Antimicrobial Coatings for Controlling Transmission of Infectious Agents:

Validation and analysis of antimicrobial copper-titania surfaces for real world and hospital infection control.

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Abstract

When patients undergo significant medical treatment, they will often spend multiple days in hospital. As a result of this prolonged stay in healthcare settings the risk of developing an infection increases. Infections which arise during a stay in a healthcare setting, or shortly after leaving a healthcare setting are commonly referred to as health care associated infections.

Health care associated infections are often bacteria surviving on many of the surfaces found in a hospital. Many of the bacteria found in hospitals belong to a group of pathogens known as the ESKAPE pathogens. The ESKAPE pathogens are a group consisting of *Escherichia coli, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterococcus sp.*. These species are all highly monitored and are often the cause of many healthcare infections.

To combat this, infection control interventions are required to break the chain of infection from health care setting to patient.

The standard material used for hospital surfaces is stainless steel due to its corrosion resistance. As stainless steel does not naturally possess antimicrobial properties, ESKAPE pathogens can reside on these untreated surfaces in between periods of cleaning. Copper is effective as an antimicrobial coating but has not yet been widely adopted because of the additional cost and the additional factors in the manufacture of copper coated items.

The objective of this study was to investigate the effectiveness of copper and titanium coated stainless steel surfaces produced using chemical vapour deposition (CVD) method in reducing healthcare associated infections caused by ESKAPE pathogens.

We hypothesize that the dual-action of $CuTiO_2$ surfaces can provide an effective way of reducing bacterial transmission in a real world environment.

$CuTiO_2$ surfaces utilize a dual action antimicrobial killing mechanism. The UV generation of free radicals and oxidative species obtained from the titanium dioxide is combined with copper, which utilises its ionic charge as a lewis acid, to kill microbes.

A new biofouling method was developed to ensure consistency in colony counts and antimicrobial surface testing. Copper and titanium were deposited onto stainless steel using CVD method to create photocatalytic surfaces. The effectiveness of $CuTiO_2$ surfaces in reducing bacterial

survival was tested using viable *S. aureus* NCTC 8532 and BacLight Live/Dead staining along with agarose plate transfer counts.

Preliminary investigations into the effects of $CuTiO_2$ surfaces on antimicrobial susceptibility of isolates exposed to $CuTiO_2$ surfaces were also investigated. Culture and 16S rRNA-based techniques were used to test $CuTiO_2$ activity in an uncontrolled real world environment, and were placed in a university toilet open to students and staff.

$CuTiO_2$ surfaces demonstrated a marked decrease in viable *S. aureus NCTC 8532* and *E. coli 01210* cells within 120 minutes compared to untreated stainless steel (p=0.021 after 1 minute, and p=0.0055 after 60 minutes). The use of UV irradiation increased bacterial killing. Analysis using BacLight Live/Dead staining confirmed a reduction in bacterial survival within 60 minutes compared to un-coated controls (p=0.0054). Preliminary investigations into antibiotic resistance of isolates exposed to $CuTiO_2$ surfaces showed increased susceptibility to certain antibiotic compounds than un-coated and standard strain controls (p=0.03 and p=0.02 respectively). $CuTiO_2$ surfaces were found to only contain isolates with natural copper resistance genes such as *copA* and *copZ*, and the main species found on these surfaces were *Micrococus luteus* and *Bacillus altitudinis* respectively.

Phylogenetic analysis revealed closely related bacterial species forming various clusters, suggesting that resistant bacteria persisted on $CuTiO_2$ surfaces but were not identical clones. Tetracycline resistance was found in most isolates, across all samples (n=14) and formed two evolutionary branches similar to the presence of *merR1*, a highly conserved mercury resistance gene.

In conclusion, copper and titanium coated stainless steel surfaces produced using CVD method have potential as simple, cost-effective, and durable antimicrobial surfaces that can reduce healthcare associated infections caused by ESKAPE pathogens. $CuTiO_2$ surfaces demonstrated antimicrobial activity against *S. aureus NCTC 8532* and *E. coli 01210* and increased susceptibility to certain antibiotic compounds. However, further research is needed to investigate the long-term effectiveness of $CuTiO_2$ surfaces in reducing healthcare associated infections in a hospital environment.

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AMR	Antimicrobial resistance
ANOVA	Analysis of variance
BCE	Before common era
CFU	Colony forming unit
CLABSI	Central line associated blood stream infection
CLED	Cysteine lysiene electrolyte deficient agar
CONS	Coagulase negative *Staphylococci*
Cu	Copper
CuO	Copper oxide
$CuTiO_2$	Copper-titanium dioxide
CVD	Chemical vapour deposition
DDD	Drug daily doses
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
ESKAPE	*Enterococcus faecium*,*Staphylococcus aureus* *Klebsiella pneumoniae*, *Acinetobacter baumannii* *Pseudomonas aeruginosa*, and *Enterobacter* species
EUCAST	European union committee on antimicrobial sensitivity testing
GASP	Growth advantage in stationary phase
GBK	Genbank
GlyNAC	N-Acetylcysteine
HCAI	Health care associate infection
HCI	Healthcare infection
IAI	Intra-abdominal infection
ICT	Infection control team
ICU	Intensive care unit
IPC	Infection prevention control
JPL-SAF	Jet propulsion laboratory - spacecraft assembly facility
LB	Luria-Bertani
MALDI-TOF	Matrix-assisted laser desorption ionization time-of-flight
MCL	Maximum composite likelihood
MDR	Multi-drug resistance
mRNA	Messenger Ribonucleic acid
MRSA	Methicillin resistant *Staphylococcus aureus*
MurNAC	N-Acetylmuramic acid
MSSA	Methicillin-sensitive *Staphylococcus aureus*
MGE	Mobile genetic elements
MB	Microbial burden
NHS	National Healthcare Service
NAM	N-acetylmuramic acid
NAG	N-acetylglucosamine
NASA	National Aeronautics and Space Administration
nBLAST	nucleotide Basic local alignment search tool
NCBI	National center for biotechnology information

PBPs	Penicillin binding proteins
PBS	Phosphate buffered saline
PD	Per Day
PCR	Polymerase chain reaction
PHE	Public health England
RNA	ribonucleic acid
rRNA	Ribosomal ribonucleic acid
RPM	rotations per minute
SEM	Scanning electron microscopy
SMRP	Small multidrug resistance proteins
SSI	Surgical site infection
TAE	Tris base, acetic acid and EDTA
tRNA	Transfer Ribonucleic acid
Ti	Titanium
TiO_2	Titanium dioxide
UTI	Urinary tract infection
UV	Ultraviolet
VAP	Ventilator associated penumonia
WGS	Whole genome sequencing
Zn	Zinc

1 Introduction

1.1 Antimicrobial resistance

Before the use of antimicrobial compounds by humans, bacteria were utilising antimicrobials against each other to fight for survival and resources. Through evolution, different species of bacteria developed new ways of killing other bacteria, which led to new antimicrobial compounds with differing mechanisms. In order to survive, bacteria developed mechanisms for resisting the destructive impacts of antimicrobial compounds.

Antimicrobial resistance (AMR) refers to the phenomenon where bacteria develop resistance to antimicrobial compounds that were previously effective in treating infections.

Antimicrobial resistance, is an ever-growing problem with the potential to cause 10 million deaths per year over the next 3 decades (**oneill2016tackling**). Since their first introduction, multiple generations of antimicrobials have been rendered inadequate for use, prompting the development of new generations and variants of antimicrobials, all of which promote growing resistance year by year (**peter2019tracking**). The continued usage of last-resort antibiotics to treat multi-drug resistant (MDR) infections leaves humanity in a critical state where drug

development and testing are lagging behind the growing resistance to each subsequent generation of antibiotic.

In 2016 the O'Neill report on tackling AMR was released as part of a parliamentary review on the effect anti-microbial resistance is having on global healthcare (**oneill2016tackling**). The report concluded that investigation into preventing the transmission of antibiotic resistant bacteria is a key step in preventing nosocomial infections, contributing to the predicted 10 million deaths. Interrupting the spread of antimicrobial infections plays a pivotal role in the patient journey and quality of care by reducing wait times for surgery or discharge, whilst also reducing further burden on already strained healthcare services (**nhsinfections**) and the overall prevention of patients developing infections.

Preventing nosocomial infections also includes the protection of staff. By increasing infection control measures and applying interventions, staff are exposed to fewer harmful microbes which may be in the hospital environment.

In total, this leads to a reduction in infection related deaths among patients, and fewer staff needing to take leave due to their own illness (**spearing2000direct**).

In order to treat infections, antimicrobial compounds are utilized to target the infectious organism. There are multiple categories of antimicrobial compound with varying mechanisms of action, but to counter these bacteria have evolved strategies that allow them to resist each category of antibiotic through mutation and acquisition of resistance genes.

Antibiotic resistance is generally characterised as intrinsic resistance, or acquired resistance (**sandner2018genomic**). Intrinsic resistance are fundamental properties of the classification of the species, such as resistance to antibiotics of certain classes due to the lack of drug targets (**cox2013intrinsic**).

Intrinsic antibiotic resistance cannot be transferred between cells, whereas acquired resistance can be transferred between bacterial cells using a variety of methods. This includes plasmid release to the environment (**rodriguez2021beyond**), conjugation of sex pilae and mobile genetic element exchange (**majdic2021escherichia**), and environmental DNA fishing by some species (**ellison2018retraction**)

Undue exposure to levels of antimicrobials below lethal doses allows sub-sets of bacterial populations to persist and develop resistance mechanisms (**manasherob2021tolerant**). This

enables survival even in the presence of lethal doses, bacteria which possess antibiotic resistance genes can survive (**palleja2018recovery**), whilst others die. This selective pressure leads to an infection caused by bacteria that are completely resistant to the chosen antibiotic (**tanwar2014multidrug**) and can leave the patient in a worse situation than before.

It is essential to monitor and track outbreaks of infection in a timely manner to disclose hot spots or areas where infection control is lacking. Detection, as well as phenotyping and genotyping to characterise resistant bacteria, and identification of areas with high transmission are crucial elements of effective surveillance, which need to be more widely implemented. Current methods for detecting antibiotic resistance present many challenges, often requiring multiple hours or days before resistance profiles can be returned to inform clinical decisions about treatments and control measures (**macgregor2020tuberculosis**).

1.2 Infections in a clinical setting.

In previous studies on infection epidemiology, within intensive care units (ICUs) 80% of infection based cases were relating to primary blood infections, respiratory or gastro-intestinal infections and urinary tract infections (**alberti2002epidemiology**).

More recent studies suggest that the lack of ICU beds also has a significant impact on the development of serious complications from infections. Even when inside an intensive care unit, 27% of ICU cases in the United Kingdom progress to bloodstream infections (**mayr2014epidemiology**). The lack of beds within hospitals prevents treatment of patients. By having patients waiting for a bed, and waiting to undergo diagnosis and treatment, undiagnosed infections have a higher likelihood of progressing to bloodstream infections, ultimately leading to the development of sepsis. If ICU beds are not available for those who are developing sepsis, they cannot be effectively treated. This leads to an overall increase in mortality rates compared to those who are quickly moved to an ICU and are able to be allocated bed space.

A recent study investigating the presence of antimicrobial resistance of Gram-negative ESKAPE infections in Asia and the Pacific found a total of 3052 abdominal infections, and 1088 urinary tract infections caused by *K. pneumoniae, P.aeruginosa and Enterobacter spp* (**karlowsky2017antimicrobial**). Of these Gram-negative species found causing infections across Asia and the Pacific, extended

spectrum beta-lactamase harbouring *K. pneumoniae* were more common in UTI's over other locations (27% of UTI (Urinary tract infection) isolates over 16% of IAI isolates) (**karlowsky2017antimicrobial**). *Enterobacter spp.* were on average more resistant to antimicrobials used in this study, with resistance to levofloxacin at approximately 18%. *Enterobacter spp.* isolated from both UTI's and IAI's only presented with susceptibility to ceftazidime in less than 60% of cases (**karlowsky2017antimicrobial**).

The ESKAPE pathogens are primarily responsible for most HCAIs (**abban2023burden**) and have a wide variety of site for infection ranging from soft tissue to urinary tract infections (**mayr2014epidemiology**). A growing number of ESKAPE pathogens are developing antibiotic resistance to at least, their first line antibiotics at an increasing rate (**chinemerem2022antibiotic**). This select group is of high clinical significance as preventing infection and the increase in resistance can reduce patient mortality (**masoud2022extent**).

Approximately 90% of wounds have been reported to contain some level of drug-resistant organism (**lutheryn2020ultrasound**). This is vital in impacting the efficiency and response of medical professionals to infections. If infected wounds are initially mistreated with ineffective antimicrobial compounds, it is possible that there may be significant secondary complications (such as septic shock) due to poor early goal-directed therapy (**gyawali2019sepsis**; **martinez2020approach**). The rate of motrality of non-AMR sepsis is as low as 41% (**gyawali2019sepsis**), AMR increases the risk of mortality significantly above 41% (**mu2023integrative**). However any specific percentage increase would be influenced by the hospital setting, treatment availability, and response times of clinical teams to emerging information on the infection.

In recent times, with the onset and continued waves of the SARS-CoV-2 pandemic, more stringent infection control and isolation of vulnerable or sick patients had the side effect of preventing other outbreaks of infection (**johanna2020mass**). The increased cleaning regimens, higher regard for sanitation and lack of visitors in hospitals has led to a general decline in outbreaks of non-Coronavirus infections (**shimabukuro2020environmental**).

1.3 Seasonal variation in HCAI pathogens

Hospital environments have been monitored for microbial presence and it is known that they are far from sterile (**cadnum2021microbial**). The type and level of microbial inhabitants can be

influenced by many external factors, such as the season of year, or severity of seasonal afflictions such as cold and flu. Thus careful monitoring is key to ensure that disinfection and control procedures are appropriate at all times during the year.

HCAI's are categorised by the routes of infection and tropism. The most common forms of HCAI are; surgical site infections (SSIs), central line-associated bloodstream infections (CLABSIs), ventilator-associated pneumonia (VAP), and catheter-associated UTIs. Across 195 National Healthcare Service (NHS) hospitals, and 8 voluntary private hospitals, Public Health England (PHE) reported 1,197 SSIs in 134,547 surgical procedures (0.899% prevalence) and upon further analysis of ten year trends, PHE described a decrease in nine of their thirteen categories of surgery, with surgical procedures resulting in SSIs falling from 8.7% to 7.7% (**PublicHealth2020**). 80% of health care associated infections (HCAI's) are caused routinely by 12-20 prevalent organisms (**haque2018health**), specifically featuring the ESKAPE pathogen group, which are becoming every increasingly drug resistant (**santajit2016mechanisms**). Approximately 80% of ESKAPE pathogen infections display some form of antimicrobial resistance (**haque2018health**), and traditional use of infection control has played a significant, albeit small role in reducing the burden of ESKAPE infections in clinical settings.

For example, it is well known that Methicillin-resistance *Staphylococcus aureus* (MRSA) can be dangerous to a patient. Vancomycin resistant *Enterrococci* became significantly apparent in North America starting in the 1980s, with over 50% of *E. faecium* isolates resistant to vancomycin by the early 2000's (**santajit2016mechanisms**).

When studying the causative organisms, PHE stated that *Enterobacterales* continued to make up the largest proportion of causative organisms across all surgical categories in 2019/20 for both superficial SSIs (29.8 %) and deep or organ based surgery (26.2%), however *S. aureus* still contributes to a large proportion of deep or organ space SSIs (24.2%) (**PublicHealth2020**). The ten year trend in SSI infections by species was analysed from 2010, through to 2020, and is displayed in figure 1.1.

It is evident that small seasonal variations through the year are lacking, but over the ten year period assessed, trends such as a slight increase in *Enterobacterales* are displayed. Prior to the fall in 2018/2019, other species notably *Pseudomonas spp.* and *Enterococcus spp.* rise before promptly falling and remaining low, potentially due to new therapies being implemented.

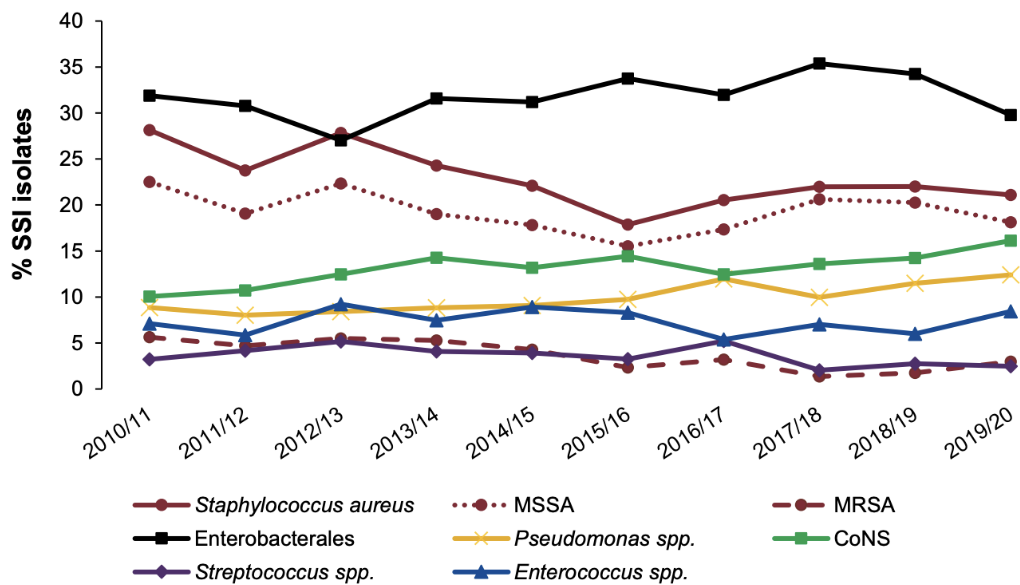


Figure 1.1: Micro-organisms reported as causing inpatient and readmission superficial SSIs in all surgical categories in NHS hospitals England from April 2010 to March 2020. Taken from data on healthcare acquired infections (**NHS2020**). The graph shows the percentage of isolates causing surgical site infections over a ten year period, and that the rates of methicilin susceptible *S. aureus* (MSSA) and regular *S. aureus* have dropped, with *Enterobacterales* increasing alongside a rise in *Pseudomonas spp.*. Coagulase negative *Staphylococcus aureus* (CoNS) has also seen a slight rise since the early 2010's but remained consistent despite a sharp rise in 2020.

1.4 The ESKAPE pathogen group

1.4.1 *Escherichia coli*

Escherichia coli, (*E. coli*) is a key part of the human microbiome and resides in the digestive tracts of all people (**aly2012antibiotic**).

E. coli is a Gram negative, motile bacterium lacking a capsule which has been well documented as a key pathogen both in the digestive tract, and in the urinary tract. Despite being part of the normal microflora of the human body, pathogenic strains of *E. coli* do exist, with serotypes such as *E. coli* O15:H7 causing severe gastrointestinal disease if ingested (**lim2010brief**). Toxic *E. coli* infections in the gut must possess fimbriae, tropic to the cells contained within wall of the gut. This allows the *E. coli* to bind to the intestine and cause a significant infection through the release of toxins in the intestinal tract. These toxins are often described as either shiga-like toxins or cholera-like toxins and result in large discharge of electrolytes and water resulting in severe internal dehydration and diarrhoea (**dupont1971pathogenesis**).

Ever-growing antimicrobial resistance in *E. coli* is of great concern to the medical community. Recent studies have shown *E. coli* resistance to conventional first line Beta-Lactams, Quinolones and the last line class of tetracycline antibiotics (**aly2012antibiotic**), the mechanisms of which are discussed later.

1.4.2 *Staphylococcus aureus*

Pathogenic staphylococci covers many species and subspecies, some of which are commensal microflora in and on humans (such as *Staphylococcus aureus* and *Staphylococcus epidermidis*) (**parlet2019commensal**). However, *Staphylococcus aureus* is one of the most important commensal species and constitutes a key member of the ESKAPE pathogen group (**pendleton2013clinical**).

S. aureus is a Gram positive cocci shaped organism, with multiple virulence factors including clumping factor coagulase (**peetermans2015coagulase**) and hemolysin (**jahn2022alpha**) and Staphylococcal Protein A (**rigi2019comprehensive**).

Antimicrobial resistance in *Staphylococci* is well documented, with a particular focus on multidrug resistance and methicillin and vancomycin resistance (**mlynarczyk2022molecular**).

Despite its presence on the skin and in the naso-pharyngeal tract of most people, *S. aureus*

infections commonly occur as both soft tissue opportunistic infections, and deep rooted bone infections (**nair2000advances**). As such, it is important to reduce the transfer of *S. aureus* in wards that deal with osteopathic surgery due to this specific tropism.

1.4.3 *Klebsiella pneumoniae*

Klebsiella pneumoniae is a common Gram negative opportunistic pathogen, often found in sputum & lung samples, blood, urine, pus & wound exudate, and cerebrospinal fluid (**el2013virulence**).

As a Gram negative rod, the spread of antibiotic resistance is of great concern, much like that of *E.coli.* In the environment, Klebsiella penumoniae is often found in bodies of natural water, as a soil commensal and often in the nose and throat of most mammals (**wyres2018klebsiella**). As one of the ESKAPE pathogens, K. penumoniae is one of the key pathogens under investigation and monitoring for both hospital and community based infections.

Once *Klebsiella spp.* infect the lungs, outer membrane vesicles are secreted which induce a mammalian immune response by releasing interleukin 1β further driving extracellular innate immunity (**lee2012klebsiella**).

After infection, lung tissue becomes swarmed with immune cells, which generates pleural effusion, vasculitis and edema. Once damage is caused trying to remove the *Klebsiella spp.*, fibrin is deposited in lung tissue as means to attempt a repair, instead of developing elastin-based tissue which further compromises lung function.

Both *Klebsiella penumoniae* and *Escherichia coli* have been documented as having high resistance to most Beta-Lactam antibiotics (**wyres2018klebsiella**) (as discussed below). The complexity of a drug-resistant *K. pneumoniae* infection has been found to increase hospital stay by an average of 9.7 days (**el2013virulence**) therefore increasing the burden on hospital resources.

1.4.4 *Acinetobacter baumanii*

Much like the majority of the other ESKAPE pathogens, *Acinetobacter baumanii* is a Gram negative organism with a coco-bacilli presentation. Identification of *Acinetobacter spp.* is often complicated without the use of gene sequencing technologies as the organism does not ferment sugars, is non-fastidious and is oxidase negative. These characteristics are common among other

Acinetobacter spp. and biochemical identification of suspected *Acinetobacter* infections stop at genus level taxonomy (**berlau1999distribution**).

However, these closely related species are variable in their required treatments (**lee2017biology**), and it is of upmost importance that suspect *Acinetobacter* infections are identified to species level quickly to aid the onset of the correct treatment (**lynch2022infections**).

Many rapid methods for detecting this have been developed, such as DNA fingerprinting, matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) and the standard use of the 16s-23s ribosomal RNA complex for genetic identification (**holmstrom2022acinetobacter**).

The opportunistic nature of *A. baumanii* was reported highly in combat zones, and it promptly earned the nickname "Iraqibacter" due to its prevalence in combat hospitals in the middle east where it was found to infect shrapnel wounds, and if left untreated, then progressed to septicemia (**sebeny2008acinetobacter**; **guerrero2010acinetobacter**).

Despite being found in war zones, *A. baumanii* is reportedly seen in patients who have stayed longer than 3 months continuously in a clinical setting. This can be especially dangerous for the patient as this can lead to longstanding gastrointestinal infections, or even develop into blood stream infections. Pathologically, *A. baumanii* does not possess any specific mechanism of toxicity, however its virulent infection of the blood and rapid production of siderophores, alongside resistance to the complement cascade makes it a dangerous pathogen in sepsis cases.

Environmental *A. baumanii* also have an increased uptake in extracellular DNA due to the many membrane-contained porins which, when combined with ComE protein, transport this DNA inside the cell. This explains the rise in antibiotic resistance within *A. baumanii.*

1.4.5 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a common hospital pathogen, with growing resistance to multiple antimicrobial groups including beta-lactams, carbapenems and DNA gyrase inhibitors (**poole2011pseudomonas**; **nasrin2022distribution**) potentially due to its large elastic genome (**pang2019antibiotic**). Multi-drug resistance (MDR) is of growing concern in Gram negative species, including *P. aeruginosa*, which has become increasingly resistant to compounds such as aztreonam (**nasrin2022distribution**) and beta-lactam antibiotics (**poole2011pseudomonas**).

P. aeruginosa posesses the type 3 secretion system, a protein complex that extrudes from the

membrane and has the potential to transport proteins into other cells. *P. aeruginosa* therefore uses this type 3 secretion system to transfer proteins into the cells of the host which prevent intra-cellular signalling, cytokine generation and can prevent apoptosis in cells. An additional way of countering the host immune response employed by *P. aeruginosa* is the production of elastase and alkaline protease, which are used to remove the bacterial flagella in order to prevent immune cell activation through flagellin based pathogen associated molecular patterns (PAMPs) or toll-like receptors (TLR's) (**casilag2016lasb**). These molecules then go on to slowly degrade the tight junctions that line surface level cells in the intestines or along mucous membranes.

P. aeruginosa also produces pyoverdine and pyocyanin, a pair of siderophores which scavenge for iron compounds, which becomes increasingly problematic in blood stream infections. Due to the high iron content of red blood cells, pyoverdine and pyocyanin begin to break down and chelate iron from the blood which negatively affects the host and provides beneficial iron for the infection (**paprocka2022pseudomonas**).

1.4.6 *Enterococcus faecalis & faecium*

Enterococcus faecalis (*E. faecalis*) is a Gram-positive commensal bacterium that predominantly resides in the gastrointestinal tract of humans. Under normal conditions, *E. faecalis* is regulated internally within the gut, and can act as a pro-biotic organism, however pro-biotic strains lack virulence genes (**zhu2019beneficial**; **are2008enterococcus**). Most of the virulence genes are associated with antibiotic resistance (**samani2021prevalence**), expressing resistance to antibiotics such as vancomycin. This then contributes to severe, hard to treat infections in the oral biome (**najafi2020oral**).

Enterococcus faecium is a Gram-positive, gamma-hemolytic bacterium. Similarly to *E. faecalis*, both organisms live as commensal flora within humans (**devarakonda2022rarely**). However in contrast to *E. faecalis*, *E. faecium* causes significant opportunistic infections in the heart and meninges rather than the gastrointestinal tract.

1.5 History of antimicrobials and antimicrobial resistance

Antimicrobial resistance has been a key challenge in infection control since the first usages of penicillin by Alexander Fleming in 1928. By 1940, Ernst Chain and Edward Abraham had discovered the that some bacteria were not killed by penicillin, and were resistant to it. Some bacteria harboured an enzyme responsible for preventing the death of the microbes. Chain and Abraham termed this enzyme penicillinase (prior to its classification as a Beta-lactamase).

Fleming made note in his Nobel prize speech that overuse of antibiotics could lead to their lack of effectiveness, and since the first commercialisation of antibiotics, humanity has been facing this problem.

In 1950, tetracycline was discovered and became a commercially available product, 9 years later, tetracycline-resistant strains of Shigella were discovered (**akiba1960mechanism**). In an even shorter period, Methicillin-Resistant Staphylococcus aureus (MRSA) was detected for the first time just 2 years after the discovery of Methicillin.

The fastest detection of antibiotic resistance was reported in 2000 when Linezolid was discovered and entered into general clinical use, less than a year later, Linezolid-resistant strains of *S. aureus* were identified (**stefani2010linezolid**).

Humanity has been constantly at war with microbes, and the drug discovery discovery pipeline has significantly slowed since the golden age of antibiotic discovery in the 1950's and 1960's followed by the development of drug modification in the 1970's and 1980's (**rinderknecht1947studies**; **herrlich1976nitrofurans**). More recently, networks for development and discovery of antibiotics has been slowly gaining momentum, with the ability to sample new parts of the globe, combined with high throughput sequencing methods, and even artificial intelligence finding new antibiotic compounds, recent years has seen an increase in antibiotic development (**melo2021accelerating**).

The prolonged issue lies in testing and approval. These compounds once discovered require extensive testing and approval in order for them to reach commercial use. Antibiotic production and utilization is lagging behind the development of resistance to commonly used antibiotics, and last line antibiotics (**yelin2018antibiotic**; **pang2019antibiotic**). It is therefore of crucial importance to explore ways of preserving the effectiveness of current antibiotics.

1.6 Classes of antibiotics, and resistance mechanisms

Antibiotics were originally produced by bacteria as byproducts of metabolic pathways, and produced an environment which benefited the producing organism. As humanity discovered the effectiveness of these compounds as treatments for infection, manipulation of antimicrobial compounds allowed the formation of man-made synthetic antibiotics.

The mechanisms of action and formulation of these antibiotics allows the separation of these compounds into both classes (mechanisms of action) and generations (newer versions of antibiotics produced through chemical manipulation).

1.6.1 Penicillins

Penicillin was first isolated by Alexander Fleming in 1928 after he extracted the compound from *Penicillium rubens*. It was later discovered that the genus of *Penicillium* produces an antibiotic compound, however only penicillin G and penicillin V have been purified and used medicinally (**mccarthy1960absorption**; **kaplan2001unexplained**; **nathwani1993penicillins**).

Later manipulation of penicillin led to the production of aminopenicillins and specific penicillin compounds such as antistaphylococcal and antipseudomonal penicillin.

Penicillin is a beta-lactam antibiotic, with 4 variations of the R group bound to a beta-lactam and a thiazolidine ring. Beta-lactams are bacteriocidal and prevents the formation of peptidoglycan layers in bacterial cell walls.

These beta-lactams prevent the formation of D-Ala-D components at the end of peptidoglycan precursor molecules (muropeptides) which allow the cross-linking and stability of the molecules.

Beta-lactams mimic the site of the D-Ala-D complex, which results in competitive inhibition of the penicillin-binding proteins (PBPs)S and disruption of the cell membrane leading to lysis and cell death. This mechanism is shown in figure 1.2.

1.6.2 Carbapenems

Another group of beta-lactams, Carbapenems act in the same way as penicillins, but are often much more effective with a lower incidence of resistance clinically and have a broader spectrum of activity.

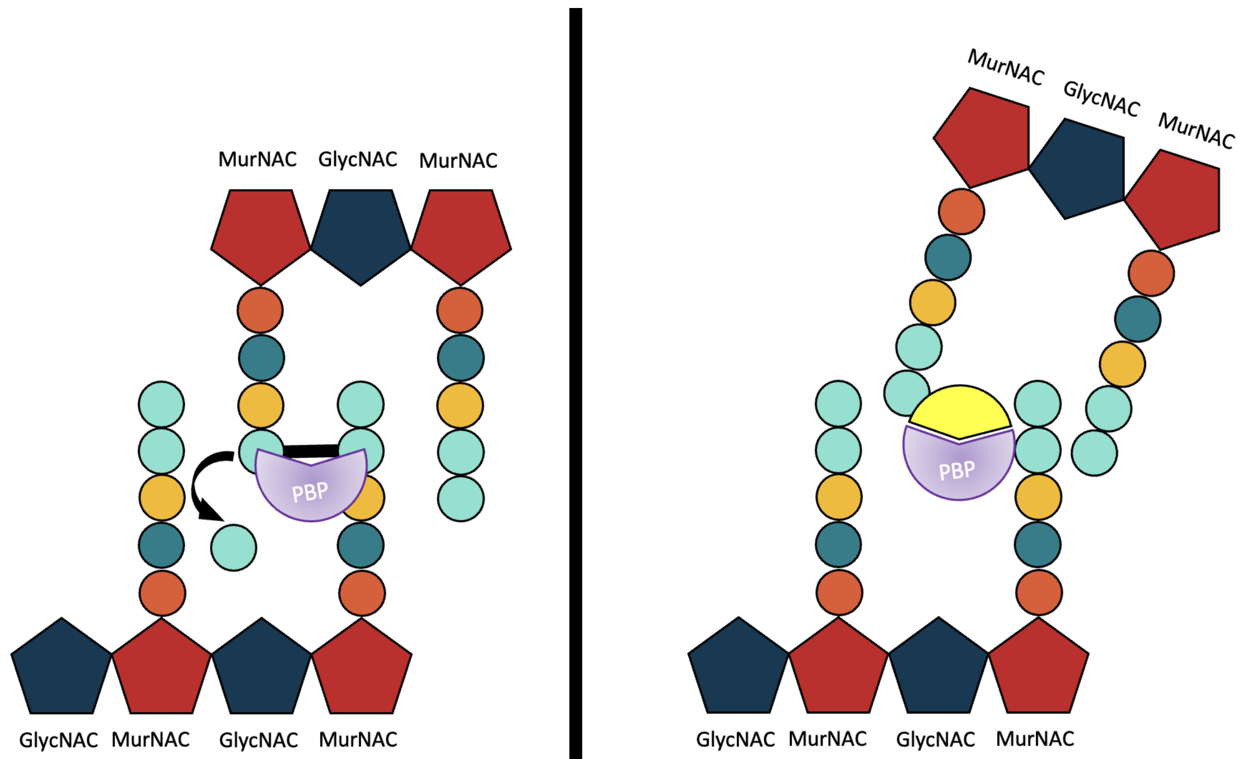


Figure 1.2: Antimicrobial action beta-lactam antibiotics through competitive binding and inhibition of D-Ala-D-Ala cross-link complexes and prevention of peptidoglycan formation. In the presence of the antibiotic (right) the beta-lactam antibiotic (yellow), binds to the penicillin binding protein (purple) preventing the formation of cross links between chains of amino acids bound to N-Acetylmuramic acid complexes. Produced using the Adobe creative cloud.

Carbapenems are typically used in incidents where the fear of multi-drug resistance is high, and the use of other beta-lactam antibiotics may be ineffective.

Carbapenems were originally derived from *Streptomyces cattleya*, however the later generational ertapenem have been used as one of the first treatments in suspected multi-drug resistant infections, especially those in the intra-abdominal cavity after surgery or wounding from bacteria such as *E. faecalis*.

Problems are arising however, with an increasing presence of multi-drug resistance, and the diminishing availability of new antibiotics, resistance to carbapenems has been well documented in *Enterobacteriaceae spp.* and *Klebsiella spp.*

Carbapenems work the same as other Beta-lactam antibiotics, as shown in figure 1.2.

1.6.3 Cephalosporins

Cephalosporins are beta-lactam antibiotics, first isolated from the *Acremonium* fungus genus in 1945. Cephalopsorins are bactericidal rather than bacteriostatic, as their mechanism of action resides in disrupting the formation of petidoglycan, just like other beta-lactam antibiotics.

Compared to other beta-lactams, Cephalosporins are more resistant to beta-lactamase enzymes produced to degrade the antibiotic, however, resistance has been found in *Enterobacter cloacae*, *Neisseria sp.*, *Escherichia coli* and *Citrobacter freundii*, with increasing levels of resistance being found in *Klebsiella pneumoniae* and *Psuedomonas aeruginosa*.

1.6.4 Fluoroquinolones

Quinolones are an antibiotic with a fairly large spectrum of activity. They are based around molecules that contain 4-quinolone, a molecule containing two conjoined rings of carbon, supplemented with nitrogen and a ketone group.

Functionally, flouroquinolones are the antibiotic form of Quinolone compounds and contain various functional groups, and the addition of a flourine atom to one of the central rings. Despite being bacteriocidal in nature, fluoroquinolones are some of the most abundant and widely used antibiotics with formulations such as Ciprofloxacin being ones of them.

Fluoroquinolones find optimal use in *Salmonella sp.* and, bone infections, especially in complex cases such as patients with blood-based abnormalities such as haemophilia or anaemia.

Ciprofloxacin has the ability to enter bone tissue without disrupting or destroying the natural hematopoiesis functions of many blood and bone cells in the area (**fong1986ciprofloxacin**; **castro2003ciprofloxacin**).

The mechanism of fluoroquinolones relies on inactivating bacterial topoisomerases (which are only present in bacteria, and therefore, pose no risk to humans or animals).

These DNA topoisomerases function by separating strands of DNA, ready for the introduction of replication forks and the generation of new cells (**blondeau2004fluoroquinolones**).However the fluoroquinolones target DNA Gyrase and topoisomerase IV, and when bound to the enzymes, it introduces structural changes to the enzymatic secondary and tertiary structure which prevents spearation of chromosomes and strands of DNA (**blondeau2004fluoroquinolones**).

1.6.5 Glycopeptides

Vancomycin is one of the most valuable antibiotics since the rise of methicillin-resistant *Staphylococcus aureus* (MRSA).Its activity against Gram-positive organisms is incredibly significant in infections with no known cause upon examination (**pace2006glycopeptides**).

Resistance to vancomycin was already documented in *Enterrococci sp.* (**pace2006glycopeptides**) however intermediary and full resistance has been documented in Gram-positive organisms. This has led to the development of a new generation of glycopeptides containing dalvancin and telavancin (**pace2006glycopeptides**).

Glycopeptides, like Beta-lactams, focus on the disruption of D-Ala-D groups in the bacterial cell membrane. However, glycopeptides focus on the glycan chains which form the predominant structure and are later converted into peptidoglycan via peptidoglycan polymerase. By preventing the conversion of these glycan chains, the cell membrane is unable to become rigid (**reynolds1989structure**).

Glycopeptides work functionally on the outside of the cell membrane, meaning resistance to them is harder to evolve and focuses mostly on potential target mutations, rather than enzyme inhibition (**reynolds1989structure**).

1.6.6 Protein synthesis inhibitors

Protein synthesis inhibitors are a group of important antibiotics that contain tetracycline, aminoglycasides, tigecycline and macrolides.

Tetracycline and Tigecycline block the transcription of mRNA into proteins through binding to the 30s ribosomal sub-unit. This blockage prevents the binding of tRNA's at the A site and blocks the elongation of the polypeptide chain (**nguyen2014tetracycline**).

Macrolides work in a similar way by targeting the bacterial ribosome, however they bind to the 50s ribosomal subunit and inhibit protein synthesis as peptides trans-locate from the A site to the P site (**vannuffel1996mechanism**).

1.7 Antimicrobial Stewardship

The incorrect usage of antibiotics has been a well-acknowledged issue since their initial introduction, and the monitoring of antibiotic utilization for therapeutic purposes has contributed to the emergence of antibiotic-resistant strains.

However, there is a clear disparity in the implementation of antimicrobial stewardship between hospitals based on hospital funding. Developed countries have the means and resources to apply antimicrobial stewardship programs, however countries which suffer from underdeveloped and underfunded hospital infrastructure, lack significant and effective antimicrobial stewardship programs (**panditrao2021impact**).

A study into antimicrobial stewardship in India by Panditrao et al assessed multiple methods of stewardship and misuse prevention such as timeouts (where antimicrobial therapy is stopped after 48 hours without empirical evidence to suggest otherwise), dosage assessment and correction, and education for clinical staff (**panditrao2021impact**).

Despite the intervention phase having a higher population of patients, there was still a significant reduction in antimicrobial administration (characterised by daily doses per 1000 patient-days), from 249.4 DDD/1000PD to 192.7 DDD/1000PD in the case of Piperacillin/Tazobactam, whilst some other alternative antibiotics saw an increase in use, consistent with the inability of first-choice antibiotics to fight infections (**panditrao2021impact**). The length of therapy was also reduced in some cases despite the increase in number of patients as shown in table 1.1.

Table 1.1: Comparison of Days of therapy/1000PD and Length of treatment/1000PD between the baseline and interventions phases conducted in an Indian hospital intensive care unit. Taken from (**panditrao2021impact**). Commonly, after the intervention of antimicrobial stewardship implementations, most days of therapy fell for each antibiotic used. Some exceptions occurred, such as the new use of Teicoplanin, or an increase in Metronidazole and Cefoperazone/Sulbactam however this could be also dependant on the causative agents of the organism changing. However after intervention, key antibiotics such as Piperacillin/Tazobactam, and Levofloxacin declined.

Antimicrobial agent	**Baseline phase**	**Intervention phase**
Piperacillin/tazobactam	249.4	192.7
Imipenem	403.7	346.8
Vancomycin	128.3	130.1
Colistin	255.2	294.1
Meropenem	25.3	19.6
Cefoperazone/sulbactam	9.01	49.7
Clindamycin	57.6	48.9
Levofloxacin	58.7	22
Amikacin	36.5	11.4
Metronidazole	34.4	68.7
Teicoplanin	0.00	21.2

This is a significant finding, displaying that even when higher levels of patients are present in the hospital intensive care units, interventions of antimicrobial stewardship policies and teams can reduce the burden of antimicrobial resistance, whilst also better managing resources and finances in the hospital (**panditrao2021impact**).

1.8 Infection prevention in clinical settings

Health Care Associated Infections (HCAIs) are a prominent problem in hospitals, and to combat this, Infection Control Teams (ICT) are employed to track and trace infectious outbreaks, monitor the rate of HCAI prevalence and produce and implement interventions to break the chain of infection and reduce the rate of HCAIs.

The official classification of an HCAI defines that any infection that appears within 48 hours of hospitalisation, or after 30 days after receiving any form of medial or health care (**stewart2021epidemiology**).

Current estimates suggest that HCAIs are among the top 10 leading causes of death in the United States (**haque2018health**) and effect 5%-15% of patients in developing countries which increases to 39% of patients admitted to intensive care units (**haque2018health**).

Stewart et al surveyed hospitals in Scotland, and found that urinary tract infections, blood stream infections and lower respiratory tract infections were the most common in Scottish healthcare settings (**stewart2021epidemiology**). Further investigation found that the most prominent causative organisms for causing these infections were found to be *Staphylococcus aureus* and *Escherichia coli* (**stewart2021epidemiology**).

Infection control teams are therefore responsible for tracking infection outbreaks, whilst also implementing preventative measures and controls, limiting the transmission of bacteria and viruses between patients, staff, and wards.

The outbreak of COVID-19 and subsequent waves of the pandemic highlighted the role of medical staff in the transmission of infectious organisms between both patients and staff (**cheng2021multipronged**). Transmission has been documented as occurring between healthcare workers and other healthcare workers as well as patients (78,834 patients and 81,955 healthcare workers in Hong Kong) (**cheng2021multipronged**). Cheng et al's study in Hong Kong showed that the increased infection control protocols during the pandemic allowed only 0.5% (n=38) of healthcare workers to contract the virus, 5 of which were documented cases between staff members (**cheng2021multipronged**).

The methods used to keep the infection rate low in staff members were implemented by the hospitals infection control teams, having learnt from previous epidemics of SARS. ICT implemented the use of hand hygiene, environmental cleaning and COVID-19 specific measures, which included an increase in laboratory surveillance of COVID-19, proactive screening for those vulnerable or deemed to be at higher risk of contracting the virus, and quarantine camps for positive testing patients (**cheng2021multipronged**). Similar strategies were then adopted by other hospitals globally.

1.9 Hospital infections post - SARS-CoV-2

In December of 2019, a new emerging pneumonia-like infection took hold in China. The virus of unknown origin was quickly identified, and through genetic sequencing the positive-sense single RNA virus was originally thought to have jumped from bats, to humans. The virus, later named SARS-CoV-2, after its genetic similarity to SARS and MERS, but also beta-coronaviruses within

the subgenus sarbecovirus.

The up-regulation in sterilization and a global health-conscious focus on preventing disease transmission has led to a level of awareness among the general population about preventing the transmission of infection.

Mask wearing, alcohol sanitizers, and one way systems became staples of many buildings including hospitals, all aimed at trying to reduce the transmission of this new infection.

This new pandemic allowed a spike in hyper-vigilance for keeping hospitals clean, not only for patient safety, but also for the safety of those working in the hospitals.

Early statistics showed that hospital transmission of the virus was possible, and that healthcare workers were 29% more likely to catch, and transmit the infection (**wee2020minimizing**; **johanna2020mass**; **cheng2021multipronged**). Dedicated COVID-19 wards were set up for positive-testing patients, and diagnostics for the virus rapidly increased to reflect the need for testing patients.

An outbreak of *Acinetobacter baumanii* was found in Hospital Juarez de Mexico in a COVID-19 intensive care ward. The infection was initially found in patients suffering with VAP (ventilator associated pneumonia) (**duran2021clonal**) but testing of staff found an identical AdeABCRS operon assembly among healthcare workers. This showed that the transmission of infectious isolates can occur between patients and staff, and vice versa, with commonly touches surfaces being a key factor in this transmission (**duran2021clonal**). Reducing this transmission therefore is a key part in developing better infection control measures and preventing the transmission of infectious organisms between hospital residents.

Hospital sanitation has increased so far that the most prevalent infection of 2020 was found to not be present on 46 sampling sites in Bellvitge University Hospital, Barcelona, Spain. Despite high touch surfaces being sampled (elevator buttons, coffee vending machines, hand rails and staff telephones) cleaning, sensitization and infection control methods used by staff were enough to prevent transmission of SARS-CoV-2, (although evidence of these surfaces acting as fomites in the transmission of SARS-CoV-2 is also minute), however there is no evidence to suggest that these cleaning regimens have an effect on hardier pathogens such as bacteria (**parvin2023biofilms**; **kourounis2023study**; **malyshev2023hypervirulent**). One other factor in this may also be the lack of evidence suggesting that SARS-CoV-2 could survive and be transmitted through

surfaces.

If current sanitation regimes can be maintained but combined with copper/ copper-titanium surfaces for areas identified as high-touch, the copper's natural antimicrobial action and the photocatalytic activation of titanium would allow the surface to be utilized over a long period of time and utilizes two different mechanisms for killing microbes. The use of titanium would also allow use in hospitals without the quality of the material diminishing over time, whilst also preventing the transmission of infection in hospitals.

Hospital infections are greatly increasing year on year, and with the impact of the COVID-19 pandemic has been shedding light on the problems associated with hospital infection control and infection transmission within the general population. COVID-19 highlighted the importance of infection control in both hospitals, and public areas (**wee2020minimizing**). With the SARS-CoV-2 pandemic falling day by day, and the general public returning to 'life as normal', face coverings and hand hygiene are slowly regressing. On public transport and in public spaces, this leads to hot-spots for the transmission of infection. Whilst the general public are returning to pre-pandemic mindsets, infection control is still a key part of the patient journey throughout NHS England & Wales.

1.10 Bacterial survival in the hospital environment

Hospital surfaces can act as reservoirs of infection, and the nature of the work undertaken in most areas of hospitals introduces multiple forms of fomite, which may harbour infectious organism (**merlin2020major**).

A considerable diversity of bacteria have been identified on a wide range of equipment used in hospitals, including scrubs and surgical gowns (**dyer2019biocide**; **malyshev2023hypervirulent**) used during surgery. Even after laundering and sanitation, *Clostridioides difficile* spores were found to attach, survive and still be recoverable from surgical gowns in the United States (**dyer2019biocide**; **malyshev2023hypervirulent**).
The various types of fomites in hospitals are displayed in figure 1.3.

Chng et al, dived deep into the issue of hospital-associated antimicrobial resistance, and the role of the environment in the transmission of both bacteria and antibiotic resistance genes

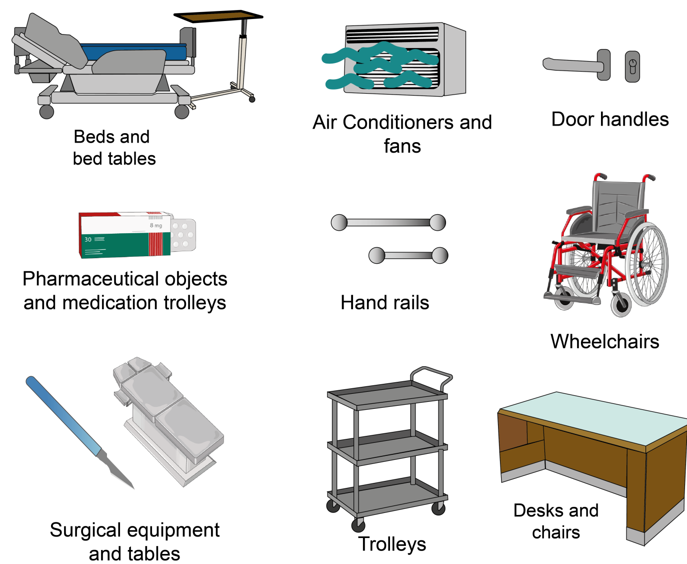


Figure 1.3: Potential sources of bacteria, adapted from work done by **merlin2020major** on a hospital ward in Cameroon. Bacteria in hospitals can be found on most places within a hospital ward, however some areas act as hot spots between staff and patients, such as door handles, beds and bed tables and trolleys used in all areas of the ward. There could be potential targets for the application of an antimicrobial coating to reduce the transmission of bacteria between patients, and between members of staff. Produced in the Adobe creative cloud suite.

(**chng2020cartography**), their use of a shotgun-based metagenomic approach revealed sites of microbiological communities forming biofilms which mediated the transfer of antibiotic resistance genes.

Further phylogenetic analysis revealed that multi-drug resistant species (such as *Cutibacterium acnes* and *Staphylococcus epidermidis*) were spread throughout hospital sampling sites and were present for over 8 years when referenced to isolates cultured from patients (**chng2020cartography**). This study sampled multiple sites in hospital rooms, including items that should undergo regular disinfection, including the bed call units and pulse oximeters used to monitor patient vital signs.

Results of this sampling showed two distinct groups through taxonomic profiling, one group of which was identified as being associated with the sink complex, the other associated with the bed rail, table and locker (**chng2020cartography**). This shows the fact that even inside a single hospital bay there are distinct microbiomes associated to separate areas, which may need different forms of individual cleaning or decontamination, which also collaborates with work conducted by **merlin2020major**. Chng's group found that, excluding door handles, the taxonomy of the cluster of species associated with the bed, table pulse oximeter and bed rail was more taxonomically diverse than the group associated with the sink (**chng2020cartography**).

This may be due to the presence of the patient, and the constant drainage of the sink providing a limited surface for certain species to survive in, or a more stable cluster of taxa which is less dynamically shifting as demonstrated by the close relatedness of species (**chng2020cartography**). This is supported by a second study, suggesting that bacteria in sinks and the plumbing network, are not aerosolized. This lack of aerosolization would explain the lack of overlap between the two taxonomic groups (**gilbert2010airborne**).

It is well known that bacteria can survive on hospital surfaces (**merlin2020major**), some even after cleaning, but the spread of genetic material between species is also common in all microbial communities. Bacteria from the skin can easily be transferred to a surface, where it may form biofilms alongside existing microbiota. This biofilm allows the transmission of genes between different bacterial flora, through the use of plasmids, and other mobile genetic elements (MGE's) (**molin2003gene**; **stalder2016plasmid**; **roder2021biofilms**).

Biofilms also form a protective and enriching environment for bacteria, and thick biofilms prevent dehydration whilst also protecting from Ultraviolet irradiation (**luo2022advances**;

carr2021lichens). MGE's facilitate the transfer of antibiotic resistance genes between organisms (**gasparrini2020tetracycline**), and some bacteria (such as *Virbio choloerae*) have even developed tools for the direct uptake of genetic material in the environment (**ellison2018retraction**).

This means that in hospitals where antibiotics are being used regularly, the transmission of microbial agents is of upmost importance, as resistance to last resort antibiotics, such as tetracycline and tigecycline is growing every year (**hsieh2021outbreak**). Tetracycline has already been demonstrated to be transferred between environment and human flora, and pathogenic species (**gasparrini2020tetracycline**; **hsieh2021outbreak**).

1.11 Antimicrobial metals as a tool in fighting hospital infections

Hospitals are primary hotspots of infection due to the very nature of the work which is conducted there. The frequency of post-surgical infections has fell over recent years, however there is a rising risk of non-surgical HAI that produce serious complications in patients. To counter this, increased sensitization and cleanliness routines can be implemented, but this does not address the issue at heart. Patients are at risk when bacteria are transmitted between healthcare professionals, patients, and the environment.

Whilst increased infection prevention methods that rely on general cleanliness, hand-washing and other essential components of the staff-patient interaction can help reduce bacterial transmission, this does not address the effect of hospital staff interacting with their non-living environment.

Preventing the transmission of bacteria between surfaces and other surfaces, or between humans and surfaces should form a fundamental part of infection control management within hospital settings. However, this can take many forms, but the most widely implemented can be the use of antimicrobial metals and antimicrobial metal-based coatings. These metals, such as silver or copper can be directly applied to surfaces, clothing, face masks or other pieces of equipment with relative ease and has become common practice for many manufacturers to produce alongside their non-coated garments (**lopez2022effectiveness**; **abazari2023fabrication**).

Not only can these metals be applied to garments given to patients or used by staff, but they can be applied to equipment found in many clinical settings (**birkett2022recent**). Birkett

et al. highlighted that across many different studies, copper-based coatings have been applied to equipment such as computer mice, hand rails on public transport, door handles, and even supermarket trolleys.

Clearly this work demonstrates the wide variety of applications for metal coatings across many different environments, however there is still a lack of uptake for the use of metal coatings in public healthcare. Many hospitals are still outfitted with standard stainless steel panels or door handles, which lack the benefits of antimicrobial coatings.

1.12 Limitations of existing clinical surfaces

Stainless steel does not naturally posses antimicrobial properties and is relatively easily scratched when in contact with other stainless steel items, allows the formation of peaks and troughs in which microorganisms can find refuge for prolonged survival.

As such, common pathogens such as the ESKAPE pathogens and watch list pathogens such as *Clostridoides difficle* and drug-resistant *Campylobacter sp.* (**tiwari2019post**), can reside on these untreated steel surfaces in between periods of cleaning. Thus a hospital policy including implementation of copper surfaces could improve infection prevention control (IPC) in clinical situations. There is an additional benefit of antimicrobial surfaces that require less maintenance and cleaning, thus saving on staff and resources that are in short supply, which is a major challenge in Global South countries. However, initial costs of manufacture and installation is perceived as a major barrier, the true cost-benefits are yet to be realised.

1.12.1 Lewis Acids and transition metal chemistry

In its most basic form, a Lewis acid is an elemental species which can accept two electrons, often referred to as a lone pair. Different transition metals retain different strengths of accepting these electrons from other chemicals in the environment, allowing the differentiation of transition metals into strong and weak Lewis acids (**PRITCHARDTHESIS**).

The strength of a metal as a Lewis acid differs across various metals, for example copper is traditionally a weak Lewis acid, whereas non-metallic fluorine is a strong Lewis acid with a high affinity for lone pairs.

This strength is partially determined by the radius of the atom, where elements such as copper, with an atomic radius of 135pm will have a weaker ability to take on lone pairs, compared to flourine which has an atomic radius of 50pm.

These metal cations when acting as Lewis acids therefore affect bacterial cells through the mechanisms outlined in figure 1.4.

Cellular membrane disruption and permeability occurs due to the binding of these Lewis acid metals to the complex protein structures, whilst also binding to the phosphate backbone on the cell's DNA cause conformational changes to the secondary DNA structure (**PRITCHARDTHESIS**).

It is worth noting that other metals, such as silver, mercury and cadmium are also Lewis acid metals, and that the dynamics of metals as surfaces when exposed to environmental elements such as oxygen and carbon can produce a surface varies in acidity as these environmental elements form more acidic or basic forms of the Lewis acid metals (**stair1982concept**).

1.13 Controlling infection transmission using copper

Despite proven antimicrobial activity, implementation of copper based antimicrobial surfaces in clinical settings has faced multiple challenges. The standard material used for hospital surfaces is stainless steel, because it is relatively strong, and does not corrode easily. Copper has been demonstrated to act as an antimicrobial agent in many forms (**schmidt2012sustained**), and with multiple mechanisms at play, however studies into the efficacy and usage in hospital specific environments are lacking, especially over prolonged periods of time.

Despite its clear efficacy, the uptake and implementation of copper based antimicrobial surfaces is fundamentally financially driven. This can often be seen as the first challenge for the widespread use of antimicrobial surfaces. Currently, small scale productions of antimicrobial surfaces drives the cost up, as with any form of manufacturing. Without investment from both the manufacturing side, along with the consumer side, companies are unwilling to spend additional funds to produce antimicrobial surfaces as products, should there be a lack of consumer demand.

Secondly, there is no demand from hospitals to take on antimicrobial coatings. Currently, hospital budgets are stretched and to implement antimicrobial surfaces across one single hospital may outweigh their justifiable returns. Hospitals may not see initial benefits from introducing

antimicrobial coatings, and the cost of doing so may make some trusts apprehensive towards their use. Making antimicrobial coatings, especially those with cheap and effective materials, such as copper commonplace is the second challenge.

Finally, the upkeep cost of many forms of antimicrobial coating is largely unknown. Some trusts have implemented plastic-based antimicrobial coatings, which possess indicator marks informing users when they need replacing, however the likelihood of this being replaced within a reasonable time-frame after their efficacy has worn down is low. Coatings will need to be cost-efficient and low maintenance, whilst also being able to withstand harsh cleaners or environments. Therefore cheaper but effective copper-based coatings provide a level of performance without high expense. Despite this overall longevity and maintenance is unknown and would need to be understood for each major type of coating used.

Cuprous ions as a tool for infection control have multiple reasons in favour of their use. Their activity is fairly broad spectrum, and their high surface to volume ratio means they have the ability to be active without requiring high concentrations (**foster2010antimicrobial**) and they work rapidly, with times as low as one minute (**santo2008contribution**; **santo2011bacterial**).

Copper can work in many ways to fight microbial agents, however there is no confirmed single method of action, different forms of copper treatment utilize different methods of antimicrobial activity, but key ideas include DNA denaturation, protein synthesis disruption and cell lysis through membrane disruption (as seen in figure 1.4).

The current school of thought is that the rapid killing of bacteria using copper relies on the Lewis acid nature reacting with the cell membrane causing increased permeability leading to cell swelling and osmolysis. Santo et al saw no evidence of membrane blebbing which would display signs of DNA damage or disruption (**santo2011bacterial**) and therefore, programmed cell death, nor did they see damage to DNA after short periods of time (such as damage to methyl or other functional groups), removing DNA damage as the immediate causes of bacterial killing (**santo2008contribution**; **santo2011bacterial**).

In ionic form, copper can utilize it's ionic charge and lack of atomic shielding to disrupt microbial cell membranes, causing perforations.

In Gram positive organisms, peptidoglycan deformation is most likely to occur at the D-Alanine, Pentaglycine, L-Lysine cross link that occurs and binds layers of N-acetylmuramic acid

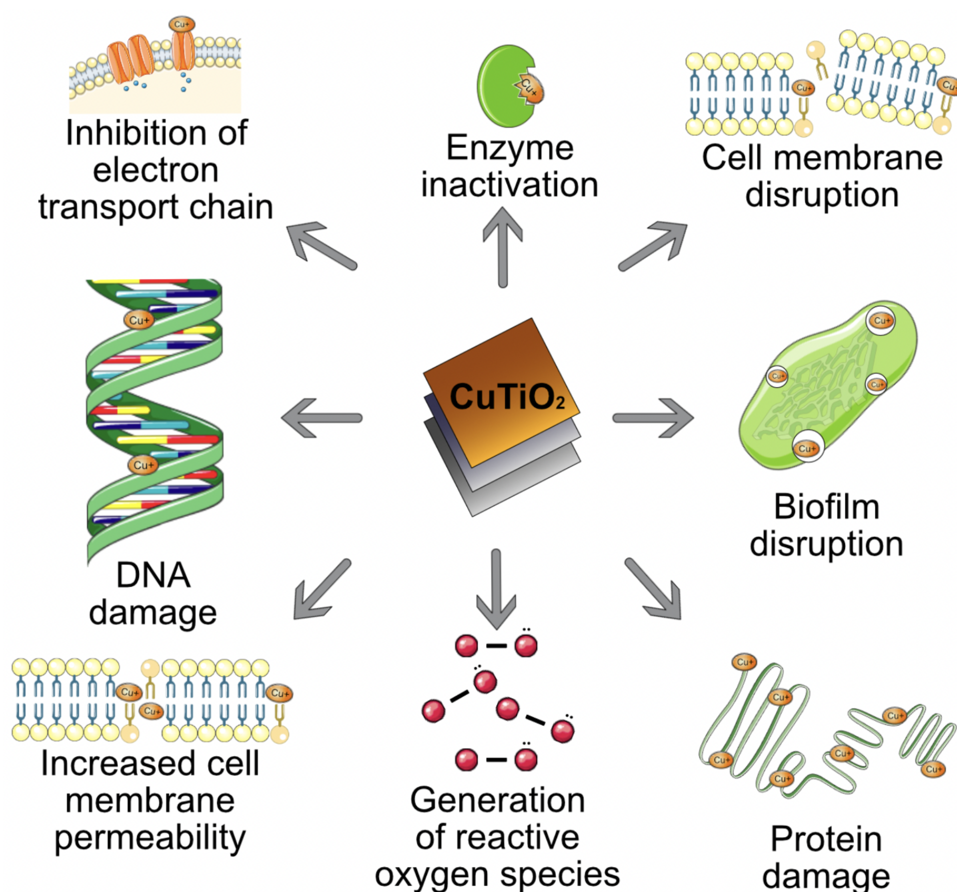


Figure 1.4: The general bacteriocidal and bacteriostatic mechanisms of $CuTiO_2$ including inhibition of bacterial proteins, DNA damage, membrane disruption and generation of reactive oxygen species. Copper can bind to the various structures in bacterial membranes both within and outside the cell, as such it can disrupt the electron transport chain which results in a lack of overall ATP synthase action and a lowering in cellular energy from this mechanism. In a similar fashion, copper particles disrupt both membrane formation and membrane stability through mechanistic binding of oxygen maintained within chemical structures. The removal of this oxygen causes membrane instability and a lack of bonding within the peptidoglycan resulting in deformed cell membranes and an increase in membrane permeability. In doing so key chemical structures are deformed due to the removal electronegatively charged areas. Similarly, the disruption of charged areas in proteins caused by copper ions can severely deform functional proteins and enzymes. This change in charge affects both secondary and tertiary structures of proteins and causes changes in conformational changes, likely affecting both structure and function. Enzymes which utilize oxygen and other electron donors as part of their binding site will also be significantly disrupted, producing problems with the critical shape for binding and disrupting overall electrochemistry. Due to all these mechanisms, overall biofilm production and maintenance is disrupted, affecting both the cells which produce the biofilm and by disturbing and deforming the extracellular matrix produced. Consequently, when in the presence of UV light, the $CuTiO_2$ surfaces produce reactive oxygen species which damage DNA causing both mutations and irreparable damage inducing cell death. These reactive oxygen species damage multiple aspects of bacteria providing multiple mechanisms of killing. Figure produced using the Adobe creative cloud.

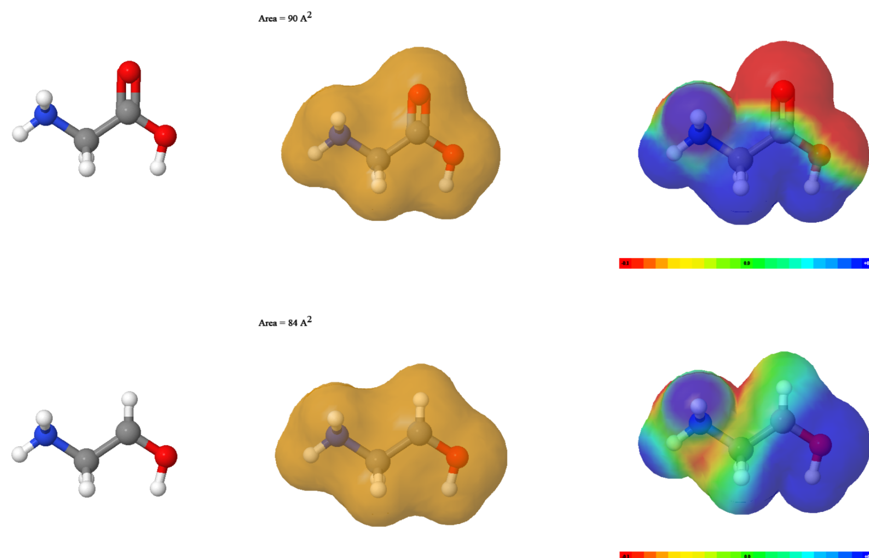


Figure 1.5: Molecular modelling of glycine with (top) and without (bottom) oxygen removal caused by the presence of copper 2+ ions in *Staphylococcus aureus*. The image displays molecule structure (left), surface area (middle), and electrostatic potential (right) and is built in ChemMagic. After loss of the pi-bonded oxygen atom, the molecule loses electronegativity in half of the molecule, which could cause problems during poly-glycine bonding, disrupting the peptidoglycan wall.

(NAM) and N-acetylglucosamine (NAG) together. A quick study of the molecules involved showed a reduction in overall area and significant changes in electrical charge of the molecules once the pi-bonded oxygen atoms were removed during the formation of copper oxides as seen in figure 1.5 when looking at glycine.

This mechanism is similar to that of lysostaphin, produced by *Staphylococcus simulans*, which targets the pentaglycine cross link and cleaves the C-teminus between glycine molecules (**barrett2012handbook**). It may be a factor that lysostaphin, produced by *Staphylococcus simulans* contains a high concentration of zinc, (reportedly 1 mol per unit of protein) which could act as a specific Lewis acid in forming ligands with the amine and thiol functional groups present within the glycine (**bochtler2004similar**).

The production of Zn(II) ions and the direct utilisation of these by the enzyme would cause breaks in the pentaglycine cross-links, therefore destroying the petidoglycan layer that surrounds *S. aureus*.

Copper, like Zinc, also has a toxic effect by damaging cytoplasmic proteins and enzymes

causing abnormal denaturation and reducing the cellular function of the bacteria at play. This again is through the attraction of copper as a chelating Lewis acid, which causes conformational changes in both secondary and tertiary protein structures.

To damage DNA, ionic copper generates a positive charge, this positive charge allows the copper to bind to, and disrupt the phosphate backbone.

The addition of titania, once activated with UV allows the formation of free radicals, which are known to damage DNA and DNA like structures even in the presence of protective mechanisms such as catalase and superoxide dismutase (**pigeolet1990glutathione**).

In fungal species, copper ions have been shown to disrupt mitochondrial function and induce the release of cytochrome C, beginning the caspase cascade, resulting in fungal cell death (**vincent2018contact**).

schmidt2012sustained studied the microbial burden (MB) of multiple sites inside hospitals, both pre and post infection control intervention through the application of copper to surfaces which were otherwise made up of wood, plastic or stainless steel (the common standard for hospital materials, purely for its ability to be autoclaved and its high tolerance to environmental damage) (**schmidt2012sustained**). Their work displayed that the implementation of copper to common hospital surfaces reduces overall microbial burden on both the direct surfaces, and the control surfaces by reducing overall transmission of bacteria from surface to surface (**schmidt2012sustained**).

borkow2004putting demonstrated that application of copper to fabrics could greatly reduce MB after two hours, demonstrating a key bactericidal effect against both *S.aureus* and *E.coli* after 2 hours (**borkow2004putting**). These copper infused fabrics (both in clothing and bedding) were shown to treat fungal infections in 50 individuals, displaying that the effect of copper is not restricted to bacteria alone (**borkow2004putting**).

Application of copper-impregnated surgical gowns and scrubs could assist in the prevention in the transmission of microbes from healthcare workers to the environment, and to patients (**dyer2019biocide**).

It has been demonstrated that copper has an antimicrobial effect on *Clostridoides difficile*, the common hospital pathogen, and that *C. difficle* can survive on hospital gowns and surfaces. Even when laundered, *C.difficile* spores were shown attached to the fibres, and were recoverable

to culture (**dyer2019biocide**).

It may well be possible that the use of copper treated clothing could be a potential method in significantly reducing the transmission of bacteria, fungi and viruses in hospital settings.

Reviewing surfaces in preventing the transmission of infection, **cortes2020use** established that copper has a prominent anti-viral activity against human respiratory viruses, with efficacy shown against many strains of influenza (such as H1N1, H5N1, H2N2, and H9N2 for example) and other human viruses, like SARS-CoV, and Human Immunodeficiency Virus.

Cortes later established that work by **van2020aerosol**. showed a reduction in transmission and infectivity of SARS-CoV-2 after 4 hours of exposure to copper as no visible virions were detected.

Copper clearly has a strong potential as an antimicrobial application to reduce microbial survival on surfaces. However, literature surrounding its activity over a prolonged period of time is limited, **schmidt2012sustained** assessed the activity of copper over 43 months, implementing the copper-based solution after 23 months.

Most studies utilize hybrid materials, such as Copper-Silver coatings, or brass alloys, which lack the independent activity of copper, and focus on costly joint materials, or without justification. Copper and silver both have antimicrobial effects, but lack hardness, meaning they tarnish, scratch and degrade faster than other metals (**bryce2022antimicrobial**). These studies also do not consider the use of silver, and the high price point associated with the utilization of this material (**natsuki2015review**).

1.13.1 Historical knowledge of copper's antimicrobial properties

The development of practical solutions to prevent the survival of bacteria on surfaces has exploited the antimicrobial activities of several metals, including copper, which have been known since the Bronze age with evidence of use as food and water storage to prevent spoilage in Ancient Egypt (**lucas1927copper**).

In fact, copper has been a staple of human life since the stone age being originally discovered in 9000 BCE, and one of the oldest copper artifacts being a copper pendant found in northern Iraq, dated to 8700 BCE, its widespread abundance was a fundamental component in its utilisation

in early tools and utensils. Copper smelting had been perfected by Neolithic standards by 3300 BCE, as Otzi the Iceman was found with copper tools.

Copper also had medicinal uses, with Ancient Egyptians and Ancient Indians using copper in medical applications. In ancient India, copper was used as part of traditional Ayurveda with surgical instruments and antiseptics being made from copper and copper alloys. Even drinking out of goblets made of specific metals such as gold, silver or copper were described in a series of detailed instructions for medical procedures in the ancient medical handbook contained within the Sashruta Samhita (**SushrutaSamhita**).

This metal possesses structural and chemical properties that are desirable to work with, being electrically conductive with good conductivity, despite being soft. The properties of copper however, depend on the size of the material. Long and thin extrusions of copper provide flexibility, without being too strong meaning it is easily manipulated into wires and flat shapes.

Copper is naturally antimicrobial as it is a Lewis acid of moderate activity. However, despite its application copper maintains an antimicrobial activity in any form through the use of charged ions and other mechanisms (see figure 1.4).

Multiple antibacterial and antiviral actions of copper have led many to purpose it as a major antimicrobial component of surfaces in public areas to reduce the risk of infection transmission. This has been successfully applied as micron thick layers on surfaces which directly target bacterial and viral viability with good results in vitro (**foster2012antimicrobial**; **foster2010antimicrobial**; **ditta2008photocatalytic**). The bonding of Cu to both metal, and borosillicate glass has been achieved in various configurations using flame assisted chemical vapour deposition (CVD) to distribute copper particles onto surfaces as a thin layer. These surfaces can later be installed in high touch areas (such as windows, doors, lifts, and hand rails for stairs) (**ditta2008photocatalytic**).

Since copper displays high antimicrobial activity, it could play an important role in strategies to tackle AMR by improving infection prevention. Combining copper with titanium would allow for a dual-action, multi-mechanistic antimicrobial surface, which would potentially last 10 years before needing replacements (Even inside the human body) (**valea2017titanium**). However, *in situ* assessment of these surfaces in point of care environments are yet to be undertaken to assess their activity and longevity.

Therefore it would be highly beneficial to investigate the implementation of copper-titanium

surfaces in hospital settings, with a view to then extend the materials out to public transport solutions. However there still remains several questions about its longevity and practicality for broad applications in public spaces.

1.14 Unknowns in the world of copper as an antimicrobial surface

1.14.1 Activity time

One key publication in the use of copper as an antimicrobial surface is based on work done by **santo2011bacterial**. Their work compared the efficacy of copper in both a liquid and a dry environment representative of potential uses of copper.

The initial work on wet copper surfaces displayed that copper ions released from the pure copper diffused into the buffer medium whilst also providing some form of contact killing for cells adhered to the surface. This was marked by a thousand fold difference in cuprous ions between stainless steel surfaces in buffer, and copper surfaces in buffer (**santo2011bacterial**).

Despite the leaching of copper ions into the buffer, **santo2011bacterial** showed wet copper surfaces provided a decline in colony forming units from 10^9 *E. coli* recoverable cells to no viable cells within four and a half minutes.

Testing *E. coli* cells on dry copper surfaces, **santo2011bacterial** found that copper ions accumulated up to 5.1x10^9 inside the cells providing lethal damage in one minute (**santo2011bacterial**).

Santo had already displayed the efficacy of copper in killing *E. coli* within one minute prior whilst evaluating the potential contributing co-factors involved (**santo2008contribution**), however, this is the effect of pure copper, which would have two large areas of activity rather than a single sided surface which would be found in real world applications.

1.14.2 Effect of copper surfaces on bacterial communities

Despite the obvious use for copper or copper coated materials to replace stainless steel in public environments, the lack of uptake has prevented research into investigating the effects of copper / copper coated surfaces on bacterial loads and the overall makeup of bacterial isolates which may be found on surfaces.

Currently little information is available on how $CuTiO_2$ surfaces shape communities of bac-

teria, potentially having more of an effect on species which are less likely to harbour antibiotic resistance genes, or metal resistance genes. Aside from the development of metal resistance or antibiotic resistance, the effects of $CuTiO_2$ on biofilms and early log-phase bacterial communities in the environment are not known.

$CuTiO_2$ could compeltely disrupt biofilm formation which would limit bacterial colony growth and inhibit the transfer of mobile genetic elements which spread antibiotic resistance. This has been explored in the context of medical devices which are used directly with patients (**tran2014antimicrobial**), however there is little knowledge on the effects of $CuTiO_2$ surfaces on biofilms. Currently there are no existing timelines for the effects of $CuTiO_2$ surfaces or how they might change the communities which eventually reside on them.

1.14.3 Length of stability, wear & tear and exposure to potential cleaning chemicals

Currently, no literature exists discussing the effects of various cleaning chemicals being used on $CuTiO_2$ coated surfaces. It is therefore important to determine whether common cleaning solutions of various compositions have a prolonged effect on the quality of the $CuTiO_2$ coatings. It may be possible that exposure to chelating agents, quaternary ammonium compounds and bleaches may have an reductive effect on the efficiency of killing, and potentially disrupt the structure of the coating.

Some research has been produced by **bryce2022antimicrobial** however, these used copper-impregnated surfaces and spray on coatings bound with nickel. **bryce2022antimicrobial** suggested that these coatings be replaced every three months, which would increase the overall cost, unlike $CuTiO_2$ CVD coatings which may last significantly longer.

1.14.4 Activity of $CuTiO_2$ surfaces over time in hospitals

Currently, $CuTiO_2$ surfaces have only had preliminary trials *in vitro* and in a single public environment. This preliminary trial displayed effectiveness against both Gram positive and Gram negative organisms in culture, but both were still detected on the surfaces in the public environment.

Hospital environments have also displayed large scale ertapenem resistance and a dominantly Gram positive bacterial community, however these surfaces are not representative of a hospital

environments where pathogens are especially virulent.

Some species may also be restricted to just the hospital as it is often rare to see *C. difficile* infections outside of a healthcare environment.

2 Aims and Objectives

This work therefore should primarily begin to address the suitability of chemical vapour deposition produced copper and titania coatings. Coatings should also be tested with their secondary UV mechanisms activated to ensure that bacterial killing still occurs to confirm the presence of the dual-killing mechanism.

$CuTiO_2$ coatings should be evaluated both in the lab and in the environment. Insights should be gained into how long the proposed mechanisms of action take to inform later testing.

Therefore the overall objectives outlined for this project are as follows:

1. Determine the efficacy of $CuTiO_2$ surfaces against artificial bio-fouling in the laboratory

2. Assess the speed of bacterial reduction to influence future work, provide insights into the mechanism(s) at work, and to provide information for downstream optimisation and usage of $CuTiO_2$ surfaces.

3. Place $CuTiO_2$ surfaces in a real world environment to establish the feasibility of their use in a public and clinical setting. Sampling of these surfaces in a real world should provide insights into any species which may persist or survive, allowing future downstream optimisation and research.

The results of this study will provide valuable insights into the potential benefits of $CuTiO_2$ coatings for use in both public and clinical settings, and could inform the development of new coatings with refined bactericidal qualities to reduce the transmission of infectious agents.

3 Materials and Methology

3.1 Bacterial Strains

Cultures of *S. aureus NCTC8532*, *E. coli 01210* and *P. aeruginosa PA01* were revived from -80°C stocks using nutrient agar plates, incubated at 37°C and were subcultured until plates showed no contamination and a single uniform morphology.

3.2 Liquid Media

3.2.1 Nutrient Broth

Nutrient broth powder (14.03g) (Thermofisher scientific CM0001B) was added to deionised water (500mL) and gently stirred using a magnetic stirrer plate. Once fully dissolved, the mixture was aliquotted into 100mL bijoux bottles after which all bottles were autoclaved at 151°C for a minimum of 15 minutes.

3.3 Solid Media

3.3.1 LB agar

Luria-Bertani (LB) broth powder (12.5g) (Neogen LAB169) was added to deionised water (500mL) and mixed thoroughly until dissolved. Bacteriological agar (5g) was then added and mixed until dissolved prior to autoclaving at 151°C for a minimum of 15 minutes. Autoclaved LB agar was then poured into sterile petri dishes.

3.3.2 10% Blood agar

Deionised water (500mL) was mixed with Columbia blood agar base (20.5g) (Neogen NCM0031A) and autoclaved at 151°C for a minimum of 15 minutes. After autoclaving, the agar was cooled to 40°C and defibrinated horse blood (50mL) was gently added to the mixture before being slowly rolled to facilitate thorough mixing.

3.3.3 Nutrient agar

For all experiments requiring nutrient agar, Nutrient agar powder(14g) (Neogen NCM0033A) was added to deionised water (500mL) and autoclaved at 151°C for a minimum of 15 minutes. After sterilisation, agar plates were produced using a sterile serological pipette to ensure plate volumes were 25mL as outlined by EUCAST standards (**EUCASTMANUAL**).

3.3.4 Agarose plates

Agarose (50g) was added to deionised water (500mL), the 10% agarose mixture was stirred using a magnetic stirrer, and gently heated to 40°C to facilitate dissolution. The mix was then autoclaved at 151°C for a minimum of 15 minutes, before being dispensed into empty petri dishes using a serologocial pipette to dispense 25mL volumes into each dish.

3.3.5 MacConkey agar

MacConkey agar powder (25g) (Neogen NCM0017A) was added to deionised water (500mL) and gently stirred using a magnetic stirrer plate. Once fully dissolved, the mixture was autoclaved and dispensed into petri dishes ensuring a 25mL volume.

3.3.6 Mannitol salt Agar

Mannitol salt agar powder (55.05g) (Neogen NCM0078A) was added to deionised water (500mL) and gently stirred using a magnetic stirrer plate. Once fully dissolved, the mixture was autoclaved and dispensed into petri dishes ensuring a 25mL volume.

3.3.7 C.L.E.D agar

Cystiene lysiene electrolyte deficient agar (C.L.E.D), (18.1g) (EO Labs MED1328) was added to deionised water (500mL) and gently stirred using a magnetic stirrer plate. Once fully dissolved, the mixture was autoclaved at 151°C for a minimum of 15 minutes and dispensed into petri dishes ensuring a 25mL volume.

3.3.8 *Pseudomonas* specific agar

Pseudomonas agar base (24.3g) was added to deionised water (500mL), this mixture was mixed until large clumps were broken up. 10mL of glycerol was then added to this mixture and gentle stirred before autoclaving.

After sterilisation from the autoclave, 1 vial of modified cfc x108 supplement was added. After gentle mixing, this media was then aloquotted into empty sterile petri dishes using a serological pipette to a 25mL standard volume.

3.4 PCR Primers

16s rRNA sequencing was conducted using primers targeted at the V3-V4 variable region originally designed by **klindworth2013evaluation** using the following sequences: Bakt 341F: 5'- CCT ACG GGN GGC WGC AG -3' and Bakt 805R: 5'- GAC TAC HVG GGT ATC TAA TCC -3'

3.5 Buffers

3.5.1 1x TAE buffer

50X TAE Buffer (Thermofisher scientific B49) was diluted 1:49 with deionised water.

3.5.2 Phosphate Buffered Saline (PBS)

5 PBS tablets (Oxoid BR0014G) were placed into deionised water (500mL) and stirred using a magnetic stirrer until the tablets had fully dissolved. The solution was then autoclaved at 151°C for a minimum of 15 minutes, prior to use.

3.6 Methodologies

3.6.1 Surface production using Chemical Vapour Deposition (CVD)

Deposition of TiO_2 occurred using Atmospheric Pressure CVD (thermally heated) and CuO via Flame Assisted CVD. Stainless steel squares were cut with surface areas dependant on use. For environmental sampling, wide rectangles with an approximate area of 6000mm^2 were used. For

Table 3.1: Layout of surfaces on mounting boards and the various chemical compositions and base substrate used.

$CuTiO_2$ Borosilicate glass	$CuTiO_2$ Stainless steel
CuO Borosilicate glass	CuO Stainless steel
TiO_2 Borosilicate glass	TiO_2 Stainless steel
Control Borosilicate glass	Control Stainless steel

transfer testing squares were cut with an approximate $600mm^2$ area whilst for live/dead analysis, an area $400mm^2$ was used in order to fit within 12-well plates used during microscopic analysis. Similar sizes were also cut from borosillicate glass.

Squares were then loaded onto a small bi-directional conveyor shelf which passed under a print head. Copper(II) sulfate was boiled within a round-bottomed flask using a heating mantle and connected to the print head via insulated and heated pipework maintained at 200°C.

As the squares passed under the flame of the print head, the copper(II) sulfate vapour was passed through assisting in the dispersal of the vapour across the stainless steel or borosillicate glass surface. The cut squares were then passed through under the print head. This process was repeated until a charted number of passes had been completed.

This process was then repeated with a liquid titanium component to replace the copper(II) sulfate.

Coated squares were then measured using digital microscopy to study the general thickness and variance the number of passes had on coating thickness, deposition and quality. Some surfaces were subject to thicker marginally coatings than others due to an increased number of passes of copper oxide passes (**foster2012antimicrobial**; **foster2010antimicrobial**; **ditta2008photocatalytic**).

3.7 Construction of surface sampling boards and locations

Four boards were produced containing 16 surfaces of various configurations of coatings. These surfaces differed in base material (glass or 304 brushed stainless steel), number of copper oxide passes and number of titanium passes. A summary of the layout and configuration of each surface is outlined in table 3.1. These boards were placed in the Gender neutral toilets, Peel building, University of Salford (displayed in figure 3.1.

Figure 3.1: In-situ photograph of surfaces placed in the gender neutral toilets, mounted vertically to the wall. Surfaces were mounted to the back boards using a strong adhesive paste, and were mounted near a hand drying vent.

3.8 Sampling of coated surfaces in situ

Surfaces were swabbed once using E-Swabs (Copan Diagnostics), the swab was saturated with amies media contained within the sample tube prior to swabbing (as this was found to be more effective by **afshinnekoo**). Surfaces were then swabbed for thirty seconds in a horizontal fashion, and then in a vertical fashion for thirty seconds as described by EN ISO 18593. Swabbing was conducted from edge to edge as to cover the entire sampling area. After sampling the swab was snapped at the break point and kept in the respective E-swab tube of amies media. Samples were then labelled and placed into a secure box for transit, and remained on ice until arrival at the laboratory.

Surfaces were samples once a week, for four weeks, producing 32 swabs.

3.9 Environmental isolate extraction for identification

Swabs were vortexed for one minute and the amies transport medium transferred to a sterile microcentrifuge tube. Tubes were centrifuged at 15,000 rpm for three minutes to form a pellet. The excess amies transport was removed (Approximately 700µL), leaving 200µL of liquid in the tube along with the pellet.

The pellet was then re-suspended via vortexing and half the suspension was pipetted (100µL) onto 10% blood agar and spread evenly before being incubated overnight at 37°C (a minimum of 12 hours). Colonies with unique and representative morphology's were then picked and patched onto a second 10% blood agar plate using a 57-grid reference pattern. These plates were then incubated overnight at 37°C (a minimum of 12 hours).

3.10 Long term storage of bacterial isolates

50% Glycerol stocks were produced of each isolate using one single colony grown on nutrient agar overnight and stored at -80°C.

3.10.1 Non-photocatalytic evaluation against *S. aureus NCTC8532* and *E. coli 01210*

The first approach to validating the effectiveness of $CuTiO_2$ surfaces utilised bacterial suspensions of each organism produced to a standard absorbance of 0.08-0.1 ABS at 625nm in a spectophotometer as recommended by EUCAST for disk diffusion assays. (**EUCASTMANUAL**). This suspension was pipetted (100μL) onto an agarose gel and spread evenly across the surface using a sterile L spreader. $CuTiO_2$ surfaces were placed onto the 5% agarose gels and left for sixty seconds to allow bacterial transfer and adherence.
After the initial sixty second contact with the agarose, surfaces were then left in a sterile petri dish for half hour time intervals (0 minutes through to 120 minutes). After the time interval, surfaces were then placed onto nutrient agar (Neogen NCM0033A) for sixty seconds, before being removed. Agar plates were then incubated at 37°C for a minimum of 15 hours, or overnight.

The second approach was replicated from **santo2011bacterial**. Using the same concentrations of bacterial cells as mentioned before, through use of a 0.5 McFarland standard, borosilicate glass and $CuTiO_2$ coated glass were placed onto 5% agarose gels which had been inoculated with the bacterial load. Surfaces were then removed after 60 seconds. One of each surface was then left for 2 minutes, 30 minutes and, 60 minutes before they were placed into ice cold PBS and beat with 20 glass beads to facilitate the removal of adhered cells.

The supernatant (100μL) was then pipetted onto a nutrient agar plate and spread with a sterile L spreader and incubated at 37°C overnight. Colonies were then counted using a colony counter.

3.10.2 Bacterial colony transfer

S. aureus or *E. coli* suspension (100μL) (produced to a 0.5 McFarland standard) was pipetted onto a 10 % agarose gel plate and spread with a sterile L spreader or a sterile loop. Once evenly distributed on the agarose plate, coupons of 304 stainless steel, copper-titania dioxide coated stainless steel, uncoated borosilicate glass, or copper-titania dioxide coated borosilicate glass, measuring 25mm^2 were then placed onto the surface of the agarose plate with sterile tweezers. No additional continuous pressure was applied to the surface, other than ensuring all four corners

were touching the agarose plate surface. After one minute of contact time, the square was lifted from the agarose plate and placed onto a nutrient agar plate and left on the surface for one minute to allow for bacterial transfer ensuring the side that was in contact with the suspension remained face down. After one minute, the squares were removed from the nutrient agar plate. Both nutrient agar and agarose plates were incubated at 37°C overnight.

Variable time points were used to establish the killing effect of the copper-titania coated surfaces by adding time between the inoculation of the surface and the placement of the surface on the nutrient agar. In this situation, the inoculated surfaces were left face up in a covered petri dish to prevent rogue contaminants coming into contact with the surface.

These experiments were conducted five times across both *S. aureus* and *E. coli*, each timepoint had a minimum of 3 coupons per repeat.

3.10.3 Colony counting and imaging

After incubation, plates were removed from the incubator, colonies were counted manually with the assistance of a colony counter (Stuart Science). Images of plates were taken using a Syngene GBox Chemi 16 and GeneSys software.

3.10.4 Baclight Live/Dead staining of coated surfaces

Live/Dead staining was used to produce images displaying cells with intact and deformed or broken membranes (Live/Dead BacLight bacterial viability kit L7007; Invitrogen). Utilization of two contrasting DNA stains: a green- fluorescent SYTO 9 stain and a red fluorescent propidium iodide stain. Propidium iodide has the ability to enter cells with damaged membranes, showing cells which have ruptured, or non-intact cell membranes caused through a variety of mechanisms. This can then bind to the DNA inside and cause a red fluorescence. However, SYTO 9, the counter stain can traverse the membrane of both living and dead cells, meaning it stains DNA in cells that are both alive and dead.

A reduction in SYTO 9 fluorescence is seen in the presence of propidium iodide, therefore showing dead cells with damaged membranes as red, and live cells as green.

However, due to the nature of image capture, the Leica DMi8 utilises separate channels for each wavelength before overlapping images, meaning cells with damaged membranes may appear

as red or yellow due to surrounding cells providing a green fluorescent output (a combination of red and green wavelengths overlapping in the final image output, and the ordering of these images may cause problems with differentiation between live cells and dead cells).

Therefore, differentiation of live bacteria with intact membranes can be done by viewing green fluorescence, while bacteria with damaged membranes fluoresce red. Cells lacking red fluorescence can therefore be deemed as alive or with intact membranes at the time of staining.

Cells were removed from surfaces as described by **santo2011bacterial** using a bead beating method however 2mm glass beads were substituted with 5mm glass beads (due to supplier availability).

For staining, supernatant cells were transferred in 100µl of 0.9% NaCl, and 1µl of the BacLight Live/Dead stain. This mixture was left in the dark for 15 minutes and then 1µL transferred onto a glass slide before being covered with a glass cover slip, and examined by fluorescence microscopy.

Images were captured at 400x magnification and 1000x magnification under oil immersion using an inverted confocal microscope (Leica DMi8). For SYTO 9, the filter cube used a filter at 485nm, and for propidium iodide, the filter changed to 493nm. Images were then captured under constant fluorescence using Leica Acquisition Software (LASX - Leica). One image per time point was captured using the automated timer function included with the Leica Aquisition Software.

3.10.5 Post processing of Live/Dead images

Post image capture processing was conducted in Adobe Lightroom Classic (Adobe Creative Cloud, Adobe USA) to remove background noise and enhance optimal contrast between red and green channels.

Images were split into respective red and green channels using the split channels command in ImageJ. Thresholds of colour were normalized using built in ImageJ standard normalization with ignore black turned on. Channels were then analysed for pixel grouping, size, and abundance (using the measure command) which gave a total count of absolute white pixels in the image, and the average size of pixel grouping, representing the amount of green and red fluorescence.

3.11 DNA extraction, purification and PCR

DNA extraction and purification of bacterial isolates was carried out using Powersoil Pro Kits (Qiagen) according to manufacturers instructions, however final elution utilised 50µL of nucleotide free sterile water for better optimisation of downstream applications.

Extraction was attempted on amies media from samples, however DNA quality and quantity was too low to provide usable samples for identification.

Post PCR cleaup was conducted using Sigma-Aldrich GenElute PCR cleanup kit (NA1020-1KT, Sigma-Alrich). Final Samples were diluted in 50 microliters Of PBS and then stored at -20°C.

3.12 Quality checking of DNA extraction

DNA samples for quantification was conducted using the Qubit dsDNA high sensitivity quantification assay kit. Standards were produced according to the manufacturers instruction for use with a Qubit fluorometer 3 (Thermofisher scientific).

3.13 Preparation of strains sent away for external strain analysis

Isolates from week 1 and week 4 (n=7 & n=8 respectively) were revived from -80°C stocks onto nutrient agar and incubated overnight at 37°C. Plates were checked for potential contaminants, isolates which showed contaminants were subcultured again on nutrient agar until pure. Isolates from week 2 and 3 were not used in this study.

After establishing a pure culture of each isolate from the -80°C stock, all colonies were scraped from the agar plate using an L-spreader, and were placed into PBS (10mL) in a Falcon tube. The falcon tube was then gently vortexed to create an even bacterial suspension.

1mL of this solution was then put aside for spectroscopy where a 1:10 dilution in PBS was used for DNA quantification. Following the protocol outlined by Microbes NG, a pellet containing between $4x10^9$ and $6x10^9$ was produced and placed into the supplied DNA buffer. These were then sent away for whole genome sequencing by Microbes NG. DNA libraries were prepared using the Nextera XT Library Prep Kit and sequencing was conducted using Illumina sequencing platforms with a 2x250bp paired-end reads protocol producing a minimum of 30x coverage.

3.14 Kirby-Bauer disk diffusion assay

Nutrient agar plates were used to isolate pure cultures of all isolates. These were then diluted in PBS until a 0.5 McFarland standard was reached. Isolates were then swabbed using a lawn technique onto another nutrient agar plate ensuring whole coverage. Antibiotic infused disks (notably; ceftazidime, tobramycin, ampicillin, tetracycline, and ciprofloxacin) were then places equidistant apart and incubated overnight at 37°C. After a night of growth, zones of inhibition were read using a digital caliper, and were compared against the EUCAST V12 guidelines for reading zones of inhibition (**EUCASTMANUAL**).

3.15 Bioinformatics

Whole genome analysis were conducted by MicrobesNG (Birmingham, England) after providing approximately 50µg of pelleted bulk bacteria. DNA extraction and library prep was also conducted by MicrobesNG along with genome assembly and annotation.

Genome annotations were then studied for antimicrobial resistance genes were tested for using ABRicate through Galaxy (usegalaxy.org). Genbank (GBK) files were studied in SnapGene for genes of interest that may not be discovered through ABRicate due to its specific affinity to AMR genes which did not include drug-resistance proteins or metallo-resistance genes.

3.15.1 Phylogeny

Potential genetic relations were explored by putting sequences of taxon similar species into MEGA X using genetic 16s rRNA sequences trimmed to a similar length and studied using a most-likelihood application of phylogenetic analysis (**kumar2016mega7**; **tamura1993estimation**).

3.16 Calculating maximum cells per surface

To calculate the efficiency of transfer for the agarose plate transfer method, the maximum potential bacterial cells per millimeter squared of the test surface area was calculated using the following formula for round plates:

$$\left(\frac{\text{Inoculant CFU per } 100\mu\text{L}}{\pi \times \text{Plate radius}^2}\right) \times (\text{Test surface Area}^2) = \text{CFU per surface}$$

4 Validation of $CuTiO_2$ surfaces against standard laboratory strains.

4.1 Agarose transfer allowed enumeration of bacteria on surfaces

4.1.1 Introduction

Accurate inoculation of surfaces needs to be developed in order to reflect accurate contamination, therefore an inert media needed to be used to prevent the growth of any infectious organism. Therefore the agarose plates were used to hold bacteria whilst the surfaces were placed on to facilitate the adhesion of bacterial cells.

In this we see that using an inert surface such as agarose, facilitated the spreading of high densities of bacterial suspensions without causing growth or death. Surfaces could then be placed on the spread suspension to cover the entire surface.

After transfer and incubation, bacterial growth maintained a normal and linear pattern of transfer and repeatability, with the removal of an anomalous repeat, all plates maintained similar recovery rates of 68% of the initial bacterial load which could be successfully transferred off the stainless steel surface.

4.1.2 Aims and objectives

This experiment aimed to demonstrate that the agarose plate transfer method can successfully and accurately transfer bacteria without increasing or decreasing the growth of bacteria applied to the surface.

4.1.3 Results

Using control 304 stainless steel and an inoculum calculated to 1 x 10^8 CFU/mL, transfer of colonies produced an estimated 1101 CFU per $25mm^2$ surface used. The recovery of each surface averaged 753 CFU $\pm$ 353 CFU. These plates were imaged (figure 4.1) and showed the distribution of liquid when in contact with agar. Excluding a single anomalous result (D1), samples were all within Log_{10} (0.5) with a minimum deviation from the estimated CFU per surface of 38 colonies

and a maximum deviation of 864 colonies. The average recovery of all surfaces was 359 CFU below the estimated maximum value of bacterial cells per 25mm surface (n=15).

Logarithmic counts were graphed to view any potential areas in variation from the testing or if any agarose plate had a significantly lower number of colonies (figure 4.1 and figure 4.4).

Colony counts between repetitions were then subject to statistical analysis in R. One-way ANOVA showed the means between surfaces were not equal ($p=0.109$), suggesting there is slight variation between agarose plate repeats. Pairwise t-testing analysis revealed a significant difference between repeat D and repeats A, B and E, (p values; A= 0.03, B= 0.009, E=0.009)

CFU counts after one-way ANOVA were plotted on a fit vs residuals graph to study homogeneity of variances and identify key outliers which may be removed from further statistical testing (figure 4.3a). The study showed a reasonable and linear model however, points 10, 14 and 15 presented as outliers. Linearity was confirmed by a normality residual plot which showed high homoscedasticity (figure 4.3b) and linearity along with homoscedasticity was still maintained after the removal of outliers. After removal of the outliers, one-way ANOVA showed that all surfaces had statistically similar colony counts ($p=0.0045$).

4.1.4 Discussion and conclusions

Testing showed that the agarose plates did not increase the number of bacterial cells recovered on the surfaces as all counts were below the estimated maximum calculated count per surface, and that this methodology of artificial biofouling can replicate incredibly high density bacterial loads. Statistical analysis showed that the method results in a normal distribution of colony counts, and that experimental error and variation can be minimised and identified, resulting in accurate transfer of bacterial cells.

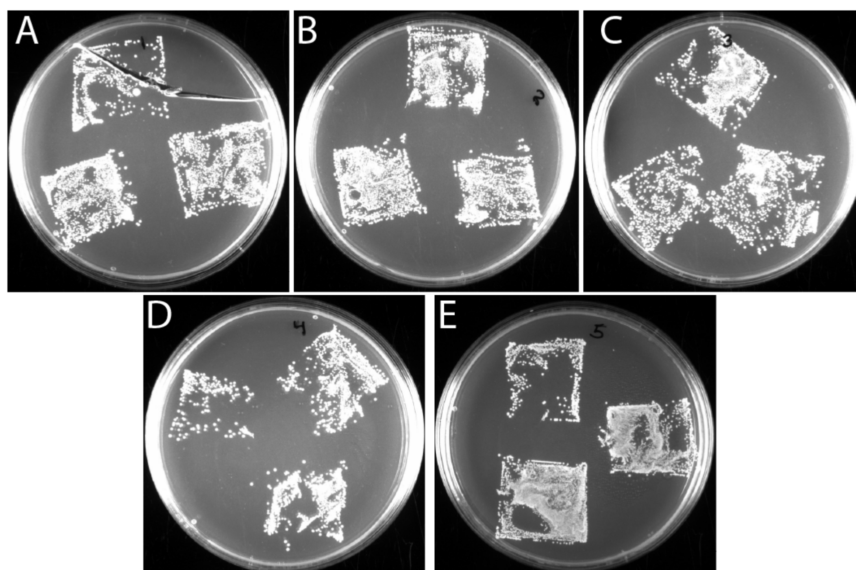


Figure 4.1: Colonies generated using the direct transfer method and an inoculum with 1 x 10^8 CFU/mL presenting with the shapes of the original surface material. These plates also show that the surface tension of the trapped liquid does not spread through to the center of the surface (as seen in figure 4.1, D and E) caused by the inability of the surface to be pressed onto the surface of the agar plate without tearing the surface.

4.2 Copper surface coatings resulted in fewer recoverable bacteria

4.2.1 Introduction

Validation of copper surfaces has already been conducted by **santo2008contribution**; **santo2011bacterial** using both a culture based approach, followed by an investigation into the potential mechanism of action using BacLight Live/Dead staining. Their work provided an established peer-reviewed methodology which allows for a direct comparison between $CuTiO_2$ surfaces produced using CVD, with pure copper surfaces.

Due to the high cost of pure copper, and ineffective idea to replace existing stainless steel surfaces with those of pure copper, it would be ideal to slowly phase in a transition from regular stainless steel surfaces into CVD coated $CuTiO_2$ surfaces, which would be more cost effective and more durable than a pure copper surface.

It first would need establishing the efficacy of $CuTiO_2$ surfaces to determine their levels of activity compared to the pure copper shown by **santo2011bacterial**. It would also be useful to gain an insight into whether the coating has any difference when applied to borosilicate glass, or

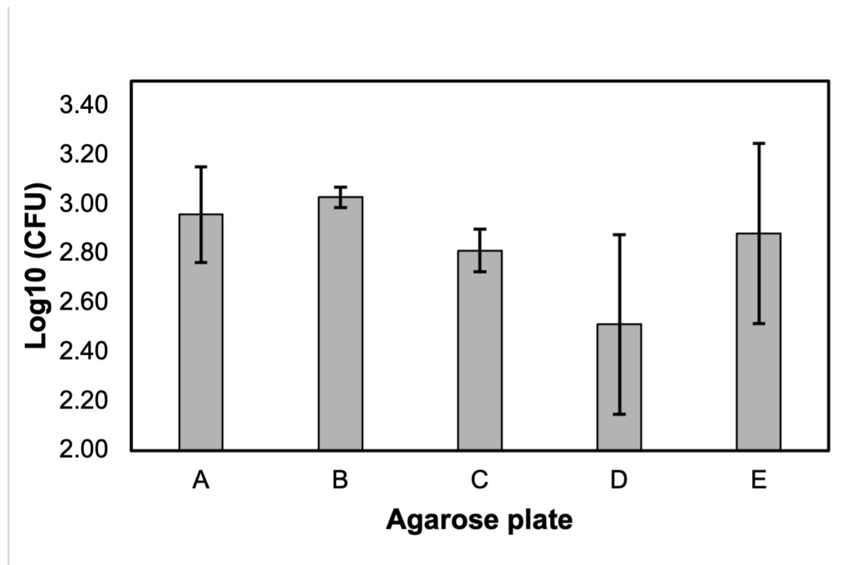
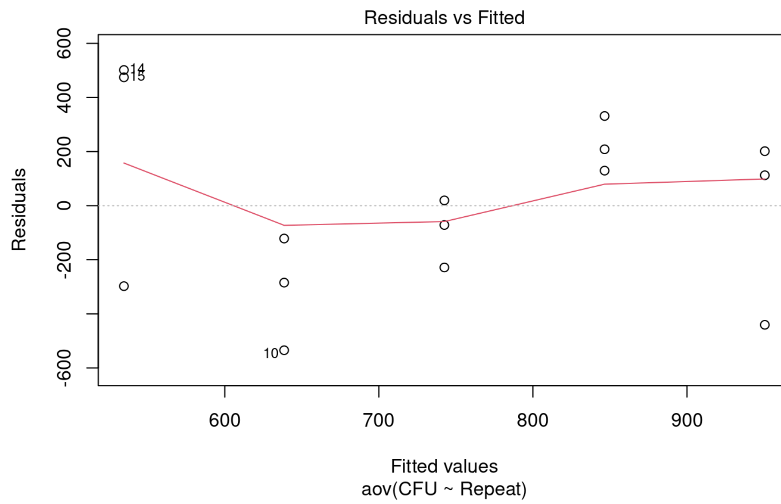
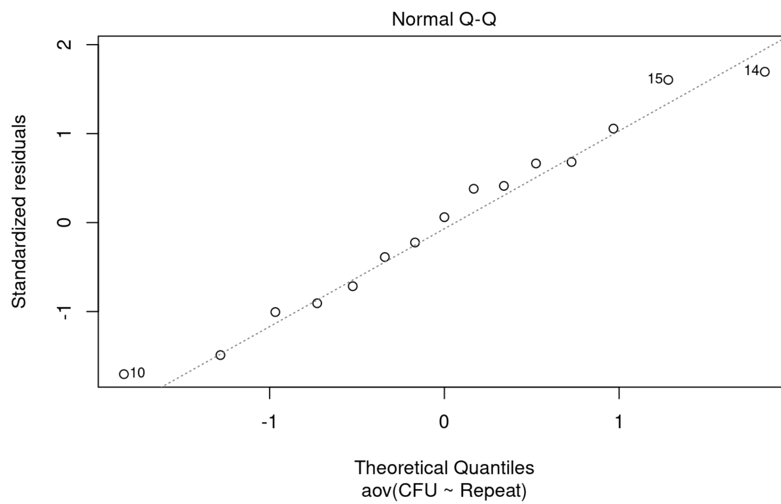


Figure 4.2: Mean logarithmic graphing of colonies recovered from a 1 x 10^8 CFU/mL inoculum across 5 different groups of stainless steel transfer tests. Each letter refers to a trio of 304 stainless steel inoculated with bacterial culture and placed onto an agar plate for one minute before being removed. Aside from repeat D, results remained consistent across all other repeats. Removal of outliers from sample D showed similar colony counts across all surfaces ($p=0.0045$). Error bars show the standard deviation in colony counts within the corresponding trio of agarose transfer counts.



(a) Homogeneity of variances analysis conducted in R.



(b) Normality plot of residuals from the 304 Stainless steel transfers.

Figure 4.3: In (a), the red trend line stays relatively central implying a linear relationship between each trio of surfaces. Outliers are seen at points 10, 14 and 15. (b) shows points 10, 14 and 15 as outliers but all other results fitting within a normal level of variance against a 45 degree reference line.

to 304 stainless steel whilst also establishing rates of killing.

After repeating the bead beating methodology with $CuTiO_2$ surfaces, one minor substitution in methodology was made, as the use of 5mm glass beads were substituted instead of 2mm glass beads due to supplier availability, however, it is unlikely that this provided any significant differences.

Once established that $CuTiO_2$ surfaces did possess an antimicrobial activity using this same method, it is vital to assess how fast this mechanism works. Work by **santo2008contribution**; **santo2011bacterial** suggested that the effective killing time of pure copper surfaces is approximately 1 minute. However, other studies in the literature suggest killing times closer to one hour (**grass2011metallic**; **luo2017killing**; **fowler2019effect**).

This therefore employed the surface transfer method stated previously, utilizing time points to assess bacterial loads after an initial coating with bacteria.

It would be a useful comparison to assess the killing time of $CuTiO_2$ surfaces compared to the pure copper surfaces to establish if they still possesses the same rapid killing.

This is shown to occur when using the agarose transfer method effectively, however the surface finish of various material substrates had an effect on the viability of bead beating as an extraction method.

Whilst using the bead beating methodology outlined by **santo2011bacterial**, only 304 stainless steel maintained a high bacterial density, even un-coated surfaces, such as plain glass prevented a high level of bacterial recovery due to its surface smoothness. Coated surfaces provided fewer bacterial cells recovered both through their surface qualities and the antimicrobial coating.

After assessing the killing effect of $CuTiO_2$ surfaces, it was apparent that significantly less bacteria survived past the 60 minute time points ($p=0.0055$), which was also echoed across all time points when compared to un-coated substrates ($p=0.0093$).

4.2.2 Aims and objectives

This experiment aimed to determine surface killing effects using an established method set out by **santo2008contribution**; **santo2011bacterial**. Afterwards, the aim changed to further use the agarose transfer method, as it provided the best results for creating bio-fouling and transferring bacteria. Antimicrobial activity has already been established by **foster2012antimicrobial**

however this has not been tested using an existing methodology for comparison.

4.2.3 Results

To determine if there was a difference in the killing effect of various coatings, time-point surface transfer was conducted on all 304 Stainless steel and borosilicate glass with different coatings. Acting as a control, 304 Stainless steel transferred the most bacterial cells and displayed no significant killing effect. The average number of cells transferred across was 13.3 CFU (n=18). Uncoated borosilicate glass transferred colonies with a standard deviation of 1.5 CFU, similar to that of the $CuTiO_2$ glass which had a standard deviation of 2.3 CFU. Copper oxide glass presented with a standard deviation of 0, the same as titania coated glass. $CuTiO_2$ coated stainless steel transferred colonies with a standard deviation of 0.57CFU, whereas 304 Stainless steel transferred the most colonies (24 CFU) out of all tests and repeats, and had the largest standard deviation at 9.2CFU.

ANOVA of different surfaces revealed a statistical significance between grouped means ($p=0.01$), and upon further review, plotting of the residuals and normalisation suggested that the only outliers were related to the 304 uncoated stainless steel substrate. Aside from these values, all other test results displayed an even level of linearity with no major variations aside from the 304 uncoated stainless steel.

Pairwise t-tests highlighted statistical significance between 304 uncoated stainless steel and every other surface tested, however no other combination of surfaces when compared displayed statistically significant differences in colony reduction.

A paired t-test was conducted between reference borosilicate glass and $CuTiO_2$ coated glass, significance was found at 0 minutes and 60 minutes ($p=0.021$ and $p=0.0055$ respectively), across all time points these surfaces displayed a significant difference between uncoated borosilicate glass and $CuTiO_2$ coated glass ($p=0.0093$).

Further to this, logarithmic reduction calculations were conducted showing the level of reduction from the inoculum. After calculating this $CUTiO_2$ surfaces displayed a 7 fold Log_{10} reduction and a 6.5 fold Log_{10} reduction at 30 minutes and 60 minutes respectively. This conforms with the guidelines set out in ISO EN 22196 regarding what constitutes an antimicrobial surface.

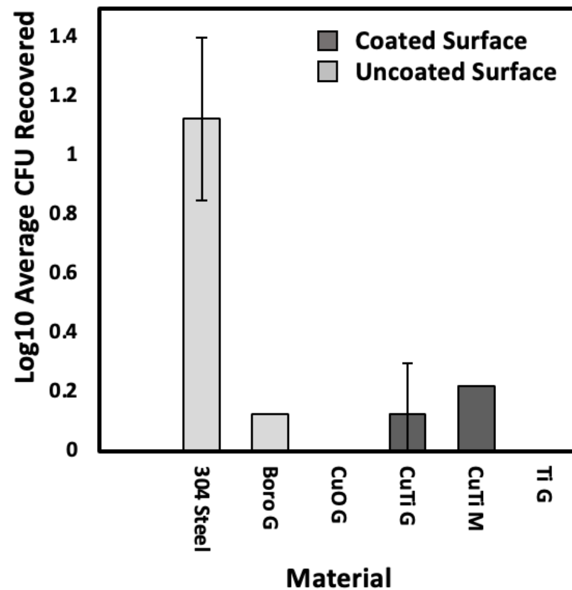


Figure 4.4: Logarithmic counts of colonies recovered from various surface coatings (n=15) on 304 stainless steel and borosilicate glass substrates. Error bars represent the standard deviation of the Log10(CFU) recovered from coated and uncoated surfaces. X axis labels refer to the substrate base (G/M) for glass or metal. This indictaes whether borosilicate glass or 304 stainless steel were coated. The coating is denoted with either CuO (copper oxide only), CuTi (copper and titania dual coating) and Ti (titania only coating). No error bars are present for $CuTiO_2$ metal, copper oxide glass, borosilicate glass and titania glass due to the low standard deviation present when producing the logarithmic calculation. These error bars are too small to display, or resulted in negative values. This figure displays a clear difference between 304 stainless steel and all other substrate surfaces used. Coated metal provides a bactericidal effect whilst the use of a glass base utilises a polished surface to prevent easy bacterial adhesion.

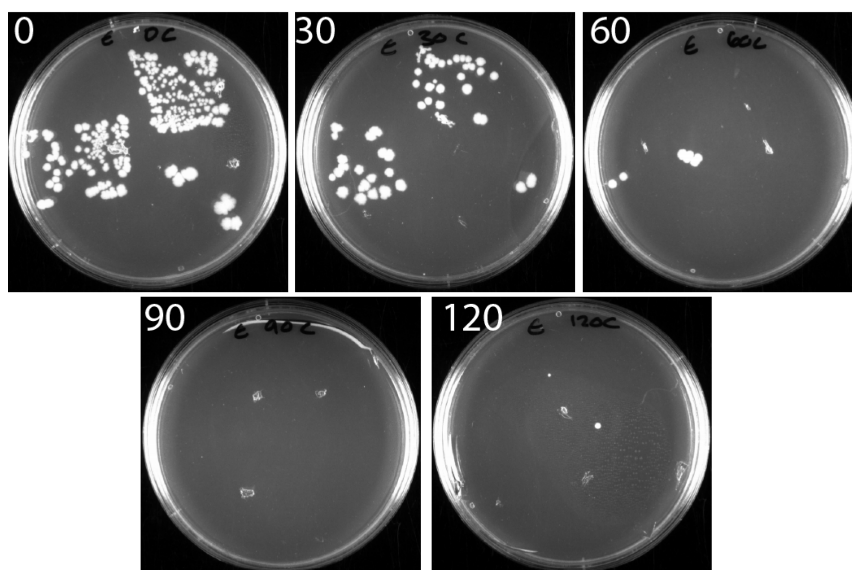


Figure 4.5: Colonies produced using the agarose transfer (n=15) method on $CuTiO_2$ surfaces between zero and two hours at thirty minute incubation time points to allow for bacterial killing. Individual colonies can be identified and traced to show the general shape of the $CuTiO_2$ surface used to inoculate the agar. It is apparent that as the length of contact between $CuTiO_2$ surface and transferred colonies increases, overall bacterial survival decreases.

4.2.4 Discussion and conclusions

Due to the similarity of results between the borosilicate glass and the coated surfaces, it is thought that the variability in the quality of the steel surface aids in the adherence of bacterial cells. The peaks and troughs of the surface provides additional surface area, whilst the borosilicate glass has a relatively smooth surface which limits overall surface area. The overall smoothness (ranging from 50Å root mean square to 20Å root mean square) can prevent bacterial adhesion and therefore would contribute to the overall lower transfer of bacterial cells, especially using this methodology. This is ultimately shown as a low transfer rate between borosilicate glass, $CuTiO_2$ glass, and TiO glass, all of which are present here. Highly polished stainless steel (often for architectural purposes), are categorised as '2k' often have a maximum root mean square of 5Å, however the type of stainless steel used during this test is categorised as '2G' which is finished with a 180 grit sandpaper.

This finish introduces a surface roughness in a unidirectional pattern which would produce an uneven surface finish with plenty of peaks and troughs for bacterial colonies to adhere to.

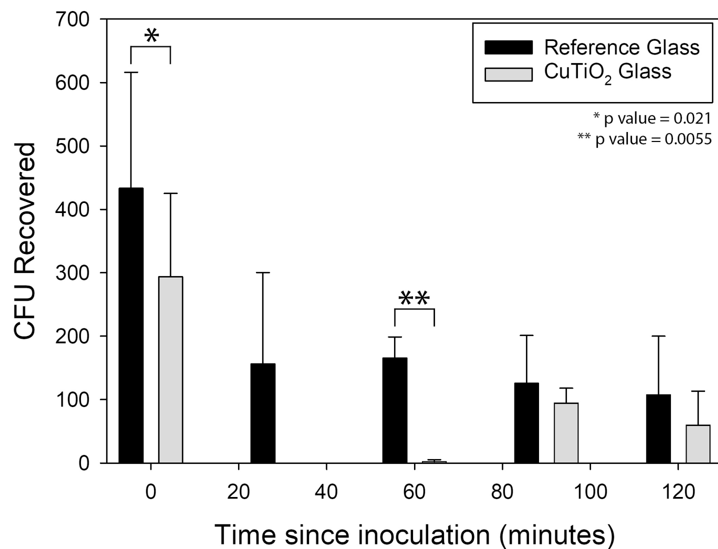


Figure 4.6: CFU counts (n=15) of colonies recovered from the agarose transfer method using a high density suspension of *E.coli* across various time points on both reference borosilicate glass and $CuTiO_2$ coated glass. This data displays the high initial transfer of bacterial cells on reference glass at the zero minute timepoint, before leveling off and remaining steady at each twenty minute time point afterwards. This is not seen in the $CuTiO_2$ coated glass, which sees a significant decrease in colony counts at the sixty minute mark as found using paired t-tests (, p=0.0055).

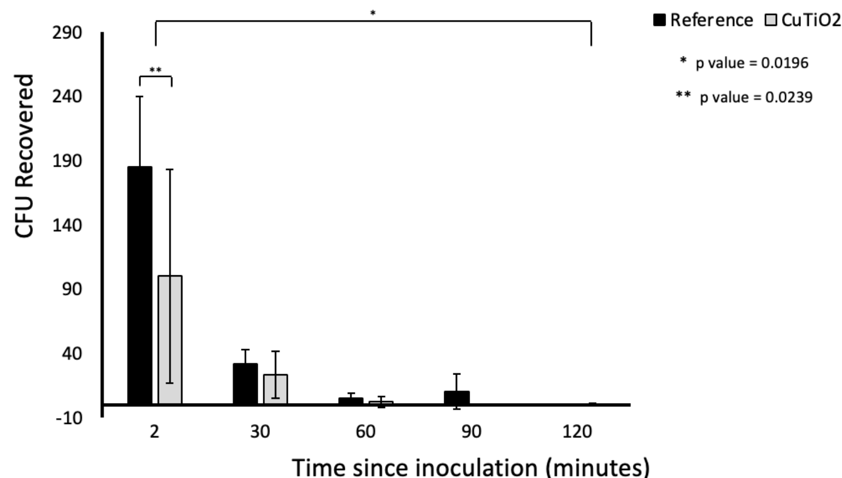


Figure 4.7: CFU counts (n=15) of colonies recovered from the agarose transfer method using a low density suspension of *E.coli* across various time points on both reference borosilicate glass and $CuTiO_2$ coated glass. Using a lower density suspension, overall bacterial recovery was lower than previous, but still showed a high rate of bacterial survival on reference glass than the $CuTiO_2$ coated glass, which paired t-tests showed statistically significant killing across all time points compared to the uncoated glass (p=0.096).

However, despite this, the data still suggests that there is a significance in the reduction of bacterial loads after inoculation between coated and uncoated metals, however more investigation is needed to distinguish between these effects on borosilicate glass.

Whilst $CuTiO_2$ coatings do display a reduction in antimicrobial activity, there is sometimes a level of re-growth after certain timepoints. This is often due to the dynamics seen within stationary phase bacteria, which is different to those in log phase.

Bacterial cells in stationary phase are not exponentially growing and as such protect their DNA through condensing the nucleoid through the up-regulation and increased presence of MrgA (**jaishankar2017molecular**; **morikawa2019happens**). As the $CuTiO_2$ coatings produce oxidative stress, through both UV activation, and the Lewis acid mechanism of copper, bacteria in the stationary phase with its condensed nucleoid would be better protected, and therefore potentially survive the extended timepoints. This could also happen real time, where during the waiting period between inoculation and transfer, the nucleoid is compressed and therefore some cells which survive are transferred onto the agar plate and allowed to grow.

This dynamic is also mirrored in the quantity of bacteria present during biofouling. High densities of *E.coli* shows an adequate level of killing, however more cells are in this survival phase and this, combined with the stationary phase dynamics allows survival and re-growth after certain timepoints.

In general, these stationary phase bacteria which have a high level of ability in the presence of adverse conditions are named 'persistor cells' and are part of the development of bacterial colonies in the stationary phase which are not present in the log phase.

Extended stationary phase cells often express growth advantage in stationary phase (GASP) phenotypes (**finkel2006long**) which would also explain the survival of cells in the presence of $CuTiO_2$ surfaces.

These mechanisms of survival and the focus of bacterial colonies can be seen in figure 4.8.

It would be apparent that within high density inoculations of *E.coli* there are enough cells in the stationary phase and later stationary phase, with enough persistor cells, that regrowth becomes apparent, which is not observed in low density *E.coli* suspensions. This effect is apparent when comparing figure 4.6 and figure 4.7.

4.2.5 Future Work

Further analysis on this method should be conducted, the surface smoothness does have an impact in reducing bacterial adhesion. Surface quality analysis and surface roughness should be mapped and correlated to bacterial recovery using the **santo2008contribution** & **santo2011bacterial** methods.

This work forms a fundamental basis for the efficacy of both the use of $CuTiO_2$ coatings, and for the use of the agarose transfer method as a way of accurately determining the antimicrobial efficacy of surfaces without producing errors due to uneven bio-fouling.

Further work should assess this method and coating in virtual environments which mimic closely, the effects of draughts, water sprays and aerosol movement around the surface which would lead to the dissemination of colonies, more akin to a real world application.

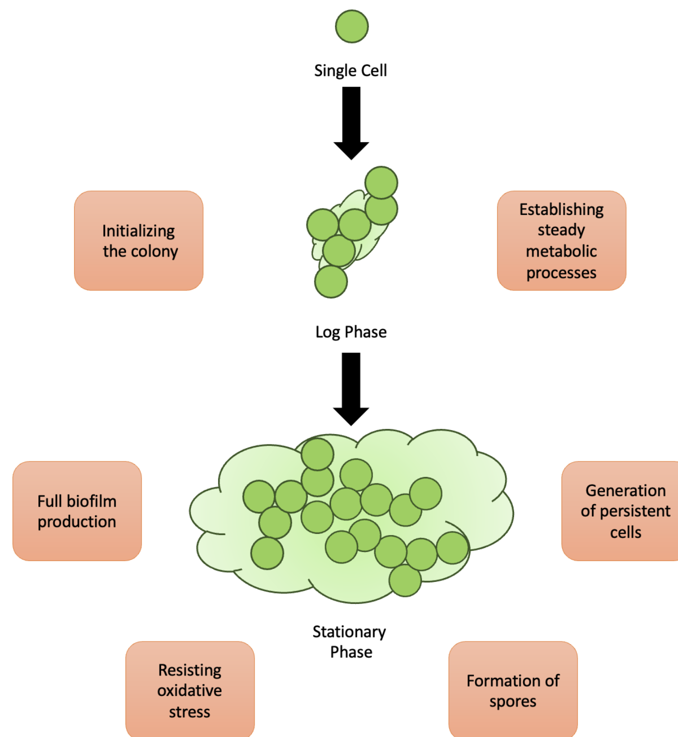


Figure 4.8: Schematic outlining the focus of bacterial colonies at different growth stages. As a single cell the focus is on replication and building the colony and starting the key metabolic processes that allow for increased survival, such as the formation of the biofilm. During the stationary phase, full and expansive extracellular matrixes are produced with a substantial biofilm, DNA is condensed and mechanisms of oxidative stress resistance are employed, persistor cells are generated, and spore forming bacteria will begin to produce endospores. Produced in the Adobe creative cloud suite.

4.3 Bactericidal effects of copper surface coatings can be visualised microscopically

4.3.1 Introduction

To determine the speed at which $CuTiO_2$ coatings could potentially kill bacteria, it was vital to conduct real-time analysis of samples. This would be incredibly hard to conduct using culture-based microbiology, and as such this is conducted using various forms of microscopy. With bacteria that are motile, arguments could be made to visualise their motility using phase microscopy until they die and they lose all motile activity, however this can often only be conducted in liquid samples which contain the antimicrobial compounds. To test this method on non-motile and non-liquid samples Live/Dead staining is used. Through Live/Dead staining, dead cells are stained with propidium iodide. This stain can enter through broken and disrupted cell membranes, aggregating inside the cell. The counter stain of SYTO9 is used due to the opposite excitation colour, but also due to the ability for it to enter cells with intact membranes. With this combination of stains, cells with intact membranes can be distinctly identified from those without, and assessments can be made to see if this changes over time due to the presence of an antimicrobial coating.

Initial Live/Dead staining was conducted on $CuTiO_2$ coated borosilicate glass using BacLight Live/Dead staining kit for microscopy (Invitrogen L7007), however excess liquid present on the surface even after removal of large volumes led to images displaying Brownian motion of live cells not adhered to surfaces (as seen in figure 4.9). This method was replaced by one previously developed by **santo2011bacterial**.

Having previously used **santo2011bacterial**'s methodology for surface transfer results, the next step was to use Live/Dead staining using the protocol also outlined. This allowed a better comparison and reference to work from but also highlighted some areas in which the **santo2011bacterial** method did not fully meet the criteria for the experiment.

This method also led the development of a new methodology which could only be completed on samples which used a borosilicate glass substrate base. This method followed the initial process outlined in the transfer plating protocol, but rather than plating these out after various incubation periods, inoculated substrates were stained using Baclight Live/Dead stain and placed

into the fluorescent microscope for visualisation. From this images were then captured for later post processing to identify the ratio of live to dead cells.

Having conducted 15 minute intervals of Live/Dead analysis on $CuTiO_2$ coated glass, cell death increased at every time point using the agarose transfer method of inoculation, which was not seen when using the original **santo2011bacterial** protocol.

4.3.2 Aims and objectives

This experiment aimed to confirm the antimicrobial activity of various surfaces and coating combinations using an existing methodology and a new adapted methodology, through the use of Live/Dead staining.

4.3.3 Results

After post processing with ImageJ (using the default parameters), the density of pixels corresponding to each colour were tabulated. The results for the Live/Dead staining assay are displayed in figure 4.10. Due to the nature of the experiment (the inability to reduce and remove brownian motion from the images) and post processing analysis, standard deviation for time points were not able to be calculated. Without further time to develop the methodology, pixel movement would contribute a large factor in the calculation of any deviations. Similarly, any repeats of this would have high levels of differentiation, even within the same sample where another group of bacteria could be images. The pixel counts from the live/dead assay failed to satisfy an Anderson-Darling test, and therefore was logged to base 10. The logged data failed the assumptions test as well as a Levene's test.

Despite following the **santo2011bacterial** method, no statistically significant difference was seen in the number of live or dead cells at each time point. However, there was a statistically significant difference in the average number of red and green pixels at each time point ($p=0.0054$) using the agarose transfer method. A pairwise t-test was used for further analysis of this resulting in no significance between any time point specifically.

A t-test found that there was a statistically significant difference between the percentage of green pixels between on the $CuTiO_2$ coated surface ($p=0.0013$) across the whole series when using the modified transfer method on $CuTiO_2$ coated glass. This significant difference therefore

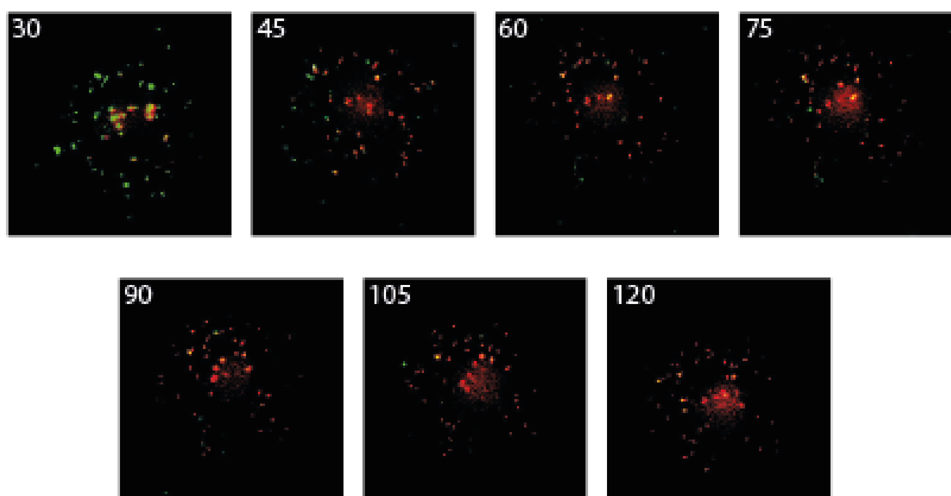


Figure 4.9: BacLight Live/Dead staining using SYTO 9 (both dead, and alive cells) and Propidium Iodide (dead cells only) to visualize cell death over a 2-hour period at 15-minute intervals. These images show the Brownian motion found when using the original Live/Dead staining method on $CuTiO_2$ coated glass, with bacterial cells rotating and aggregating in the center of the frame. Quantification of staining allowed for a basic classification of live/dead cells showing the change in pixel colour over time displaying a significant change from mostly green to red pixels on the $CuTiO_2$ coated glass. Images for each time-point were split into respective red and green channels. Thresholds of colour were normalized using built in ImageJ default normalization. These channels were then analyzed for pixel grouping, size, and abundance.

shows that as the time increased from 30 minutes to 120 minutes, the number of live cells (as depicted by the green pixel count, and percentage composition of the image) decreased, and that dead cells (red pixel count) increased indicating that killing occurred within the 120 minute period.

4.3.4 Discussion and conclusions

These results demonstrate that after 45 minutes, a significant reduction in live cells can be seen, which is fitting with work by Santo el Al. As described the overall trend showed a significant difference between live and dead cells, however some individual timepoints did not display a statistical significance prior to 90 minutes.

This method however, has its limitations where an excess of liquid can cause brownian motion which results in live cells being washed in and out of the frame. Depending on the level of green pixels in the image when it is taken can cause variation in the results. This could be limited by

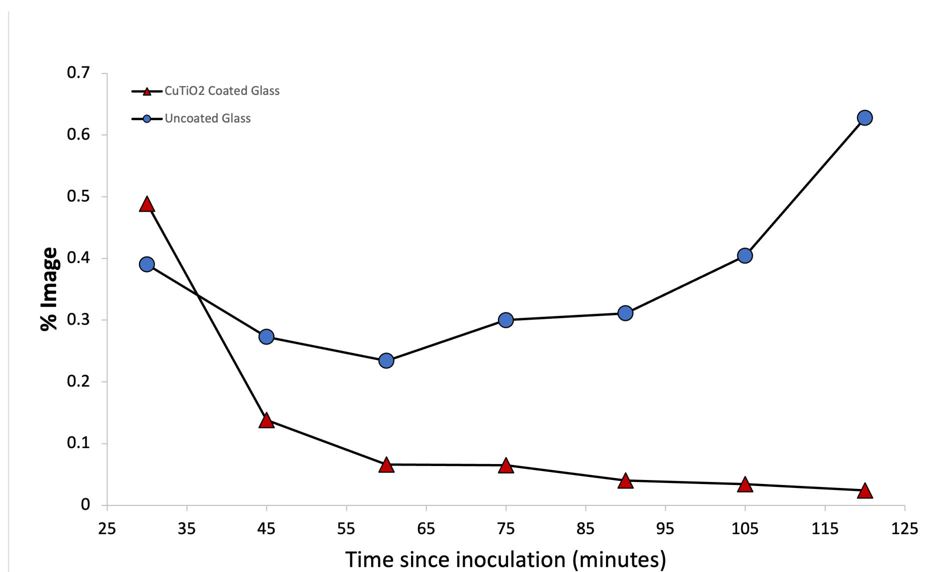


Figure 4.10: BacLight Live/Dead staining using SYTO 9 (both dead, and alive cells) and Propidium Iodide (dead cells only) to visualize cell death over a 2-hour period at 15-minute intervals despite brownian motion. After post processing, pixel counts were conducted and graphed as a percentage area of the total image. The $CuTiO_2$ borosilicate glass (red triangle) showed a reduction in bacterial load compared to the uncoated glass (blue circle). Testing began at 30 minutes due to the time taken between exposing the bacterial cells to the antimicrobial surface, removing them from the surface, followed by staining cells and placing them under the fluorescent microscope for imaging.

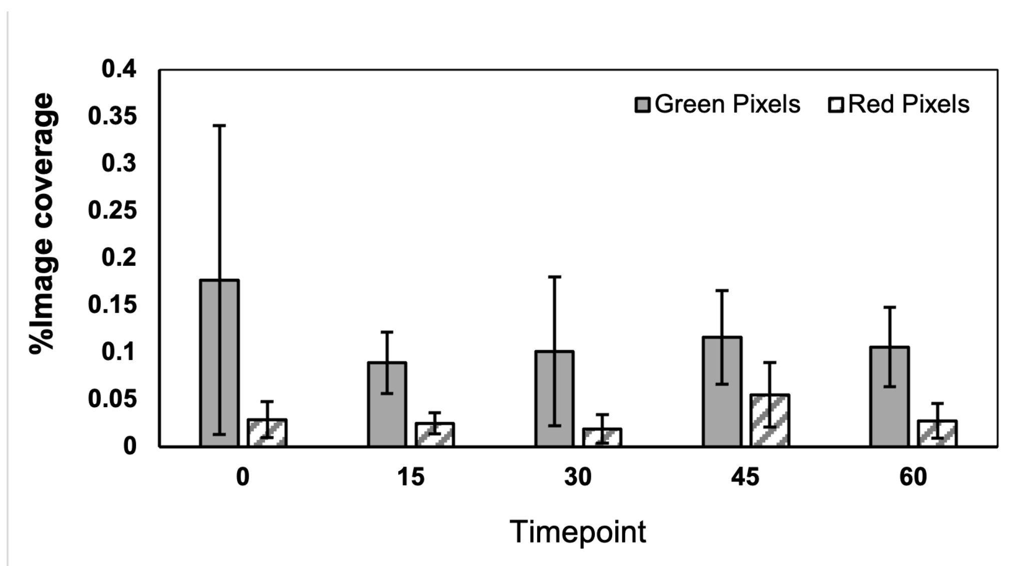


Figure 4.11: Percentage of the image covered by the two colour pixels used in live dead staining. Using the Santo method, it is clear that there is a constant level of green pixels through the time series and no significant change in the variation of red pixels. Error bars display standard deviations of each timepoint.

taking multiple images at each timepoint and utilizing the average to plot and test for significance.

After testing the **santo2011bacterial** method, we believe that it only works for surfaces that are rough in comparison to borosilicate glass. This experiment was conducted on coated borosilicate and un-coated borosilicate glass and presented problems with the extraction of cells from the surface. The method relies on a bacterial suspension being pipetted onto the surface, allowing a time for adherence, and then removal of the cells into a salt solution. This would cause a fundamental problem for the use of glass surfaces as they are extremely smooth compared to those of processed and manufactured metals. This low level of adhesion could be a significant factor in the lack of change between time points, as there were less cells present to convert from living to dead (as seen in figure 4.11). This also re-enforces the use of the agarose transfer method due to it's greater ability to transfer bacterial cells on borosilicate glass.

4.3.5 Future Work

After this experiment we believe it would be important to fully assess the mechanism of bacterial killing at play. Clearly there is a reduction in bacterial load compared to uncoated surfaces, but

this does not elude to the true mechanism of bacterial killing. Therefore future work should conduct a similar experiment and compare the reduction of $CuTiO_2$ surfaces with other known mechanisms such as acidic and basic attack, liquid antimicrobial compounds, lysis beads, and heat denaturation. This would potentially show whether the direct mechanism at play is similar to an existing mechanism.

However, we believe based on the timeframe studied, and the rapid killing of these bacterial cells, this mechanism cannot be related to DNA mutation or any modification of the genetics within the bacterial cell. This also prevents the theory that killing occurs during any phase of binary fission, such as disruption of the Z-ring or protein damage to *FtsZ*, the protein responsible for the production of the Z-ring used by bacteria to divide.

This killing time would also be too fast to fully account for complete metabolic shutdown of bacteria, it would be potentially a factor in long term exposure of bacterial cells to $CuTiO_2$, however I do not think that internal ionic concentrations of copper would reach a level significant enough to disrupt all the potential sites of oxidative phosphorylation.

I would therefore suggest that this mechanism is clearly responsible for membrane disruption, but this would need to be investigated further using Live/Dead staining, followed by scanning electron microscopy to potentially identify and visual disruptions to cell membranes, which would not be visible under standard microscopy.

4.4 UV activated Ti surfaces provided a mechanism of bacterial killing

4.4.1 Introduction

After establishing the antimicrobial activity of CuTi surfaces, where both the copper Lewis acid and free radical producing titanium are present, it was important to validate the efficacy of the titanium coating itself to establish whether it contributed to the overall antimicrobial killing of bacterial loads present. Whilst it is known that titanium is able to produce free radicals, its efficacy in this coating is only just being established.

After exposing Ti coated surfaces to UV overnight, time point analysis using Live/Dead staining was conducted between 304 stainless steel and 304 Ti coated steel. Using green pixels as a marker of live cells, UV activated Ti surfaces has s significantly reduced number of green pixels at every time point, meaning Ti surfaces once activated with UV provided a mechanism for high bacterial killing.

4.4.2 Aims and objectives

This experiment aimed to assess whether UV activation acts as a secondary mechanism in the killing of bacterial cells adhered to the coated surfaces. This could be an additional dual-mechanism to provide overall better killing for some bacterial isolates who may be resistant to copper or heavy metals.

4.4.3 Results

Pixel density analysis corresponding to each colour was analysed using the methods outlined above using ImageJ. A paired t-test found that there was a statistically significant difference between the percentage of green pixels (live cells) in the images ($p=0.001$) between UV activated Ti coated surfaces and non activated Ti coated surfaces across all time-points. The same significance ($p=0.001$) was found for red pixels (dead cells) , implying there was a significant difference in the means between activated and non activated Ti coatings ($p=0.0004$). By studying the decrease of green pixels, and the increase of red pixels, it can be determined that bacterial cells were consistently killed after exposure to TiO surfaces at all time points. The increase in red pixels demonstrates that this was not just a reduction in green pixels through experimen-

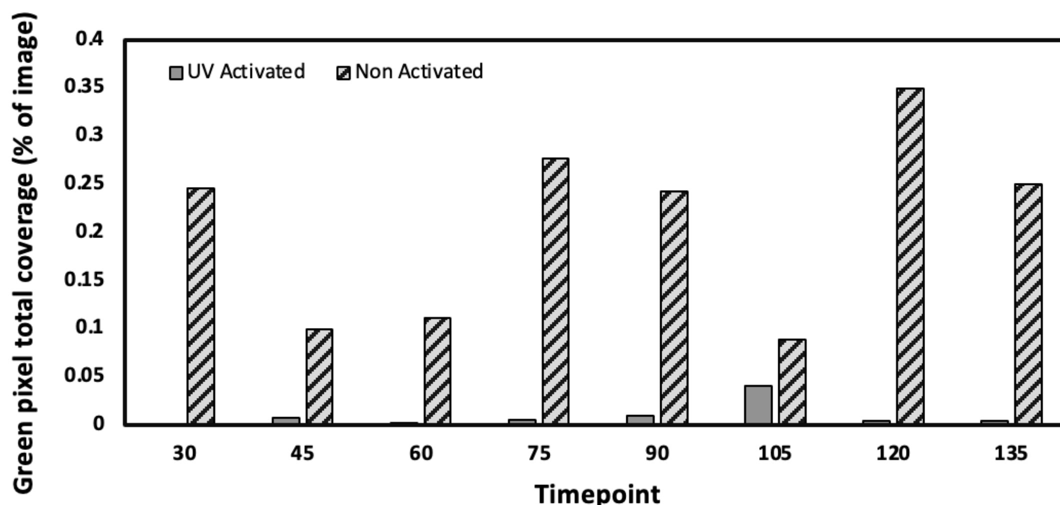


Figure 4.12: Graphical comparisons of UV activated and non UV activated titania coatings on 304 stainless steel after capturing live/dead staining images at fifteen minute time points. Green pixels act as an inference of live bacterial cells. No standard deviation is available as repeats of this experiment would not yeild similar enough results due to the different compositions of the images taken. Whilst multiple repeats displayed similar effects, overall green pixel coverage varied largely from repeat to repeat.

tal measurement, but that propidium iodide stain was actively passing through perforated and damaged bacterial cell membranes, and fluorescing. This shows that bacterial cells were dying actively whilst being observed and whilst being exposed to the free radicals produced by the titania coating.

Plotting this data showed a visible difference between the time points with UV activation and without UV activation (as seen in figure 4.12).

4.4.4 Discussion and conclusions

Ti coatings do show a substantial effect when activated with a high enough strength of UV and with a correct wavelength of UV. UV radiation in the 100nm to 280nm wavelength is often filtered out by the atmosphere, however this test was performed using a wavelength of 368nm with a small peak at 404nm, which falls under UV radiation that passes down to the surface.

It is important to note that the strength of the UV was also a key factor measuring $0.5 mW/cm^2$, however this strength of UV radiation is higher than normal UV radiation found in the environ-

ment and therefore efficacy of Ti surfaces could be much lower when normal environmental levels of UV radiation are tested. It should be noted that the UV irradiation was not put directly onto surfaces that had been subject to bacterial cultures and UV exposure was conducted prior to biofouling.

4.5 Effect of $CuTiO_2$ coatings on antimicrobial resistance

4.5.1 Introduction

If there is an end goal of implementing these surfaces in hospitals, it is also important to investigate whether they will upset the natural dynamics of the hospital resistome. If exposure to $CuTiO_2$ surfaces leads to fluctuations in the use of antibiotic resistance genes, or contrary, metabolic focus is driven towards protecting the cell from copper ions through the employment of the *cop* and *sil* operons.

It could be possible, that the presence of a $CuTiO_2$ coating promotes protective mechanisms against all forms of cellular stress, but it could also act in the opposite way, by causing damage to bacterial cells which allows antibiotics to work more effectively.

After recovering a survivor *E. coli* colony exposed to $CuTiO_2$ glass, and one from un-coated glass, these colonies were subject to an antibiotic panel consisting of 5 antibiotics with various mechanisms. Utilising the original strain as a third reference, the colony subjected to the $CuTiO_2$ coating trended towards being more susceptible to Ampicillin, Tobramycin, and Tetracycline that the colony recovered from the un-coated glass or the reference colony from the freezer stock.

4.5.2 Aims and objectives

This experiment aimed to take the sole survivor of one agarose transfer method experiment, which yielded one colony each on both the $CuTiO_2$ coated glass, and one colony from the uncoated borosillicate glass and put them through a Kirby-Bauer disk diffusion assay using standard antibiotic compounds. This was then compared to the original strain used in the inoculation, and against the EUCAST breakpoint table.

As a preliminary experiment, the overall objective is to elude to what effect $CuTiO_2$ surfaces have on antibiotic resistance profiles.

4.5.3 Results

This experiment displayed a potential trend towards increasing susceptibility to certain antibiotics after $CuTiO_2$ exposure. A significant difference was observed in this isolate in the total resistance profile (zones of inhibition across all antibiotics) against the surface-less control and

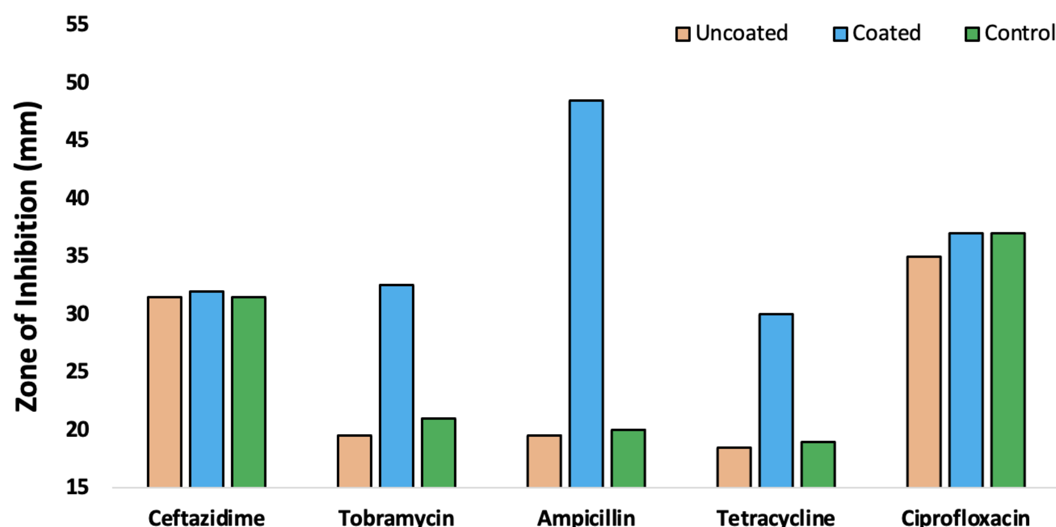


Figure 4.13: **Antibiotic profile of *E. coli* after antibiotic exposure.** Zones of inhibition of *E. coli* after exposure to a $CuTiO_2$ surface, control borosilicate glass, and a control without any surface (n=1). It can be seen that after exposure to $CuTiO_2$, isolates trend towards higher levels of susceptibility towards some antibiotics. As the number of tests conducted was 1, standard deviations are not calculated. It is likely that multiple repeats of this would fall within 1mm variance of each other due to the nature of repeated kirby-bauer disk diffusion assays.

the glass control (p=0.03 and p=0.02 respectively) and no significant difference between the glass control and surface-less control (p=0.43).

This isolate was more susceptible to ampicillin, tetracycline and tobramycin compared to the control and compared to the EUCAST standard.

4.5.4 Discussion and conclusions

This preliminary experiment does show some promise, if these trends are correct it could show a new mechanism in fighting antibiotic resistance. A combined $CuTiO_2$ coating can facilitate a synchronistic antimicrobial effect, with both traditional antimicrobials, and antimicrobial coatings working in tandem to prevent the spread of infection, or reduce the burden of infections in hospitals.

By using $CuTiO_2$ surfaces, it could be possible that infections contracted in hospitals are therefore more susceptible to first generation and second generation antibiotics, which are currently rendered ineffective. This could also prevent the redundancy of older antibiotics, which

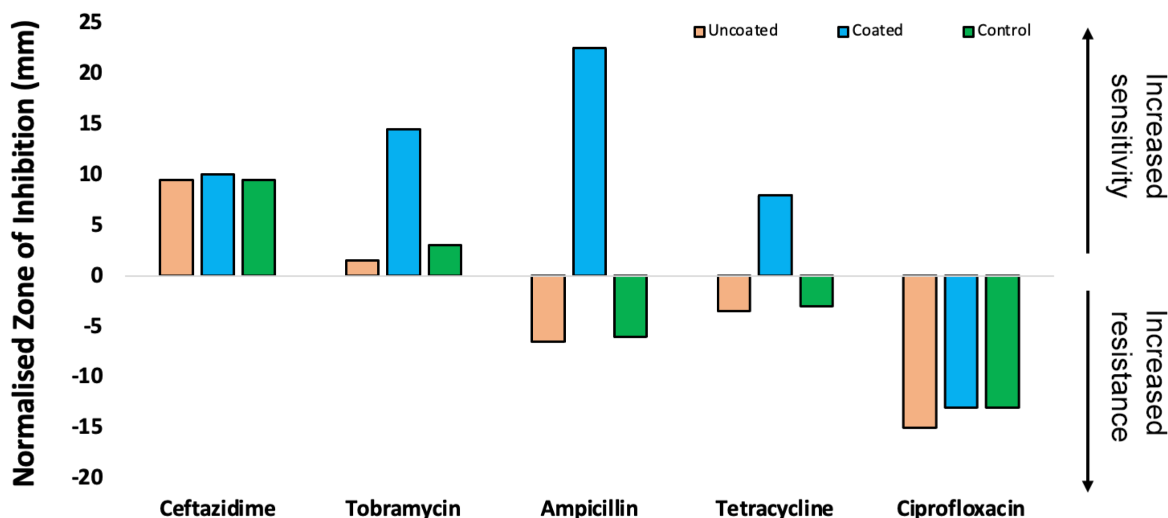


Figure 4.14: **Normalised antibiotic profile of *E. coli* compared to EUCAST expected values.** Normalised zones of inhibition generated by subtracting the value used for determining sensitivity towards each antibiotic. Positive values suggest more susceptible than the EUCAST species standard. As n=1, standard deviations are not calculated due to this being the difference between the expected EUCAST resistance value, and the acquired value for a *E. coli* control strain.

are seen as clinically useless, as the initial resistance mechanisms produced by bacterial species, such as target modification in penicillin-binding proteins is now largely rendered ineffective.

This could help prevent the rise in antibiotic resistance and flatten the curve by which antibiotic resistance is increasing.

This work however is on a single isolate of *E. coli* and therefore would need multiple repeats, and multiple species analysed to ensure that this mechanism can actually be employed. Different species of interest, such as other members of the ESKAPE pathogen group may have a different reaction to antibiotic exposure after exposure to $CuTiO_2$ coatings. It is possible that $CuTiO_2$ coatings provide a beneficial effect to antibiotics which rely on membrane disruption (such as ampicillin, and other Beta-lactam antibiotics), or the ability of the antibiotic to enter the cell, such as tetracycline. Specific antibiotics which rely on membrane damage, such as colistin, may also see increased efficacy, and a reduction in resistance as membrane integrity is already compromised.

If metabolic activities were disrupted by the $CuTiO_2$ surface, it could be possible that this

allowed the antibiotic in question to work more effectively.

Membrane formed pores caused by the disruptive copper would likely allow more antibiotic into the cell. For ampicillin, it is possible that these membrane formed pores would allow the beta-lactams to disrupt the function of the penicillin binding protein enzyme responsible for the cross-linkage of D-Ala-D-Ala complexes. However this is yet to be confirmed or investigated further.

4.5.5 Future Work

This work in future will need to be repeated with multiple repeats, across multiple species and strains to fully validate this preliminary experiment. As this experiment contained one single isolate, repeats are a must in order to be able to fully assess this potential mechanism of action.

5 Efficacy of $CuTiO_2$ surfaces of bacterial load reduction in a real world environment.

5.1 Introduction

Having validated the activity of CVD coated surfaces *in vitro*, the next step was to study these surfaces in the environment. In order to do this, boards comprising of various glass and 304 stainless steel surfaces with different coatings were placed in a gender-neutral public toilet at the University of Salford.

Initial sample recovery and antibiotic resistance data processing and analysis was conducted by (R.K), however Live/Dead staining & Scanning Electron Microscopy was conducted to validate the mechanisms of action along with 16s sequencing to get accurate identities of bacterial isolates was conducted to further validate the efficacy of these surfaces.

5.2 Scanning Electron Microscopy Imaging (SEM) showed amalgamation of debris

5.2.1 Introduction

Eight surfaces with various combinations of copper and titania were produced using flame assisted chemical vapour deposition (as outlined in table3.1). Once mounted to a wooden board, these surfaces werev then fixed to the wall of a gender neutral toilet. After four weeks these surfaces were removed and studied.

After a month of in-situ application, it was important to check on the coating applied to the surface and potentially look for areas of damage, corrosion, or significant bio-fouling. To do this, sections of the borosilicate glass based samples were cut from the original surfaces and provided for scanning electron microscopy (SEM).

Microscopic analysis showed areas of potential corrosion or staining as a result of water droplets from landing on the surface. Using SEM, nanoscopic analysis of the coating could be conducted and referenced to images taken after the coating had been produced, yet not exposed to the environment.

Notably, as these water droplets were studied, SEM images highlighted that where many droplets had fallen, there was a large number of debris found within those droplets. It was found that more debris was found within these droplets than outside, suggesting that this may be bacteria, transported through aerosol droplets.

This could also be explained as these surfaces were placed opposite a toilet (which are known for their ability to form microscopic water droplets containing bacterial cells), and next to a hand dryer, which could displace the water from the user's hands, and onto the coated surfaces.

By using SEM, bacteria sized debris could be identified in these droplet deposits, however there is no conformation as to whether these are bacterial cells or other forms of debris.

Scanning electron microscopy was carried out by the Salford Analytical Services, and false colouring was applied afterwards.

5.2.2 Aims and objectives

After a month in situ, this analysis aimed to check on the condition of the antimicrobial surfaces and check if any bacterial cells were present and identifiable.

In doing so this would allow us to determine if there were any significant problems with the durability of the $CuTiO_2$ coating, or if it was easily discernible to identify bacterial cells which managed to adhere and survive on any of these coated surfaces.

By comparing SEM images of the surface coating before and after environmental exposure, information regarding their real-world applications would also inform future experimentation. If SEM images showed high levels of damage or disruptions to the $CuTiO_2$ coating, further assessments could be made to improve this coating by any means.

Typically, SEM is used in the areas of antimicrobial coatings, to study if the composition and deposition of the materials have generated any interesting or unique properties or patterns. This is also the case if a coating protocol is designed and made to produce a desired topology, such as designing and producing titanium nano-spikes (**sjostrom2016bactericidal**; **mathew2023nanospikes**).

In studying the coating under SEM, calculations relating to particle size can be conducted and used to further improve antimicrobial coating development. In a similar fashion this can also be used to visualise surface roughness, which is a key factor in bacterial adhesion and biofilm

generation.

5.2.3 Results

These images provided a visual way to check on the quality of the surfaces. Large areas of the surfaces showed areas where water droplets have hit the surface which would otherwise be invisible. These droplets appeared as wide regions of darkened colour with soft edges, and showed no damage to the nanoscopic structure of the $CuTi_2$ coating. This could also be characterised by the technical staff operating the electron microscope. Within some of these areas, large clumps approximately 5um-10um can be seen below. Some of these have been coloured with false colouring to provide contrast. It is not yet clear whether these are bacterial biofilms or other forms of contamination.

The use of SEM also allowed us to understand the deposition of the coating and how that may be changed over time. Despite exposure to the environment, damage to the coating at a nanoscopic level pertained mostly to scratches within the glass surface itself which had also taken away part of the $CuTiO_2$ coating.

Upon analysis of these images, multiple spheroids were identified with an approximate size ranging from 8µm to 12µm and were not part of the original coating.

5.2.4 Discussion and conclusions

SEM imagery highlighted multiple instances of debris or bacterial cells, however no clear cell shape was observed that could be categorised as definitively bacterial and therefore potential bacterial cells were inferred based on shape and size. Shapes that were approximately 10 microns wide and roughly spherical with no sharp or defining edges were suspect to be bacterial cells, however this is only for qualitative analysis rather than quantitative analysis. Despite this, SEM showed clear areas where water droplets, invisible to the eye or under high magnification, were present from the use of the toilet, sink and hand dryer. These appeared as darkened areas as seen in the top right corner of figure 5.1b.

This reinforced the potential idea that bacterial cells were being caught in aerosol droplets and transferred onto these surfaces. Large numbers of potential bacterial cells and surface debris was contained primarily in these darkened areas as seen in figure 5.1b.

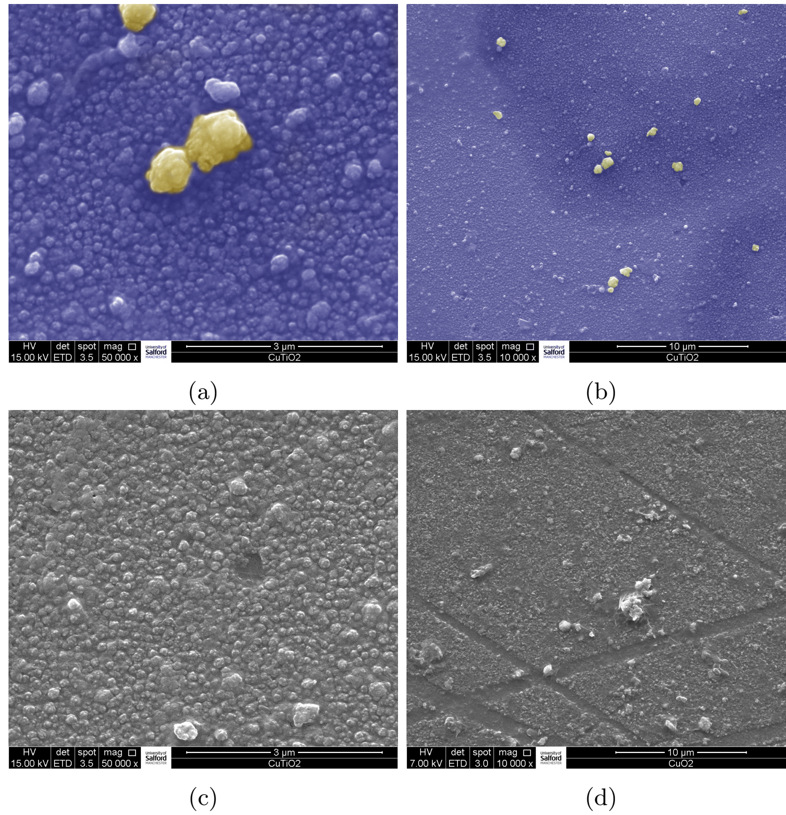


Figure 5.1: (a) False colouring of SEM with potential cocci based on size and shape present. (b) Bacterial or debris particulates (coloured yellow) on the surface on the CuTiO2 surface. The darker area is the remnants of liquid droplets landing on the surface and drying. (c) Standard CuTiO2 coating on 304 stainless steel under SEM. Individual islets of copper can be seen as nanometer sized spheroids. (d) Bacterial colonies and debris on CuO2 surface also showing damage to the coating done during removal.

It is well known that bathroom fixtures, such as toilets are rampant aerosol producers. Therefore it is not beyond reason to suspect that bacterial cells from the toilet or hand drier within the proximity of the wall-mounted surfaces would be the result of the use of both these amenities.

This gives additional reason for the use of $CuTiO_2$ surfaces for hospital bathrooms and toilets where multiple patients with various different microbiomes and infections, which could be passed to other persons within the hospitals through this mechanism.

From this circumstance, it would be expectant to see normal human microflora, such as *S. aureus*, which may be some of those bacteria sized objects identified on the false colour SEM. It would also be plausible to see other genera such as *Klebsciella spp.* and *Escherichia spp.*. Notably some patients in hospitals are overtly exposed to *C. difficle* which may also be seen in surfaces used within the hospital environment.

However, despite these common flora being prevalent, they must also contain copper resistance genes which facilitate their survival when exposed to high concentrations of copper ions.

5.3 Quantification of DNA from direct swab extracts of surfaces captured bacterial DNA

5.3.1 Introduction

After saving the swab used for sampling each surface, the remaining liquid after the initial extraction of bacteria, was used for the extraction of DNA from the surface. This would give a representation of bacteria that did not provide viable cells, and it is important to discover any bacteria which may not be cultured but may be present on the surface of the swab and therefore on the surface itself. By doing this it may lead to an understanding of bacteria which did not survive in the presence of certain metal concentrations and different combinations of metal.

5.3.2 Aims and objectives

The aim of this experiment was to successfully extract DNA from the swabs and use that to determine if any species are present but not cultivatable. To do this, it requires successful extraction of DNA from the swab, successful amplification of the 16S RNA gene and the successful sequencing of the 16S RNA gene. Therefore, the main objectives were to complete DNA extraction of the swabs and attempt to sequence any 16S RNA present.

5.3.3 Results

After extracting DNA using the Powersoil Pro kits, PCR of the 16S V3-V4 sub region were detected in all but one surface swab (n=31) as seen in figure 5.2. Whilst this confirmed the presence of bacteria, it did not confirm any viable species.

After cleaning up these samples, Qubit quantification was conducted, however all but two swabs were below the threshold for detection on the Qubit fluorometer using the high specificity kits, therefore sequencing of these isolates could not be completed.

5.3.4 Discussion and conclusions

Due to the lack of sequencing, it is therefore uncertain whether these bands produced were a product of un-culturable bacteria or DNA picked up by the swab, or by bacterial isolates that have already been established in culture. Further work could attempt to re-extract DNA from

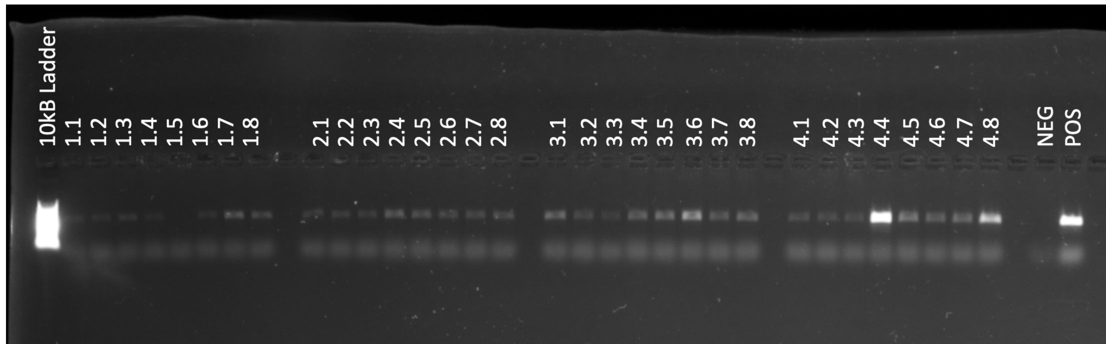


Figure 5.2: 16s Gel for post-PCR confirmation of gene isolation. Samples were tested against a known positive from *Bartonella* spp. to confirm gene presence rather than the use of the ladder. Numbering above each lane denotes the week and surface number for this test allowing for cross reference with the master spreadsheet.

these swabs and follow the direct PCR & sequencing protocol set out by Oxford Nanopore which could provide more data on the bacterial communities found on these surfaces.

6 Whole-genome analysis of sequenced isolates

6.1 WGS 16s analysis of isolates from surfaces display 4 genera of interest

6.1.1 Introduction

After placing surfaces in situ within gender neutral toilets, it becomes important to identify which bacterial species are present. This could be the presence of live cells which were recovered from the surface, or dead cells of which DNA was still obtainable using swabs.

This could help suggest species which $CuTiO_2$ coatings are effective against, and those which they may be less effective. This would also allow further cross-referencing with antibiotic resistance data gathered, which may display an important link between antibiotic resistance genes or protective mechanisms, and Lewis acid protection mechanisms.

6.1.2 Aims and objectives

Sequencing these genomes using whole genome sequencing would provide multitudes of useful information regarding the efficacy of $CuTiO_2$ surfaces, therefore the main objective of this experiment was to successfully extract and sequence DNA from isolates recovered from in-situ experimentation. The overall aim of sequencing was to produce genomics data to allow for the identification of species recovered. These 16s rRNA sequences were extracted from whole genomes and cross referenced with the NCBI nBLAST database, eluding to a potential match, and allowing us to determine the species isolated from the surface.

By taking whole genome sequences, annotations were attached by MicrobesNG, which displayed 16s variable regions, these regions were then compiled into a .fasta file and matched to existing data in the NCBI nBLAST database.

6.1.3 Results

We saw four genera of bacteria, with differences in populations over time identified on these surfaces as seen in table 6.1. The distribution of these across week 1 and week 4 is visualised in figure 6.1.

These changed in number as seen in figure 6.1 . Despite CFU counts remaining the same, the species isolated from each surface changed on two of the seven surfaces.

Between week 1 and week 4, the diversity of the species found changed, whilst both weeks had a similar number of isolates sequenced (8 and 7 respectively), variation on genus changed. No *Staphylococci* were identified in week 4 despite being present in week 1, *Micrococci* became more prevalent in week 4 along with the emergence of *Corynebacterium*.

6.1.4 Discussion and conclusions

The lack of overall bacteria recovered from these surfaces does imply that this novel $CuTiO_2$ coatings may work well within the environment. Similarly, the genus and species identified are those which have been contextually found to be resistant to many environmental influences such as plant based environments, soil ecosystems or general human flora. These isolates will have developed natural mechanisms to resist copper and other metals through competition in these environments. This selection process in the environment explains why most isolates found contained some level of copper resistance, notably CopA/CopZ, and are also found in extremely controlled environments due to their ability to survive most modern human cleaning regimes (**link2004extreme**).

It is important to recognise that studying WGS for bacteria isolated from surfaces can provide a window into both the presence of AMR genes, and also metal resistance genes. These insights give light to the persistence and virulence of bacteria on surfaces, and it must be utilised in further experimentation to develop a complete understanding of the factors surrounding $CuTiO_2$ surfaces, especially when considering their potential effects on AMR.

Despite this, as WGS was only conducted on isolates from week 1 and week 4, any intermittent isolates which may have appeared in weeks 2 and 3 have no data or analysis conducted. In these weeks, there may be bacterial species which are present, but were not detected, which may be virulent human pathogens, or may be bacteria with other key traits.

Furthermore, by only being able to test DNA from isolates which grew, but not from any amies media remaining after sampling, there is an incomplete picture regarding the microbial communities which reside on these $CuTiO_2$ surfaces. Without being able to gain seqeunces or identify bacteria from the liquid, only bacteria which provided colonies upon plating could

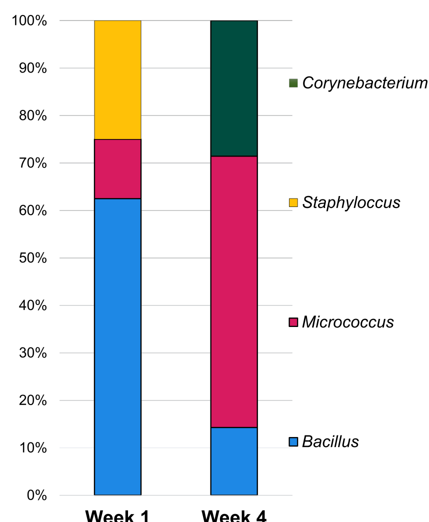


Figure 6.1: Combined taxonomical analysis of Family present within the sample between week 1 and week 4. There is a large influx of *Microccous* in week 4. The level of *Bacilli* dropped between weeks 1 and 4, whilst the number of *Micrococcus* rose significantly. *Staphylocci* also disappeared in week 4. There's no evidence to suggest why this may happen, however it may require further investigation into if long-term DNA damage does occur over a period of weeks which may cause the drop in *Staphylocci*.

be tested, and therefore the overall genetic profile and results of whole genome sequencing are skewed. By developing a protocol to isolate and sequence nucleic acids from the post-plating amies media, bacteria which died on the $CuTiO_2$ surfaces could potentially be detected and studied. This could provide a possible reason as to their demise (such as those lacking copper regulation genes) and would provide a more accurate snapshot regarding common bacteria found on $CuTiO_2$ surfaces, which could not survive. This evidence could also bolster the efficacy and profile of $CuTiO_2$ surfaces, as it may provide evidence of other key bacterial species which have not been tested against, or outlined in this body of work.

Table 6.1: 16s results conducted using NCBI nucelotide BLAST with sequences identified and trimmed in Snapgene. Sequence loci were given by MicrobesNG and were blasted using the standard nucleotide collection databases with highly similar sequences selected.

Isolate Number	**Species**
1.1, 1.6, 4.5	*Bacillus altitudinis*
1.2, 4.6, 4.8, 4.9, 4.10	*Micrococcus luteus*
1.3, 1.4, 1.9	*Bacillus pumilus*
1.11	*Staphylococcus equorum*
1.13	*Staphylococcus capitis*
4.7	*Bacillus safensis*
4.14, 4.15	*Corynebacterium aurimucosum*

6.2 Phylogeny and relationships of bacterial isolates provides insight into survival of bacteria over time.

6.2.1 Introduction

With multiple isolates of the same species present on the real world surfaces, investigating if any of these isolates are related genetically can be an important tool in identifying if the coated surfaces produce genetic or adapted responses. By cross referencing phylogenetic analysis of isolates and antibiotic susceptibility testing data, we can begin to investigate whether the coating could produce species with higher levels of passive mechanisms to deal with Lewis acids and free radicals.

6.2.2 Aims and objectives

This analysis aimed to investigate any links between isolates extracted from the surfaces placed in situ. This would help determine if there were passive mechanisms present which may allow isolated to survive the antimicrobial coating, or if there were common species amongst isolates which were more often transferred onto the surface.

6.2.3 Results

After identifying species from WGS, species clusters (minimum number of 3 isolates) were subject to phylogenic tree building in MegaX using a maximum likelihood method.

Isolates identified as *B. pumilus* displayed two isolates (1.4 and 1.9) being a different branch

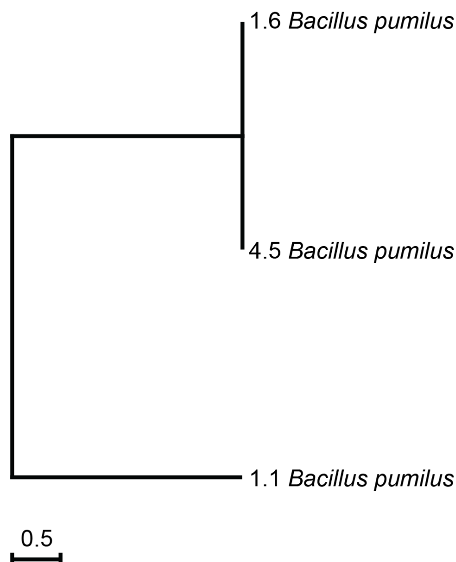


Figure 6.2: Phylogenetic analysis of *B. pumilus* isolates using the Maximum Likelihood method and Tamura-Nei model (**tamura1993estimation**) and Neighbor-Join and BioNJ algorithms were applied to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach to generate the tree (**kumar2016mega7**; **mavsek2017developmental**).

to isolate 1.3, suggesting a common ancestor without them all being directly related. This can be seen visually represented in figure 6.2.

Micrococcus luteus isolates were also found and displayed a much wider genetic variation in phylogeny. These isolates separated into two main clusters, however interestingly one cluster contained isolates from both week 1 and week 4. Their phylogenetic tree can be seen in figure 6.3.

Bacillus altitudinis also showed a similar feature where isolates from week 1 and week 4 were found within the same cluster along with another cluster which can be seen in figure 6.4.

6.2.4 Discussion and conclusions

This work highlights the similarities between bacterial isolates, which may have been isolated weeks apart. Isolates taken in week 1, such as 1.6 *Bacillus pumilus* and isolates taken in week 4 such as 4.5 *Bacillus pumilus* are related through a common ancestor, more-so than the third *Bacillus pumilus* isolate found. This insists that there is a high level of survival and persistance

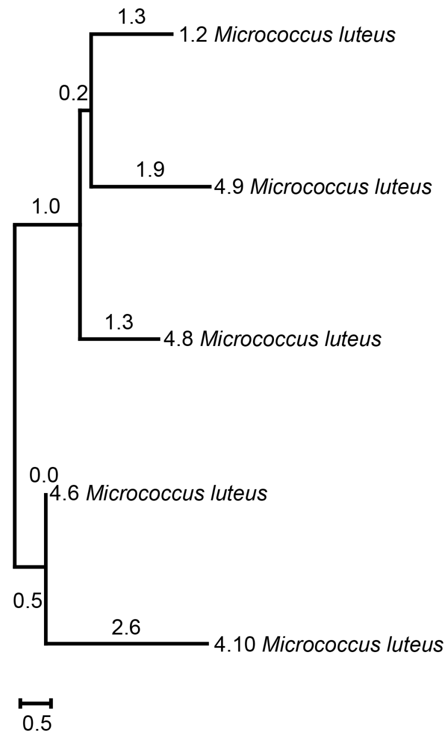


Figure 6.3: Phylogenetic analysis of *M. luteus* isolates for relationship using the Maximum Likelihood method and Tamura-Nei model (**tamura1993estimation**) and Neighbor-Join and BioNJ algorithms were applied to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach to generate the tree (**kumar2016mega7**; **mavsek2017developmental**). Here we see two distinct groupings, however some isolates from one group are seen across the 4 week time-frame signifying some level of continuous growth.

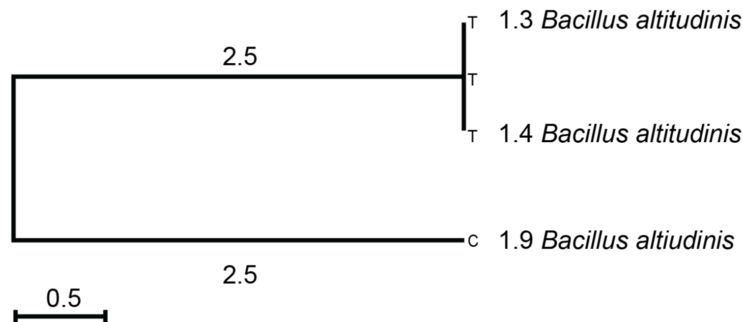


Figure 6.4: Phylogenetic analysis of *B. altitudinis* isolates using the Maximum Likelihood method and Tamura-Nei model (**tamura1993estimation**) and Neighbor-Join and BioNJ algorithms were applied to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach to generate the tree (**kumar2016mega7**; **mavsek2017developmental**). Here two distinct clusters are formed with isolates being seen from both week 1. Of the three isolates a small group is formed denoted as *T* and **C**. This signifies a level of difference between the two isolates, representing more divergence within the isolates recovered.

of bacteria on these surfaces. This is also confirmed in the *Micrococcus luteus* tree as multiple branches feature isolates that are closely related from different weeks. By having multiple species related and branching from the same ancestral lineage, with some isolates being present on week 1 and week 4 being of the similar, but not identical genetic sequences, it must be concluded that these cells have survived on the surface across all 4 weeks.

Figure 6.3 also highlights that there is a branch of isolates from week 4 that are discernible from the others. These two isolates (4.6 and 4.10) are potentially related but do not form part of the main cluster.

In contrast to this, figure 6.4 shows all isolates being closely related, however two isolates being paired together, more-likely to be closely related daughter cells with a minor base pair mutation. All isolates were found in the same week and were not prevalent in the following weeks.

These trees highlight that despite multiple species being isolated, they are not direct clones of each other, which may explain the variance in some isolates possessing different quantities or forms of antimicrobial and metallotolerant genes.

6.3 Antimicrobial resistance & metal resistance gene analysis highlighted ubiquitous Tetracycline resistance and Copper resistance

6.3.1 Introduction

Whilst studying isolates from the first week of testing and the final week of testing, antimicrobial resistance data changed significantly. Antimicrobail resistance gene diversity became lower during the final week of testing, similar to the reduction in overall bacterial species diversity. This change was surprising and potentially showed a lack of species and genetic variation, as such investigating this change could be an important step in understanding how the $CuTiO_2$ coating affects microbial communities and, potentially if the surface produces adverse developments in bacterial resistance of characteristics.

6.3.2 Aims and objectives

After studying the diversity of bacterial species present, those of interest could possess genes which are either beneficial or detrimental to survival on $CuTiO_2$ surfaces. Establishing whole genomes would allow cross referencing with known genomes to determine if any mutations or genes are present that aid in virulence.

6.3.3 Results

Initial analysis of AMR genes was conducted using ABRicate conducted on Galaxy servers. ABRicate utilised the NCBI 'default' database, and highlighted AMR genes within this database that provided an accurate match. This found 11 different types of AMR gene within the set of sequences, of which, type A-6 chloramphenicol O-acetyltransferase cat86 and class D beta-lactamase BPU-1 were the most prevalent. The results of the ABRicate report can be seen in figure 6.5.

This database did not pick up some AMR or metal resistance genes, found present in the whole genome sequence therefore manual analysis for all sequences was conducted. Using common sequences for multiple metal resistance genes, such as CopA and CopZ, searches were conducted to find any matches within the WGS. This was also repeated for potential antimicrobial genes of interest, such as tetA or tetB. Tetracycline resistance genes were chosen as isolates presented with

high levels of tetracycline resistance when using kirby-bauer disc diffusion assays. By searching each sample manually for matched sequences within Snapgene, matching sequences could be identified and cross referenced with the NCBI nBLAST default database, confirming the suspect gene as a tetracycline resistance gene. A similar process was conducted using merR1, which promotes mercury resistance in the environment, and is another key factor in environmental bacterial survival (**PRITCHARDTHESIS**).

After manual analysis, all isolates from week 1 displayed a form of tetracycline resistance, 7 of the 9 isolates contained *tetA*, whilst two isolates contained the *tetAB* hybrid multi-gene. The similarity and evolutionary maximum likelihood tree can be seen in figure 6.6.

All but three isolates contained merR1 genes, conferring resistance to mercury, these isolates formed two evolutionary clusters, however no sequences were identical. This maximum-likelihood tree is shown in figure 6.7

Phylogenetic analysis of the *tetA* gene sequences produced the evolutionary tree seen in figure 6.6.

Multiple metal resistance genes were found, including some which function on copper, cadmium, arsenic and mercury. These genes, and their functions can be seen in table 6.2.

Table 6.2: Metal resistance genes identified during WGS. Their targets and function are explained alongside. The most common genes found in this sample are highlighted and induce copper resistance through genetic up-regulation. Other mechanisms feed into the CopAZ mechanism or work in a similar way through the use of membrane bound efflux pumps.

Gene symbol	Protein/Function	Reference
copZ	**Copper chaperone CopZ**	**corbett2011combined**
copA	**Copper-exporting P-type ATPase**	**corbett2011combined**
csoR	**Copper-sensing transcriptional repressor**	**corbett2011combined**
cutC	**Copper homeostasis protein cutC**	**gupta1995identification**; **calafato2008kno**
copB	Copper-exporting P-type ATPase	**ge2021influence**
mmcO	Multicopper oxidase MmcO	**rowland2013multicopper**
ricR	Copper sensing transcriptional repressor	**shi2014copper**
ycnJ	Copper transport protein ycnJ	**chillappagari2009copper**
actP	Copper-transporting P-type ATPase	**jiang2021characterizations**
merR1	Mercuric resistance operon regulatory protein	**schaefer2004role**; **PRITCHARDTHESI**
merA	Mercuric reductase	**schaefer2004role**; **PRITCHARDTHESI**
merB	Organomercury lyase	**schaefer2004role**; **PRITCHARDTHESI**
modB	Molybdenum transport permease protein ModB	**delgado2006functional**
arsR	Arsenic resistance operon repressor	**shen2013contribution**
arsD	Arsenic resistance operon trans-acting repressor ArsD	**ben2018distribution**
acr3	Arsenical resistance protein Acr3	**ben2018distribution**
cadC	Cadmium resistance transcriptional regulatory protein CadC	**silver1996bacterial**

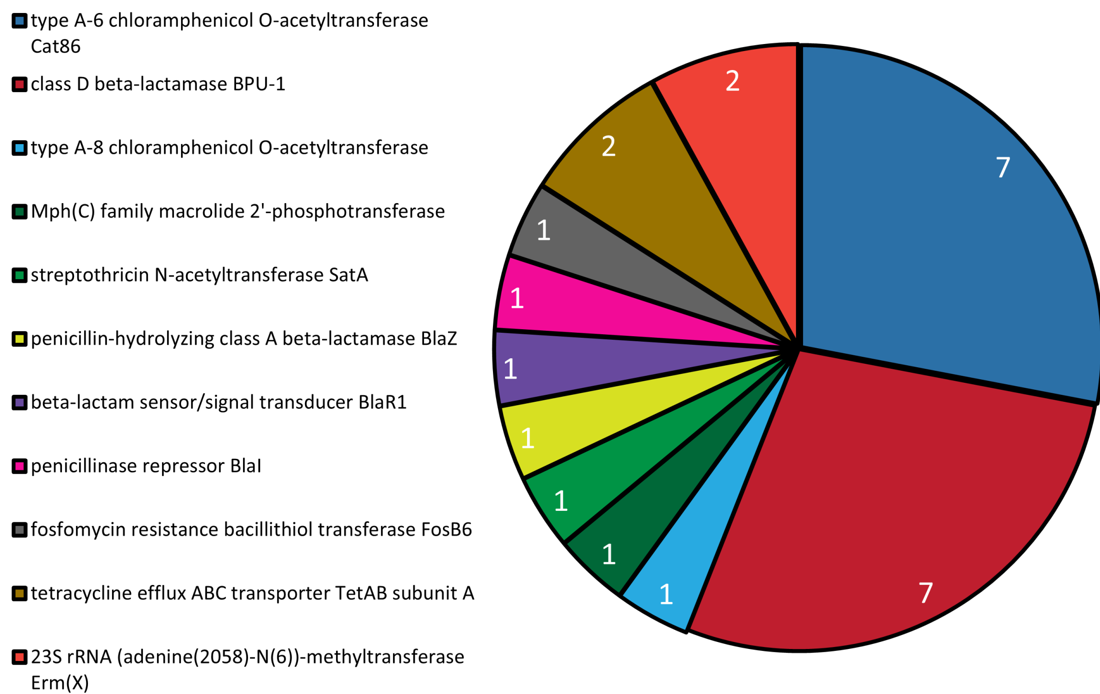


Figure 6.5: Pie chart depicting the antimicrobial resistance genes found using the NCBI database and Galaxy ABRicate. The two dominant AMR genes found using the NBCI database and ABRicate pipeline were a type A-6 chloramphenicol O-acteyltransferase Cat86 and a class D beta-lactamase BPU-1. Surprisingly this did not align with in-depth genome analysis or phenotype resistance testing using disk diffusion assays.

6.3.4 Discussion and conclusions

Within the isolates recovered form the surfaces placed within the environment, tetracycline resistance was highly prevalent. Initial reporting on tetracycline resistance was produced specifically on the basis of suspected genus produced by RK's gram staining and identification. However after WGS it was apparent that some of these isolates were drastically different from their suspect and were therefore reported on incorrectly. For example, both *Cornyebacterium* isolates were originally reported as suspect *Staphylococcus*, and therefore only one isolate qualified as resistant, however according to the new WGS identification and EUCAST breakpoints v13 (**EUCASTMANUAL**), both isolates display tetracycline resistance despite not having *tetA/B*.

Isolate 1.13 was identified as *Staphylococcus capitis*, and despite having *TetA* present in WGS, phenotypic results to tetracycline disk diffusion borders on intermediate sensitivity, with sensitivity achieved in higher doses (**bennett2019mandell**). This bordered close to resistance, being only 2mm in diameter away from resistance.

No breakpoints exist for tetracycline testing of *Bacillus spp.* according to EUCAST, therefore resistance can only be inferred from a standardised criterion. Despite this, all isolates except one were phenotypically susceptible Tetracycline. Isolate 1.4 was the only isolate presenting as a *Bacillus spp.* that was fully resistance to tetracycline (zone of inhibition diameter =0mm).

Despite the presence of *tetA* genes in most isolates, it appears that many were not mechanically active within the cells, repeat exposure to tetracycline could induce tetracycline resistance.

It is highly expected to find mercury resistance genes, such as Mercuric resistance operon regulatory protein (merR) and Mercuric reductase (merA). It is widely known that these genes are highly conserved among bacterial species, especially in those present in the environment (**PRITCHARDTHESIS**). However it is surprising to see that despite the sequences being highly conserved, there are no identical sequences even within the same isolate species group. It is plausible that this variation may only be present as single nucleotide polymorphisms which do not generally affect the overall sequence of amino acids built by the ribosome.

copZ is a chaperone protein with the primary function of transporting, collecting and delivering cellular copper to the CopA transmembrane ATPAse for export. Due to their synergistic nature, *copZ* and *copA* are part of the same operon complex and are activated together through

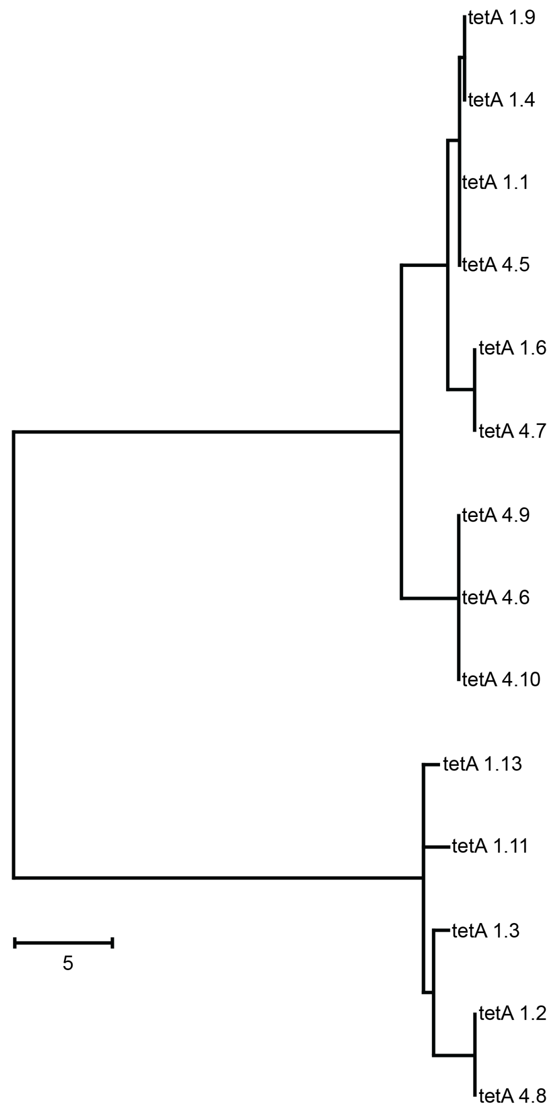


Figure 6.6: Analysis of tetA sequences taken from isolate WGS. Evolutionary history was inferred using the Maximum-Likelihood method and Tamura-Nei model (**tamura1993estimation**). The tree with the highest log likelihood is shown (-11476.4). Initial tree structure was produced using the Neighbor-Join and BioNJ algorithm to a matrix of pairwise distances estimated using the Tamura-Nei model, and then selecting the topology with the superior log likelihood value. The tree is drawn to scale, branch lengths denoting the number of substitutions per site. This tree contained 14 nucleotide sequences. The final dataset used a total of 1188 positions. Evolutionary analysis was conducted in MEGA X (**kumar2016mega7**)

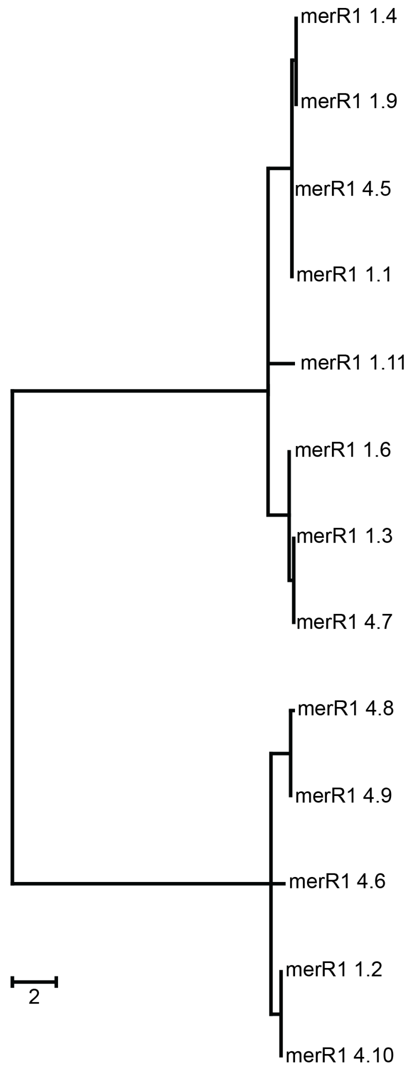


Figure 6.7: Analysis of merR1 genes isolated using WGS. The tree was built using a Maximum-Likelihood method and Tamura-Nei model (**tamura1993estimation**). The tree with the highest log likelihood is shown (-11476.4). Initial tree structure was produced using the Neighbor-Join and BioNJ algorithm to a matrix of pairwise distances estimated using the Tamura-Nei model, and then selecting the topology with the superior log likelihood value. The tree is drawn to scale, branch lengths denoting the number of substitutions per site. This tree contained 13 nucleotide sequences of different species. Evolutionary analysis was conducted in MEGA X (**kumar2016mega7**)

activation by CueR. As such *copZ* and CopZ (the respective protein produced) is key for bacterial copper ion homeostasis in both high and low copper concentrations (**utz2019cu**).

copA much like *copZ* is a homeostatic regulator of copper ions within the microbial cell. As such in high internal concentrations of Cu^{+} ions, *CopA* proteins export copper complexes out of the cell through the use of active transport and the hydrolysis of adenosine triphosphate (ATP). This is seen in many species however, knockout trials conducted by Zheng et al showed that in ΔcopA cells, the inability of cells to export Cu(II) ions resulted in bacterial cell death (**zheng2019copa**).

Therefore we believe it these bacteria with *copA* and *copZ* genes that have survived on these $CuTiO_2$ surfaces, and future investigations cna be conducted on these isolats to both confirm the presence of copA and copZ using PCR and agarose gel electropheresis, but also potentially, the use of disruption and insertion testing with a confering plasmid, to test the uptake and implementation of these genes, and whether this confers resistance to the high levels of copper found in $CuTiO_2$ surfaces.

By conducting transformation experiments, known strains can be challenged in the presence of $CuTiO_2$ surfaces, and these transformed bacteria can be tested for their copper tolerance, before and after transformation. Transformation could be conducted using ampicillin positive selection, ensuring that testing is done with competent bacteria which have taken on the selected genes.

CsoR is a copper sensing protein produced by *csoR*, which was commonly found in multiple samples (**liu2007csor**; **marcus2016csor**). Each isolate containing *copAZ* also maintained a level of *csoR*, however it was also found in isolates that did not contain *copAZ*. This means that despite their similarities in copper regulation, *csoR* acts independently of *copAZ* and is not singularly associated with the *copAZ* export system. As a copper sensing protein, CsoR regulates genomic integrity through binding to the cso promoter region of the bacterial genome (**liang2016functional**; **marcus2016csor**). In high concentrations of copper, CsoR is fully removed through disassociation (**teramoto2012corynebacterium**). This then exposes the promoter region for transcription, leading to copper homeostatic mechanisms present on the cso regions to be translated. Due to its structure, CsoR is found to bind to a CG rich specific promoter sequence, 5'-GTAGCCCACCCCCAGTGGGGTGGGA-3'.

With the cso region acting as the promoter, the disassociation of CsoR allows the downstream translation of *cueA* which encodes another form of ATP hydrolysis copper exporting membrane complex (**schwan2005mutations**; **liang2016functional**; **teramoto2012corynebacterium**).

CsoR promoter regions are also often found above *copAZ* sections of the genome, suggesting that CsoR does regulate both *cueA* and *copAZ* responses to high levels of intracellular copper (**dwarakanath2012response**; **teramoto2012corynebacterium**; **marcus2016csor**). A generalised mechanism of this can be seen in figure 6.8.

cutC is part of the six-gene family found in many bacterial species which regulate copper homeostasis. A similar homolog is found in humans, however both proteins function in a similar way, through the binding of one Cu ion each (**gupta1995identification**). *cutC* produces a 147 length amino acid protein, which features a single copper-bindigg domain, unlike CsoR which features at least two. Despite functioning similar to CsoR, the regulation of *cutC* and therefore CutC protein levels has been found to be dependant on RNA regulation, rather than protein disassociation in the genome. MicL is transcribed from the *cutC* promoter region and affects the transcription of envelope lipoproteins through the targeting of Lipoptotein lpp mRNA (**guo2014micl**). Therefore, by sequestering and removing lipoprotein mRNA, MicL prevents vulnerability to copper along with many antibiotics such as vancomycin (**mathelie2020lipoprotein**).

cutC has been found itself to be a mutant of other genes in the *cut* family, however *cutC* and *cutF* must both be found in an isolate in order for it to be seen as copper tolerant (**gupta1995identification**). Mutations in *cutC* and *cutF*, along with mutations in MicL sequences all displayed higher levels of copper sensitivity.

Despite the overall low prevalence of general antimicrobial resistance genes, other genes of interest to this project were also found. In some samples, *quacA* and *sugE* were found, which encode proteins facilitating quaternary ammonium compound resistance. This is especially important as most cleaning solutions both inside and outside the clinical settings utilise these quaternary ammonium compounds. Another gene of interest found is *sepA* which encodes the multi-drug resistance efflux pump SepA. SepA has been found to help mediate ciprofloxacin resistance (**hassanzadeh2017frequency**), tetracycline resistance (**truong2005mgra**).

Multiple isolates contained the *ykk* family of multi-drug resistance proteins. This family acts as drug exporting membrane proteins (**bay2008small**). *ykkC* and *ykkD* are found on the same

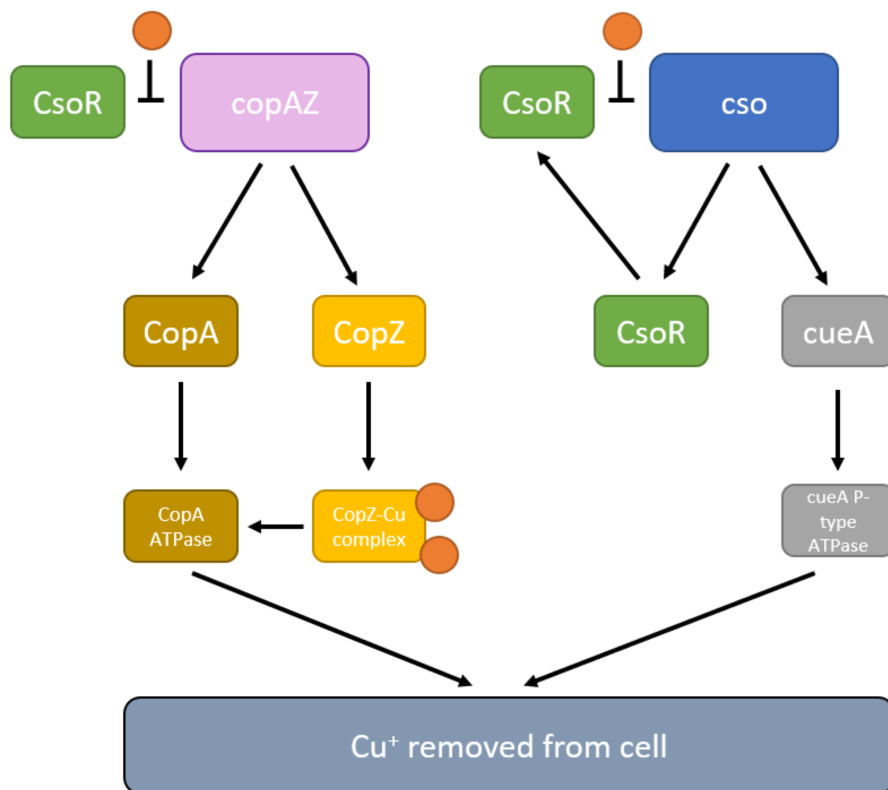


Figure 6.8: Cellular signalling mechanism of CsoR when activated in the presence of excessive copper ions. Copper ions prevent CsoR from binding to the copAZ promoter region and the cso promoter region, as such CsoR cannot act as an inhibitor. This allows the downstream production of CopA and CopZ mRNA which is translated down stream into the CopA ATPase efflux pump and the CopZ transporter complex. CopZ actively binds up cellular copper and transports it ot the vacinity of the CopA efflux pump which removes the copper into the extracellular space. CsoR is then also up-regulated by the transcription of cso, which produces more CsoR and cueA. cueA is later translated to produce another membrane bound ATPase which pumps out copper from the cell. When the levels of copper begin to fall below quantities needed to prevent the binding of CsoR to the copAZ and cso promoter regions, down-regulation of cellular CsoR occurs and new efflux pumps are not produced. (Figure produced in the Adobe creative cloud suite).

operon and work in tandem as small drug exporters. Interestingly, these genes have been found to display genetic plasticity, with these genes being found both integrated into genomic DNA and on plasmids (**bay2008small**). This group, termed 'small multidrug resistance proteins' (SMRP) also includes the proteins EbrA and EbrB, which were also found in isolates extracted from these $CuTiO_2$ surfaces.

6.4 Future Work

Future work on this could adapt to timed gene expression or RNA expression assays to uncover the short term and long term functions of some of the identified genes, proteins and mechanisms that allow bacterial species to confer resistance to copper and other metallic systems in real time. This WGS analysis also displayed the issues with using whole genome analysis to test for antibiotic resistances. Whilst many samples contain *TetA* genes, most samples were susceptible to the drug during antibiotic sensitivity testing. Whilst the gene may be present, secondary factors such as the effect of the sampling surface or location (such as the $CuTiO_2$ coated surfaces used here) could disrupt the nature of expressing those genes.

7 Discussing the overall effects and use of $CuTiO_2$ surfaces and final conclusions.

7.1 Validation of $CuTiO_2$ surfaces within the laboratory

7.1.1 Liquid Transfer vs Media Transfer provided more consistent bio-fouling

Existing methods for inoculating antimicrobial surfaces often use the pipetting of a standardised culture onto that surface and attempting to spread it either using the pipette itself, or the use of a secondary utensil. These methods are seen in work by **santo2011bacterial**; **santo2008contribution** which were a starting point for this analysis. As a peer reviewed method if formed a foundation for testing $CuTiO_2$ surfaces against an existing protocol. However it was apparent that this methodology provided inconsistent results when attempting to produce time based experimentation on the activity of $CuTiO_2$ surfaces or bacterial recovery from borosillicate glass.

This new surface transfer method, outlined in Chapter 3, in which an agarose intermediate surface is used to foul the tested surface, minimises loss of bacteria whilst covering the whole surface with an even distribution of bacteria, without controlling for adding or removing liquid from the surface. Leaving liquid on the surface can often be complex, and the surface will often be still damp with the inoculate. The use of an intermediary agarose plate allows the dispensing of the inoculate and contact with a surface, without running the risk of leaving residual liquid on the surface which may skew results. Existing methodologies that use this may therefore, be trending towards results which are artificially inflated due to the presence of pure inoculating culture and may not represent even distribution or transfer of bacterial cells.

After analysis, agarose transferred plate results with and without outliers still remains normal with a linear distribution implying that the higher the level of bio-fouling, the higher the recovery of bacterial cells when using 304 stainless steel.

This method therefore, outlines the best possible way (compared to methods used by **santo2011bacterial**) to investigate the antimicrobial action of various surfaces when attempting to discern their suitability in controlling bacterial colonies and preventing the transmission of bacteria between people. However this does not cover natural methods of bio-fouling. From the initial stage, fouling

and inoculation of the potential surface, using the agarose transfer method displays a linear, repeatable and consistent method (see figure 4.3a and 4.3b).

Furthermore, using the agarose transfer method with borosilicate glass prevents the need to use any form of bead beating for cell retrieval. This is especially important when studying the surface efficacy through fluorescence microscopy. Bead beating in itself, may skew potential results in favour of low bacterial cell survival. The mechanical action of bead beating can lead to bacterial cell death, which would almost certainly skew the perceived effectiveness of any antimicrobial surface undergoing testing. Additionally, the variability in bead beating protocol can cause variation in both success and cell recovery.

In summary, this means that liquid transfer results could lead to inaccurate colony counts. Liquid based inoculation cannot provide adequate surface tension to fully coat the surface without the intervention of another inert surface. This would dramatically skew any potential killing activity per mm^2 calculations. Agarose transfer methods prevent this surface tension problems by inoculating an agarose plate which provides a firm surface to place the antimicrobial surface on without causing tearing, and prevents hot-spots of bacteria or dead-zones where bacterial inoculate could not reach due to surface tension.

Both methods however are limited by surface finish, however this is universal across all surfaces. The liquid transfer method also provided large amounts of excess liquid when quantifying killing using BacLight Live/Dead staining which contributed to the brownian motion seen. This was not as apparent on agarose transfer plates when using translucent surfaces such as $CuTiO_2$ coated glass.

7.1.2 Problems with existing methods of bio-fouling are limited by surface texture at the microscopic level.

The nature of this work focuses primarily on the antimicrobial effects of various surfaces, however surface smoothness is a key factor in both the transfer of bacteria and the survival of bacteria on surfaces. As such surfaces that are generally considered 'smoother' will prevent bacterial transfer due to the lack of crevasses for cells to reside in, and rougher surfaces provide space for cells to sit in whilst also potentially being shaded by UV irradiation.

It should also be considered for future work in this field, that the quality of surface finish has a

significant effect on bacterial transfer. Smooth surfaces, such as $CuTiO_2$ coated steel, along with the base borosilicate glass substrate have a harder time facilitating bacterial adhesion. Their rougher counterparts such as 304 stainless steel possess large peaks and troughs leading to an overall greater surface area. Troughs are often large enough to capture bacteria and provide areas of shelter, unlike their counterparts.

It is evident that un-coated stainless steel retains and transfers a high amount of bacteria naturally which poses multiple problems in a healthcare setting. Primarily this is due to the surface finish and irregularities with the type of finishes commonly applied to stainless steel (**medilanski2002influence**; **hilbert2003influence**; **wu2018role**). Most stainless steel sold has a microscopically rough surface finish, and as such this can lead to an increase in bacterial residence within the troughs on the surface, and thus would lead to an increase in bacterial transmission.

To reduce the effect of the surface irregularities, further processing would have to be done during the production phase and would therefore increase the cost of the product. Provided that the surface finish has gaps wider than approximately 10µm, it is highly possible for bacteria to reside within these troughs and therefore be transferred to other people or surfaces. This is not a seen in other materials where the surface finish may have peaks, but they are significantly smaller than that on 304 stainless steel, for example the use of titanium can produce nanospikes, prevent bacteria from adhering to the surface. Without further processing it is impossible to attain a similar result with 304 stainless steel.

Currently, stainless steel is the de facto material used in clinical settings, despite evidence in the literature pointing to the beneficial implementation of other materials which could prevent bacterial transmission (**muller2016antimicrobial**; **balasubramaniam2020antibacterial**; **wang2021antibacteri**
and surfaces used in clinical settings are microscopically rough, which facilitates bacterial hiding to both UV exposure, chemical cleaning (such as the use of QAC's) allowing the survival of bacteria depending on the surface finish.

In summary, surface roughness is a key factor in the transfer of bacteria, this can be seen in figure 4.4 where glass, even un-coated provided a low level of recovered bacteria compared to 304 stainless steel. This effect can also be seen inducing some variation in the colony counts from the $CuTiO_2$ coated glass due to the small introduction of surface roughness from the CVD process.

7.1.3 Real-time analysis of killing effects using Live/Dead staining provides insights into activity time.

Similarly to **santo2011bacterial**, BacLight Live/Dead staining was employed to assess both efficacy and to discern if any mechanisms were obviously apparent. As such images were observed at verious intervals to discern the ratio of live to dead cells, and were converted to a calculable valuable using ImageJ to assess pixel colour, density and percentage within the image. These values could then be used for statistical analysis to confirm any potential conclusions drawn from the raw images.

Experimental analysis of $CuTiO_2$ surfaces, compared with un-coated borosilicate glass showed a significant killing effect within an hour and a half. This characterisation is slower than previously described by (**santo2011bacterial**; **santo2008contribution**) however this is possibly due to the use of a stainless steel or glass substrate which is coated, rather than pure copper surfaces.

Due to this fast activity time, the general hypothesis around the mechanism of action can be inferred. This killing time is too fast for DNA-based damage to be the sole killing mechanism at play. As such there are multiple mechanisms which would facilitate killing between instant dehydration and long-term DNA damage, as outlined in figure 1.4.

In a clinical setting, it is possible that $CuTiO_2$surfaces could kill off susceptible bacteria within a few hours, reducing the transfer of bacteria and therefore creating a reduction in bio-fouling.

This is very important for determining the real world case use of $CuTiO_2$ surfaces where prompt killing of bacteria is important, such as surgical wards or wards where patients may be severely immunocompromised. These situations require effective, prompt and continuous killing of bacteria to reduce the transmission of bacteria and prevent the contraction of HCAI's in patients who are highly susceptible to them.

In summary, $CuTiO_2$ surfaces provided a killing effect within an estimated thirty minute period (as seen in figure 4.10, after which killing levelled off unless the surfaces were exposed to UV irradiation prior (displayed in 4.12). This was only shown when using the agarose transfer method, as no signifcant changes were observed when using the **santo2011bacterial** method as outlined in figure 4.11.

7.1.4 UV light causes a reduction of bacteria on $CuTiO_2$ surfaces.

To fully harness the power of $CuTiO_2$ surfaces, the titanium dioxide component requires UV activation to begin the generation of free radicals. This mechanism is often described as being 'self cleaning' in the antimicrobial surface production industry (**kolev2023physicochemical**). This property increases bacterial killing, and is a core component behind the use of a copper/titania combination, rather than copper by itself.

UV radiation is specific in the wavelength and type required to generate free radicals, UV-A and UV-B would be potentially useless as it shows no clear effect in the generation of free radicals or the killing of bacteria (**link2004extreme**). The mechanisms of UV killing are indirect, and the subsequent activation of titania by UV irradiation causes the production of free radicals which assists in the killing of bacteria (**kanata2023photo**; **kolev2023physicochemical**). This is a key secondary mechanism which is especially effective on metal surfaces which have variable surface qualities with undulations where bacteria may hide or be shielded from any direct UV-C radiation.

UV-A in itself has the potential to kill bacteria (**kanata2023photo**), however this does not constitute a core function of the $CuTiO_2$ coating.

In some cases UV irradiation is required for significant reductions in all coatings containing titania, however there is little to no effect on coatings without titania. However various configurations of both coatings can be produced meaning there may be a difference between coatings, often these configurations depend on the number of passes applied during each part of the coating process. Coatings with more titania may produce better results under UV irradiation, but produce worse results without UV. Future work could potentially aim to find the optimal number of passes for each coating.

Despite the action of the copper, the aim of $CuTiO_2$ coatings is to utilize both mechanisms in addition. This UV activated titania mechanism utilizes the free radical generation (**kolev2023physicochemical**), and thus those free radicals cause damage to intracellular structures. Despite the production of free radicals, there is some evidence to suggest that bacteria can utilise their environmental protective mechanisms to resist the free radical generation (**pigeolet1990glutathione**). Some of these species were present in our environmental sampling,

and are often documented as being resistant to UV-light, such as *B. pumilus* (**link2004extreme**; **handtke2014bacillus**).

However, results highlighting the survival of *B. pumilus* and other isolates were conducted in an environment, where UV light would be diminished. As such, survival of *B. pumilus* on titania surfaces may be killed by the copper's lewis acid mechanism.

The experiment to directly test the use of the UV-induced secondary mechanism (in chapter 4.4) used long, overnight activation by UV radiation, as such there was a substantial, high power on the surface for a minimum of 8 hours. This kind of UV power would be highly unlikely to occur in the environment naturally and any attempts to utilize solar UV radiation would be minimised by shade caused by buildings and cloud cover. The inside of buildings would be highly shaded from natural UV radiation, and the overall effect reduced due to the protective nature of glass in filtering out UV radiation. This would mean for UV-activation to be effective, UV radiation must be added artificially, which reduces the overall passive effect of the copper/titania dual action.

In summary, despite the long intensity of UV irradiation, a significant contrast was seen between UV activated and non-UV activated surfaces n the level of bacterial reduction (as seen in figure 4.12). Therefore, further clinical or public implementations of $CuTiO_2$ surfaces should consider the availability of UV irradiation mechanisms, be that artificial or natural. Future work should be focused on investigating the efficacy of various coating combinations which may show the optimal coating for UV and non-UV activated killing. Similarly, further testing should be done on UV activated surfaces, in a dedicated and specific testing environment to determine if free radical generation can occur at naturally occurring strengths of UV radiation.

7.1.5 Effectiveness of $CuTiO_2$ coatings demonstrates quick mechanistic killing.

It is clear the $CuTiO_2$ surfaces do have a positive effect in the reduction of bacterial loads, however, this reduction is traded off with variability in the survival of persistent bacterial cells which are less resistant to the other active mechanisms.

As discussed in the introduction and throughout, no exact mechanism for the bactericidal effect of copper exists, and multiple theories have been explored. Some mechanisms, such as DNA damage have been ruled out by work done by (**santo2008contribution**; **santo2011bacterial**),

as their results displayed killing in a time period shorter than that expected of DNA induced death. This does not mean that over a long period of time DNA damage cannot occur, however it is not the mechanism at play when showing significant killing within 60 minutes as seen here.

Therefore, due to the quick killing, it is probably that the mechanisms at play for the cell death are the increase in membrane permeability, cell membrane disruption, generation of reactive oxygen species, and an increase in protein damage (as shown in figure 1.4).

It is possible, that in real world environments, bacterial cells do not have a foothold in developing a protective biofilm, and fulfilling the overall needs of establishing a colony, (as depicted in figure 4.8), however intrinsic resistance to oxidative stress, and the presence of the *cop* operon in the *sil* operon facilitate resistance to copper and copper ions.

This was confirmed by environmental isolates containing one form of copper resistance gene, which would enhance survival on $CuTiO_2$ surfaces. So whilst the presence of a copper resistance gene may not be necessary, there is evidence that $CuTiO_2$ surfaces are less effective towards those isolates which possess resistance genes.

DNA analysis of *S. aureus NTC8325*, the reference strain in this project, showed no evidence of copper resistance genes but still had surviving colonies after exposure. Despite a majority of cells being killed, enough cells survived for form significantly viable counts. As such it may be possible that bacteria lacking copper resistance genes could survive on the surface depending on their phase of growth which would therefore lower the overall efficacy of $CuTiO_2$ surfaces without activation of the secondary UV mechanism.

There are multiple mechanisms of copper and silver tolerance only induced in the presence of copper, and therefore the use of copper surfaces could provide an increase in clinical copper and silver resistance and an increased virulence.

Whilst quick copper killing does occur, the exact mechanism is still not known and the presence of a copper resistance gene is not required for survival as non-genetic survival mechanisms can generate tolerance to copper, however the presence of a resistance gene does ensure a higher level of survival on $CuTiO_2$ surfaces.

Correlations between the use of $CuTiO_2$ surfaces and antibiotic resistance still remains largely unstudied. It would be vitally important to assess whether further use of $CuTiO_2$ surfaces produce resistance to a broad range of antibiotics, not just those studied and seen here. This

future work would need to develop resistance to copper initially, before testing against a wide variety of antibiotics to determine any correlation between copper resistance development, and resistance to antibiotics.

7.1.6 Possible effects of $CuTiO_2$ coatings on metal tolerance and antimicrobial resistance.

After studying the survivors of an agarose plate transfer experiment, a surviving *E. coli* colony was then used for kirby-bauer disk diffusion with promising results displaying an increased sensitivity to some antibiotics. It is clear that after exposure to $CuTiO_2$ surfaces, *E. coli* showed a higher level of sensitivity to antibiotics compared to the control or the glass survivor and that this was not universal to all classes of antimicrobial, but was primarily found to those which required access to the cell membrane or internal cellular machinery. This is fairly similar to work conducted by **hernandez2008antimicrobial** however, their application of the metal are as nanoparticles, rather than a coating.

Interestingly there was a large increase in effectiveness of ampicillin and tetracycline, both of which are different classes of antibiotic, and work differently, however both still had an increased sensitivity in the presence of $CuTiO_2$.

This could mean that infections which are a result of $CuTiO_2$ surfaces may be easier to treat than those transferred from stainless steel and current materials used currently in hospitals (**hernandez2008antimicrobial**). This could greatly reduce the overall impact of infections on wards and prevent cases of HCAI's worsening and developing into sepsis.

This work is preliminary at best, and therefore this effect should be studied further to investigate if there is a significant effect across multiple antibiotics and multiple species.

7.2 Use of $CuTiO_2$ surfaces in the real world.

The application of $CuTiO_2$ coatings is difficult but can be done on various surfaces including panels, handles, taps etc but similar processes are already in use in various industries, providing existing pipelines and production methods which can be further built upon. Using $CuTiO_2$ surfaces in the real would could provide a method of reducing antimicrobial resistance, decreasing

bacterial transfer, and prevent the transmission of infection in a clinical setting.

In a clinical setting, due to under-staffing, increased demand and lack of available equipment, despite procedural advice and recommendations, it is highly unlikely that cleaning on equipment is missed between the use of some equipment between its use on patients. For example, patient slides, which are used for the movement of patients between beds are not routinely cleaned between patients. As such, this is one area in which $CuTiO_2$ coating could be easily applied and prevent the transmission of bacteria between patients and hospital workers.

Again in a clinical setting, observation equipment is used frequently on every patient almost hourly depending on the ward. As such between patients these individual items of equipment are cleaned between patients, however the trolley used for carrying this equipment is not cleaned between nursing staff who may use it, or between the areas or side rooms of a ward which may house patients with differential microflora.

As such, $CuTiO_2$ coatings, whilst not being able to be applied to the observation equipment itself, could be applied to other areas of the clinical setting which would otherwise act as a vector in the transmission of bacteria, especially those which are cleaned infrequently.

UV light in real world environments at specific concentrations and of sufficient power may be hard to come by and may require specific tools to utilize the full killing effects of the titania within the coating. High intensity UV provided large amounts of killing (as mentioned above), this could be relative as the UV present in clinical settings would be extremely limited as most areas in the clinical environment where $CuTiO_2$ coatings could be applied would lack significant forms of UV irradiation.

In the real world, the efficacy of the coating can also be reduced by damage. This could be caused by many factors in the real world, which includes incorrect cleaning, or damage with tools or equipment.

Damage can still be done to the coating revealing the surface below making it potentially ineffective if places in high touch areas which may be damaged by items such as clipboards, beds, trolleys on doors and other objects. As revealed by the SEM analysis, damage provides areas without bactericidal properties and therefore would reduce the overall efficacy of $CuTiO_2$ surfaces. Over enough time, the coating could become eroded enough to limit the use of the $CuTiO_2$ surfaces and therefore be less cost-effective than initially planned.

It is not yet known how $CuTiO_2$ surfaces respond to clinical and industrial grade cleaners. Laboratory use has shown that short term use of ethanol for up to one day allows for sterilisation without causing significant degradation to the $CuTiO_2$ coating. Industrial based cleaners are significantly stronger and more damaging to stainless steel surfaces than ethanol. Substances such as Klercide sporicidal active chlorine and Klercide quat when left on surfaces for periods of time eat away and destroy stainless steel surfaces. Currently, it is not yet known the overall durability to harsh cleaners which may affect overall longevity.

7.2.1 Whole Genome Sequencing insights provided information on the level of bacterial survival.

It should be noted that many isolates found on the environmental surfaces were isolates primarily those found in the natural world, with only a few having any correlation with human skin flora. As these surfaces were in a toilet, it was initially expected that any isolates found would be those commonly found on or in humans. Despite this, many of the isolates found were not common skin bacteria. Seven of the isolates matched species known to live on humans, whilst many other are putative opportunistic pathogens or are bacteria known to survive in the environment.

Many bacterial isolates contained genes that aid normal survival in the natural world, and as such they possess qualities when facing antimicrobial surfaces. These genes are key in the rhizosphere however, many of the genes present such as *merR* and the *cop* family also provide metal tolerance to human made environments.

Whilst also being key for general survival, it is clear that many metal tolerance and resistance genes are highly conserved and developed due to the competitive nature of the wild environment. This dynamic of the real world environment, such as the use presence of copper, mercury or silver in the rhizosphere or other soil ecological environments can impact bacterial tolerance to human-implemented materials or processes (**PRITCHARDTHESIS**).

It is also likely that isolates that possess metal-resistance genes, whilst also possessing other virulence factors and antimicrobial resistance genes which may be selected for by the presence of antimicrobial surfaces.

As such this may negatively affect the use of antimicrobial treatments should any bacteria cause infection, or possibly reduce the efficacy of many tactics employed to kill them. To build on

this further, future work should investigate the effects of $CuTiO_2$ surfaces on the susceptibility of many key WHO watch list, or ESKAPE pathogens to ensure that exposure to $CuTiO_2$ surfaces do not reduce the effectiveness of antibiotics, as stated previously.

In conclusion, few isolates were found on the $CuTiO_2$ other CVD surfaces placed in the real world, given their larger surface area and the number of surfaces swabbed. The bacteria captured by swabbing possessed metal tolerance mechanisms, possibly from the environment, which facilitated their survival. There is also a possibility that in a clinical environment, $CuTiO_2$ surfaces may select for virulent bacterial isolates which may cause problems with treating HCAI's, however this will need further investigation.

7.3 Survival of bacteria on $CuTiO_2$ surfaces may select for hardy species of bacteria.

Some isolates found are extremely hardy to UV, metal, antibiotics and therefore survive well on $CuTiO_2$ surfaces due to their genetic makeup. It would complicate the cleaning of surfaces in the real world due to some isolates having resistance genes to Quaternary Aqueous Solutions of Chlorides. Use of cleaners and coatings together could select for isolates that would be hard to clean by many industrial standards.

Species such as *C. difficile*, which is a routine hospital pathogen, and often contains genes attributed to bacterial survival in clinical environments, can be easily transmitted between persons and could survive for longer periods of time and allow the transmission of infection, however this is hypothetical and has not been tested.

Despite the proposed effectiveness of $CuTiO_2$ surfaces, a few hardy bacteria were found. These species are described below, and analysis of their characteristics may provide ideas as to their ability to survive in harsh environments.

Bacillus altitudinis is commonly found in the air (**shivaji2006bacillus**) and as such, it is expected to be seen in an uncontrolled environment. It has been found in the depths of lakes, all the way up to the high atmosphere (**rozanov2018draft**).

Bacillus pumilus is a key constituent of the rhizosphere and is commonly found in soil and soil samples (**masood2020bacillus**). Due to their exposure to hardy soil environment full of compet-

ing bacterial species, *B. pumilus* has developed highly adaptive reactive mechanisms to exposure to bactericidal byproducts of both plants and other bacterial species (**handtke2014bacillus**). This includes the presence of peroxide reductases (**handtke2014bacillus**). *B. pumilus* has also developed resistance to UV light, as such, it has interestingly been found in areas of harsh UV light including deserts (**handtke2014bacillus**) and found in the North American Space Association Jet Propulsion Laboratory (NASA JPL-SAF) despite their intense clean room practices (**link2004extreme**). NASA's JPL-SAF lab utilizes a significant proportion of biocides and UV light in order to clean their work spaces, and as such the presence of *B. pumilus* on these $CuTiO_2$ surfaces without the presence of UV light could potentially show a category of isolates which may be immune to both killing mechanisms present.

Bacillus safensis similarly to *B. pumilus* is another rod shaped Gram positive organism found within the environment with hardy spores. Due to their high similarity, *B. safensis* has also been found in clean rooms in space-craft assembly (**satomi2006bacillus**).

Corynebacterium aurimucosum has been identified as a coliform and pathogen (**bernard2012genus**; **lo2015urinary**; **lefevre2021clinical**). Therefore it is highly possible these isolates were found as part of human interaction with the surfaces.

Staphylococcus equorum is surprisingly found within the dairy industry as it has been reportedly found in various cheeses (**meugnier1996identification**), however isolates of *S. equorum* have been found as part of clinical samples (**novakova2006staphylococcus**). One of these isolates presented as a blood stream infection in an elderly male, and therefore *S. equorum* can be considered a putative pathogen.

Staphylococcus capitis is normal human skin flora, often taking up residence in areas of the body such as the face and scalp (**cameron2015insights**). As such it often is tolerated by the body when in a regular dynamic with the immune system, however *S. capitis* has been found in cases of ineffective endocarditis (**sandoe1999staphylococcus**; **bennett2019mandell**).

Micrococcus luteus is a common putative human pathogen. Historial analysis have shown that *M. luteus* has been a human pathogen since the dawn of humans (**greenblatt2004micrococcus**; **lemma2008attempts**). In modern day medicine, *M. luteus* can be found causing soft tissue infections and endocarditis (or similarly related cardiac infections) (**albertson1978septic**; **seifert1995micrococcus**). As part of human flora, it is pleasible that these isolates arrived on

these surfaces through human interaction and use of the toilets and hand-dryer.

7.3.1 Relationships of isolates highlighted species-level clustering over four weeks.

Multiple isolates, often of the same species displayed different levels of relationship within their own groups. For example, *B. pumilus* isolates from week 4 and week 1 were closer related than that to another isolate found on week 1. This is not always the case as *M. luteus* isolates showed two isolates being closely related (4.6 and 4.10), whilst also being less related to isolates found in week 1.

B. altitudinis however was only present in week 1, and still two isolates were more closely related (1.3 and 1.4), than the third isolate (1.9).

It is expected to see high levels of similarity in *merR* sequences. The ubiquitous nature and importance of the gene ensures that it is highly conserved. Effectively, here we see three main clusters with marginal numbers of base substitutions, still encoding an effective *MerR* protein. Links are still yet to be drawn between overall relationship of species and the similarities in *merR* sequence, as this could be an explanatory factor as to why isolates are grouped accordingly.

We see a similar feature in the clustering of *tetA* genes, although on a larger scale where sequences contained more base pair changes from the ancestral predicted source. Surprisingly, *tetA* is not as ubiquitous or essential as *merR* or *copA/Z*, its large presence within the sample taken is significantly interesting and future work could be done on these isolates to determine how this sequences were acquired.

7.4 DNA evidence of survival.

7.4.1 Prevalence of metal tolerance genes were highly conserved across all present species.

Every isolate possessed a form of copper resistance gene. This is to be expected due to the environmental role of copper, especially in areas such as the rhizosphere. However without the ability to identify WGS of isolates that did not survive on $CuTiO_2$ surfaces, we are limited in suggesting that the mechanisms outlined are the only mechanisms of survival.

Other metal resistance genes were present, interestingly the presence of *arsD*, *cadC* and

modB, which were not part of the antimicrobial surfaces tested. It is clear that these isolates have a significant potential to survive adverse conditions, and this adaptability allowed them to not only survive long enough to be transferred to antimicrobial surfaces, but to also form biofilms on them.

However, despite the presence of metal tolerance genes found in every isolate cultured, no testing has been conducted sampling the entirety of the surface for DNA which may have come from bacterial cells which died upon exposure to the various antimicrobial coatings. Therefore it is not possible to determine whether metal tolerance genes are widespread amongst all bacterial populations, or are only found in cultivable isolates which use them to survive on $CuTiO_2$ surfaces long enough to be captured during swabbing.

As such the presence of metal resistance genes poses a challenge for the use of $CuTiO_2$ surfaces for the use of reducing transmission of bacteria. Environmental isolates with a high likelihood of containing metal tolerance and resistance genes would be primed for survival on $CuTiO_2$ surfaces in a clinical setting. Whilst reducing the general number of bacteria, this gives rise to hot-spots of bacteria with a higher virulence than others. It is also found that metal resistance genes are found in conjunction with antimicrobial resistance genes (**PRITCHARDTHESIS**), and therefore transmission of an isolate from $CuTiO_2$ surfaces could propose a serious infection which could be resistant to antibiotics.

7.5 Challenges facing $CuTiO_2$ surfaces.

7.5.1 Lack of real world dynamic testing.

After demonstrating efficacy in reducing bacterial counts in the real world, where only hardy bacteria possessing copper resistance genes could survive, it would be vital the enhance this work by studying the feasibility in areas of mass public use and in a clinical setting before ensuring validation and accreditation for the respected ISO's is sought after. Despite experimentation, the real world dynamics of laboratory environments are lacking. Biofouling was not representative of the real world due to the high densities of the inoculum.

This bacterial inoculation was also administered through a form of liquid suspension, real world testing should utilise vaporised or aerosols. This is confirmed through SEM analysis which

highlighted the fact that most debris and contaminants were found within microscopic spots of water.

For the environmental aspect of this work, surfaces were placed in a discrete toilet, which often lacks thorough use or airflow. $CuTiO_2$ surfaces should be placed in a more open environment rather than in an isolated room. This would allow for further analysis, preferably an area with a larger quantity of foot traffic. This would allow for a better approximation of $CuTiO_2$ surfaces in the real world. As such all experiments act as an initial fact finding expedition, and lack further verification and follow up.

7.5.2 Longevity of $CuTiO_2$ surfaces are untested in real-world environments.

It is clear from all forms of testing, both inside and outside the laboratory that the longevity of surfaces can be challenging. Exposure to the elements over a long period of time, such as water droplets, can lead to disruption of the coating, although it is not known to an extent this damage occurs. If water droplets contain minor levels of salt or other metallic compounds, it is possible that during standard laboratory cleaning with autoclaves, galvanic electrolysis can occur. This was found through the initial autoclaving of surfaces within aluminium foil. After this was discovered, sterilisation was changed to ethanol baths, however prolonged exposure to ethanol (approximately three weeks of continuous submersion), also caused degradation. Not only are $CuTiO_2$ surfaces challenged through laboratory cleaning, but there is no data on their response to industrial or health care facility cleaners.

The $CuTiO_2$ surfaces coating itself can be quite fragile, as shown by the effects of removing small sections of glass substrate from larger sections, this caused damage prior to SEM imagery, which is visible in figure 5.1d as large grooves or channels.

These channels could provide areas for bacteria to reside in, depending on the depth and severity of the damage, and as mentioned above, surface finish and smoothness is critical in the reduction of bacterial transfer, especially if the coating in these damaged sections has been removed.

In summary, $CuTiO_2$ surfaces, whilst effective at killing bacteria, may be easily damaged depending on their location and application. The cleaning regimes required by clinical and industrial settings may also cause further damage, however this has not been studied and will

need to be assessed prior to any further clinical use.

7.6 Suitability of surfaces against ESKAPE pathogens and common hospital pathogens.

These experiments have displayed a level of efficacy against two common hospital pathogens, both from the ESKAPE group, however further testing and analysis is needed to cover the whole group. As such, conclusions can only be extrapolated for their use against *Staphyloccus aureus* and *Esherichia coli.* Despite this, $CuTiO_2$ surfaces show positive effects in the reduction of both bacteria, suggesting that activity is not specific to Gram positive or Gram negative organisms.

Both high density and low density *E. coli*, once inoculated onto the surface showed a significant reduction of viable cells after 60 minutes and through the whole time series. Only two of the ESKAPE group pathogens have been tested. As such to truly estimate their clinical efficacy, all the group should be tested, however results from *S. aureus* and *E. coli* show promising results against bacterial species that are not inherently resistant to metals such as *B. pumilus.*

Currently work is being undertaken to fully discern the efficacy of $CuTiO_2$ surfaces against all ESKAPE group pathogens as a starting point before extending this into other key clinical isolates. Future work would benefit from analysis of $CuTiO_2$ against other clinical bacterial species, such as *Klebsiella pneumoniae*, *Clostridium difficile*, and the highly multi-drug resistant *Neisseria gonorrhoeae.* These bacterial species pose neumerous threats to human health, and are well documented in being found in clinical environments. Aside from *E. coli* and *S. aureus*, no other ESKAPE pathogens were tested., and future investigations should aim to test all of the ESKAPE pathogen group within the scope of their work.

Whilst showing efficacy in reducing bacterial transmission, this can not be applied to the rest of the ESKAPE group, or potentially environmental ESKAPE pathogens which may possess virulence factors not contained within the standard laboratory strains.

7.6.1 Key bacterial species not tested against.

In this body of work, only two bacterial species have been tested against in the laboratory. As such there is a wide variety of other bacterial pathogens that have not been exposed to

$CuTiO_2$ coatings. Further work should be done establishing baseline effects against various putative pathogenic human flora, as well as microbes of clinical significance, such as *Mycobacterium tuberculosis*, *Clostridoides difficle*, and *Legionella pneumophila*.

To develop $CuTiO_2$ surfaces even further, a general consensus on the usefulness of $CuTiO_2$ surfaces against most clinically seen pathogens, and variations of those pathogens (such as MRSA as a variation of *Staphyloccus aureus*) should be tested.

These clinical variations could retain virulence factors which may produce tolerance to $CuTiO_2$ surfaces, therefore lowering overall efficacy. It would be a significantly useful study to compare the results from testing standard control strains of *E. coli* and *S. aureus* with clinical isolates found both within the hospital environment, but also isolates which have been the causative organism for human infection. Control strains have a limited level of virulence, whilst these wild-type clinical isolates may have other compounding factors, such as the uptake of MGE's from the environment which changes their behaviour when exposed to $CuTiO_2$ surfaces. Furthermore it would be of interest to determine if control strains of these tested isolates contain copper resistance genes, and should they lack them, they should be introduced and tested.

7.7 Conclusions.

To conclude, this body of work has described the context and necessity for the use of antimicrobial surfaces along with their implementation.

Antimicrobial resistance is on the rise, along with an increase in septic infections developing from health care associated infections. Antimicrobial surfaces could potentially reduce the burden of bacteria in clinical setting by reducing the rates of HCAI's and potentially eliminating sources of bacteria within a clinical environment.

Reducing the level of biofouling on surfaces can not only be applied to the clinical setting, but also to public environments, reducing transmission of bacteria between healthy people and those who are vulnerable, such as the elderly.

One key factor in the transmission of bacteria has been concluded to be surface finish, as such 304 stainless steel was found to transfer more bacteria than $CuTiO_2$ coated 304 stainless steel, both due to the smoother surface finish created by the CVD process, but also due to the bactericidal killing of any bacteria present.

Laboratory testing has shown that $CuTiO_2$ surfaces, both with and without UV light activation significantly reduces viable bacterial counts. This activity was then tested at various time intervals using Agarose plate transfer and BacLight Live/Dead staining to determine the rate of bacterial reduction and establish an effective working time.

After establishing that $CuTiO_2$ work within 60 minutes, this rules out which potential mechanisms of bactericidal killing could be at play. From this, we believe that the active mechanisms at play relate to the generation of free radicals under the presence of UV light, and the reductive reaction of copper ions within the cell membrane.

To explore this further, colonies subjected to $CuTiO_2$ surfaces were breifly tested against common antimicrobial compounds. After which, preliminary results found that exposure to $CuTiO_2$ coatings increased sensitivity to various antimicrobial compounds, such as ampicillin and tetracycline potentially providing a benefit that any infection picked up from a $CuTiO_2$ surface may be easier to treat. However this does not correspond to environmental isolates who all possessed tetracycline resistance genes able to prevent this benefit.

Environmental analysis provided an insight into the usefulness of $CuTiO_2$ within a real-

world environment. 16s analysis detected isolates that were both environmental in nature, and some which were common human flora. These isolates possessed copper resistance genes which facilitated their survival despite the effective killing of the $CuTiO_2$ coating.

Whole genome sequencing provided an insight into the exact copper resistance genes, where isolates possessed a *cop* or *ycn* family copper resistance gene.

Not only were copper resistance genes present, but a multitude of antimicrobial resistance genes were found, most commonly *tetA*, which was found in almost every sample. It is possible that due to the usefulness of tetracycline resistance, it is commonly conserved among isolates found. This was also found when searching WGS for mercury resistance, another heavily conserved sequence due to the tolerance it provides to environmental mercury.

Phylogenetic analysis of these isolates recovered showed similarity in their ancestral development, with isolates from different weeks being closely related, and therefore not forming groups based on the week they were collected upon. This implies that despite the time difference between sampling, isolates are still persistent on $CuTiO_2$ surfaces if they have protective mechanisms. It is also plausible that the same isolates are being continuously deposited on the $CuTiO_2$ surfaces from the same reservoir.

As such it is clear that against most bacteria, $CuTiO_2$ surfaces do provide a reduction in bacterial concentration and therefore reduce the transfer of bacteria. $CuTiO_2$ surfaces possess multiple mechanisms, which allows them to provide a significant reduction in bacterial survival within sixty minutes. Isolates able to survive on these surfaces from the environment contained some form of copper tolerance mechanism, likely acquired from their origins as an environmental bacteria. Despite this, few bacteria were recovered from surfaces placed in environmental areas, potentially due to the $CuTiO_2$ surface killing those which do not possess any forms of copper tolerance. This means that $CuTiO_2$ surfaces show an effective method of reducing bacteria in the environment for those which are naturally susceptible to the mechanisms used by $CuTiO_2$ surfaces to kill.

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