechanism in their environment. The results from this analysis were not conclusive and showed intermediate positive correlations (+0.47 and +0.57, respectively) between regular and copper sink drain air or drain sampled hospital room locations. When the average % GI-7 detection values were compared between isolates from regular sink drains to copper sink drains, an 11.5% decrease in detectable GI-7 was seen for copper air samples, while a 15.4% increase was seen for copper drain samples (Table 4.2). However, if we exclude the 0% values for copper air samples, which were generated from a very limited number of samples and therefore may not represent actual GI-7 presence, we see an increase in copper air samples in comparison to regular air samples of 18% (Figure 4.2). Despite this, no statistically significant differences were noted between these sampling groups (Table 4.2). In conclusion, there may be weak correlations present but the limited sampling of hospital rooms with copper sink drains limits our ability to make strong conclusions. However, the increase in % GI-7 detection amongst copper sink drain samples provides promising support for our hypothesis that isolates from copper sink drain rooms will select for GI-7.

As a final part of the BCIP study analysis, the most prevalent *Pseudomonas* MLSTs and the presence of detectable GI-7 in these MLSTs were determined. MLST is a strain-typing method most commonly used to organize bacterial isolates within a species based on comparisons of housekeeping gene loci which represent a small portion of their conserved genome (126). The

most prevalent MLSTs in each sampled hospital location is summarized in Figure 4.3 and Table 4.3. The most abundant MLSTs identified from the BCIP study isolate groupings (irrespective of GI-7 presence or absence) were ST253, ST175, ST381, and novel MLST(s) (Figure 4.3, Table 4.3). In general, no clear trends towards any particular MLST were noted when comparing hospital clinical and environmental (air or drain) sample locations. However, the same dominant MLST was noted among environmental air and drain sampled locations within each hospital, aside from hospitals D and E (Figure 4.3, Table 4.3). This indicates that in the BCIP study, dominant environmental MLST isolates collected from air and sink drain samples at each hospital corresponded to each other, but not to the dominant clinical MLST in the hospital's clinical samples.

Next, we wanted to determine if GI-7 sequence detection was associated with any of the most abundant MLSTs, and a summary of these results is shown in Table 4.3. *Pseudomonas* MLSTs with the highest GI-7 sequence detection values were identified in ST253, ST309, and ST381, regardless of their clinical or environmental sampling location (Table 4.3). Other MLSTs that were most abundant (e.g., ST175, ST179, ST298) lacked detectable GI-7 entirely, suggesting that GI-7 detection may be specifically enriched by or transmitted between particular *Pseudomonas* MLSTs. Ideally, further *Pseudomonas* genome isolate sequencing studies and surveillance will focus on MLST and GI-7 co-associations to determine if these trends persist in other studies.

To conclude, the presence and detection of GI-7 within *Pseudomonas* isolates collected as part of the BCIP Ontario hospital sink drain showed that this genomic island was spread across clinical and environmental isolates at similar rates, with the potential for isolates possessing GI-7 to be enriched in copper sink drains. When MLST type was considered as part of this analysis, BCIP data showed that the dominant environmental MLST isolates collected from air and sink drains samples at each hospital did not correspond to dominant clinical MLST isolates collected from patients in the study. However, there does appear to be an association between GI-7 detection among *Pseudomonas* isolates of a particular MLST (i.e., ST253, ST309, and ST381) regardless of their sampling location within BCIP study hospitals. While clear, statistically significant associations with MLSTs or certain environments, such as copper sink drains, were

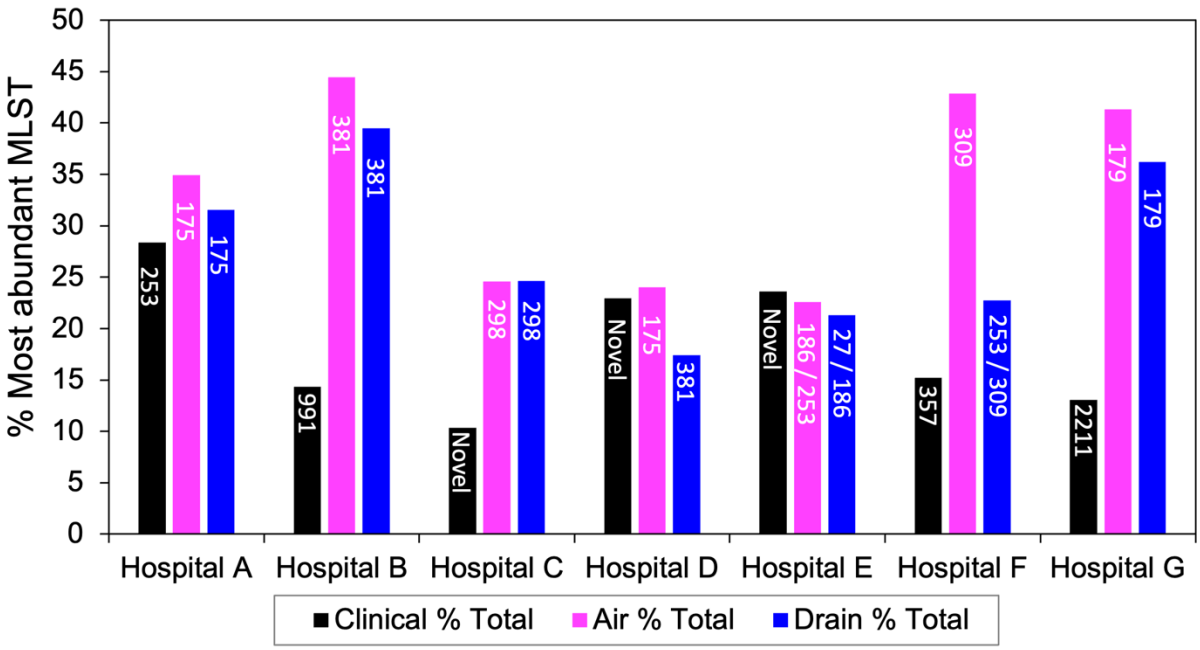

Figure 8.3 A summary of the most abundant *Pseudomonas* MLSTs from BCIP hospitals. Samples are separated by hospital and according to the type of sample (clinical, air and drain). Most abundant MLST(s) are shown in text on each bar, and the percentage of isolates that were of that MLST is represented on the y-axis.

Table 8.3 The percentage of GI-7 detection in the most abundant MLST type in each BCIP hospital's clinical, air, and drain samples.

	Most abundant MLST and GI-7 detection								
	Clinical			Air			Drain		
Hospital	MLST	Number with GI-7	% Total	MLST	Number with GI-7	% Total	MLST	Number with GI-7	% Total
A	253	36	100	175	0	0	175	0	0
B	991	0	0	381	8	100	381	21	70
C	Novel	1	5	298	0	0	298	0	0
D	Novel	1	2	175	0	0	381	12	100
E	Novel	20	40	186/253	7	50	27/186	0	0
F	357	0	0	309	12	100	253/309	40	100
G	2211	0	0	179	0	0	179	0	0

MLST indicated as 'Novel' refers to unclassified or unknown MLST types.

difficult to make due to the size of sampling, particularly in copper sink drain room isolates, continued surveillance of GI-7 in hospitals with regular and copper sink drains may help to further uncover trends in GI-7 presence and its importance to *P. aeruginosa* within sink drains.

4.2.2 Growth media acidification by copper salts

Based on the findings from the bioinformatic BCIP study survey of genome sequenced *Pseudomonas* isolates for the presence of the copper tolerant genomic island GI-7, we recognized the need to study copper tolerance phenotypically. However, when conducting copper tolerance testing in growth media, a limiting factor is the acidification caused by increasing concentrations of copper salts. When $CuSO_4 \bullet 5H_2O$ is added to an aqueous solution, such as water or growth media, it dissociates into Cu^{2+} and sulfate (SO_4^{2-}) ions (125). The SO_4^{2-} ions associate with H^+, forming sulfuric acid (H_2SO_4) and acidifying the solvent. A similar phenomenon occurs with $CuCl_2 \bullet 2H_2O$, which forms HCl when it dissociates in solution (127).

While previous studies have also explored the acidification of growth media by $CuSO_4 \bullet 5H_2O$ (65, 80), we have found that many copper susceptibility testing studies do not address this acidification effect and acidification by itself may be a major cause of the antimicrobial effects of copper. To explore this phenomenon further, we examined the extent of growth medium acidification by $CuSO_4 \bullet 5H_2O$ in various media, specifically MHB (as previously described (80)), LB, and in water (dH_2O; with no buffer adjustments) to mimic sink drain water. Although $CuSO_4 \bullet 5H_2O$ is the most common copper salt used when testing copper tolerance based on previous studies (80), we also chose to test the pH of media supplemented with $CuCl_2 \bullet 2H_2O$, as other copper tolerance studies have tested this copper salt (128). Using a 2-fold dilution series, 0-32 mM of either copper salt (0-8 mg/mL $CuSO_4 \bullet 5H_2O$; 0-5.46 mg/mL $CuCl_2 \bullet 2H_2O$) was added to MHB, LB, or dH_2O, and the pH was measured at each concentration tested. Results were based on an average of three separate experiments, and three pH measurement times. We chose to forgo pH testing in minimal media, as the addition of $CuSO_4 \bullet 5H_2O$ to Davis-Glucose (DG) and M9 minimal medias resulted in copper precipitation (Figure 4.4A). This precipitation was most likely caused by a reaction between the $CuSO_4 \bullet 5H_2O$ and sodium phosphate and/or potassium phosphate present in both DG and M9 media. These compounds undergo a double displacement reaction, forming copper phosphate as an insoluble precipitate (125).

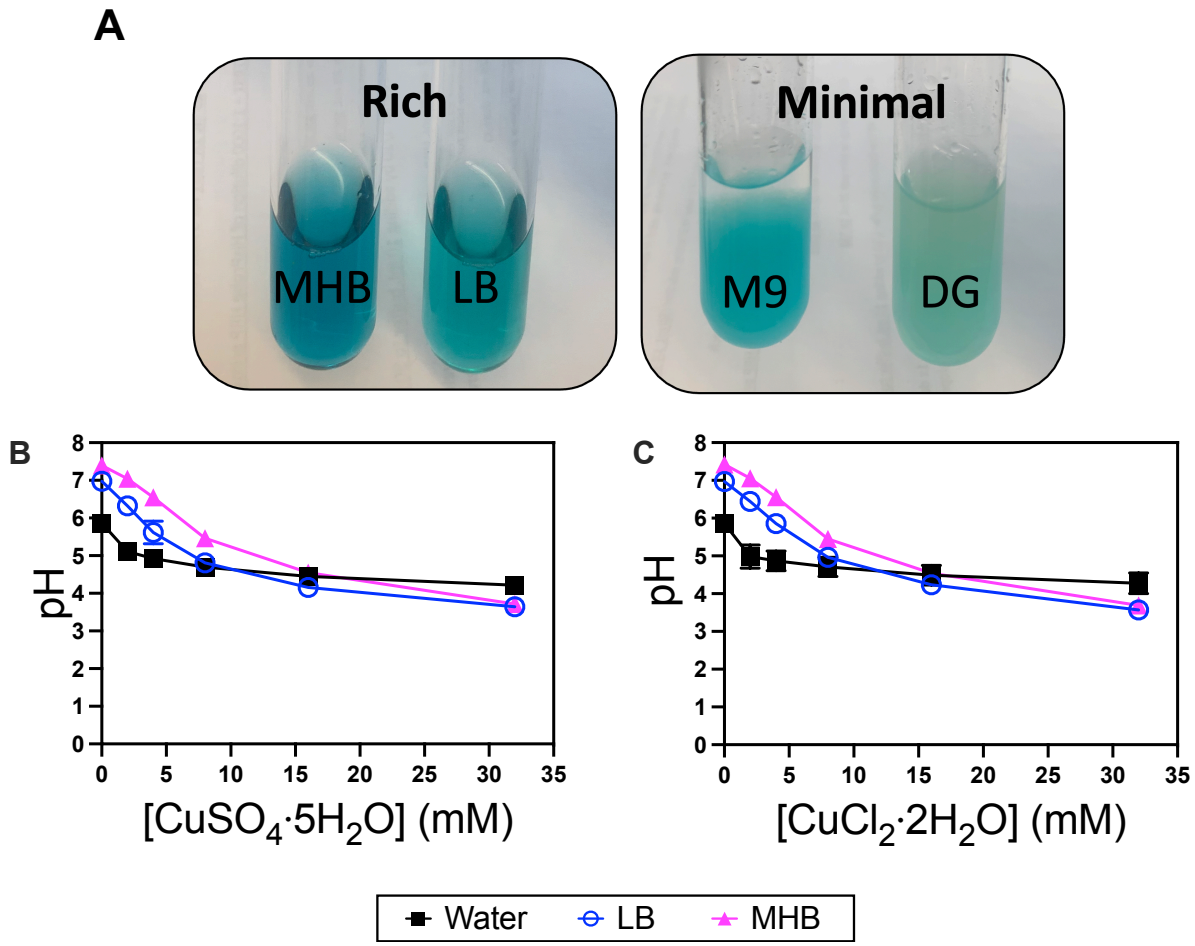

Figure 8.4 Solubility and acidity of copper salts in various solvents. **A**) Solubility of 32 mM (8 mg/mL) $CuSO_4 \bullet 5H_2O$ in rich growth media (left) and minimal media (right), with minimal media showing visible cloudiness and separation indicative of precipitation. **B&C**) Show pH of LB, distilled water, and MHB amended with 0-32 mM $CuSO_4 \bullet 5H_2O$ (0-8 mg/mL) (Panel B) or $CuCl_2 \bullet 2H_2O$ (0-5.44 mg/mL) (Panel C). Data represents the average and standard deviation of three separate experiments. All pH values of increasing copper concentrations in each solvent were found to be significantly different than the control (no copper salt added), as determined by Student's t-test ($p<0.0001$).

As shown in Figure 4.4 B-C, changes in pH were similar across both copper salts and in all three tested solvents. With no copper addition, MHB and LB had a neutral pH of 7.0, while dH_2O pH was 6.0. At the highest concentrations of copper salt tested (32 mM; 8 mg/mL), the pH value dropped to 3.5 in MHB and LB, and 4.5 pH in dH_2O (Figure 4.4 B-C). At all copper concentrations tested, the pH was significantly different than the positive control that had no copper salt added ($p<0.0001$; Figure 4.4 B-C). Similar results were seen by Hasman *et al.* 2009 (80), who reported pH values slightly above 3.5 at 30 mM $CuSO_4$ when added to MHB. This pH effect has also been seen in copper tolerance studies with other types of growth media. For example, Griffin *et al.* 2018 (65) reported that the addition of 5 mM $CuSO_4$ to four types of growth medium agar often used to grow environmental *Pseudomonads* resulted in a significant drop in pH (up to 2.75 pH units). Hence, our analysis reconfirms that $CuSO_4 \bullet 5H_2O$ and $CuCl_2 \bullet 2H_2O$ acidify media and water, which may have an impact on copper susceptibility testing outcomes as described in the experiments below.

4.2.3 P. aeruginosa copper and acidic pH susceptibility testing

Based on the findings from testing high concentrations of copper salt addition to media, which caused acidification, we sought to determine whether this acidification influenced copper tolerance testing results of various *P. aeruginosa* reference isolates with and without GI-7 sequences. To date, there are no studies available that directly compare copper susceptibility and its acidic pH. Copper tolerance susceptibility testing provided in previous studies is often measured using broth dilution assays, where a range of 0-40 mM copper salt is added to growth media similarly to AST methods (80). Given that the 0-40 mM copper salt range is known to acidify media, likely outside of the ideal pH range for *P. aeruginosa* growth (pH 4.5-9.5 (94)), we wanted to determine what effect, if any, pH had on *P. aeruginosa* planktonic and biofilm inhibition in copper microdilution assays. To compare this, MHB was supplemented with 0-8 mg/mL (0-32 mM) $CuSO_4 \bullet 5H_2O$ or its pH was chemically adjusted with 1 M HCl to match the pH of each copper supplemented media solution. Since both copper salts similarly affected pH, these studies focused on $CuSO_4 \bullet 5H_2O$. Using this copper salt, we subjected *P. aeruginosa* reference strain PAO1 to increasingly acidic pH conditions without added copper to discern whether acid or copper (or both) had measurable effects on copper susceptibility testing. As will be described

below, we first determined the pH of MHB before and after PAO1 growth in copper or in media adjusted to the same final pH concentrations as induced by that copper concentration. Afterward, analysis of both planktonic and biofilm growth inhibition of *P. aeruginosa* strains by copper and acidic conditions.

4.2.3.1 Media pH before and after planktonic growth in copper supplemented or acidified growth media

We initially measured growth media pH before and after growth of *P. aeruginosa* PAO1 in copper salt supplemented and pH adjusted media to determine what effects it had on the growth of *P. aeruginosa* after 18 hrs (Figure 4.5 A-B). The pH of all media was measured prior to bacterial inoculation ('before'), and 18 h after inoculation ('after'). In these experiments, the 'after' growth media pH was increased to an average of 8.39 in MHB supplemented with 0, 0.5, or 1 mg/mL $CuSO_4 \bullet 5H_2O$ (0, 2, or 4 mM) from initially measured pH values of 7.5, 7, or 6.5 respectively until $\geq$ 2 mg/mL $CuSO_4 \bullet 5H_2O$ values were obtained where its corresponding pH values began to descend below pH 6 (Figure 4.5 A). In MHB pH adjusted only (which matched each copper salt concentration pH value) to 7.5, 7.0, 6.5, or 5.5 respectively before growth, the 'after' growth pH values similarly increase in pH to 8.39 as was observed for PAO1 with $CuSO_4 \bullet 5H_2O$ (Figure 4.5B). These findings confirm that acidification caused by $CuSO_4 \bullet 5H_2O$ addition or pH adjustment alone both had a significant and similar effect on 'before' and 'after' *P. aeruginosa* growth pH measurements. There appeared to be one copper salt concentration where 'after' growth pH values were intermediate as compared to the pH-adjusted (5.5), specifically at 2 mg/mL $CuSO_4 \bullet 5H_2O$ (Figure 4.5A). *P. aeruginosa* PAO1 after growth pH values at 2 mg/mL $CuSO_4 \bullet 5H_2O$ showed a reduction in pH from 8.3-8.4 to 7.0, indicating this concentration of $CuSO_4 \bullet 5H_2O$ was unable to reach a basic pH caused by *P. aeruginosa* growth. 2 mg/mL $CuSO_4 \bullet 5H_2O$ may also be an ideal pH value to monitor and potentially discriminate copper addition from and pH only adjusted effects. This is explained by differences between the 2 mg/mL $CuSO_4 \bullet 5H_2O$ 'after' pH value and its matching pH-adjusted only 5.5 pH value 'after' growth; the pH-adjusted 5.5 value showed no difference from other higher pH-adjusted 'after' growth pH values (7.5-6.5; Figure 4.5 B), yet the 2 mg/mL $CuSO_4 \bullet 5H_2O$ showed a drop in after growth pH

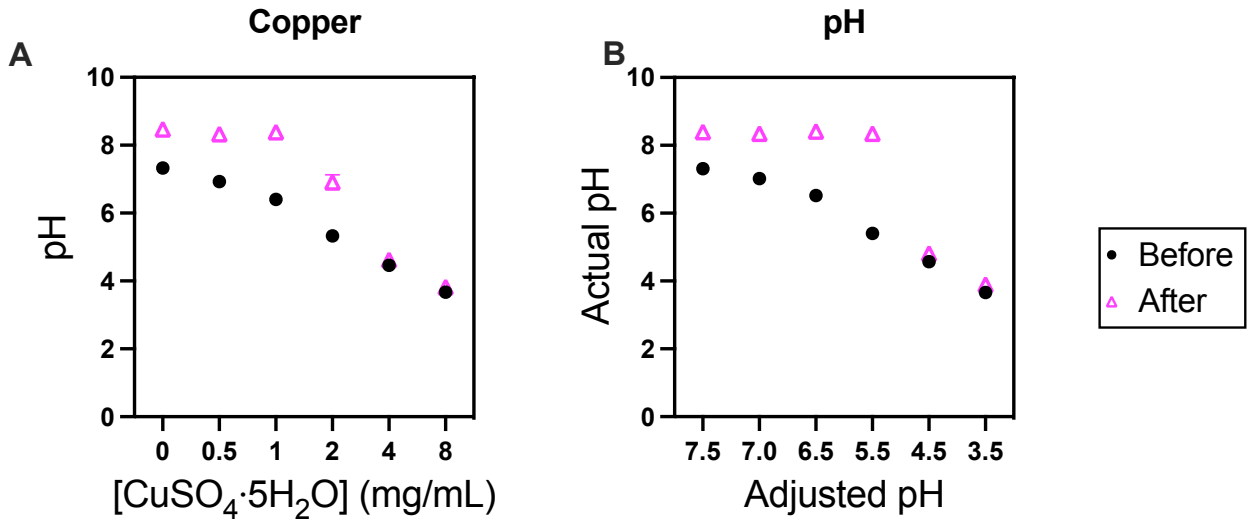

Figure 8.5 Media pH before and after growth of *P. aeruginosa* PAO1 in copper supplemented or pH-adjusted growth media. Results show the pH of MHB amended with 0-8 mg/mL (0-32 mM) $CuSO_4 \bullet 5H_2O$ (A) and pH adjusted MHB (B) before (black circles) and after (pink triangles) 18 h growth of *P. aeruginosa* PAO1. Growth data represents the average of three biological replicates. Error bars on each plotted data point indicate standard deviation.

from 8.3 to 7.0 (Figure 4.5 A). Overall, copper salt addition to *P. aeruginosa* PAO1 above 2 mg/mL $CuSO_4 \bullet 5H_2O$ resulted in more acidified media after bacterial growth, similar to pH adjusted media that matched acidification due to added $CuSO_4 \bullet 5H_2O$.

4.2.3.2 Planktonic copper and acidic pH susceptibility testing

To determine growth inhibiting $CuSO_4 \bullet 5H_2O$ values, copper susceptibility testing using broth microdilution assays were performed. As previously reported in Chapter 1, there is no standardized method of copper susceptibility testing, therefore, any reported copper susceptibility value/ MIC for a copper salt can be significantly impacted by media type, copper concentrations ranges tested, and copper exposure time, and varies greatly between studies (Table 1.1). We chose to use the most common broth microdilution AST method to measure copper susceptibility/ tolerance concentrations, where increasing concentrations of an antimicrobial ($CuSO_4 \bullet 5H_2O$) are added to growth media prior to inoculation (55). After a defined exposure period (18 h), we determined the lowest concentration of copper salt that inhibited growth by comparing the OD_{600nm} of the positive control (no antimicrobial added) to each antimicrobial condition. We chose to perform all copper susceptibility experiments with nine strains of *P. aeruginosa*, including previously reported copper tolerant/susceptible strains PA-8 (42) and BES-4 (44) and their respective GI-7 deletion mutants (PA-8 ΔGI-7 (42); BES-4 ΔGI-7 (44)), after exposure and growth at 0-8 mg/mL (0-32 mM) $CuSO_4 \bullet 5H_2O$ in MHB. In addition to copper susceptibility testing, how pH acidification caused by $CuSO_4 \bullet 5H_2O$ addition to growth media was also experimentally measured to determine growth inhibition of *P. aeruginosa.* To account for pH effects in MHB, no copper salt was added, and instead, these experiments were repeated in pH adjusted MHB that matched the pH of each copper salt concentration added to the first experiment above. The results of planktonic growth copper susceptibility and pH testing are presented in Figure 4.6.

Based on the results from Figure 4.6, similar planktonic growth susceptibility trends were seen across all nine *P. aeruginosa* reference strains tested with increasing copper exposure (Figure 4.6). Each strain showed a significant reduction in growth as determined by the OD_{600nm} values at and below 2 mg/mL $CuSO_4 \bullet 5H_2O$ concentrations when compared to the positive control

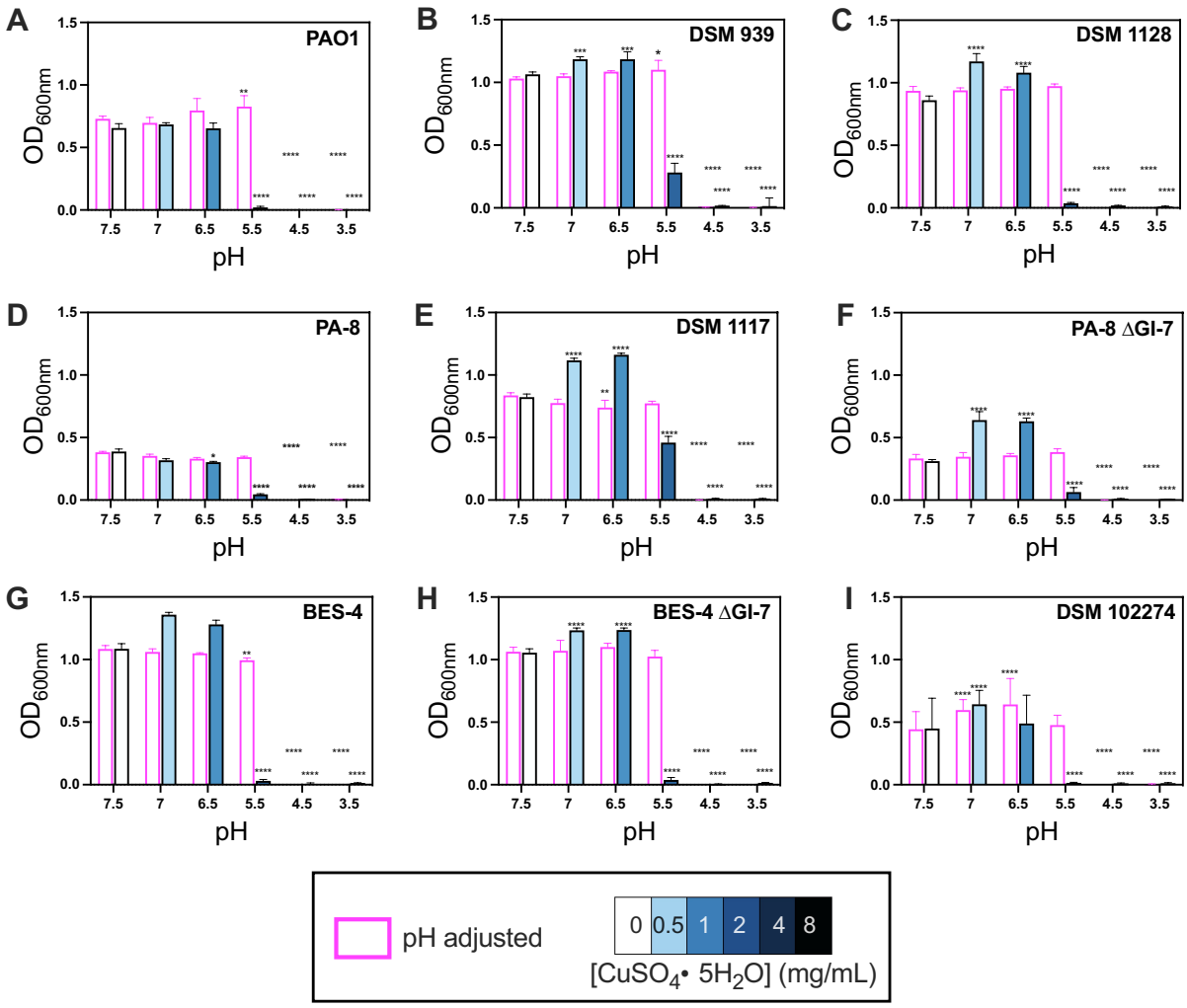

Figure 8.6 Endpoint planktonic growth assays of *P. aeruginosa* reference isolates when media was adjusted by acidification or by the addition of $CuSO_4 \bullet 5H_2O$. In all panels, the average OD_{600nm} was measured for *P. aeruginosa* isolates grown planktonically for 18 h in MHB amended with 0-8 mg/mL $CuSO_4 \bullet 5H_2O$ (refer to in panel legend) or adjusted to pH 3.5-7.5 (+/- 0.2; shown as pink unfilled bars). Data shown in each panel are representative of 5-6 biological replicates. Statistically significant differences between positive controls (0 mg/mL $CuSO_4 \bullet 5H_2O$ or pH 7.5) and copper or pH exposed cultures were determined using a 2-way ANOVA with Dunnett's multiple comparisons test. P-values from this statistical analysis are indicated by asterisks (*=0.0332-0.05;**= 0.0021-0.0331;***= 0.0002-0.0020;****=<0.0001); bars with no asterisks indicate that no statistically significant difference between the positive control and the experimental value was noted (p>0.05).

(0 mg/mL $CuSO_4 \bullet 5H_2O$; Figure 4.6). However, in MHB adjusted to the same pH as the inhibitory $CuSO_4 \bullet 5H_2O$ concentration, there was no significant difference in OD_{600nm} as compared to the MHB for these particular *P. aeruginosa* strains, or it may reflect different growth properties/ physiology or secreted products from these cultures. Additionally, these strains were particularly viscous and more prone to biofilm formation within planktonic culture than the other strains, so the lower OD_{600nm} values may be due to the inability to accurately measure the $OD_{600\ nm}$ of their planktonic cultures.

As media acidified to pH 4.5 and lower, all *P. aeruginosa* strains we tested had significantly decreased growth from their pH 7.5 growth controls based on their OD_{600nm} values (Figure 4.6). Similar results were noted for all strains grown at $CuSO_4 \bullet 5H_2O$ values of 4 mg/mL or higher, likely due to a combination of $CuSO_4 \bullet 5H_2O$ antimicrobial effects and the acidic environment at pH 4.5 and 3.5 (Figure 4.6). These results were expected, as it has been previously reported that the ideal pH range for *P. aeruginosa* growth is 4.5-9.5 (94). In conclusion, all *P. aeruginosa* strains we tested with and without GI-7 were inhibited by $CuSO_4 \bullet 5H_2O$ concentrations of 2 mg/mL. This indicates that antimicrobial-based copper susceptibility testing methods for discriminating copper toxicity could not detect differences in copper tolerance, even among so-called copper tolerant reference strains (PA-8 and BES-4) or copper susceptible strains.

4.2.3.3 Biofilm formation inhibition by copper and acidic pH

To determine if $CuSO_4 \bullet 5H_2O$ and pH influence *P. aeruginosa* growth as a biofilm, we also tested the biofilm formation inhibition in media supplemented with $CuSO_4 \bullet 5H_2O$ and adjusted to acidic pH values. Three *P. aeruginosa* strains (PAO1, PA-8, and PA-8 ΔGI-7) were initially prepared using the same planktonic copper susceptibility testing conditions or pH adjusted growth media as described above. We chose to focus on these three strains due to time constraints and the increased cost of biofilm experiments. All biofilm inhibition experiments were performed in microplates where a standard, sterile MBEC biofilm pegged lid was inserted into the plate wells to provide a surface for biofilm growth. After 24 h of exposure to copper or acidic pH, the biofilms that formed on pegs were CV stained, where the CV stain absorption was measured at A_{550nm} and converted to the molar concentration of CV. CV staining acts as a method

to measure and compare biofilm biomass formation between isolates, as it stains both bacterial cells and secreted components of the EPS matrix (71).

The overall findings from these biofilm formation inhibition assays of *P. aeruginosa* strains PAO1, PA-8, and PA-8 ΔGI-7 grown as biofilm for 24 h showed that biofilm biomass formation occurred up to final $CuSO_4 \bullet 5H_2O$ concentrations of 2 mg/mL and pH adjusted MHB values of pH 5.5 (Figure 4.7). A significant difference in comparison to the positive controls (0 mg/mL $CuSO_4 \bullet 5H_2O$) of the molar concentration of CV was identified at 4 mg/mL $CuSO_4 \bullet 5H_2O$, which corresponds to a $CuSO_4 \bullet 5H_2O$-induced pH of 4.5 (Figure 4.7). These results indicate that biofilm resulted in a 2-fold increase in copper tolerance as compared to planktonic *P. aeruginosa.*

Examination of biofilm biomass formation in increasingly acidic pH adjusted media revealed that PAO1 biomass showed no significant differences as compared to the positive control in MHB adjusted to pH 4.5 (Figure 4.7 A). However, PA-8 and PA-8 ΔGI-7 were significantly different than the control at this pH and showed limited CV staining and were therefore inhibited at the same pH as the inhibitory $CuSO_4 \bullet 5H_2O$ concentration (4 mg/mL) (Figure 4.7 B-C). These results indicate that biofilm biomass increases PAO1 tolerance to acidic 4.5 pH in comparison to when it is grown planktonically, but that PA-8 and PA-8 ΔGI-7 biofilms do not have increased tolerance to acid in comparison to planktonically grown cells (Figures 4.6-4.7). These results are interesting as increased acid tolerance in biofilms as compared to planktonic cells has been reported in the literature in *P. aeruginosa* (102).

Hence, the main findings from biofilm formation inhibition assays of PAO1, PA-8 and PA-8 ΔGI-7 examination show significant differences in CV-stained biomass formation as compared to the positive control conditions at values of 4 mg/mL $CuSO_4 \bullet 5H_2O$ for all strains, and for PA-8 and PA-8 ΔGI-7, inhibition was seen at the corresponding pH value of 4.5 in pH adjusted MHB. This experiment once again underscores the importance of these $CuSO_4 \bullet 5H_2O$ and pH values in distinguishing pH from copper-mediated growth inhibition effects by the tested *Pseudomonas* strains. Additional differences were noted for some strains, such as PAO1 which was the only strain to have increased biomass similar to pH 7.5 at acidic pH 4.5, suggesting this strain has greater acid tolerance than PA-8 or PA-8 Δ GI-7.

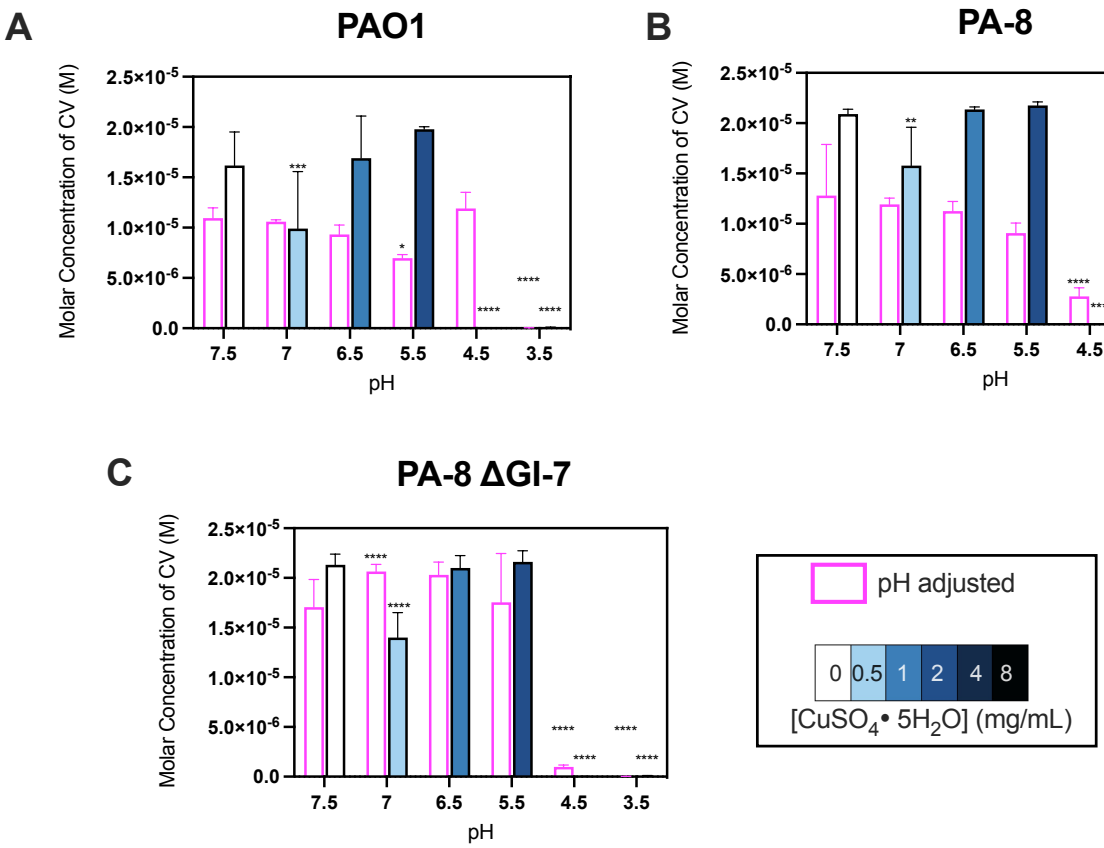

Figure 8.7 Biofilm inhibition by $CuSO_4 \bullet 5H_2O$ or acidic pH. The average molar concentration of CV (M) of *P. aeruginosa* strains grown as biofilms for 18 h in MHB amended with 0-8 mg/mL $CuSO_4 \bullet 5H_2O$ or adjusted to pH 3.5-7.5 (+/- 0.2). Data is representative of 4 biological replicates. Significant differences between positive controls (0 mg/mL $CuSO_4 \bullet 5H_2O$ or pH 7.5) and copper or pH exposed cultures within each strain were determined by 2-way ANOVA with Dunnett's multiple comparisons test. P-values from this statistical analysis are indicated by asterisks (*=0.0332-0.5;**= 0.0021-0.0331;***= 0.0002-0.0020;****=<0.0001); bars with no asterisks indicate that no statistically significant difference between the positive control and the experimental value was noted (p>0.05).

4.2.4 P. aeruginosa planktonic copper susceptibility testing in water to mimic sink drain conditions

The final experiment to distinguish the antimicrobial effects of copper from pH was by performing an experiment to alternatively test bacterial low-level copper tolerance by prolonged copper exposure as a technique adapted from Jeanvoine *et al*. 2019 (42). In this adapted experiment, we exposed standardized mid-log cultures of three *P. aeruginosa* strains (PAO1, PA-8, and PA-8 ΔGI-7) to 600 µg/L (0.0006 mg/mL) $CuSO_4 \bullet 5H_2O$ in water for 24 h at 25 °C in order to mimic sink drain conditions. The concentration of $CuSO_4 \bullet 5H_2O$ that was used was based on our preliminary experiments that showed that this concentration of $CuSO_4 \bullet 5H_2O$ was required to detect any significant differences between our selected three *Pseudomonas* strains. While 600 µg/L is higher than the concentration tested in the original Jeanvoine *et al*. 2019 (42) study (150 µg/L $CuSO_4$), our 600 µg/L copper salt concentration remains a reasonable concentration based on Health Canada's current standards for maximum acceptable concentrations of copper in drinking water, which is 2000 µg/L (129). Additionally, 600 µg/L is 833 times lower than the lowest concentration of $CuSO_4 \bullet 5H_2O$ used in our broth microdilution copper susceptibility experiments (0.5 mg/mL), indicating that the change in pH caused by the addition of $CuSO_4 \bullet 5H_2O$ to aqueous solution is essentially unmeasurable. As an added positive experimental control condition (not included in the Jeanvoine *et al*. 2019 (42) study), cultures were incubated in water without copper supplementation for the same time period to determine bacterial viability in water only. The addition of this control experiment allowed us to confirm that changes in copper survival were not simply due to the lack of nutrients in distilled water used in the experiment. A summary of the CFU/mL findings and statistical analysis from this experiment involving each of the three strains in the pre-incubation, control, and copper-exposed conditions is shown in Figure 4.8, Table 4.4, and Table 4.5.

Significant differences in CFU/mL values of PAO1 and PA-8 ΔGI-7 pre-incubation and control conditions based on Student's t-test were identified (Table 4.4), the reasoning for which will be discussed further in section 4.3.4. *P. aeruginosa* incubation for 24 h in water containing 600 µg/L $CuSO_4 \bullet 5H_2O$ was sufficient to completely kill PAO1 based on CFU/mL values as no viable cells were detected following dilution and agar plating of any copper exposed PAO1 cultures (Figure 4.8; Table 4.4). In contrast, both PA-8 and PA-8 ΔGI-7 strains incubated in water

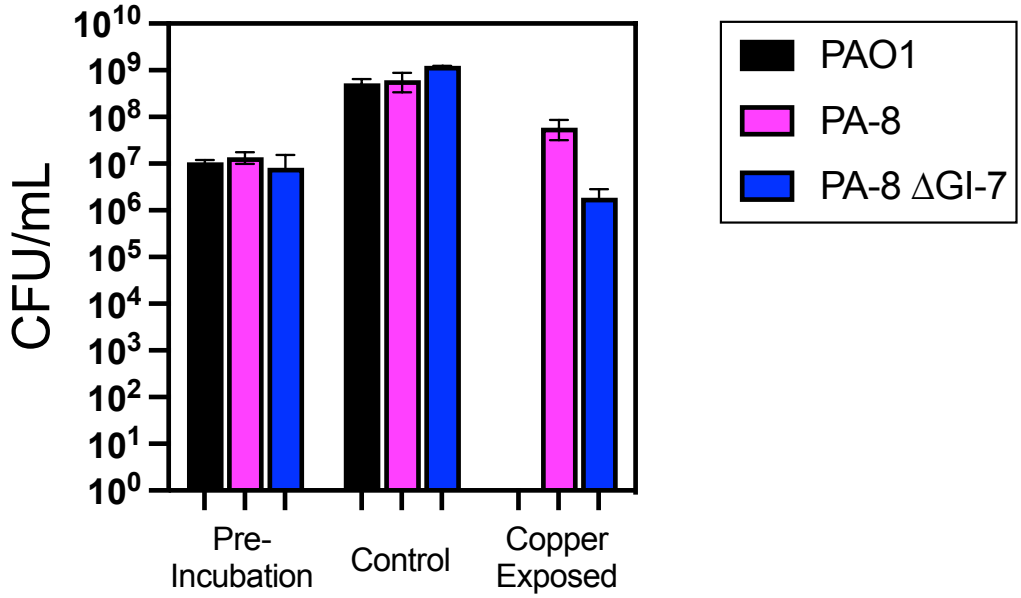

Figure 8.8 *P. aeruginosa* survival assay in copper supplemented water. CFU/mL of three *P. aeruginosa* strains prior to (pre-incubation) or after 24 h incubation at 25 °C in water (control) or water amended with 600 µg/L $CuSO_4 \bullet 5H_2O$ (copper exposed). Results are representative of the mean and standard deviation of three biological replicates for each strain. Statistical analysis of these results is available in Tables 4.4 and 4.5.

Table 8.4 Statistical analysis of *P. aeruginosa* survival assay in copper supplemented water. Statistical analysis was performed by comparing pre-incubation, control, and copper exposed CFU/mL values within each strain using Student's t-test (Panel B); significance ($p<0.05$) is indicated by bolded numbers.

	PAO1			**PA-8**			**PA-8 ΔGI-7**		
	Pre-Incubation	Control	Copper	Pre-Incubation	Control	Copper	Pre-Incubation	Control	Copper
Control	**0.0215**			0.0618			**1.979E-06**		
Copper	**0.0067**	**0.0207**		0.0979	0.0699		0.2705	**4.732E-05**	

Table 8.5 Survival ratios of *P. aeruginosa* PA-8 and PA-8 ΔGI-7 survival assay in copper supplemented water. Survival ratios of PA-8 and PA-8 ΔGI-7 were calculated as a ratio of copper exposed CFU/mL: pre-incubation CFU/mL. A Student's t-test was conducted and determined that there was a significant difference in survival ratios between the two species ($p<0.05$).

	Survival Ratio (Copper Exposed:Pre-Incubation)		
	Bio Rep 1	Bio Rep 2	Bio Rep 3
PA-8	4.3516	2.6055	5.7705
PA-8 ΔGI-7	0.3063	0.1817	0.3719
Student's t-test:	0.0491		

with 600 µg/L $CuSO_4 \bullet 5H_2O$ showed high CFU/mL values with no significant difference as compared to pre-incubation CFU/mL titres (Figure 4.8). This indicates that with and without GI-7, PA-8 can tolerate copper exposure concentrations that would be naturally present and released from sink drain plumbing. Since no statistically significant differences were noted between PA-8 and PA-8 ΔGI-7 CFU/mL in water with added copper as compared to their respective pre-incubation CFU/mL, PA-8 and PA-8 ΔGI-7 likely have additional copper tolerance genes/ factors other than those found in GI-7.

To determine if GI-7 provided any additional survival benefit to PA-8 in the 24 h copper water exposure experiment, a ratio calculation comparing the pre-incubation CFU/mL counts and copper exposed CFU/mL counts for each biological replicate was determined and summarized in Table 4.5. A two-tailed Student's t-test was used to determine if any significant differences were noted between the survival ratios of PA-8 and PA-8 ΔGI-7 (Table 4.5). This calculation accounted for any initial differences in pre-exposure cell counts for each of the strains and revealed a significant difference between the survival ratios ($p<0.05$), where PA-8 had a higher ratio of CFU/mL survival than the PA-8 ΔGI-7. This finding indicated that GI-7 does indeed contribute to a small but detectable proportion of copper tolerance in the PA-8 strain (Table 4.5).

Therefore, the low concentration copper water exposure method modified from Jeanvoine *et al*. 2019 (42) circumvents the copper medium acidification caused by high concentrations of copper addition to aqueous solutions to reveal copper susceptible and tolerant strains (i.e., PAO1 versus PA-8) (Figure 4.8; Tables 4.4-4.5). It also revealed that the loss of the GI-7 genomic island had only modest impacts on copper tolerance, suggesting PA-8 may possess additional genes outside of the genomic island that influence copper tolerance phenotypes.

4.3 Discussion

4.3.1 Although Pseudomonas isolates sampled from rooms with copper drains had greater % GI-7 detection values, no statistically significant trends between copper sink drain use and GI-7 presence in Pseudomonas genomes could be determined from the BCIP study.

The results from assessing all BCIP study hospital *Pseudomonas* isolates with and without GI-7 will be discussed first. It has been previously shown that GI-7 possesses many copper tolerance genes (42, 43); Figure 1.3). *Pseudomonas* isolates that possess GI-7 may benefit when

they grow in environments that are more commonly exposed to copper, in comparison to clinical isolates that would not benefit from copper tolerance genes. However, after examining total clinical, air and sink drain isolates from each Ontario hospital, it may come as a surprise that there is not a significantly higher proportion of GI-7 in the environmental isolates as compared to clinical isolates, i.e., rates of GI-7 detection were similar among isolates across all sampling locations (Table 4.1). However, based on past knowledge that sink drains act as bacterial reservoirs within hospitals, and sinks often lead to nosocomial outbreaks (27), it is possible that the Ontario hospital isolates that acquired GI-7 in the sink drain spread to patients, explaining the lack of significance in GI-7 detection between clinical and environmental (air and sink drain) isolates. Current studies of GI-7 presence have focused on single isolates, such as PA-8 (42), BES-4 (44), DHS01 (43), and PA834 (130), with comparisons to previously sequenced *P. aeruginosa* genomes available online to determine if GI-7 was present in these published genomes. Thus, our study is the first to look at GI-7 presence across a large group of isolates collected in a controlled study from similar environments and with known copper sink drain exposure. We are therefore unable to determine if the proportions of GI-7 present in clinical and environmental isolates are comparable to other areas of the world. However, PA834 (130) and DHS01 (43) were collected from patients while PA-8 (42) and BES-4 (44) are copper sink drain isolates, indicating that GI-7 can be present in both clinical and environmental isolates. Additionally, the spread of *P. aeruginosa* isolates harbouring GI-7 from sink drain to patients was determined with BES-4, as this isolate was the cause of a 12-year nosocomial outbreak and was believed to have colonized the hospital drainage system before its opening (44). However, given that this is a single case, continued work must be done to determine if the spread of isolates carrying GI-7 from sink drain environments to patients is a common phenomenon.

Another finding of interest was the lack of negative correlations between % GI-7 detection in regular and copper air isolates or regular and copper drain isolates (Table 4.2). As previously discussed, because GI-7 harbours copper tolerance genes, we would expect that environmental isolates from copper sink drain rooms would select for and have increased detection of GI-7 as compared to isolates from regular sink drain rooms, leading to a negative correlation. Unfortunately, none of the previously mentioned studies compared GI-7 presence in isolates

from copper and regular sink drains, and thus we are unable to determine if this finding aligns with the literature. However, we can first speculate that this finding may have been due to limited *Pseudomonas* isolates from copper sink drain rooms. If copper sink drains were under sampled, this may not paint a clear image of GI-7 presence within the copper sink drain rooms. Thus, future studies with increased sampling may be required to determine whether this hypothesis is true. However, this limited number of samples may also be evidence that copper sink drains are effective at reducing *Pseudomonas* in sink drains if the regular and copper sink drains were sampled the same amount.

When we compared the most abundant MLST identified from BCIP *Pseudomonas* isolates and the % GI-7 detection in sequence isolate genomes, none of the dominant BCIP MLSTs matched ST308 or ST395 (Figure 4.3, Table 4.3), which were the MLSTs of PA-8 and BES-4, two high-risk clones isolated from copper sink drains in France that contain GI-7 (42, 44). The most abundant MLSTs identified from the BCIP study isolates (irrespective of GI-7 presence or absence) were ST253, ST175, ST381, and novel MLST(s) (Figure 4.3, Table 4.3). *P. aeruginosa* ST253 is a common isolate in the Northern hemisphere and was one of the most frequently identified MLSTs among individuals with cystic fibrosis or chronic obstructive pulmonary disease based on a study of 2,882 *P. aeruginosa* isolates collected worldwide between 1980-2020 (131). It was also shown that ST253 chronically colonizes the lungs and airways of patients carrying this *P. aeruginosa* MLST for periods spanning decades (131). The ST253 carries at least two genomic islands that contain multiple antibiotic resistance genes (132), indicating that this is a highly concerning MLST from a clinical perspective. *P. aeruginosa* ST175 is another high-risk international clone frequently classified as being extensively drug resistant (XDR) and has been identified in multiple European countries (133, 134). In a study of *P. aeruginosa* isolates from environmental, animal, and clinical cystic fibrosis and non-cystic fibrosis sources collected between 2006-2009, ST381 was identified in all settings, indicating that this is a diverse MLST capable of surviving in both the environment and in patients (135). Finally, while characterization of the isolates with novel MLSTs has not been conducted, these isolates may harbour unique AMR genes that provide benefit in the environment and/or patients.

Next, we looked at isolates based on the most abundant MLST present in the air, drain, and clinical samples of each hospital (Figure 4.3). We know that sink drains are a reservoir for nosocomial infections, and thus we hypothesized that if *Pseudomonas* is spreading from sink drains to patients, we would see isolates with the same most abundant MLST across air, drain, and clinical sampling sites within the same hospital. When comparing the most abundant MLSTs in environmental air and drain isolates, we saw the same most abundant MLST in all air and sink isolates from the same hospital, except for in Hospital D (Figure 4.3). These results are understandable given that air samples were taken adjacent to the sink in each hospital room. It has been shown that bacteria within a sink drain can disperse up to 30 inches from the drain, so an air sample taken adjacent to the sink may capture the same *Pseudomonas* MLSTs as those that were collected directly from the sink drain (136).

However, in all cases, we saw different most abundant MLSTs between environmental and clinical isolates from the same hospital (Figure 4.3). This finding can be justified because we know that sink drains are not the only source of *Pseudomonas* within a hospital. *Pseudomonas* species are known for their ability to survive in unconventional environments, and within a healthcare setting can be carried by healthcare workers or can spread via touch surfaces and contaminated medical equipment (137). Although we know sink drains to be a source of nosocomial infections, current literature has not determined the proportion of infections that are attributed to sink drains in comparison to other sources (138), so it is important to not rule out other areas of *Pseudomonas* colonization as contributors to the most abundant MLST in clinical samples. Additionally, clinical samples were collected as routine surveillance via rectal swab from patients being admitted to, or already patients at, each of the participating hospitals. Because some of the clinical samples represent isolates acquired in the community before hospital admission, they do not only represent the *Pseudomonas* strains and MLSTs that may have been acquired in the hospital and thus may not align with the most abundant MLSTs present in the sink drain.

With respect to the BCIP study findings related to dominant MLST and % GI-7 detection (Table 4.3), a few trends were noted. Interestingly, none of the clinical, air, or environmental ST175 isolates had detectable GI-7 (Table 4.3), despite it being classified as a high-risk

international clone (133) which harbours at least two antibiotic resistance genomic islands (132). However, in the BCIP study, GI-7 was identified in the abundant MLSTs ST253, ST309, and ST381, with 50-100% of all isolates from each isolate grouping by hospital and isolation location having detectable GI-7 (Table 4.3). Jeanvoine *et al.* 2019 (42) reported that a BLAST search of the NCBI database revealed the presence of detectable GI-7 sequences in isolate genomes of ST253 and ST309, which were abundant and contained GI-7 in our samples (42). This indicates that GI-7 may be widespread in these MLSTs. ST309 has also been reported to be XDR and is a concerning emerging pathogen in the United States because it harbours GES-19 and GES-26, two extended spectrum β-lactamases (139). Thus, these clinically concerning MLSTs may be of even greater concern due to the presence of GI-7, which may allow isolates of these MLSTs to persist in copper sink drains or on copper-coated surfaces within the hospital and continue causing nosocomial XDR outbreaks.

4.3.2 The influence of increasing copper salt acidification on final pH measurements before and after P. aeruginosa PAO1 growth

As shown in Figure 4.5, the alteration of growth media pH with PAO1 growth to values of 8.3-8.4 at low copper salt addition (0-1 mg/mL) and its corresponding pH values (7.5-6.5) is understandable, given that the *P. aeruginosa* optimal pH range for growth is 4.5-9.5 (94). As values reached higher $CuSO_4 \bullet 5H_2O$ ($\geq$4 mg/mL) or descended below adjusted pH values of 6.5, the 'after' *P. aeruginosa* PAO1 growth pH values showed no differences from the before pH values measured (Figure 4.5). Although we did not test basic pH ranges (values above pH 7.5) and values above 8.3 pH were not reached by PAO1 after its growth, it is clear that the PAO1 strain alters the final growth medium to a more basic pH in either copper salt adjusted media at low concentrations of $CuSO_4 \bullet 5H_2O$ (0.5-1 mg/mL) or in more neutral- to weakly acid adjusted media at values of 7.5-6.5. This likely reflects secreted compounds by the organism created during its growth in these media to maintain homeostasis under acidic-neutral growth conditions that enhance basic pH values. These basic compounds may reflect by-products or secondary metabolites naturally secreted as part of their inherent growth and nutrient utilization in MHB. Mechanisms of maintaining pH homeostasis during neutral to acidic growth conditions are paramount to the survival of *P. aeruginosa* during infection as pH fluctuations can be

encountered throughout the body, including at the pancreatic duct in the gastrointestinal tract (140). Future studies may explore changes in pH throughout growth in a time course experiment as was done by Sánchez-Clemente *et al*. 2018 (141), who looked at changes in pH during the growth of environmental species *E. coli*, *Pseudomonas putida*, and *Pseudomonas pseudoalcaligenes* in LB and M63 minimal media with the addition of various carbon sources and found that different phases of growth in the different media types were more acidic or basic than others.

4.3.3 Copper and pH planktonic and biofilm growth testing of various P. aeruginosa strains identified key differences in growth at 2 mg/mL $CuSO_4$•$5H_2O$ and pH 5.5 values.

A comparison of copper salt and pH adjusted susceptibility testing revealed that 2 mg/mL $CuSO_4$•$5H_2O$ and adjusted pH 5.5 were useful concentration and pH values to discern the growth inhibitory effects of copper versus pH on *P. aeruginosa,* as all strains were inhibited by this copper concentration but not by its matched pH adjusted media (Figure 4.6). This was unexpected since PA-8 and BES-4 have both been reported to be copper tolerant due to the presence of genomic island GI-7 and were also shown to be copper tolerant phenotypically using a method of copper exposure in water (42, 44). In addition to inhibition by 2 mg/mL $CuSO_4$•$5H_2O$, all strains were significantly inhibited by acidic pH values of 4.5 and 3.5 (Figure 4.6) Thus, it is evident that both pH and copper contribute to bacterial inhibition, but copper may have additional antimicrobial impacts due to it causing inhibition at 2 mg/mL $CuSO_4$•$5H_2O$ while its pH-matched counterpart did not. Given that *P. aeruginosa* was inhibited at a pH that growth media can reach with copper concentrations commonly tested during copper susceptibility testing, we can speculate that acidic pH may have contributed to the inhibition seen in other studies of copper tolerance as well. For example, Benhalima *et al*. 2019 (64) performed copper susceptibility testing of *P. aeruginosa* clinical isolates from Algerian patients with $CuSO_4$•$5H_2O$ concentrations of 0.125-1.6 mg/mL; the maximum tested value of 1.6 mg/mL $CuSO_4$•$5H_2O$ approaches the inhibitory copper concentration we observed in our experiments and matching pH.

Results from biofilm biomass formation measurements of PAO1, PA-8 and PA-8 ΔGI-7 after 24 h $CuSO_4$•$5H_2O$ exposure showed that all strains tolerated 2-fold higher concentrations of $CuSO_4$•$5H_2O$ (4 mg/mL) as compared to planktonic cultures. This 2-fold increase in copper

tolerance by PAO1, PA-8, and PA-8 ΔGI-7 biofilm biomass at 2mg/mL $CuSO_4 \bullet 5H_2O$ versus their respective planktonic growth values makes sense based on past studies. As discussed in Chapter 1, biofilm growth can enhance resistance and tolerance to antimicrobials, including copper (66, 87). It has been shown that when grown as a biofilm, the tolerance of *P. aeruginosa* to copper can increase up to 600-fold (66, 87).

Additionally, while not significant, there appeared to be higher biofilm biomass formation in all three strains when grown with increasing amounts of $CuSO_4 \bullet 5H_2O$ (1-2 mg/mL). These findings may be influenced by a variety of explanations. Firstly, biofilm formation is often variable, meaning that certain biological replicates may have formed more biofilm than others. When grown on orbital shakers, it is possible that some species will not consistently form the same amount of biofilm on each peg (67). This also provides some explanation for the larger size of some error bars seen in these experiments, as well as the significant decrease in biofilms formed by PAO1 and PA-8 strains seen at 0.5 mg/mL $CuSO_4 \bullet 5H_2O$ which becomes non-significant at 1 mg/mL for all three strains (Figure 4.7A-C). Given that plates were set up in the same format for each strain, it is also possible that the wells used for the 0.5 mg/mL $CuSO_4 \bullet 5H_2O$ concentration did not have sufficient airflow and resulted in asymmetric biofilm growth as compared to the other pegs (67). While four biological replicates of each strain were used for this experiment, results may be improved with less error by increasing the number of biological and/or technical replicates in future experiments.

Secondly, enhanced biofilm biomass formation by PAO1 and PA-8 in the presence of copper may be due to the subinhibitory concentrations of the copper antimicrobial added. Subinhibitory concentrations of an antimicrobial have been shown to enhance biofilm formation, as has been demonstrated for *P. aeruginosa* when exposed to aminoglycosides (142). Various stress response pathways can be induced in bacteria when exposed to subinhibitory concentrations of an antimicrobial, causing cells to upregulate mechanisms that provide protection against antimicrobials (143). As previously discussed in Chapter 1, biofilm formation is a major AMR growth physiology used by *P. aeruginosa*, so exposure to subinhibitory concentrations of copper may trigger the production of higher biofilm biomass as part of the stress response.

Lastly, the increased CV molar concentrations recorded for PA-8 and PAO1 strain biofilm biomass at higher copper concentrations versus pH adjusted MHB medium may be due to interactions between copper and CV dye used for biomass staining. When grown in the presence of copper, previous studies have reported that biofilms can accumulate layers of copper within the biofilm, as was determined in *P. putida* CZ1 through transmission electron microscopy in combination with X-ray techniques (144). Additionally, copper coordination polymers have been identified and shown to adsorb CV, which may effectively remove CV from aqueous solution (145). Thus, copper precipitates in the biofilm biomass itself may adsorb extra CV during biofilm staining, allowing the biofilm to acquire more stain. This would make the apparent molar concentration of CV, and consequently, the amount of biofilm, appear greater than it actually is, and this effect may increase with increasing, subinhibitory copper concentrations.

The findings from pH-adjusted biofilm biomass formation showed that PAO1 had no significant difference in biomass as compared to the control (pH 7.5) in MHB adjusted to pH 5.5, while PA-8 and PA-8 ΔGI-7 were inhibited at this pH (Figure 4.7). Previous studies of *P. aeruginosa* have shown that increased acid tolerance occurs in bacterial biofilms when compared to their growth planktonic cells including (102), and while this was true for PAO1, PA-8 and PA-8 ΔG-7 were inhibited at the same pH as their matched planktonic cultures. Given that PA-8 is a copper sink drain isolate, we would expect that the harsh environmental conditions experienced in the sink drain due to exposure to copper and other antimicrobials that are flushed down the sink would lead to the activation/ repression of a diverse set of copper tolerance genes, including those for mechanisms required for acid tolerance. However, as described by Wang *et al*. 2020 (146) in their study of lactic acid tolerance of *E. coli* O157:H7, exposure to pH 4.0 had a similar effect on both planktonic and biofilm cells, indicating that biofilms are not always more tolerant to antimicrobials such as acid.

4.3.4 Pseudomonas exposure to physiologically relevant copper concentrations in water that mimic sink drain conditions highlights the copper tolerance of the PA-8 reference strain

The experimental findings determined from the modified Jeanvoine *et al*. 2019 (42) experiment of 24 h exposure to low levels of copper in water revealed important differences in final CFU/mL values of pre-incubated cells, control cells, and most importantly, cells exposed to

600 µg/L of $CuSO_4 \bullet 5H_2O$ (Figure 4.8; Table 4.4-4.5). This experiment avoids the use of high copper concentrations that would be required in standard AST assays which acidify media and instead exposes bacteria in water to more relevant concentrations of copper that would be present and leach from a copper sink drainpipe. This experiment demonstrated a noticeable difference in copper tolerance by the three *P. aeruginosa* strains tested, where PAO1 was killed after copper exposure of mid-log cultures in water after 24 h, while PA-8 and PA-8 ΔGI-7 showed no significant reduction in viable cells (Figure 4.8; Table 4.4). The findings from this study had two interesting observations.

First, significant differences between CFU/mL values within each strain's dataset were noted for pre-incubation and control samples of PAO1 and PA-8 ΔGI-7. Initially, this finding was interesting given that the water control condition is incubated in for 24 h without any nutrient sources, so changes in CFU/mL were not expected. However, *P. aeruginosa* is known for its ability to survive in unconventional conditions due to its ability to utilize diverse carbon energy sources and this species has been shown to remain viable and even grow in distilled water within a hospital (147). Additional reasons for the significant increase of cells within the preincubation and control conditions may be that mid-log cells continued to divide during centrifugation despite using cold isolation conditions to slow cell growth. These values may also be influenced by residual media carryover into distilled water after centrifugation which provided sufficient nutrients for additional growth in distilled water.

Secondly, although 24 h copper exposed PA-8 and PA-8 ΔGI-7 strains both showed no significant difference in CFU/mL values as compared to their pre-incubation condition CFU/mL counts, PA-8 still had greater survival in the copper solution as compared to PA-8 ΔGI-7 (Table 4.5). We determined this by calculating a survival ratio of copper exposed: pre-incubation CFU/mL counts for each strain's biological replicates, and then conducting a Student's t-test to compare the survival ratios generated for PA-8 and PA-8 ΔGI-7 (Table 4.5). Our results showed that the survival ratios for these two strains were significantly different ($p<0.05$), indicating that PA-8 showed greater survival after 24 h exposure to copper in water as compared to PA-8 GI-7 (Table 4.5). Based on this experiment it seems clear that the loss of the GI-7 genomic island from PA-8 ΔGI-7 had some effect on copper tolerance in PA-8 ΔGI-7, but that this genomic island is not

the only factor that contributes to copper tolerance in PA-8. There are likely explanations for this at the genetic level. The presence of additional copper tolerance genes in PA-8 and PA-8 ΔGI-7 may include those involved with biofilm formation, as it is known and we have shown that biofilms increase tolerance to copper (23, 66) (Figure 4.7). Additional copper transporter systems absent from the GI-7 genomic island but encoded chromosomally in *Pseudomonas* spp. include the *cusCFBA* copper efflux system (57) or the plasmid-borne operon *pco,* which utilizes both soluble proteins and copper pumps as copper tolerance mechanisms (59). Thus, it is possible that these copper tolerance systems outside of GI-7, and potentially copper tolerance systems that have yet to be discovered, are involved in the copper tolerance of PA-8.

While this low concentration copper water exposure method used by us and Jeanvoine *et al*. 2019 (42) was effective in identifying differences in copper tolerance between *P. aeruginosa* reference strains tested herein, and effectively eliminates the issue of media acidification by copper salts, it does have some caveats that limit its use as a high-throughput assay. First, this method was not suitable for high-throughput copper tolerance screening of the 2467 BCIP isolates, given the resources and time required to complete it. To properly count CFU/mL, multiple biological and technical replicates were required, as well as a wide range of bacterial dilutions to ensure that plates with a countable number of colonies were obtained. In the case of the BCIP study *Pseudomonas* isolate dataset, it would require tens of thousands of agar plates and months of work for each batch of isolates to be tested. It would also require a defined control strain representing well-defined copper tolerant and copper susceptible reference strains for comparison. Our attempts to complete more rapid viable cell counting methods to avoid multiple agar spread plates involved spotting 5 µL culture dilutions onto agar and counting colonies to determine CFU/mL using an ImageJ plugin (148). Unfortunately, we found this spot plate method to be just as time consuming as standard viable cell counting methods. Additionally, using fluorescent dye-based cell viability assays such as SYTO9/propidium iodide (PI) (LIVE/DEAD™) staining are not suitable or reliable with *P. aeruginosa*. Both SYTO9-PI staining kit used with *P. aeruginosa* has shown an 18-fold stronger fluorescent signal from dead cells as compared to live cells (149). This is due to the thick cell walls of live *P. aeruginosa,* and its lower permeability to many compounds, including dyes, which prevents the penetration of live cell stain SYTO9 in

comparison to the dead cell PI stain (149). Additionally, *P. aeruginosa* has high background intrinsic fluorescence due to the presence of many pigments that give this species its characteristic red colour and name, such as Aeruginosin A (150). The natural fluorescence of *P. aeruginosa* can also be impacted by the production of the siderophore pyoverdine (151) which may obscure and overlap with fluorescent dye emissions from SYTO9/PI and resazurin.

Metabolic assays (e.g., BacTiter Glo™, resazurin) were also explored as part of this thesis work to determine copper susceptibility in the water exposure method modified by Jeanvoine *et al*. 2019 (42). Unfortunately, we could not get these assays to reliably detect cell viability, likely because of the cell envelope impermeability of *P. aeruginosa*, but also because cells are measured in a medium with low added nutrients. Since this low concentration copper exposure assay is conducted in distilled water only, cells are less metabolically active, and therefore, below reliable threshold limits to be comparable to cells grown in a rich medium such as MHB or LB as are typically used in metabolic assays (152).

Finally, the low concentration copper water exposure method of Jeanvoine *et al*. 2019 (42) does not accommodate accurate testing of biofilm tolerance, which was the original goal of the BCIP study's isolates for copper tolerance testing. Initially, we sought to complete the same copper exposure experiment in water with pre-established 24 h biofilms. To determine cell viability after copper exposure, sonication would be used to dislodge biofilms from pegged lids into recovery media so that the cells could be diluted and used to determine CFU/mL (67). However, we found that both PA-8 and PA-8 ΔGI-7 formed thick biofilms that were impossible to completely dislodge from the pegs, even after long periods of sonication. If there were minimal dislodging of highly adherent biofilms from the pegs, it would result in lower CFU/mL counts and these isolates would appear less viable than others. Differences in the amount of biofilm formation and biomass adherence to pegs are likely and expected in such a large and diverse set of *Pseudomonas* environmental and clinical isolates. Hence, this low concentration copper water exposure method would not be feasible without further experimental optimization or considerable automation for future studies of biofilm copper tolerance with the BCIP isolate collection.

4.4 Concluding Remarks

Exploration of the presence of GI-7 in sink drain isolates showed that GI-7 was widely spread across clinical samples and various environmental sources, including in hospital rooms with regular and copper sink drains. While our results did not show significant differences in GI-7 presence between different isolation locations as we hypothesized, further sampling of copper sink drains may reveal a heightened presence of GI-7 in this environment.

These results also supported our hypotheses that media acidification by copper salts impacts bacterial growth during copper susceptibility testing and that exposure of *P. aeruginosa* to copper salts in water would be a more accurate method of discerning copper susceptibility between strains. However, further work is required to study the effect of acidification during copper exposure and to better adapt the method of copper exposure in water for high-throughput experiments. Overall, our findings are supportive of the development of more standardized genomic and phenotypic methods of studying copper tolerance.

Chapter 5: Final Summary, Limitations, & Future Directions

5.1 A summary and impact of the findings from Chapter 3

Bacterial AMR is a rapidly progressing concern, where new and improved methods to detect and discern AMR mechanisms of action and resistance associated with alternative antimicrobial compounds such as copper metals are needed. Methods examining how alternative antimicrobials act to prevent and eradicate bacterial biofilm formation, which is a major AMR physiology, is also paramount (153). In this thesis, these facets of AMR were examined using the antimicrobial metal copper, where copper tolerance in *P. aeruginosa* isolates and reference strains was explored. Copper is an antimicrobial metal commonly used as an antimicrobial on hospital touch surfaces (37), and as a plumbing material within sink drains (38), to prevent bacterial colonization and biofilm formation to prevent the spread of nosocomial infections such as multi-drug resistant *Pseudomonas* species. As observed with other antimicrobials over time, concerns are emerging that *P. aeruginosa* can develop tolerance to copper metal and its ions. For these reasons, we must have reliable methods for generating biofilms in the lab that are suitable for high throughput testing of environmental (sink drain) and clinical *Pseudomonas* isolates. Additionally, we must establish robust and accurate methods of testing copper tolerance which allow for the identification of copper tolerance phenotypes and to associate phenotypes to copper tolerance genes/ genomic sequence data. Improved methods are necessary when conducting phenotypic studies of copper tolerance using genome sequenced isolates. Finally, reliable methods that mimic the environmental growth conditions of many nosocomial *Pseudomonas* spp. infections (e.g., sink drains) are also needed and these techniques need to be properly assessed to account for the many confounding factors related to copper metal chemistry, such as its ability to acidify aqueous solutions and its variable solubility and oxidative states when used at higher concentrations.

In Chapter 3 of this thesis, a method of generating and measuring greater static bacterial biofilm biomass, known as the deep well biofilm device, was compared to the standard MBEC biofilm device. By assembling a deep well device using commercially available deep well plates fitted with an inserted semi-skirted PCR plate as a pegged lid, our results showed that *E. coli* and *P. aeruginosa* formed more biofilm on the deep well biofilm device as compared to the standard

device as expected for a device designed to increase biofilm biomass surface area. This analysis also unexpectedly revealed that when comparing biofilm biomass formed per mm^2 by each species, *E. coli* formed more biofilm on the standard device (Figure 3.1, Table 3.1). These results likely reflect a material preference for biofilm growth and/ or adherence to the different plastics used for each device; the standard device is composed of polystyrene in comparison to the deep well device's polypropylene composition. Additionally, our comparison of both devices confirmed that each biofilm device could be used to determine a species' MBEC value as was demonstrated for the disinfectants BZK and bleach but resulted in different MBEC values within each strain when grown on the standard and deep well devices (Figure 3.2). For example, *P. aeruginosa* and *E. coli* had a 2-4 fold lower MBEC value for bleach on the deep well device as compared to the standard device. Additionally, for BZK, *P. aeruginosa* had an 8-fold lower MBEC value on the deep well device as compared to the standard device, while *E. coli* experienced a broad, inconsistent range of MBEC values on both devices. Thus, it is important that future users of the static biofilm deep well and standard methods only compare MBEC results of different strains grown using the same type of device.

Current phenotypic growth methods for culturing biofilms include biofilm reactors for continuous long-term biofilm formation which are limited to testing a few species, to more high-throughput and rapid static standard biofilm devices, useful for AST and determining biofilm formation rates. The need for more high-throughput devices is clear since few diagnostic labs can afford the time or the need to establish the long-term biofilms for routine rapid clinical or environmental diagnostic analyses. Concerning static standard biofilm devices, its practical use is limited by its high costs/ individual device and the amount of biomass that can be formed and tested on the device's pegged lids (1).

The deep well biofilm device designed and tested in this thesis provides an alternative lower cost device and method for biofilm growth and antimicrobial MBEC determination by allowing increased biofilm biomass formation. Additionally, growth on the deep well device's polypropylene pegs is more representative of commonly used medical equipment plastics that biofilms often form on. With this consideration in mind, the deep well device tested in this thesis could have more useful future applications for larger scale studies of biofilm biomass formed by

clinically relevant hospital isolates. This technique may also have additional uses for research experiments requiring greater biomass quantities for downstream molecular analyses (e.g., multi-omics techniques) as discussed in section 5.4 below.

5.2 A summary and impact of the findings from Chapter 4

In Chapter 4, we examined *Pseudomonas* copper tolerance attributed to the genomic island genotypically and phenotypically using a two-aimed approach. Our first aim employed a bioinformatics surveillance approach, where *Pseudomonas* isolates were collected and WGS from an environmentally sampled Ontario Hospital room sink drain study (2017-2019) known as the BCIP study. The detection of GI-7 sequences among the collected and genome sequenced BCIP clinical and environmental *Pseudomonas* samples was conducted to ascertain the extent of GI-7 presence in Canadian ICU isolates. This study also offered a unique opportunity to infer the role of GI-7 in copper tolerance since the BCIP study collected isolates from regular sink drains and sink drains made of copper pipes. While we hypothesized that this copper tolerance conferring GI-7 genomic island would associate more frequently with *Pseudomonas* isolates collected from copper sink drains rather than regular non-copper pipes, insufficient isolate sampling from copper sink drains prevented our use of analytical methods with sufficient statistical power to draw conclusions on GI-7 and its association to copper tolerance and copper sink drains.

Despite sampling limitations, a few interesting main conclusions and associations were observed from the BCIP isolate study regarding the role of GI-7. Firstly, the detection of GI-7 sequences among BCIP clinical and environmentally sampled isolates generally occurred at similar rates (20-30%), but isolates possessing the GI-7 sequence increased in environmental locations where copper sink drains were used based on linear correlation analyses of different sampled isolate genomes (Table 4.1; Table 4.2). To delve deeper into the *Pseudomonas* isolate data using MLST data collected from the BCIP study, an association between GI-7 detection among *Pseudomonas* isolates of particular MLSTs (i.e., ST253, ST309, and ST381) was noted, regardless of their clinical or environmental sampling location within BCIP study hospitals (Table 4.3). This finding suggests that GI-7 acquisition may be biased toward particular MLST types in Canadian hospitals. These three MLSTs identified from this thesis' findings differed from

previously determined MLSTs from international isolates that reportedly encoded a GI-7 sequence, such as ST308 (42) and ST395 (43), which were not identified as the most abundant MLSTs from our various isolation locations. Our findings that ST253, ST309, and ST381 have high rates of GI-7 are important to note since many of these *P. aeruginosa* MLSTs are of global concern due to their known AMR, were dominant MLSTs in Ontario hospitals, and had high proportions of GI-7 detection (50-100%; Table 4.3).

In the second aim of Chapter 4, we examined *P. aeruginosa* phenotypes based on currently used methods of copper tolerance phenotypic testing. This analysis primarily focused on the study of various *P. aeruginosa* reference strains (PAO1, DSM 939, DSM 1117, DSM 1128, and DSM 102274) and copper tolerant strains with (PA-8, BES-4) and without (PA-8 ΔGI-7, BES-4 ΔGI-7) GI-7 sequences to establish optimal methods that can eventually be used in future testing to assess BCIP study isolate copper tolerance. Our findings showed that broth microdilution assays with high concentrations of copper salts at or above 1 mg/mL resulted in aqueous medium acidification that inhibits both planktonic and biofilm growth (Figures 4.4, 4.6, and 4.7). As a result, copper susceptibility testing methods using copper salt concentrations at inhibitory high concentrations (1-8 mg/mL) cannot be used to distinguish between copper tolerant and susceptible strains due to pH acidification. These findings indicate that copper acidification is a confounding factor in typical AST assay formatted copper testing studies. Finally, while copper susceptibility testing of *P. aeruginosa* isolates did reveal some key significant differences in planktonic growth of *P. aeruginosa* isolates at copper salt concentrations of 1 mg/mL and its corresponding pH of 5.5 (Figures 4.6), distinguishing copper tolerant GI-7 possessing strains from copper susceptible strains required the use of low concentration copper water exposure assays modified by the method of Jeanvoine *et al*. 2019 (42). Copper water exposure testing demonstrated clear differences in PAO1 and PA-8 CFU/mL survival after 24 h copper water incubation, where only PA-8 and PA-8 ΔGI-7 survived prolonged low concentration copper salt (0.0006 mg/mL $CuSO_4 \bullet 5H_2O$; Figure 4.8, Table 4.4, and Table 4.5). This copper exposure method in water not only prevented copper-induced media acidification by using low concentrations of copper salt, but this method was also more representative of the sink drain water conditions experienced by BCIP study isolates and other environmental *P. aeruginosa* strains. Unfortunately,

copper water exposure findings from our study have not determined whether this method is feasible for high-throughput testing and more optimization and experimental analyses are required to test the copper susceptibility of BCIP study isolates.

Past analyses of the copper tolerance genomic island GI-7 have been limited to a few studies of select environmental *P. aeruginosa* isolates from France and Brazil. Our study bioinformatically assessed sequenced genomes of 2467 *Pseudomonas* isolates for the presence of GI-7, making it the largest study of this genomic island. The findings from this project serve as a foundational study for future surveillance of copper tolerance related to GI-7 and potentially other unexplored copper tolerance genes in Canadian hospital sink drain isolates. Additionally, the phenotypic copper testing methods to ascertain copper tolerance among reference *P. aeruginosa* strains reaffirms and highlights the need to consider the confounding chemical properties and limitations of using copper salts. This is especially concerning given that most current studies do not consider or account for the acidic effects of high concentration copper salt addition to growth media when they assess copper susceptibility and that experimental methods of studying copper tolerance are poorly standardized between different labs and even studies. Our findings from the copper water 24 h exposure experiment developed by Jeanvoine *et al.* 2019 (42) with copper susceptible and tolerant *P. aeruginosa* reference strains provides further support for this method as being an accurate but laborious technique to determine copper tolerance. Ideally, more experimental optimization and reference strain testing will make this method more suitable to adopt as a universal robust standard for copper tolerance testing in the near future.

5.3 Study Limitations

Several limiting factors changed the goals for the phenotypic 'wet lab' characterization of *P. aeruginosa* isolates and strains for this project. Based on the outcomes of many preliminary experiments (discussed in detail in Chapter 4.3), the project had to change frequently from its proposal to its final completion as described here. Our initial goal was to pre-screen all 2467 BCIP sink drain isolates for their copper tolerance using 96-well broth microdilution assays with high concentrations of $CuSO_4 \bullet 5H_2O$ (0.5-8 mg/mL). However, as was thoroughly discussed in Chapter 4.2, media acidification by copper salts impeded plans for reliably using this 96 well broth

microdilution testing method as all strains were susceptible to copper due to its high acidity and propensity for copper precipitation when buffered to counteract the acidity. After completing planktonic copper susceptibility screening of reference *P. aeruginosa* strains, we intended to perform copper susceptibility testing of selected BCIP isolates, particularly those identified as being more copper tolerant or possessing known copper resistance genes/ GI-7. Although 24 h copper water exposure assays resolved the copper acidification issues when we tested reference strains PAO1 and PA-8, it also required a considerable amount of nutrient agar plates for accurate CFU/mL dilution spread plate measurements, which to the best of our abilities could not be streamlined into spot plating for more efficient high-throughput BCIP isolate analysis (as discussed in Chapter 4.3). Due to the costs and labour of the 24 h copper water exposure assay method this testing was not performed for BCIP isolates.

As part of copper tolerance testing, *P. aeruginosa* reference strain biofilm growth was also examined and encountered several technical challenges limiting its application for high-throughput testing BCIP isolates. Instead of using the 96 well pegged lid biofilm inhibition methods discussed in Chapter 3, we originally hoped to examine biofilm biomass formation directly on pegged lids or coupons made from a copper alloy. Survival and growth of planktonic and biofilm cultures on copper surfaces has been tested in past studies, primarily using copper metal coupons (60, 76); however, high-throughput applications or modifications of copper materials have yet to be successfully described since the initiation of this study. Hence, our initial goal at the start of this project was to create a copper coated pegged lid device for high-throughput biofilm screening of the reference strains and BCIP isolates. However, in the planning and development of this copper pegged lid device, we discovered several issues. Firstly, the cost of fabricating such devices would have been beyond the budget of this project, and the estimated cost became more concerning when we considered the reusability of the copper pegged lid device. When copper tolerant biofilms grow on copper surfaces, they can cause a specific type of microbial-induced corrosion known as pepper-pot pitting (154). In practice, this type of corrosion can take a long time to develop such as within a sink drain pipe. However, we performed some preliminary testing of the copper peg materials and based on these initial tests we expected that repeated exposure of copper pegs to growth media and biofilm biomass formed on the pegs by

P. aeruginosa species would accelerate this corrosion. This pitting causes differences in copper peg surface area, and copper release/leaching after repeated uses, making the costly copper peg lids unsuitable for repeated biofilm use. Additionally, the removal of highly adherent *P. aeruginosa* strain biofilms from the copper peg surfaces between experiments was another concerning limitation, as most methods of biofilm growth on pegged lids utilize disposable plastic pegged lids to avoid the build-up of prior biomass that cannot be completed removed from the peg surface after repeated uses (1). Thus, due to the anticipated costs of developing and replacing these devices, and their lack of reliable biofilm formation when repeatedly used, we did not follow through with this copper biofilm pegged lid device method.

5.4 Future Directions

As noted in the sections above, the future uses of the deep well biofilm device discussed in Chapter 3 are broad. These pegged lid biofilm devices are suitable for a variety of downstream experiments that require greater amounts of biofilm biomass for analysis of many different genera/ species, such as metabolomics, proteomics, and mass spectrometry. While our study explored *E. coli* and *P. aeruginosa* biofilm eradication by the disinfectants BZK and bleach, future studies may examine the use of the deep well biofilm device to explore biofilm inhibition by these compounds and other disinfectants or to test biofilm eradication and inhibition of other species by antibiotics and other antimicrobials. Additionally, the deep well biofilm device may be suitable for metabolic assays currently used on standard device biofilms, such as the dye resazurin. Resazurin is metabolized by metabolically active bacteria to yield a fluorescent by-product which can be measured to determine how metabolically active the cells are (155). These assays may perform better due to greater sensitivity gained from increased biofilm biomass formed on the deep well biofilm device. Finally, it would be important that before using the deep well biofilm device on species other than *E. coli* and *P. aeruginosa* that growth is compared on either the standard or the deep well devices with a biofilm-forming reference strain to ensure that the species is suited for biofilm growth on the polypropylene deep well device.

Future directions from the findings of the BCIP study are manyfold. This study provided a wealth of samples and genotypic data that still have many possible avenues for future analysis. Future bioinformatic genomic studies of the BCIP isolates could delve further into the analysis

and separation of *Pseudomonas* isolates by their hospital collection date and hospital rooms, which were available datasets but not assessed in our analysis in Chapter 4. This analysis was excluded due to a lack of time to complete and include this as part of the thesis. Assessing samples collected from sink drain rooms over time may provide more insights on whether GI-7 is gained by MLSTs over time within the BCIP isolate sink drain study and if rooms where copper pipes were installed had an increase in GI-7. Unfortunately, the number of isolates collected from copper pipe sink drain installation rooms was quite low, so it may be more challenging to assess statistically robust trends in this specific analysis.

Additionally, we were not able to complete phenotypic copper susceptibility testing of any BCIP isolates due to the challenges associated with establishing a reliable copper susceptibility/ exposure test. We hope that future studies/ students on this project will be able to complete copper tolerance testing of the BCIP isolates, ideally using the 24 h copper water exposure method and using a statistically powered and representative proportion of isolates from the BCIP study. By using a smaller sampling of the isolates that represent particular MLSTs, isolates with GI-7 presence and absence, and isolates from specific sampling locations, the 24 h copper water exposure experiments will be more manageable. This statistically significant sampling of isolates may also include a random sampling or a more biased but targeted sampling (as discussed above) by eliminating isolates that were collected on the same day from the same hospital room, or by selecting a subset of isolates ±GI-7 to see if this copper tolerance genomic island contributes to copper tolerance in the BCIP isolates.

Finally, as briefly discussed in Chapter 2, transcriptomic analysis was initiated to begin the comparison of *P. aeruginosa* reference strain PAO1, PA-8, and PA-8 ΔGI-7 gene expression when grown in standard pH 7.0 MHB (control), MHB amended with 2 mg/mL $CuSO_4 \bullet 5H_2O$, and MHB adjusted to pH 5.5. This analysis was initiated in March 2022 and sent to LC Sciences for RNA-seq analysis to determine total mRNA expression changes by these strains when exposed to various copper and pH stresses. Unfortunately, this analysis is ongoing and we have yet to receive the bioinformatic results summary of this experiment from LC Sciences. Thus, continuation of the RNA-seq project will involve the analysis of transcriptomic data to determine which genes are up- or down-regulated by copper and/or acidic pH tolerance in *P. aeruginosa*. Overall, we expect

to see overlap in up-regulation of general stress-related genes, while there will likely be differences in expression in genes that are specific to copper or pH tolerance. Ideally, we will see up-regulation of GI-7 genes in PA-8 but not in PAO1 or PA-8 ΔGI-7, such as cupredoxins, the Cop transport system, CopZ, CzcABC, and plastocyanins (Figure 1.3). As discussed in Chapter 4, PA-8 ΔGI-7 appeared to still have some tolerance to copper despite having a deletion of GI-7. This transcriptomic analysis may uncover alternative copper tolerance genes not found in GI-7 that are present and upregulated in the PA-8 strain during copper stress. Finally, because the copper exposure condition also results in acidic pH, we would expect to see that all genes upregulated during exposure to pH 5.5 would also be upregulated during 2 mg/mL $CuSO_4 \bullet 5H_2O$ exposure. These acid stress response genes may include those discussed in Chapter 1, such as the periplasmic chaperones HdeA, Skp, DegP, and SurA (99).

5.5 Main Conclusions

Based on the outcomes of this project we designed and tested a method of forming greater biofilm biomass, known as the deep well biofilm device, which allows for a lower cost alternative for standard high throughput biofilm growth experiments on pegged lids. Additionally, we looked at copper tolerance associated with the GI-7 genomic island in *Pseudomonas* reference strains and BCIP isolates. We found that GI-7 was present in a proportion of isolates from the BCIP study hospitals and that some of the most abundant MLSTs contained GI-7 and are already of concern worldwide due to resistance to antibiotics. Finally, by studying current methods of copper susceptibility testing and the inherent challenges imposed by the chemistry of copper, we were able to identify growth conditions distinguishing the effect of copper salt-induced acidity on the susceptibility of *P. aeruginosa* strains to $CuSO_4 \bullet 5H_2O$. We performed 96 well broth microdilution copper susceptibility testing experiments as well as 96 well biofilm formation assays with copper tolerant and susceptible *P. aeruginosa* reference strains (PAO1, PA-8, PA-8 ΔGI-7), as well as adapted a previously described method of 24 h copper water exposure testing of *P. aeruginosa.* Findings from the copper water exposure assay were the most reliable for discerning copper tolerant (PA-8) from susceptible (PAO1) strains of *P. aeruginosa*. This copper water exposure method may be the most reliable technique to use for

future BCIP collection sink drain isolate phenotypic testing to determine copper susceptibility of the BCIP isolates.

Lastly, future research should explore additional connections between GI-7 and unassessed data collected for the BCIP isolates, including the date of collection and hospital room associations. Additionally, a continuation of copper tolerance testing should be undertaken with the BCIP isolates using the copper water exposure assaying method with low concentrations of $CuSO_4 \bullet 5H_2O$ in water to avoid acidification by copper salts. Finally, future work will analyze the transcriptomic changes for *P. aeruginosa* strains exposed to copper or acidic pH growth conditions to determine which genes/ stress pathways are involved in *P. aeruginosa*'s response to these potentially different antimicrobial conditions.

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Appendix A: Fasta file for GI-7

Fasta file of the GI-7 sequence determined from PA-8 (ST308) provided by Dr. Didier Hocquet (Besançon, France).

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>GI7_in_ST308_full Concatenation of 2 sequences
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