Establishing the Virucidal Properties of a Copper-Nickel-Zinc Alloy using a Genetically Modified Insect Virus

by

Sadru-Dean Walji

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I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

Healthcare associated infections (HAIs) cause a significant financial burden on the health-care industry. To reduce incidence of HAIs, strategies for cleaning and infection control have been developed to target common transmission routes. Surfaces that are frequently touched, so called high-touch surfaces, have been identified as a key transmission route. High-touch surfaces are cleaned inconsistently, and frequent disinfection of these surfaces is impractical.

Copper and its alloys have been shown to have contact biocidal properties and pose a solution to inconsistent and infrequent cleaning of high-touch surfaces. However, the virucidal properties of these surfaces are poorly understood due to variability in literature methods. The goals of this thesis are assess the virucidal properties of a copper(65)-nickel(18)-zinc(17) alloy designed to replace stainless steel high-touch surfaces, investigate factors that may diminish the virucidal properties, evaluate virucidal activity of each alloy component, and characterize leaching of metals from the alloy.

The copper alloy was shown to have a strong virucidal activity under clean and moderate soiling conditions ($>$4-log) for virus droplets or dried virus onto the surface. Multiple exposures of the surface to virus found that the surface was unable to inactivate virus droplets ($<$1-log) while dried virus was repeatably inactivated ($>$3-log), regardless of no or moderate soiling. Heavy soiling reduced inactivation below an acceptable efficacy threshold ($<$1-log). Copper, nickel, and zinc were identified as the primary metals being released (leached) by the surface and causing virucidal activity. These metals were further investigated as ion solutions.

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Virucidal tests of copper, nickel, and zinc ions found that copper and nickel were significantly virucidal (Cu,Ni p<0.05; Zn p>0.1). Concentration of the leached metal ions was dependent on the solution applied to the surface. The driving force behind leaching could not be identified but osmolarity, chlorine content, and protein load of the solution were ruled out.

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Dedication

This is dedicated to my parents. Your love, support, and motivation makes me who I am today. I am forever thankful.

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Abbreviations

AIV avian influenza virus

AiV Aichi virus

APMV avian paramyxovirus

BSA bovine serum albumin

BSL biosafety level

CAP cold atmospheric plasma

CDC United States Centers for Disease Control and Prevention

CFU colony forming units

CHIKV Chikungunya virus

CPE cytopathic effect

DI-viruses defective interfering viruses

DMEM Dulbecco's Modified Eagle Medium

DNA deoxyribonucleic acid

ECO electrochemical oxidant

ELISA enzyme-linked immunosorbent assay

EPA United States Environmental Protection Agency

EPDA endpoint dilution assay

F-TiO$_2$ fluorinated TiO$_2$

FAC free available chlorine

FBS fetal bovine serum

FCV feline calicivirus

GDA glutaraldehyde

HAdV-1 human adenovirus serotype I

HAI healthcare associated infection (also referred to as nosocomial infection)

HIV human immunodeficiency virus

HSV herpes simplex virus

HuNoV human norovirus

ICP inductively coupled plasma optical emission spectroscopy

InfA human influenza virus

MERS Middle East respiratory syndrome

mKOκ monomeric Kusabira orange κ fluorescent protein

MNV murine norovirus

MOI Multiplicity of Infection

NEW neutral electrolyzed water

PAA peracetic acid

PBS phosphate-buffered saline buffer

PEV porcine enteric virus

PFU plaque forming units

QAC quatenary ammonium compounds

qPCR quantitative polymerase chain reaction

RNA ribonucleic acid

RNS reactive nitrogen species

ROS reactive oxygen species

RT-qPCR reverse transcription quantitative polymerase chain reaction

SARS severe acute respiratory syndrome

SDC silver dihydrogen citrate

SDS-PAGE sodium dodecyl sulfate polyacrylamide gel electrophoresis

Sf9 *Spodoptera frugiperda* clonal isolate 9

SwIV swine influenza virus

TGEV transmissible gastroenteritis virus

TX-100 Triton X-100

UNS unified numbering system

UPW Type I ultra-pure water

WHO World Health Organization

Chapter 1

Introduction

1.1 Nosocomial Infections

Healthcare associated infections (HAIs), also called nosocomial infections, are infections in patients that occur while in hospital care and originate from pathogens within the hospital. These infections are a source of financial strain to healthcare institutions, costing billions of dollars annually [Magill et al., 2014]. HAIs are a ubiquitous problem and still occur in some of the most developed nations, with an adult patient infection rate of 4% in the United States [Scott II, 2009] and 10% in Canada [Magill et al., 2018]. Some of these infections are considered preventable; approximately 80% are spread by person-to-person contact [Butler-Jones, 2014]. Preventable HAIs are estimated to rival the cost of treatments such as stroke and diabetes complications [Scott II, 2009]. As a result, curtailing of HAIs has been investigated intensively [Scott II, 2009; Butler-Jones, 2014; Magill et al., 2018; Bender

and Holyoke, 2018].

HAIs are not a new or novel issue, but long-established strategies [Struelens, 1998] have not been sufficient to control the problem [Butler-Jones, 2014]. Control measures have grown from reactive procedures to a meticulous prevention system enforced by policy and informed by interdisciplinary research [Scott II, 2009]. The most prevalent tools used by current infection control practices center around surveillance of antimicrobial resistant pathogens and hand hygiene [Gebel et al., 2013; Magill et al., 2018; Butler-Jones, 2014]. To help supplement current hand hygiene practices, common sources of contamination can be identified.

Surfaces that are touched frequently by patients and health care workers are an important focus of HAI prevention methods [Gebel et al., 2013]. So called high-touch surfaces are tested with new [Park et al., 2014] and old [Boyce, 2018] disinfection technologies. These technologies will be covered in Chapter 3. A particularly interesting approach is to have a surface that passively inactivates pathogens. These surfaces have particular properties that causes disinfection. Some surfaces use unique geometry with nano-scale spikes [Tripathy et al., 2017] or slowly release bactericides, such as copper ions [Noyce et al., 2006]. Copper alloy surfaces are likely the best candidate for surface disinfection as they have been shown to have a broad range of efficacy and are robust enough to resist damage from wear [Warnes and Keevil, 2013; Noyce et al., 2007; Michels et al., 2015]. CuVerro Shield^{TM}, a copper alloy containing 65% copper, 18% nickel, and 17% zinc, is one such product currently sold as an antimicrobial surface in Canada [Aereus Technologies, 2019]. While these approaches have been shown to be effective for bacteria, the research on virucidal activity is limited.

Viral infections make up a small portion of HAIs, but their etiology is difficult to decouple from bacterial infections that typically follow [Chow and Mermel, 2017]. Recent studies have shown viral infections cause the highest rates of morbidity in neonatal intensive care units [Zinna et al., 2016]. Further, viral HAIs have been shown to extend patient stay and complicate patient diagnosis in adults [Bender and Holyoke, 2018], although they have not been shown to cause mortality [Hong et al., 2014]. Surface disinfection of viruses is an inconsistent and under-investigated field, yielding a precedent for further investigation.

1.2 Properties of Viruses and Their Quantification

As mentioned previously, viruses are an underestimated threat in HAIs [Hong et al., 2014]. However, studying viruses is a difficult task and some of these challenges will be outlined in the following sections. First, the physical characteristics of viruses will be described. Then, the quantification of viruses will be discussed, followed by the use of surrogate viruses. Next, the specific virus used in this thesis is discussed. Finally, the issue of organic soiling interference with disinfection is addressed.

1.2.1 Principles of Virus Structure and Infections

Viruses are defined as obligate intracellular parasites, requiring host cells for protein production and genome replication [Acheson, 2011]. Viruses are typically much smaller than their host cells, with viruses ranging from 0.02 to 0.5 µm and most cells ranging from 0.4 to 50 µm [Acheson, 2011]. All viruses encase their genome in a protective protein layer,

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called a capsid [Acheson, 2011]. Unlike traditional parasites, viruses must shed their protective coating and expose genetic material directly to the host cell to propagate. Viruses are difficult to categorize phylogenetically, but do have a few general phenotypes by which they can be categorized [Acheson, 2011].

Two core phenotypic factors that are used to categorize viruses are the type of genomic nucleic acid (DNA or RNA), and single or double stranded genome. These factors are the basis for the Baltimore classification system, one of the most popular virus classification systems [Acheson, 2011].

Another phenotypic difference, enveloped versus non-enveloped viruses, plays the biggest role in determining environmental persistence [Acheson, 2011; Steinmann, 2001]. Enveloped viruses coat their capsid in a lipid membrane that is taken from the host cell upon budding of virions (extracellular, infectious virus). When virions are released without a lipid envelope, they are 'naked' or non-enveloped viruses. Non-enveloped viruses have better resistance to environmental factors as the lipid envelop is more susceptible to chemical disinfectants, as with host cells. The lack of lipid membrane is why some viruses can require longer disinfection times or higher concentrations than bacteria [Terpstra et al., 2007].

1.2.2 Methods for Virus Quantification

Virus quantification can be performed in many ways. Three of the most common methods are binding assays, infectivity assays, and genome quantification. Method selection is dependent on the virus and the goal of quantification, as each method has a unique set of

limitations.

Binding assays rely on the virus's native cell entry system. Using host cell receptors, the virus binding can result in a physically quantifiable response. The most widely used example of binding assays is the heamagglutination assay [Pankaj, 2013]. In this assay, the heamagglutinin protein on the virus surface binds to sialic acid residues on the surface of red blood cells. This binding results in agglutination, a loosely packing form of the red blood cells, and appear as a pink coloured shell in the bottom of multi-well plates [Pankaj, 2013]. Cells that do not agglutinate will form a denser red pellet at the bottom of the well [Pankaj, 2013]. This method is specific to viruses that use sialic acid receptors as binding sites, and so is limited.

Of the quantification methods, infectivity assays have the most sensitivity and can discriminate for active virus [Murhammer, 2016]. These assays use a dilution series of virus exposed to host cells and rely on physical evidence of infection. The most commonly used infectivity assays are the plaque assay and the endpoint dilution assay (EPDA) [Murhammer, 2016]. The plaque assay is considered the 'gold standard' of infectivity assays and measures voids created by lysis in host cell monolayers. However, cells that do not grow in a monolayer are difficult to use for these tests. Alternatively, EPDAs are used by measuring cytopatheic effect (CPE). While judging CPE, viruses can be engineered to express a reporter protein (such as fluorescent proteins), which are easier to score [Murhammer, 2016]. A general issue with infectivity assays is virus unculturability. If an appropriate host cell cannot be cultured or if the virus itself does not replicate under cell culturing conditions, infectivity assays cannot be used.

In cases where binding assays and infectivity assays are not possible, genome quantification methods must be used. Depending on the virus' genome, either quantitative polymerase chain reaction (qPCR) and reverse transcription qPCR (RT-qPCR) can be used to approximate genome concentration. PCR methods work by amplifying a known section of the genome with a fluorescent marker and using standards to determine the concentration [Pankaj, 2013]. Alternatively, genomic material can be stained using fluorescent dyes and concentration determined using flow cytometry [Pankaj, 2013]. These methods cannot discriminate infectious from noninfectious virus without some pre-processing. For example, samples can be incubated with DNase or RNase to degrade unprotected genomes before quantification [Manuel et al., 2015]. However, even with these preparations, methods are not as accurate at determining virus concentration [Manuel et al., 2017].

When trying to quantify virus, infectivity assays are the optimal method. However, issues such as virus unculturability prevent utilization of these tools. In some cases, viruses that are similar, either phylogenetically or physically, can be used instead.

1.2.3   Surrogate Viruses

Several studies have been performed to evaluate the strengths and weaknesses of using viruses or their surrogates. However, only a few key examples will be presented so that a general understanding of the challenges is achieved.

Use of surrogates is required when virus cannot be propagated in cell culture. This 'unculturablility' requires use of phylogenetically related or physico-chemically similar viruses [Cromeans et al., 2014]. Having an accurate surrogate of the target virus is an important

step in validating disinfection and infection control strategies. Alternatively, surrogate viruses can be used when the virus is highly pathogenic, and so for safety reasons, it is better to use a less pathogenic surrogate [Cook et al., 2015]. Further, highly pathogenic viruses require access to high biosafety level labs, but access is restricted and limits research. For example, there are only a few biosafety level (BSL) 4 facilities in the world, but there can be numerous BSL 2 facilities within a research institution [Federation of American Scientists, 2013].

Norovirus is the world's leading cause of acute gasteroenteritis, and has caused numerous outbreaks globally [Moorman et al., 2017]. As such, disinfection of this virus is important to study. However, unculturability of this virus means many surrogate virus options have been investigated. Some of these options are MS2, murine norovirus (MNV), feline calicivirus (FCV), Aichi virus (AiV), and porcine enteric virus (PEV). An excellent study by Croomeans *et al* compares the physical characteristics of these viruses [Cromeans et al., 2014]. Some of their results are discussed in Section 3.

Surrogates are also used for the Coronavirus family, of which severe acute respiratory syndrome (SARS) and Middle East respiratory syndrome (MERS) are the most prominent. The surrogates typically used are mouse hepatitis virus and transmissible gastroenteritis virus [Goyal et al., 2014] [Boyce, 2018]. Using these surrogates allows establishing infection control methods to help hospitals prepare for outbreaks. Having surrogates that exhibit similar physical properties is important for determining the effectiveness of a proposed control method.

1.2.4   Baculovirus

Baculovirus are viruses that infect insects; these viruses have been studied extensively in cell culture. The baculovirus and insect cell culture system have found use in three areas: protein synthesis, pesticide synthesis, and as a model system [Murhammer, 2016]. The most studied baculovirus is *Autographa californica* multiple nucleopolyhedrovirus (AcM-NPV), and is known to infect many insect species in addition to some mammalian cell lines [Murhammer, 2016]. An advantage to using this system is that it is cheaper and simpler to maintain than mammalian cell culture, while retaining some protein synthesis complexities lost to the simpler bacterial cultures [Murhammer, 2016]. For this reason, insect cell and baculovirus systems have been adopted for manufacturing of some therapeutics [Murhammer, 2016].

Baculoviruses are enveloped viruses, with a relatively stable envelope [Jarvis and Garcia, 1994]. A study on storage conditions of baculoviruses found that they were stable for a wide range of temperatures, multiple freeze-thaw cycles, and only lost infectivity after exposure to light [Jarvis and Garcia, 1994]. Influenza virus, an enveloped virus that infects five million people a year [Mistilis et al., 2017], requires stabilizers for long term storage when used in vaccines [Coenen et al., 2006] and is very sensitive to temperature and freeze-thaw cycles [Patois et al., 2011]. These properties suggest that baculovirus would make a conservative surrogate for the studies of enveloped virus disinfection. A further advantage is that lab-strain AcMNPV is certified as Biosafety Level 1 (BSL-1), meaning it is safe to handle and does not require expensive facilities to maintain safety. For these reasons, AcMNPV was selected for the work performed in this thesis.

1.3 Soiling and Disinfection Interference

In a general sense, soiling is the contamination of an object by foreign matter and can interfere with the normal function of the object. Organic soiling is when the contamination is made of organic matter, and 'dirties' an object. One routine method of organic soiling generation is as a normal part of infections, whereby healthy cells are destroyed and release proteinaceous and nucleic acid debris.

In the context of disinfection, soils are an interfering factor that can reduce the efficacy of a disinfectant [Rutala et al., 2019] [Steinmann, 2001]. This occurs when disinfectants are quenched by soils before neutralizing the pathogens. Therefore, testing disinfectants on pathogenic samples with a representative level of organic soiling is critical to understanding the actual-use efficacy. This is particularly important for lab preparations of viruses, where virus stock must be purified (clarified) as too much cell debris can significantly degrade virus stocks or hinder infection [Murhammer, 2016]. This process does not remove all the debris and problems in testing with this purification method is discussed further in Chapter 4.

1.4 Hypothesis

A copper-alloy surface designed to reduce healthcare associated infections (HAIs) has already been shown to exhibit antimicrobial properties [Aereus Technologies, 2019]. The goal of this thesis is to determine if the alloy also has virucidal properties, and what factors are important to achieve the greatest inactivation from the surface.

1.5 Objectives

The objectives and structure for the thesis are as follows:

1. Analyze literature in antimicrobial studies to establish a suitable virucidal surface test methods (Chapter 2 and 3);

2. Evaluate the virucidal properties of copper-alloy coating, and any pertinent factors interfering in said properties (Chapter 4);

3. Quantify alloy metal ions (namely copper, nickel, and zinc) to understand their role in virus inactivation (Chapter 5); and

4. Determine matrix properties that allow for release of virucidal ions from alloy surface to establish clinical relevance (Chapter 6).

Chapter 2

Review of Standard Methods to Evaluate Self-Sterilizing Surfaces

The following section is written as a manuscript evaluating disinfection by self-sterilizing surfaces. Currently, there are no standard test that explicitly measure the virucidal properties of self-sterilizing surfaces. However, standard methods that perform similar tests are compared for their applicability to known interfering factors of virus surface disinfection.

2.1   Introduction

Healthcare associated infections (HAIs) are a problem in health-care across the globe. These infections are contracted while a patient is already in hospital leading to complications, longer stay, and delayed treatment of other patients [Bender and Holyoke, 2018]. In

the United States, the HAI Prevalence Survey project conducted by the Center for Disease Control (CDC) found that from 2011 to 2015, there was a 3.2% drop in number of HAI cases but the mortality rate remained approximately the same at 11% [Magill et al., 2018]. In Canada, from 2013 to 2017 there has been a decrease in incidence for two of the three major HAI-causing antimicrobial-resistant bacteria [Program, 2017]. A breakdown of incidence-by-ward illustrates how extensive infection rates can be, with some European intensive care units reaching close to 50%. HAI contraction rates become even more alarming when looking at developing countries, with some reaching a fatality rate of approximately 20% [Allegranzi et al., 2011].

The HAI Prevalence Survey also found that up to 70% of HAIs can be avoided with better surveillance [Magill et al., 2014]. These infections have a significant economic burden, with some estimates putting the cost of HAIs in the range of billions of dollars per year in the US alone and warrant cost-saving interventions [Arefian et al., 2016] [Fukuda et al., 2011]. Causes of HAIs are well known, with a multitude of studies performed to further assist in decreasing the rate of occurrence [Struelens, 1998; Gaynes et al., 2001; Allegranzi and Pittet, 2009]. As HAIs are a broad category of infections, many disciplines have collaborated to target different sources. Results of these studies help in the ever-advancing pursuit to eliminate HAIs and have yielded a multitude of procedures, guidelines, and even legislation [Stone et al., 2015].

One cause of HAIs is through transfer of pathogens from common touch surfaces. These surfaces are a reservoir for pathogens and infections can be transmitted and contracted by patients, health-care workers, and visitors. Studies have shown that cleaning in hospitals is inconsistent, with thorough cleaning only feasible after patient discharge (terminal

cleaning) [Manian et al., 2011]. Further, some studies have shown that even cleaning with disinfectants only provides temporary relief, with bioburden levels returning to pre-cleaned levels within 2.5 hours [Attaway et al., 2012]. Given the rapid bioburden restoration and variability in cleaning effectiveness, a passively cleaning surface is beneficial.

Copper has been shown to be an effective passively cleaning surface capable of reducing the high rate of bioburden restoration seen on hospital surfaces [Schmidt et al., 2013]. The US Environmental Protection Agency (EPA) allowed copper and its alloys to claim antibacterial properties starting in 2008 [Environmental Protection Agency, 2016]. In the ensuing years, research evaluating these properties for copper-alloys has increased greatly, studying the interactions between surface and bacteria. While no single mechanism for inactivation has been determined, biocidal properties are likely due to a multi-faceted attack involving loss of membrane integrity, interference with cellular machinery, and disruption of DNA and RNA structure. A standardized protocol is currently in development to help validate antibacterial claims; however, a standard test for virucidal properties is lacking. Showing that such surfaces can obtain virucidal activity should be an important part of claiming HAI prevention as viral infections reportedly make up 60% of global infections [Vasickova et al., 2010].

Several reviews have been performed to show the antimicrobial properties of copper surfaces to prevent hospital-acquired infections and charted the progress since they became EPA approved [Villapun et al., 2016; Chyderiotis et al., 2018; O'Gorman and Humphreys, 2012; Grass et al., 2011]. Briefly, these reviews have found that copper likely reduces HAIs and has been shown to be effective against a variety of microbial organisms in the lab. However, no reviews have been performed for virucidal properties of these surfaces, partly

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due to the handful work focusing on this subject. The goal of this review paper is to develop a set of recommended parameters to evaluate virucidal activity of self-sterilizing surfaces, particularly for those composed primarily of copper. This will be achieved by reviewing antimicrobial standard protocols, critically evaluating protocols currently used by researchers for virucidal tests, and assessing related virucidal standard protocols.

2.2   Methods

Three databases (Scopus, PubMed, and Web of Science) were queried using a form of the string: (((nosocomial) OR (hospital infection*) OR (health care infection*) OR (healthcare infection*) OR (HAI) OR (HCAI)) AND (((metal)OR(copper)OR(alloy))) AND(((virus)OR (virucidal)OR(viruscidal)OR(antiviral)OR(viruc*)))). Articles were only selected if they matched the strict criteria of: virucidal properties of copper surfaces designed to prevent healthcare associated infections. General searches of the literature were also performed to find related articles and standard protocols.

2.3   Existing Antimicrobial Standard Tests

There are many standards organizations that seek to create technical standards. These standards allow for consistency of products across an industry. There are many standards organizations, but a few of the prominent ones are ASTM International (ASTM), International Organization for Standardization (ISO), and Japanese Industrial Standards (JIS).

A review by Villapun et al. [2016] identifies standards from the EPA, JIS (Z 2801:2010), and ISO (22196:2011) as the most suitable methods for antimicrobial surfaces considered self-sterilizing [Villapun et al., 2016]. Briefly, the review identifies the EPA protocol, the only one designed specifically for copper surfaces, as the best method to use. This section seeks to expand on their review and highlights shortcomings with some of the mentioned protocols. Further, an in-depth analysis of the EPA protocol will be used to construct a platform for virucidal assessment copper surfaces.

EPA, ISO, and JIS protocols test one Gram-positive (*Staphylococcus aureus*) and one Gram-negative bacteria. The EPA protocol uses *Pseudomonas aeruginosa* instead of *Escherichia coli* as the Gram-negative bacteria as some strains are antibiotic resistant and therefore of clinical concern [Villapun et al., 2016]. It should be noted that the ISO adopted the JIS protocol, and so they are the same [Villapun et al., 2016]. Both the JIS and ISO test 6 x 10^5 cells/mL on the surface as well as a known non-antibacterial surface as negative control (such as stainless steel or polypropylene Petri dish) [Villapun et al., 2016]. These are incubated at high relative humidity (>90%) for 24 hours at 37°C [Villapun et al., 2016]. One of the main concerns with these two protocols is that these conditions are not reflective of a hospital environment. As an example, when testing antimicrobial activity of silver-containing surfaces, there will be discrepancies between field tests and standard tests. Following the JIS Z 2801 protocol at high humidity (> 90%), silver surfaces have high inactivation, but at lower humidities (tested at 50% and lower) silver surfaces lose almost all antibacterial properties [Michels et al., 2009]. The high relative humidity and temperature do not represent conditions in hospitals and is the true weakness of this method. Both temperature and humidity are strictly controlled in hospitals. The Facility Guidelines In-

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stitute (FGI)'s guidelines for the design of hospital and care-facilities is hugely influential in the US. The FGI Guidelines have even been adopted, in varying degrees, by state legislation for building compliance [Facility Guidelines Institute, 1999]. These evidence-based guidelines, have determined a recommended humidity between 30 to 60 % and temperature between 20 to 24 °C [Wong et al., 2017]. Testing at these conditions would allow a better understanding of how the surfaces might perform when implemented in hospitals.

Parameters tested in the EPA protocol are more extensive than those in the ISO and JIS protocols. The EPA protocol tests resistance to routine abrasion and cleaning in addition to the antimicrobial properties [Environmental Protection Agency, 2016]. Surfaces are tested against 200 cycles of abrasion and cleaning at ambient temperature and humidity over 8 weeks, using three commonly used cleaning agents. They are then tested for antimicrobial activity and compared to unabraded surfaces [Environmental Protection Agency, 2016]. Additionally, the EPA protocol requires a soiling test. Tested organisms are centrifuged to remove growth media, resuspended in PBS, and mixed with an organic soil. This organic soil is composed of BSA, mucin, and yeast extract to simulate potentially interfering organic debris [Environmental Protection Agency, 2016]. After being incubated, the surface is submerged in 20 mL of neutralizer solution to inactivate the surface and resuspend the surviving bacteria [Environmental Protection Agency, 2016]. In addition to comparison with stainless steel, four controls are required: inoculum purity, soil load sterility, surface sterility, and neutralizer sterility [Environmental Protection Agency, 2016]. Overall, this protocol addresses the weaknesses of the ISO and JIS. Additionally, the abrasion and cleaning agent tests simulate the long-term efficacy of the surface from use-degradation.

The EPA protocol is still under development and is working with ASTM to create a

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standardized method [Rogers, 2010], but no official protocol has been published by either agency. The protocol is currently on the second round of public input on the method [Environmental Protection Agency, 2016], and is specifically designed for copper surfaces [Villapun et al., 2016]. No such standard tests exist for virucidal properties of surfaces, however tests do exist for virucidal properties of surface disinfectants.

2.4 Virucidal Standard Protocols for Non-Copper Surfaces

In order to develop a protocol for virucidal properties of copper surfaces, currently employed standard tests of surface disinfectants can be of use. ASTM International was selected as the database from which to evaluate standard methods. As sample preparation can be drastically different between virus and bacterial samples, related virucidal assessment protocols will be examined. Additionally, nuances of sample handling, testing, and recovery to achieve high quality results will be reported as part of the effort to establish protocol guidelines. Currently ASTM lists seven current protocols within the ASTM Subcommittee E35.15 on Antimicrobial Agents that address virucidal activity, four of which pertain to surface disinfection. They are:

- E1052-11 Standard Test Method to Assess the Activity of Microbicides against Viruses in Suspension [ASTM International, 2011a]

- E1053-11 Standard Test Method to Assess Virucidal Activity of Chemicals Intended

for Disinfection of Inanimate, Nonporous Environmental Surfaces [ASTM International, 2011b]

- E2197-17e1 Standard Quantitative Disk Carrier Test Method for Determining Bactericidal, Virucidal, Fungicidal, Mycobactericidal, and Sporicidal Activities of Chemicals [ASTM International, 2017]

- E2721-16 Standard Practice for Evaluation of Effectiveness of Decontamination Procedures for Surfaces When Challenged with Droplets Containing Human Pathogenic Viruses [ASTM International, 2016b]

ASTM E1052-11 was designed to test the effectiveness of microbicides for viruses in suspension [ASTM International, 2011a]. Briefly, the test mixes one part virus to nine parts microbicidal mixture for as long as the manufacturer directs [ASTM International, 2011a]. The microbicide is then neutralized according to manufacturer directions and virus is titered using an appropriate method [ASTM International, 2011a]. Virus can be prepared using an organic soil, to simulate contamination that may interfere with the microbicide's effectiveness [ASTM International, 2011a]. While this method does not have much translatability to self-sterilizing surfaces, it does provide a comprehensive matrix of clinically important non-enveloped and enveloped viruses and their recommended host cell lines [ASTM International, 2011a]. These combinations are recreated in Table 2.1. All listed viruses do not have to be tested, but it is recommended that at least one non-enveloped virus is, due to their longer environmental persistence [ASTM International, 2011a]. This protocol is unsuitable for self-sterilizing surfaces because it tests virucidal activity of virus in solution. Aside from the virus and host cell line matrix, this protocol

does not provide much other information that would be applicable to self-sterilizing surface standards.

ASTM E1053-11 is used to test virucidal products after the virus is dried onto a non-porous surface [ASTM International, 2011b]. To assess virucidal activity, at least 10^4 infectious units per surface are required. The suggested volume is 200µL, but additional volume can be used to meet the minimum infectious units requirement. The chosen volume is dried onto a glass petri dish, forming a film. 2mL of working-concentration virucidal agent is applied to the film for the agent's directed contact time [ASTM International, 2011b]. Immediately after the contact time expires, 2mL of neutralizing agent is added and a cell scraper used to resuspend the virus film [ASTM International, 2011b]. This mixture is then serially 10-fold diluted using a buffer and titered with four technical replicates [ASTM International, 2011b]. For virus selection, the same matrix of viruses and appropriate host cells in E1052-11 is provided [ASTM International, 2011b]. Unlike E1052-11, E1053-11 requires an organic soil as part of the virus preparation, and describes a specific formulation [ASTM International, 2011b]. Stock solutions of bovine mucin (0.004g/mL), BSA (0.05g/mL), and tryptone or yeast extract (0.05g/mL) are all dissolved in PBS at pH7.2 [ASTM International, 2011b]. These stock solutions are then combined with the virus in the following concentration: 20% bovine mucin, 5% BSA, 7% tryptone or yeast extract [ASTM International, 2011b].

ASTM E2197-17e1 is a test of biocidal sprays, and covers bactericidal, virucidal, fungicidal, mycobactericidal, and sporicidal properties of disinfectants [ASTM International, 2017]. Seven test viruses are listed (Human Adenovirus 5, Hepatitis A virus, Canine Parvovirus, Feline calicivirus, Human Rhinovirus, Human Rotavirus, and Murine Norovirus)

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[ASTM International, 2017], but none of them are enveloped. Rationale for selection of these viruses is not clear, but all either are, or are surrogates for, commonly occurring infections. It may be useful to include enveloped viruses as well, as they may behave differently from non-enveloped viruses. One small, but important, weakness of the protocol is the method of harvesting the virus. After using freeze-thaw cycles to release the virus, a low-speed centrifugation is used to remove large cell debris, (ultracentrifugation is only recommended for concentration of virus) and the organic soil is added [ASTM International, 2017]. While suggested growth medium is the same for all host cell-lines [ASTM International, 2017], a clearer declaration of virus matrix is required. A procedure similar to the proposed EPA protocol [Environmental Protection Agency, 2016] would be best, whereby virus is resuspended in PBS after purification (either through dialysis or ultracentrifugation) and then spiked with the organic soiling mixture. A minimum threshold of infectious units should be used to allow detection of a 4-log decrease in viral titer, and so the purification methods can also be used to concentrate the virus. Although this sample preparation may seem extensive, it would be essential to minimize any variability of conditioned growth medium being confounded with interference from soiling. Additionally, purification would avoid the variability of using the various growth medias for respective host cell lines.

Of the mentioned protocols, ASTM E2721-16 is the closest to providing a viable protocol to assess virucidal properties of self-sterilizing surfaces [ASTM International, 2016b]. This protocol is designed to test decontamination of surfaces when exposed to aerosolized droplets of human respiratory viruses [ASTM International, 2016b]. In fact, it even mentions that it may be used for self-decontaminating materials [ASTM International, 2016b].

Application of the virus to the surface is performed using a spraying device (which can be readily assembled with the provided details) and which accurately mimics dispersion from humans [ASTM International, 2016b]. However, this protocol cannot be considered a *de facto* method for self-sterilizing surfaces because of its specificity. Viral spread occurs through either aerosolization (respiratory viruses) or the fecal-oral route, both of which use touch-surfaces as reservoirs. A second component, with higher levels of organic soiling seen in fecal matter [Rose et al., 2015] would be required for a comprehensive study. The organic soil used in E2721 [ASTM International, 2016b] is an artificial saliva, as opposed to the simpler soils in E1053 and E2197 [ASTM International, 2017], and should be used as the organic soil used in the study of respiratory viruses. By no means should this protocol be disregarded until a more comprehensive method is developed, instead it should be actively used until the new guidelines are established.

In summary, the currently employed ASTM protocols are not applicable to test for virucidal properties of copper surfaces. All protocols are deficient in humidity and temperature control [ASTM International, 2011a,b, 2017, 2016b]. These are important factors that can have an effect on a surface's performance [Michels et al., 2009], and are tightly controlled in hospitals [Facility Guidelines Institute, 1999; Wong et al., 2017]. These standards do provide an accurate, light organic soil and method of testing aerosolized virus [ASTM International, 2016b], but are lacking in testing heavier soiling rates found in viruses spread along the fecal-oral route [Rose et al., 2015].

Table 2.1: ASTM 1052-11 recommended test viruses and their host cell lines [ASTM International, 2011a]

Virus Type	Virus[*]	Host Cell Line
Enveloped	Cytomegalovirus, strain AD-169	Human diploid lung (MRC-5 or WI-38)
	Herpes Simplex Virus, Type 1, strain F(1)	Vero Hep-2
	Influenza A, strain Hong Kong/8/86 or PR/8/34	Madin-Darby Canine kidney Rhesus monkey kidney
	Respiratory syncytial virus, strain Long	Hep-2 MRC-5
	Vaccinia, strain WR	VERO Hep-2
Non-Enveloped	Adenovirus Type 2 or 5	Human Lung Carcinoma (A549) Hep-2 Vero
	Canine Parvovirus, Cornell-780916-80	A-72
	Feline calicivirus, strain F-9	CRFK
	Hepatitis A Virus, strain HM-175	FRhK-4
	Murine Norovirus	RAW 264.7
	Rhinovirus, Type 14 or 37	MRC-5 WI-38 HeLa T4+
	Rotavirus, strain Wa	MA-104 CV-1

[*] All suggested viruses are BSL-2

2.5 Virucidal Assessments Used in Literature

While there is a current standard protocol [ASTM International, 2016b] with the ability to test virucidal properties of self-sterilizing surfaces, none of the reviewed publications have employed it. A few example publications have been selected to show the diversity with which virucidal properties are currently tested and reported. Using methods in the following articles, the antimicrobial testing standards previously discussed, and the virucidal tests for disinfectants, a protocol specifically to test virucidal properties of non-porous, hard surfaces can be developed. The test conditions are summarized in Table 2.2 and will be discussed here.

A general weakness among the studies was no control of humidity [Campos et al., 2012; Hodek et al., 2016; Michels et al., 2009; Noyce et al., 2007] [Park et al., 2014; Villapun et al., 2016; Warnes and Keevil, 2013; Warnes et al., 2015b]. Based on data from our lab alone (unpublished), humidity in the culture lab can vary from 70% RH on a rainy spring day, to as low as 23% RH on a winter day. While copper has been shown to retain biocidal properties at a range of humidities, there is evidence that silver can have drastically varying antimicrobial activity with varying humidity [Michels et al., 2009]. Therefore, to allow for accurate assessment against competing technologies (like silver), humidity is a variable that should be controlled. If abrasion is to be included in the test, then humidity will play a part in surface degradation. While the humidity should fall into a relevant range at ambient conditions, it should be explicitly reported.

Time and temperature were in a relevant range for most of the studies. Only one study did not test at or around ambient temperature [Noyce et al., 2007]. All studies tested a

relevant time scale. The only study that went up to 24 hours was part of a time course where relevant times were sampled and assayed [Noyce et al., 2007].

Only one study accounted for soiling effects and mentioned the ISO guideline on creating such a factor [Campos et al., 2012]. Campos et al. [2012] found that soiling can have an impact on the long term stability of the virus [Campos et al., 2012], which therefore may also play a role in the virucidal properties of the surface. In conjunction with soiling, the virus matrix also plays an interfering role. This leads to the issue of virus preparation.

Three of the studies used PBS [Campos et al., 2012; Warnes and Keevil, 2013; Warnes et al., 2015b], but one diluted the PBS virus solution with media [Warnes and Keevil, 2013]. One did not say what the virus was resuspended into after purification [Park et al., 2014], one did not purify virus (but it should be noted that this experiment predates conception of the EPA protocol) [Noyce et al., 2007], and one did not explicitly report the conditions but is likely filtered cell growth media (DMEM supplemented with L-glutamine, 10% FBS, and antibiotics) [Hodek et al., 2016]. Resuspending virus in PBS is significantly harder than performing the equivalent for bacteria, with techniques such as dialysis ultra-filtration and ultra-centrifugation requiring specialized equipment. However, if the starting concentration of virus is high enough, it can be diluted into the appropriate matrix such that any interfering factors are too dilute to have any effect. This allows a much simpler preparation where amplified virus stock is clarified (removal of host cells) via low-speed centrifugation and followed by dilution to create a working stock. However, filtration of the virus using a 0.2 µm filter prior to dilution would be necessary to remove any of the larger debris as clarification via centrifugation does not remove all large debris.

The most obvious issue here is that no two protocols for virucidal activity were the same, even studies from the same group used different virus preparation methods [Warnes and Keevil, 2013; Warnes et al., 2015b]. Lack of consistency among testing protocol makes comparisons between papers near impossible preventing accurate comparisons of different surfaces or alloys. Therefore, it is strongly urged that some general testing parameters to operate within are agreed upon. It would be beneficial to develop a protocol that tests the wide survivability properties of viruses, beyond just testing the enveloped and non-enveloped categories. Further investigation is required to determine the parameters of the guidelines, some of which may include exploring DNA versus RNA viruses, single versus double strand, and capsid compositions. Enveloped viruses should have similar envelop compositions since they are all human-derived viruses, and so that should not be as great a concern. A challenge of having many viruses to test means that the academic labs may struggle to provide comprehensive data, as it would be unlikely for academic labs to have the breadth of these viruses and their host cell lines.

Table 2.2: Selected references showing variability in virus inactivation methods

Virus	Test	Temperature	Humidity	Time	Soiling	Purified	Reference
APMV	Biocide spray	20°C	NR	1, 5 min	Yes	Yes, PBS	[Campos et al., 2012]
MNV	Copper surface	Ambient	Ambient	120 min	No	Yes, PBS	[Warnes et al., 2015b]
MNV	Copper surface Wet and dry	37 and 4°C	NR	0-30 min (dry) 0-120 min (wet)	No	Yes, PBS Wet diluted with media	[Warnes and Keevil, 2013]
Influenza A	Copper surface	22°C	50-60%	1-24h	No	No	[Noyce et al., 2007]
MS2, FCV, MNV	UV activated F-TiO2 surface	Ambient	Ambient	120 min	No	Centrifugation and/or filtration	[Park et al., 2014]
HIV, Coxsackie B3, HSV1, Influenza A, Dengue type 2	Ag, Cu, Ni sol gel coating	NR	NR	240 min	No	NR	[Hodek et al., 2016]

2.6 Conclusions and Recommendations

This review initially sought to summarize protocols used for assessing virucidal properties of self-sterilizing surfaces, with a focus on copper and copper-alloys. However, very little work has been done in this field and works have had little consistency. This forced a change of the scope to encompass some closely related works, including standard antimicrobial copper protocols and established protocols for virucidal disinfectants.

For antimicrobial standard tests, there are directly equivalent protocols for testing the antimicrobial properties of self-sterilizing copper. The protocols issued by JIS and ISO do not adequately represent environmental conditions found within a hospital [Facility Guidelines Institute, 1999]. The draft protocol proposed by the EPA in conjunction with ASTM holds the greatest value in terms of what factors need to be tested using virus. Literature showed little consistency between published methods, making comparisons between the different surfaces types difficult. However, each piece of this puzzle provided insight into which factors should be considered in a comprehensive analysis of virucidal properties of self-sterilizing surfaces.

The final experimental guidelines determined by analyzing these studies and protocols are humidity, temperature, organic soiling, viral species, and appropriate controls. Humidity and temperature are tightly regulated in hospitals and therefore testing conditions should replicate them. Temperature is held within a narrow range of 18 to 24 °C, and so any value within this range can be deemed appropriate. Humidity experiences a far greater range, with recommendations being 30 to 60 % and, in practice, reaching approximate ranges of 16 to 46 %. It would be prudent to test at the ends of these ranges: 16%

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and 60%. Selection of viral species requires reaching a difficult balance between accessibility and comprehensiveness of the protocol. Minimum requirements should include a prototypical enveloped and non-enveloped viruses as established by the most frequently occurring infections of those viruses. Studies should be undertaken to evaluate if virus capsid composition, nucleic acid type, and strandedness can reduce or prevent disinfection. Finally, testing of appropriate controls is important to establish applicability. Surfaces tested should include those that are being sought to be replaced (i.e. control stainless steel for antimicrobial copper bed rails and door knobs) and determination of virus persistence under the tested conditions (i.e. not subjected to a surface). Using these guidelines will help to ascertain efficaciousness as well as cost-effectiveness of alternative surfaces.

Chapter 3

Literature Review

Nosocomial infections are a source of significant strain on health-care resources. In Canada, nosocomial infection rates for patients affect 1 in 10 adults and 1 in 8 children, with an estimated 80% of these infections spread by person-to-person contact [Butler-Jones, 2014]. Several control and disinfection strategies have been developed to help combat transmission of pathogens [Magill et al., 2014], with viral nosocomial infections not uncommon [Hong et al., 2014].

Viral infections do not constitute the largest proportion of nosocomial infections, but represent a 'hidden danger' as they are under-reported [Chow and Mermel, 2017] and underestimated [Hong et al., 2014]. Further, viral nosocomial infections have the highest rates of morbidity in neonatal intensive care units [Zinna et al., 2016]. While some viral infections are not fatal in adults [Hong et al., 2014], they do result in complication of diagnoses [Bender and Holyoke, 2018], extension of patient stay [Zinna et al., 2016], and treatment

delay of other critical patients [Bender and Holyoke, 2018]. These factors increase costs and waiting times at hospitals, causing back-logs across the health-care system [Bender and Holyoke, 2018][Zinna et al., 2016]. Controlling the spread of these viruses would aid in reducing viral nosocomial infections.

High-touch surfaces have been identified as a key mode of transmission for nosocomial infections [Gebel et al., 2013]. Evidence shows that fecal-oral route viruses, like norovirus [Warnes et al., 2015b], and respiratory viruses, like coronavirus [Warnes et al., 2015a], retain infectivity for many days after deposition on these surfaces. These studies give precedence to evaluating virucidal properties of disinfection methods.

Traditional infection control barriers utilize disinfectants. These disinfectants are applied to surfaces in a variety of methods, such as spray, vapour, or light. Spray and vapour disinfectants are chemical agents that can disrupt the pathogen's viability through membrane and/or genomic damage [Zonta et al., 2016a], whereas ultraviolet (UV) light primarily causes genomic damage [Health Quality Ontario, 2018]. However, extensive use of disinfectants such as bleach [Gallandat et al., 2017] and UV are known to damage surfaces. Therefore, it is important to continue refining disinfectant technology.

An emerging complementing disinfection method is the use of self-sterilizing surfaces, which disinfect pathogens on contact using unique surface properties. Disinfection can be achieved through a variety of ways that include unique surface geometries and leaching of disinfecting agents. Interest in this area has been aided by the United States Environmental Protection Agency (EPA) certifying copper as a contact antimicrobial in 2008 [Environmental Protection Agency, 2016], and the previously established properties of an-

timicrobial silver [Rai et al., 2009]. These surfaces are used in conjunction with existing control and disinfection protocols, and aim to provide a comprehensive barrier to surface transmission of disease.

A general issue with disinfection, is the reduction in efficacy of disinfectants by organic soiling [Rutala et al., 2019]. Soiling represents myriad of organic molecules originating from patients. Pathogens are suspended in bodily fluids that are high in organic matter, which can protect pathogens from disinfectants. In lab cultures, the pathogens are typically purified before any further work is done, and would not be representative of the in-use scenarios [Steinmann, 2001] [Rutala et al., 2019]. To avoid this, many standard tests include an organic soil after virus harvest and purification (e.g. [Environmental Protection Agency, 2016], [ASTM International, 2016a]).

Antimicrobial studies have dominated the academic space, both for traditional disinfectants and self-sterilizing surfaces; however, comparatively little has been done to explore and evaluate virucidal properties. Further, the methods surrounding virucidal properties of disinfectants in particular is inconsistent in literature, perhaps in part to a variety of possible methods from major standardization bodies. Although important parameters such as test type (suspension versus carrier) and soiling have long been established as important factors [Steinmann, 2001], ill-suited methods are frequently employed. Further, some notable reviews of self-disinfecting surfaces were

This literature review will focus on virus disinfection of high-touch surfaces, specifically with disinfectant treatments of stainless steel (a common high-touch surface) or self-sterilizing surfaces designed to replace them, and published in the last five years (2014-

2019). 25 studies met the inclusion criteria, with 19 testing disinfectants and 6 testing self-sterilizing surfaces. The goal of this review is to provide an update of the literature, briefly discuss how those technologies work, and evaluate the merits of the test methods.

3.1 Methods

Three databases (Scopus, PubMed, and Web of Science) were queried using a form of the string: (TITLE-ABS-KEY (virucide OR virucidal OR virus) AND (TITLE-ABS-KEY (surface AND (disinfectant OR sterilization)) OR TITLE-ABS-KEY (self AND sterilizing))) AND (LIMIT-TO (PUBYEAR , 2018) OR LIMIT-TO (PUBYEAR , 2017) OR LIMIT-TO (PUBYEAR , 2016) OR LIMIT-TO (PUBYEAR , 2015) OR LIMIT-TO (PUBYEAR , 2014)). Only articles testing disinfectants for healthcare use were included. General searches of literature were also performed to find related articles.

3.2 Standard Tests

Validation of commercial disinfectants is achieved by accredited standardized tests published by standard test organizations. There are many industry-established standard tests to evaluate virucidal properties of non-porous surfaces using disinfectants (called carrier disinfection tests) in a hospital setting. These carrier disinfection tests utilize a sample surface (called a carrier or coupon) onto which the virus and disinfectant are applied. To determine if the product is virucidal, the required reduction is dependent on the standard

test and ranges from a 4-log reduction [EPA, 2015] to a 3-log reduction [Codex Alimentarius, 2012] [AFNOR, 2007]. For the purposes of this review, a 3-log reduction will be considered the threshold for an effective virucidal agent.

Currently, standard tests are underutilized in literature. Access to these tests typically requires a subscription to each organization, and may be a reason for their limited use. Further, some methods are not representative of in-use conditions, and lead to new methods being developed [Rabenau et al., 2014]. These new methods may better test actual conditions, but their obscurity and slow acceptance lead to further variation in testing methods.

Additionally, materials and methods sections in literature do not provide an adequate description of virus preparation, soiling conditions, or environmental conditions. These factors in particular have been shown to change the required time and/or concentration of disinfectants [Michels et al., 2009] [Hodek et al., 2016]. Even in standards tests, there does not appear to be a consensus level for these factors. An evidence based justification of these levels would help qualify use of the test and better understand the limitations of use.

Standardization of disinfection protocols in literature needs to occur so that the broader trends of disinfectants can be elucidated. There have been previous calls for a consensus protocol in 2013 [Gebel et al., 2013] and 2001 [Steinmann, 2001], yet no protocol testing actual conditions has been developed.

3.3 Overview of Established Disinfection Methods

Some disinfectants have been used for many decades with information about them found in regulatory body guidelines and literature summaries in review papers. Guidelines by the United States Centers for Disease Control and Prevention (CDC) and literature reviews will be used to summarize disinfection technologies that have been tested for antibacterial efficacy only.

The CDC keeps an updated list of recommendations on currently employed disinfectants in the Guidelines for Disinfection and Sterilization in Healthcare Facilities originally compiled in 2008 [Rutala et al., 2019]. In this guideline, high-touch surfaces are a component of environmental surfaces and are in the noncritical items tier. This classification is defined as surfaces contacting unbroken skin, and are considered the lowest risk. Of the 16 listed methods of disinfection, 6 are appropriate for high-touch surface disinfection: alcohols, chlorine and chlorine compounds, hydrogen peroxide, phenolics, quaternary ammounium compounds (QACs), and ultraviolet (UV) radiation. Of these, no studies testing phenolics or UV radiation met the inclusion criteria of this review (virucidal properties of disinfectants on a stainless steel surface). For this reason, a brief note of the technology will be presented here.

Phenolics and UV radiation have both been shown to disinfect virus [Rutala et al., 2019]. Phenolics pose a danger to infants, requiring very careful use [Rutala, 1996]. The safety around penolics use may be a reason for slow development of the technology. UV radiation has been used extensively in other fields, such as waste water treatment and pharmaceutical industries. However, in healthcare disinfection, UV radiation cannot practically be used

for multiple occupancy rooms and particularly liable to organic soiling [Rutala et al., 2019]. Additionally, the efficacy of portable devices reducing HAIs is still unclear due to lack of high-quality research [Health Quality Ontario, 2018].

3.4 Virus Disinfectant Plasmas, Vapours, and Solutions

Advances in surface disinfection have expanded beyond the traditional chemical agent spray, introducing vapours and plasmas as alternative modes of disinfection. Briefly, vapours create a mist of disinfectant solution, which then settles on all surfaces and disinfects the room [Nayak et al., 2018]. This method necessitates an unoccupied room, with the device disinfection times specified by the manufacturer. Plasma disinfection for surfaces is a relatively new prospect, and is translated from food sterilization technology [Nayak et al., 2018]. This method uses electric current to ionize a gas, generating reactive oxygen species which then flows to the surface and causes disinfection.

3.4.1 Cold Atmospheric Plasma

Generation of reactive oxygen and nitrogen species (ROS and RNS respectively) from cold atmospheric plasma (CAP) has been proposed as a method to achieve surface sterilization. A system using dielectric barrier discharges was designed for sterilization of bioreactors, but the technology also has a capacity for hospital surface disinfection. Testing was performed by drying feline calicivirus (FCV) onto a stainless steel disc for 30 minutes in a biosafety

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cabinet. Disinfection was performed by exposing the surface to effluent gas from the CAP process, without directly exposing the virus to the plasma. Dried virus did not achieve more than a 1-log inactivation, but wet virus had all detectable virus inactivated ($\geq$5-log) after a 3 minute exposure [Nayak et al., 2018]. CAP is more appealing than hydrogen peroxide vapor, as it requires a much shorter time to achieve the critical inactivation threshold of $\geq$3-log. However, its inability to inactivate dried virus means that it is unsuitable for terminal cleaning.

3.4.2   Hydrogen Peroxide

In an effort to reduce inconsistent disinfection by health-care workers [Manian et al., 2011], automated hydrogen peroxide vapour devices have been developed. Hydrogen peroxide vapour systems have been shown to be more effective at reducing bioburdens of antimicrobial resistant pathogens [Manian et al., 2011].

Disinfection by a condensing hydrogen peroxide vapor system was evaluated on five viruses (feline calicivirus (FCV), human adenovirus serotype 1 (HAdV-1), transmissible gastroenteritis virus (TGEV), avian influenza virus (AIV), and swine influenza virus (SwIV)) and showed a reduction of all detectable virus. The authors do acknowledge the need to evaluate this technology with higher organic soiling, but state that it is intended for use after initial cleaning of the surfaces. In terms of utility however, it carries a high time-cost, with the procedure requiring 2 to 3 hours [Goyal et al., 2014]. Another system using hydrogen peroxide vapour tested disinfection of HuNoV surrogates, FCV and murine norovirus (MNV) [Zonta et al., 2016b]. Again, virus was reduced significantly at the end of

the exposure period, but this was only seen in infectious titers [Zonta et al., 2016b]. Virus quantification using qPCR did not detect as large of a decrease in genome copy number. These results indicate that hydrogen peroxide reduces infectivity by damaging the capsid, rather than genomic material [Zonta et al., 2016b].

Hydrogen peroxide vapor technology has been shown to be quite effective at reducing even the persistent viruses, as indicated by the norovirus surrogates tested [Zonta et al., 2016b]. However, because the systems require a long time to reach the required levels of virucidal activity, they are limited to being used solely for terminal cleaning [Rutala et al., 2019]. Even more restricting, this method of terminal cleaning requires an unoccupied room, leading to difficulty in implementation for many rooms at hospitals [Rutala et al., 2019]. Finally, with conventional terminal cleaning requiring 25-45 minutes [Coppin et al., 2019], it is not optimal for health-care facilities to double or triple the time a room remains unoccupied when already under pressure to reduce these times [Rutala et al., 2019].

3.4.3   Silver Nanoparticles

Silver nanoparticles are widely recognized as an antimicrobial agent, utilizing production of reactive oxygen species. Silver, therefore, would make a good disinfectant candidate, but the ions alone are unstable in solution [Manuel et al., 2017]. One method of overcoming the instability is by addition of citric acid in the patented formulation, silver dihydrogen citrate (SDC). In tests with and without a standardized soiling agent, inactivation of human norovirus (HuNoV) was evaluated. SDS-PAGE showed that SDC targeted viral envelope proteins exclusively, further confirmed by detection of RNA after RT-qPCR on

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challenged virus without RNase pre-treatment. Both carrier and suspension studies saw soiling decrease efficacy to non-statistically significant level, less than a 1-log reduction [Manuel et al., 2017]. While the limits of silver nanoparticles are shown by Manuel et al. [2017], there are further concerns about the environmental persistence of nanoparticles, in addition to long-term exposure leading to damage in humans [Rezvani et al., 2019], that indicate silver nanoparticles disinfection technology still faces several major hurdles.

3.4.4 Chlorine-Based Solutions

Chlorine-based solutions encompass utilization of sodium hypochlorite (bleach) to achieve disinfection through oxidative damage. Sodium hypochlorite has been a staple of disinfection procedures due to its strong and broad ranging effectiveness.

Zonta et al. [2016a] evaluated the disinfection of FCV and MNV dried onto stainless steel with a light soil (0.3% BSA) using a commercial bleach product (5000 ppm). After a 5 minute exposure, the bleach was found to have a significant inactivation of the virus [Zonta et al., 2016a]. As this test accounts for soiling (as recommended by standards tests) and uses a generic formulation of sodium hypochlorite, these results can serve as a reference-point for comparison of other chlorine-based solutions discussed in this section.

Cromeans et al. [2014] used a 5 minute exposure of 1000 ppm sodium hypochlorite solution to inactivate FCV. The corresponding RT-qPCR results showed a 2.5-log reduction lower than the infectivity assay, indicating that damage primarily occurs to capsid integrity [Cromeans et al., 2014]. This comparison also highlights the difference in result when comparing the two methods. While results both Zonta et al. [2016b] and Cromeans et al.

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[2014] indicate that 5 minutes may be sufficient for FCV inactivation in the presence of soiling, four other norovirus surrogates were unable to reach an appreciable reduction (all >1.5-log reduction) [Cromeans et al., 2014]. These results indicate that virus type may both influence the inactivation.

Enveloped viruses are not usually studied in disinfection tests, as their envelop makes them more susceptible to environmental stresses than non-enveloped viruses [Hodek et al., 2016]. However, due to recent outbreaks, there has been significant interest in disinfection of Ebola virus, an enveloped virus. Cook et al. [2015] showed Ebola virus was inactivated by sodium hypochlorite only at higher concentrations (>1000 ppm) [Cook et al., 2015]. All variants (Makona, Kikwit, Mayinga) were able to achieve complete disinfection (>6-log) in 5 minutes with a 5000 ppm sodium hypochlorite solution [Cook et al., 2016]. However, when exposed to higher levels of soil, as expected in field clinics, no effective inactivation (<3-log) was achieved [Smither et al., 2018].

Further work was done by Gallandat and Lantagne to develop an Ebola virus surrogate, ultimately selecting bacteriophage Phi6 as a conservative BSL-1 alternative [Gallandat and Lantagne, 2017]. The utility of establishing a surrogate was shown in a follow up study where Gallant *et al* were able to test different methods of sodium hypochlorite generation for field clinics [Gallandat et al., 2017]. Results indicated that solid pellets used to generate sodium hypochlorite achieved a similar inactivation of virus to that of liquid solutions, allowing clinics to reduce storage space and shipping costs [Gallandat et al., 2017].

When using chlorine as a disinfectant, current United States Centers for Disease Control and Prevention (CDC) recommendations are 1000 ppm for clean surfaces and 5000 ppm

for heavily soiled surfaces. However, with concentrations greater than 500 ppm causing corrosion, Moorman et al. [2017] argue enriching the amount of free available chlorine (FAC) should bring the required concentration below the corrosive levels [Moorman et al., 2017]. To this end, neutral electrolyzed water (NEW) can be used to generate a solution with 250 ppm of FAC. NEW is made by electrolysis of a dilute sodium chloride solution. A neutral pH is maintained so that the dominant species is the hypochlorite ion (OCl^-). Testing NEW found that binding receptors and capsid proteins were targeted soon after exposure [Moorman et al., 2017]. Again, it was shown that soiling will significantly reduce efficacy, with no appreciable reduction (<0.5-log) with up to 30 minutes of exposure [Moorman et al., 2017].

Another type of chlorine disinfectant generated by electrolysis is electrochemical oxidant (ECO) solution. Aside from generating hypochlorite species, the process also generates ROS and has a high pH ($\sim$10). This method is believed to have increased efficacy when compared to similar FAC levels in bleach. A benefit to these systems is that ECO can be made simply and on-site. A study by Julian *et al* found that bleach was more effective at lower concentrations ($\leq$ 500ppm), but at higher concentrations ECO was more effective [Julian et al., 2014].

Overall, chlorine is an effective disinfectant in light soiling conditions. However, when there is heavy soiling, a high concentration of sodium hypochlorite is necessary. These higher concentrations can lead to corrosion of surfaces, shortening their lifespan and increasing surface area that can be a reservoir of pathogens. Additionally, high concentrations may temporarily prevent use of the area as toxic fumes are present. Some alternatives to traditional bleach have been proposed and tested, but these types also struggle to disinfect

in the presence of organic soils.

3.4.5 Peroxy Acids

A well explored type of disinfectant is peroxy acids, specifically peracetic acid (PAA). This class of disinfectants has been proposed as an alternative to chloride-based disinfectants as it has fewer harmful by-products and likely no harmful effects to humans, while still achieving broad disinfection [Dominguez Henao et al., 2018].

In a multicenter study, human adenovirus and four animal parvoviruses were tested against a 5 minute exposure to various concentrations of PAA [Rabenau et al., 2014]. Parvoviruses had variable disinfection by PAA, and required a higher concentration than HAdv to achieve inactivation [Rabenau et al., 2014]. Data from the different test centers had variable PAA concentrations to achieve effective disinfection, varying from 0.02% to 0.1% [Rabenau et al., 2014]. Unlike chlorine-based solutions, it appears that peroxy acids are less susceptible to soiling, as they require much lower concentrations (approximately 100-fold) to achieve effective disinfection after 5 minutes [Vimont et al., 2014]. In cases of extremely high soiling, Ebola virus required a higher concentration when dried in blood plasma compared to cell culture medium [Smither et al., 2018]. Even though a higher concentration was required, PAA was able to inactivate Ebola virus dried in blood plasma, while sodium hypochlorite was not [Smither et al., 2018].

Like sodium hypochlorite, using a solid source of PAA helps decrease shipping costs, storage space, and mitigating some of corrosive hazards. Dagher et al. [2017] showed that Bioxy, a powder formulation to be dissolved in water, was able to achieve effective

disinfection. Another powder source of PAA, PES-Solid, effectively inactivated MS2 after 15 minutes [Buhr et al., 2014].

While it appears that PAA is less susceptible to soiling than sodium hypochlorite, PAA does have some limitations. These limitations include neutralization by metals and organics, storage in vented containers, and evolution of noxious fumes [Dagher et al., 2017]. For this reason, dry pellets like the Bioxy or PES-Solid pave the way for a safer utilization of this disinfectant.

3.4.6   Alcohols

A comprehensive review by Boyce in 2018 covers most of the important features and applications of alcohols as a disinfectant for healthcare settings. Briefly, there are three types of commonly used alcohols: ethanol, isopropanol, and n-propanol. These alcohols are effective when mixed with water, generally needing to be between 60% and 90% v/v, and are effective against a broad range of pathogens, including viruses. Considering the virucidal data, the authors found a general theme for alcohols where ethyl alcohol was more effective against non-enveloped viruses than isopropyl alcohol [Boyce, 2018]. Below are results that were not part of Boyce's review.

In contrast to the findings in Boyce's review, some high-risk enveloped viruses (Chikungunya virus (CHIKV), Ebola virus, Middle East respiratory syndrome coronavirus, Modified Vaccinia Virus Ankara, and Zika virus) were all found to be more susceptible to propanol than ethanol. Further, a commercial multi-alcohol cleaning solution was able to effectively disinfect Chikungunya virus, the most resistant of the tested viruses, in 1

minute of exposure [Franz et al., 2018]. When a heavier soil load is used, longer exposure is required to achieve disinfection even with a more susceptible virus [Cook et al., 2015].

Long required contact times are a major disadvantage of alcohols as the rapid evaporation rate prevents practical use. Instead, alcohols are commonly used as a sterilizing wipe for lower-level disinfection of skin and noncritical items. In fact, the CDC has recommended against using alcohols for surface disinfection, citing the rapid evaporation rate shortening contact time [Rutala et al., 2019]. As such, only a small fraction of EPA registered solutions are purpose-made for disinfection of environmental surfaces [Boyce, 2018].

3.4.7   Other Disinfectants

There are many instances where only one study has looked at a particular disinfectant in the scope of this review. This section brings attention to these methods, but as with other sections, variability in methods makes it difficult to draw direct comparisons with other disinfectants unless they were performed in the same study.

An investigation of commercial disinfectants' effectiveness against CHIKV found that both an oxidizer and QAC solution were able to effectively inactivate dried virus in light soiling in under one minute [Franz et al., 2018]. However, the QAC cleaning solution had cytotoxic effects on the cells used in the infectivity assay. This is consistent with the general hazards of using QAC, although there are methods of developing QACs with lower toxicity [Forman et al., 2016].

In a multicenter study on disinfection by glutaraldehyde (GDA), inactivation of four parvoviruses and human adenovirus (HAdv-5) was evaluated [Rabenau et al., 2014]. Under

light soiling, the parvoviruses required a much greater concentration (2000ppm) than HAdv (125-500 ppm) to achieve effective disinfection, showing the breadth of disinfection resistance of non-enveloped viruses [Rabenau et al., 2014].

Like the work by Rabenau et al. [2014], Zeitler and Rapp [2014] have shown that enveloped viruses also exhibit a variability in disinfection. Glucoprotamin was proposed as an environmentally safe and broad-ranging disinfectant in the 1990's [Disch, 1994]. In an effort to evaluate glucoprotamin's disinfection using standard testing procedures, Zeitler and Rapp [2014] found differing effectiveness for enveloped viruses. Vaccinia virus was resistant up to the maximum time tested (30 minutes) but Influenza A virus reached effective disinfection after 15 minutes [Zeitler and Rapp, 2014].

Combinations of disinfectants are likely to have better efficacy than utilizing a single disinfectant. Commercial disinfectants typically are novel combinations of the discussed disinfectants. Zonta et al. [2016a] evaluated the disinfection with some of these types of commercial disinfectants on norovirus surrogates and found MNV was inactivated by a wide variety of disinfectants tested, but FCV was not. Namely, one of the two mixtures of QAC + alcohol + GDA (Kenocid 210®), and both mixtures of chlorhexidine + isopropanol (Alcocid®, Kenosept G®) were not able to effectively disinfect FCV [Zonta et al., 2016a]. Both surrogates have been used in the past to evaluate disinfectant candidates. Some research suggests that MNV is a better surrogate than FCV [Sattar et al., 2011] in terms of physico-chemical properties. These results highlight the importance of choosing the a suitable surrogate so as to not disqualify viable disinfecant agents.

3.4.8 Virucide Disinfectant Testing Method Variability

As previously mentioned, variability in methods may have some impact on validity of disinfectant for in-use efficacy [Steinmann, 2001]. Table 3.1 highlights the variability for the methods discussed in this section.

The results are broken down into seven subsections, as they were discussed in this section. In each of those subsections, a list of the disinfection methods (challenges) are described for the number of viruses, temperatures, humidities, and soil types used in the studies. Italicized values represent the values of each subsection.

In total, 32 viruses were studied across the 19 articles reviewed, with eight studies only evaluating one virus. 11 studies did not report a temperature and 14 did not report a humidity. Nine different soiling agents were used, with only Smither et al. [2018] comparing multiple soiling agents.

The greatest variability occurs in chlorine-based disinfectants section. Here, 18 types of viruses were tested, using seven different soiling agents in 10 different studies. The variability in soiling has been shown to decrease the efficacy of disinfectants, but soiling is particularly liable to this interference [Cromeans et al., 2014]. A table showing all the tested conditions of Table 3.1 can be found in Appendix Table A.1.

Table 3.1: Disinfectants Tested for Virucidal Activity on Stainless Steel

Challenge	Unique Number of				
	Viruses	Temperatures	Humidities	Soil Types	Studies
Plasma					
CAP	1	1	1	1	1
Hydrogen Peroxide					
Vapour	6	1	1	2	2
Silver					
SDC	2	1	1	1	1
Chlorine-Based					
ECO	2	1	1	1	1
Fresh NaOCl	1	1	1	1	1
HTH	1	1	1	1	1
Kenochlore (NaOCl)	2	1	1	1	1
NaDCC	1	1	1	1	1
NaOCl	15	4	4	6	7
NEW	2	1	1	1	1
8	*18*	*4*	*4*	*7*	*10*
Peroxy Acids					
Bioxy (commecial PAA)	1	1	1	1	1
PAA	7	3	2	5	3
PES-Solid in DSS	1	1	1	1	1
PES-Solid in SDX-12	1	1	1	1	1
PES-Solid in water	1	1	1	1	1
PPA	1	1	1	1	1
6	*9*	*4*	*3*	*7*	*5*
Alcohol					
1-propanol	5	1	1	1	1
2-propanol	5	1	1	1	1
ethanol	10	3	2	2	4
3	*10*	*3*	*2*	*2*	*4*
Other					
Alcocid (alcohol, biguanide)	2	1	1	1	1
Bacillol AF (commercial multi-alcohol)	1	1	1	1	1
GDA	5	1	1	1	1
Glucoprotamin	2	1	1	1	1
Kenocept G (alcohol, biguanide)	2	1	1	1	1
Kenocid 210 (QAC, alcohol, aldehyde)	2	1	1	1	1
Kenocid 2100-2 (PAA + H2O2)	2	1	1	1	1
Peform (oxidizing non-ionic surfactant)	1	1	1	1	1
QAC	1	1	1	1	1
Virocid (QACs, alcohol, aldehyde)	2	1	1	1	1
10	*10*	*4*	*1*	*1*	*4*

3.5 Self-Sterilizing Surfaces

As previously mentioned, disinfection performed by cleaning staff has been shown to be inconsistent [Manian et al., 2011]. Further, a recent clinical trial in an out-patient facility found that many high-touch areas cleaned using an ethanol cleaning product was not able to achieve effective disinfection of a tracer virus [Reynolds et al., 2018]. A complement to these procedures would be to have a self-sterilizing surface, such as biocidal metal alloy, or development of novel surfaces. Self-sterilizing surfaces utilize unique surface properties that have contact biocidal properties. These self-sterilizing surfaces are not designed to replace routine cleaning procedures, but to act as a complement to help reduce the spread of pathogenic material from high-touch surfaces.

3.5.1 Metal-Alloy Surfaces

Since this is a relatively new design space compared to disinfectants, there are standard protocols for microbicidal investigation of these surfaces, but none for virucidal investigations. Even among the established antimicrobial protocols there are problems with suitability. For example, antimicrobial silver is very effective ($\geq$ 5-log) under the high humidity and temperature outlined in JIS Z 2801 (Antibacterial products - Test for antibacterial activity and efficacy) [Michels et al., 2009]. However, it is ineffective (<0.3-log reduction) when tested against bacteria at ambient temperature and humidity, as shown by Michels et al. [2009].

Copper alloys have been use to deter biofouling for millennia, but only in the last two

decades has it been purposefully investigated as a self-sterilizing surface. Pure copper surfaces are shown to be effective at virus disinfection [Bleichert et al., 2014] [Warnes et al., 2015a] [Warnes et al., 2015b]. However, using pure copper as a replacement for high-touch surfaces is unfeasible as it is not robust enough to resist wear and deformation from frequent touching. Instead, alloys with lower corrodibility and malleability are better suited as high-touch surfaces. Some recent reviews have been performed on biocidal properties copper surfaces and with a focus on the antimicrobial properties, but none have focused on virucidal studies [Weber and Rutala, 2013; Villapun et al., 2016]. Generally speaking, it has been shown that a minimum copper concentration of 79% in nickel alloys and 70% in zinc alloys is required for effective disinfection [Warnes et al., 2015a] [Warnes et al., 2015b]. Enveloped virus (human coronavirus-229E) required at least 40 minutes [Warnes et al., 2015a] and non-enveloped (MNV-1) required at least 30 minutes [Warnes et al., 2015b] to reach the threshold. Characterization of the inactivation shows that copper ions are likely destabilizing the outer layer (envelope or capsid) [Warnes et al., 2015a] [Manuel et al., 2017], and also directly damaging the nucleic acid [Manuel et al., 2017]. Further, it is likely that Cu(I) and Cu(II) ions both play a role in the inactivation and ROS only aids in inactivation when copper alloys are used [Warnes et al., 2015a].

These studies show great potential for copper alloys as replacements to stainless steel high-touch surfaces. However, early implementation of these surfaces has found a significant difference between efficacy in lab settings and field use [Michels et al., 2015]. An essential part of determining the validity of this technology is to bring testing in line with more rigorous testing conditions, such as vircudial activity with soiling and repeated exposures [Environmental Protection Agency, 2016].

3.5.2 Modified Surfaces

Copper alloy surfaces are more expensive than the stainless steel surfaces they seek to displace. However, initial cost is not the only factor to consider, as these surfaces have the potential to reduce costs incurred by the healthcare institutions that utilize them. Initial costs should not discourage development of novel surfaces. Some of these recent explorations are discussed below.

A fluorinated TiO_2 (F-TiO_2) surface coating was shown to exhibit virucidal properties, using UV-light generated from fluorescent bulbs to generate reactive oxygen species (ROS) [Park et al., 2014]. Infectivity assays showed that MS2 and MNV-1 were effectively inactivated after 2 hours and both followed similar inactivation kinetics. However, no degradation of genetic material was found for MS2 or two HuNoV GII strains. In another test, MS2 on F-TiO_2 surface was placed on a table in an office to simulate realistic lighting conditions. Results showed that MS2 decreased below the detectable threshold after 12 hours ($\geq$5.1-log reduction) [Park et al., 2014]. One major limitation to this technology is the push towards energy-efficient LED lights, which do not produce UV-light at the same scale.

Feuillolay et al. [2018] investigated the virucidal activity of zinc and magnesium oxide microspheres impregnated into polypropylene (PP) surfaces. The microspheres are made of Zn and Mg oxides, and are engineered to facilitate production of ROS. Additionally, since these microspheres are already accepted additives in European, American, and Japanese Pharmacopeia, implementation of this technology requires fewer safety studies. The authors were able to integrate microspheres into the plastic without any changes to

the manufacturing process. While this surface did not achieve disinfection by 24 hours for Influenza A virus H1N1 (InfA H1N1) or HSV-1, it does pose an interesting avenue of exploration [Feuillolay et al., 2018].

Using sol-gel synthesis, Hodek *et al* were able to create a coating containing copper, nickel, and zinc cations. The virucidal activity was tested against HIV-1, InfA H1N1, dengue virus type 2, HSV-1, and coxsackie B3 virus. Only HSV-1 was reduced effectively, with HIV-1 close to achieving effective disinfection as well [Hodek et al., 2016].

These surfaces are a step in the right direction to help reduce spread of nosocomial infections. However, none of these tests have investigated the impact of soiling. Novel surfaces have the potential to radically change infection control and prevention strategies, as well as reduce financial burden caused by nosocomial infections. Further innovation is required in the prevention of surface contamination for healthcare institutions, but healthcare is not the only industry that requires infection control measures.

3.5.3   Virucide Surface Testing Method Variability

The method variability seen in Section 3.4 is continued in virucidal surface tests. In this section, 11 viruses were investigated in six studies. Both temperature and humidity were studied at four different levels but since no single study tested multiple levels, the effect cannot be determined. Six different soil levels were investigated, with Manuel et al. [2015] and Feuillolay et al. [2018] testing two levels. Three of the studies did not explicitly report the soiling agent. A table showing all the tested conditions of Table 3.2 can be found in Appendix Table A.2.

Table 3.2: Virucidal Stainless Steel Surface Alternatives

Challenge	Unique Number of				
	Viruses	Temperatures	Humidities	Soil Types	Studies
Copper Alloys					
Brass	4	2	2	3	3
Bronze	3	1	2	3	2
Copper	6	3	3	3	4
Copper-nickel	4	2	2	3	3
Muntz metal	2	1	1	2	1
Nickel	1	1	1	1	1
Zinc	1	1	1	1	1
7	*6*	*3*	*3*	*3*	*4*
Modified Surfaces					
F-TiO2	4	1	1	1	1
PP metal microsphere	2	1	1	2	1
2	*6*	*2*	*2*	*3*	*2*

3.6 Disinfection in Related Fields

Many other industries struggle with surface contamination being a vector in disease transmission, and face similar challenges in creating safe and effective disinfectants. Such industries include veterinary clinics, livestock handling, food preparation, water sterilization, pharmaceutical manufacturing. The various constraints faced by different industries pushes them to look for innovative virucidal solutions and surfaces from green tea extract to sterilize food-contact surfaces [Randazzo et al., 2017] to novel disinfectant combinations in water treatment [Sun et al., 2016]

A recent study looked at designing a specific virucidal disinfectant. An oligothiophene compound, named **4sc**, was shown to have strong specificity to influenza A viruses, independent of strain type. **4sc** also demonstrated a high selective index, a ratio of therapeutic dose to cytotoxicity [Shen et al., 2018].

A combination of octaethylene glycol monododecy ether and di-n-decyldimethylammonium chloride, referred to as $[\mathbf{C_{12}E_8}]$ and $[\mathbf{DiC_{10}}][\mathbf{Cl}]$ respectively, showed a synergistic increase in disinfection of enveloped viruses. Interestingly, the authors also found that the critical concentration to achieve a 6-log reduction of the virus was strongly associated with the size of the viruses [Nardello-Rataj and Leclercq, 2016].

Another study combining traditional disinfectant methods looked at UV and hydrogen peroxide working in tandem to water and wastewater treatment [Sun et al., 2016]. They found that this combination was significantly better than UV alone [Sun et al., 2016], which could lead to testing tandem UV and hydrogen peroxide vapour devices for hospital use.

Inspired by plants' defensive use of polyphenols, a class of secondary metabolites, Catel-Ferreira et al. [2015] impregnated Kimwipes® with polyphenols to confer virucidal properties. They were able to show specificity for phage but not $E.~coli$, and required long contact times to achieve effective disinfection. While they acknowledge that this experiment is more in line with a contact study than a wiping simulation, they argue that because it was tested at a high concentration of virus, it still has potential as a disinfectant when used as a wipe [Catel-Ferreira et al., 2015]. Plant-derrived polyphenols have been well studied for their biocidal properties. In fact a recent review focused on polyphenols as an alternative source for virucidal disinfectants with lower environmental impact [D'Souza, 2014].

The lack of standard virucidal tests is not unique to medical applications. Morin et al. [2015] sought to create a quantitative carrier test method to test surface disinfectants in the food industry. Importantly, they also included a soiling concentration from a standard

suspension test already used in the industry [Morin et al., 2015]. It is also important to note these results are translatable to results discussed in this review, as the disinfectants tested (sodium hypochlorite, peracetic acid mixture, potassium monopersulfate) were all tested on stainless steel surfaces under similar conditions to those described in the above sections.

Another food industry study investigated the critical role of soiling in the effectiveness of disinfectants. Kingsley et al. [2014] propose an interesting approach where a virus stock is heat-neutralized, sanitizer is added, and then the effective concentration of the sanitizer is measured. This approach may reduce variability in results from soils, as the soil will quench disinfectant. Their work showed that FAC was reduced approximately 6-fold in a semi-purified stool sample of HuNoV [Kingsley et al., 2014]. They paired this with a novel detection method to approximate the remaining infectious virus of the unculturable HuNoV. The binding assay, developed by Tian et al. [2008], uses a porcine receptor conjugated to magnetic beads to increase detection sensitivity.

3.7 Conclusions

The goal of this review was to assess strategies employed to reduce viral nosocomial pathogens on high-touch surfaces in the last five years. Specifically, disinfectants tested on stainless steel, or self-sterilizing surfaces designed to replace stainless steel, were included in the review. The methods used to prepare virus samples and addition of soiling were also of interest.

Variability in methods testing virucidal efficacy is very high. In the section on disinfectants, 19 references were analyzed. Of those, 32 types of virus with 10 different purification methods and 11 different soiling methods were used. In the studies investigating self-sterilizing surfaces, 6 references were analyzed. Of those, 11 viruses were tested with 6 purification methods and 6 different soiling methods were used.

Many of the studies have found varying degrees of efficacy, but due to the variation in testing, it is difficult to assess efficacy as a whole. Virus preparation and addition of soiling have been shown to impact the efficacy, but is a factor that is not investigated in self-sterilizing surface studies, and inconsistently tested in disinfectant studies. With studies showing lower efficacies in hospitals for both disinfectants and self-sterilizing surfaces, more fundamental research needs to be performed on the interaction of soils and sterilizers.

Chapter 4

A Study on a Virucidal Copper Alloy and the Effects of Virus Matrix on Disinfection

A study was undertaken to evaluate the virucidal properties of a copper-alloy designed to reduce nosocomial infections. This surface was designed by Aereus Technologies, and the antimicrobial properties had been confirmed before our work began.

In preliminary work, virucidal properties were inconsistent as shown in Figure 4.1. Our first investigation found an approximate 98% reduction in virus. However, the control virus concentration increased by almost a factor of 5. These results indicated that the copper-alloy was a viable candidate for virucidal properties, but that humidity control was required to avoid concentrating virus.
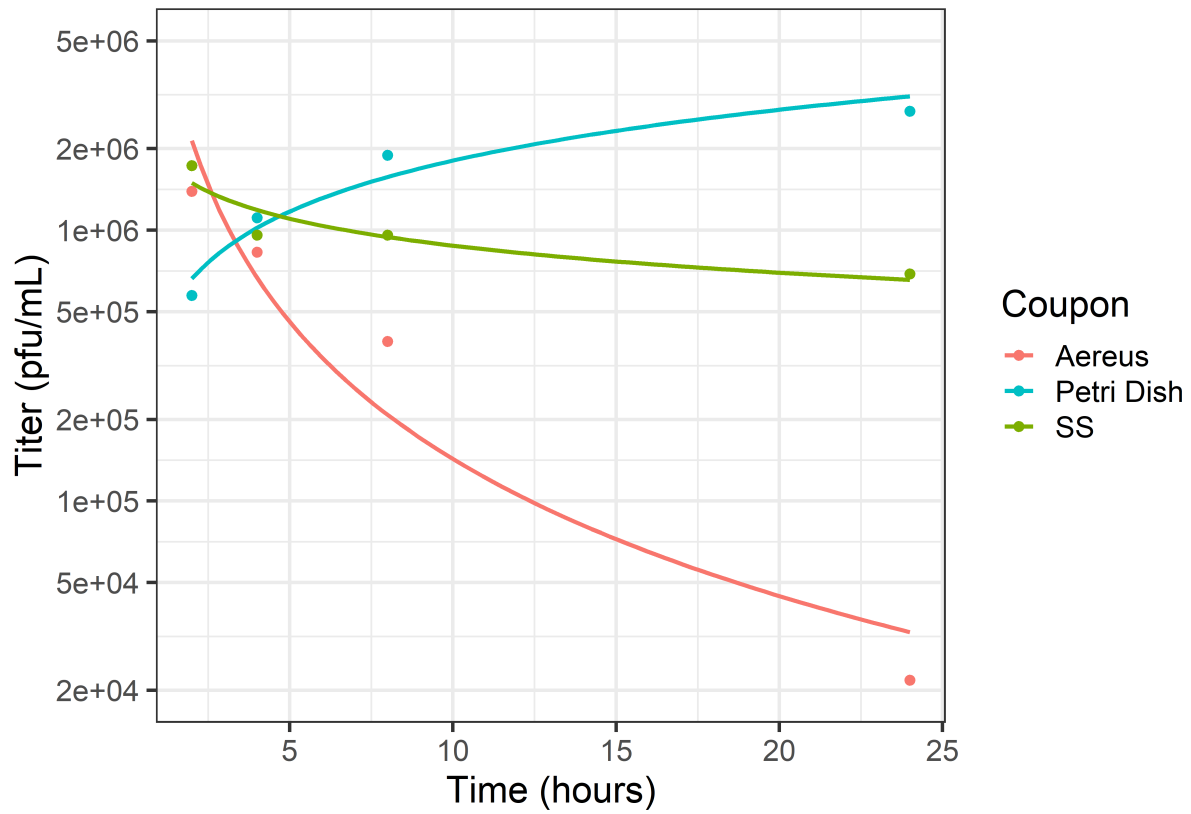
Figure 4.1: Virucidal activity of various surfaces to determine inactivation by surface. SS represents the stainless steel coupon. Lines are to help visualize the trends in data and generated using the 'geom_smooth' function in the ggplot package for R.

In follow-up experiments, it was determined that unfiltered virus stock was not inacti-vated by the surface. Further tests showed that a 1:10 dilution into fresh media allowed a $\geq$4-log inactivation by 24 hours, as compared to stainless steel (Figure B.2). These prelim-inary studies indicated that organic debris generated in the infection process may interfere in the surface's virucidal properties.

The remainder of this section is a manuscript for an original research article evaluating virucidal properties of a copper-alloy. This alloy is intended to replace high-touch surfaces to help reduce nosocomial infections. High-touch surfaces have been identified as an im-portant vector of transmission in health-care institutions, and likely cause more nosocomial infections than reported. While the antimicrobial protocols to test these surfaces are well explored, the protocols for virucidal properties are relatively unexplored.

4.1 Introduction

Nosocomial infections are a large burden on heath-care systems, with the American Center for Disease Control (CDC) estimating one in every 25 patients acquiring one. In 2009, a CDC summary indicated that a conservative cost-estimate of preventable nosocomial infections ($5.7 - $6.8 billion) was comparable to the cost for treating stroke, complications from diabetes, or chronic obstructive pulmonary disease [Scott II, 2009]. These types of infections are known to occur through medical devices or environmental transmission, and strategies have been developed to reduce them.

The key to environmental transmission is an intermediary reservoir, and transmission barriers can be developed to target these reservoirs. Currently, there are two major avenues: increased hand-washing compliance, and routine cleaning/disinfection protocols. The first avenue is strongly focused on by healthcare professionals, and simply seeks to increase hand-washing compliance through monitoring and awareness [Beggs et al., 2015]. While this does address a key transmission route for nosocomial infections, compliance is inconsistent and requires observation [Kovacs-Litman et al., 2016]. The second avenue, disinfection and cleaning, also faces consistency problems, with inconsistency in method by cleaning staff [Manian et al., 2011]. A newer avenue is to design surfaces that are biocidal against key pathogens. This method may add initial costs, but it does allow for greater consistency and has potential to decrease costs incurred in treatment of nosocomial infections. Development of these surfaces does not endeavour to displace established cleaning and disinfection procedures, but to supplement them and create a comprehensive defence against environmental transmission.

Copper-based surfaces have antimicrobial properties, something known for several centuries. However, formal recognition was only obtained in 2008, when the United States Environmental Protection Agency (EPA) recognized copper as an antimicrobial surface. Initial literature has shown copper to be an effective antimicrobial, even showing complete reduction of copper-resistant bacterial species [Espirito Santo et al., 2010]. Further research has shown that pure copper performs better than alloys [Michels et al., 2008]. However, using pure copper is impractical as it is soft, malleable, and tarnishes. It would be practical to use an alloy robust against the high-frequency touching these surfaces incur.

While work has been done to show the antibacterial properties of copper, comparatively little has been done to show virucidal effects of such surfaces. Although, bacteria and fungal spores are considered the primary threat for nosocomial infections, viral infections make up an estimated 60% of all infections [Vasickova et al., 2010]. Further, recent work shows that viral nosocomial infections are underestimated [Hong et al., 2014] and under-reported [Chow and Mermel, 2017]. Virus, like bacteria, can be transferred to multiple surfaces, with a study finding a clean hand could spread norovirus from a contaminated surface to multiple subsequently touched surfaces [Barker et al., 2004]. This spread of virus further adds to a precedent for exploration of self-sterilizing surfaces to replace currently used stainless steel for high-touch surfaces.

A problem with evaluating virucidal properties of these alloys is that no purpose-made standard tests are available. This leads to difficulty in comparisons of efficacy to established disinfectants. The EPA is currently developing a standard test that evaluates the antimicrobial activity under actual use conditions [Villapun et al., 2016; Environmental Protection Agency, 2016], but does not test the virucidal properties of the surface. Al-

ternatively, existing protocols for disinfectant sprays can be adapted to investigate the virucidal properties of self-sterilizing surfaces. However, the danger of using these parallel protocols was demonstrated by Hodek et al. [2016], where virucidal silver was effective in the standard's test conditions, but not in use-conditions. The lack of clear guidance has lead to a variety of methods being tested in literature.

A major source of variability in protocols arises in soiling of the sample. Organic soiling is generated naturally in all infectious material as a part of the matrix itself and a result of cellular damage. Soiling is a known interfering factor of disinfectant tests [Steinmann, 2001], and is included in several parallel standard tests. However, the quantity and type of organic soil varies between standard organizations and intended use of the disinfectant.

The goal of this study was to first determine the virucidal activity of a copper-nickel-zinc alloy. Then, the effect of soiling and virus preparation were investigated. Finally, repeatable inactivation by the surface was explored. Using the BSL-1 *Autographa californica* multiple nucleopolyhedrovirus, baculovirus, we investigated the above parameters either by allowing the droplet to stay wet using humidity control (standard test conditions), or by drying a virus droplet (actual conditions) onto the alloy and stainless steel.

4.2   Materials and Methods

4.2.1   Cell Culture and Virus Production

Spodoptera frugiperda cells (Sf-9, ATCC®CRL-1711™), able to support the replication of baculovirus, were grown in serum-free SF-900 III (Gibco BRL, Life Technologies, Burling-

ton, Ont., Canada). Cells were routinely maintained in 125 mL glass shake flasks (Corning GlassWorks, Corning, NY) with a working volume of 30 mL at 27°C and agitated at 130 rpm. Cells were subcultured twice per week to maintain the cell density between 0.5 and $5x10^6$ cells/mL. Cell densities were assessed using a Countess^{TM}II FL Automated Cell Counter (Invitrogen, FisherSci, Ont., Canada). Cell viability was determined via the trypan blue exclusion method.

Baculovirus (*Autographa californica* multiple nucleopolyhedrovirus) was engineered to express the fluorescent protein mKOκ under the late and strong viral p10 promoter using the Bac-to-Bac^{TM}Baculovirus Expression System (Fisher Scientific, Ontario, Canada). A passage 2 working stock of the virus was made by infecting $2x10^6$ cells/mL of exponentially growing Sf-9 cells with recombinant virus at a MOI of 0.01. Cell viability was measured daily until it was between 80 and 60% (72-96 h post-infection), after which virus supernatant was harvested, purified by low speed centrifugation (800 x g, 10 minutes) and filtration through a 0.2 µm filter. Purified virus was stored at 4°C until use.

The enveloped baculovirus was selected for it's safe handling and ease of manipulation. Using genetic manipulation to express a fluorescent protein allows a more consistent and accurate judgment over cytopathic effects when scoring end-point dilution assays. Further, red fluorescent protein was selected to avoid the auto-fluorescence of growth media when exciting green fluorescent protein. Auto-fluorescence can mask fluorescence from infected cells, thereby lowering accuracy of the end-point dilution assay.

4.2.2 Test Surface Preparation

Aereus Technologies provided copper-alloy (UNS C75200) coated stainless steel and stainless steel (UNS S30400) coupons (1 x 1 x 1/16 in). Alloy-plated coupons were created using a patented liquid-spray application process and had a semi-polished finish on both sides. The alloy-coating material is made from 65% copper, 18% nickel and 17% zinc. Before use, surfaces were cleaned using 70% ethanol followed by a soak in 70% ethanol for at least 5 minutes immediately before use. For repeated uses, immediately after virus incubation surfaces were cleaned with 70% ethanol, followed by a warm deionized water rinse with light scrubbing. Before the repeated incubation, the surface was prepared as before.

4.2.3 Surface Tests

4.2.3.1 Wet Virus Droplet

For wet virus droplet tests, coupons were placed in a 60 mm Petri dish and held at >90% humidity in a sealed container. 750 µL of virus solution was pipetted onto the coupons, creating a pool on top of the coupon. A 10 µL sample was collected at various times and stored in 990 µL SF-900 III media (10^2 dilution) at 4°C until titering. All tests were performed in a biosafety cabinet in a temperature controlled room set to 21°C.

4.2.3.2 Dried Virus Droplet

For dried virus droplet tests, 25 µL of virus was dried onto the surface in a bio-safety cabinet at ambient lab conditions (20-22°C, ~35%RH). Virus was resuspended in 25 µL

of fresh media by pipetting 10 µL of resuspended virus was added to 990 µL SF-900 III media (10^2 dilution) and stored at 4°C until titering.

4.2.4   End-Point Dilution Assay

Sf-9 cells from an exponentially growing suspension culture were diluted to 2.0×10^5 cells/mL, and 100 µL of this dilution was seeded into each well of a flat-bottom tissue-culture treated 96-well plate (Fisher Scientific, Ontario, Canada). The cells were given at least one hour to attach, while kept inside of a humidified box at 27°C. The virus was serially diluted in the range of 10^{-2} to 10^{-8}, creating seven test dilutions. After the cells attached, 10 µL of the serial dilutions was added to each column, creating 12 replicates in one plate. The diluent, media, was used as a negative control and also added in 12 replicates. One 96-well plate was created for each experimental condition. Plates were incubated for seven days at 27°C, after which they were checked for red fluorescence using a fluorescent microscope. Results were reported as plaque forming units per mL (PFU/mL).

4.2.5   Wet Ashing

Virus samples were diluted (0.5mL harvested virus sample 9.5mL type-I ultra-pure water (UPW)) and placed on a heating block set to 110°C in a fume hood. 1 mL of aqua regia acid (3:1 molar ratio of hydrochloric acid to nitric acid) was slowly added and the mixture was allowed to reflux for 15 minutes. A further 2 mL of aqua regia was added slowly to the sample and refluxed for an additional 30 minutes. A digestion blank of 10mL UPW

was performed along-side the digestion to match the sample preparation. After wet-ashing was complete, samples were cooled to room temperature and filtered.

4.2.6 Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES)

Samples were analyzed for 20 commonly occurring elements. Calibration curves with a range 0 to 100 ppm for each element allowed quantification. Samples were loaded in with an internal 10 ppm yttrium standard to adjust any changes in intensity cause by matrix effects using a Prodigy High Dispersion ICP (Teledyne Leeman Labs, New Hampshire, USA).

4.3 Results

4.3.1 Virus Droplet Tests For Inactivation

Viruses droplet tests were performed at $\geq 90\%$ relative humidity to prevent drying. These tests do not mimic realistic scenarios as virus will dry and a lower relative humidity is expected in hospitals. However, this method mirrors those outlined in a current standard tests evaluating surface disinfectants (JIS Z 2801, ISO 22196) and follows the methods described in Section 4.2.3.1.

4.3.1.1   Cell Debris as a Soiling Agent

To create a testable virus stock, virus was amplified by infecting a host cell culture and resulted in an approximate 10-fold increase in titer. When harvesting the amplified virus, cell viability was approximately 75% and density at 4×10^6 cells/mL. These parameters were selected to minimize cell debris and virus degradation from the proteins released by lysed cells. Low speed centrifugation was used to remove cells and large debris. Smaller cellular debris typically remains and therefore the supernatant was filtered using first a 0.4 µm and then a 0.22 µm filter.

Using the virus amplification parameters, 1×10^6 cells/mL in exponential growth phase were collected and resuspended in either fresh media or conditioned media. Conditioned media is media in which cells have been cultured and contains metabolites and extracellular matrix proteins excreted by cells. Conditioned media was taken from exponentially growing cells and low speed centrifugation was used for clarification (removal of cells and large cell-related debris). A further 1:10 dilution of the sonicated cells was also performed. These solutions, along with fresh and conditioned media without sonicated debris, were used as diluents to investigate interference of the copper alloy's virucidal effects by cell debris and growth media. The tested conditions are outlined in the diagram in Figure 4.2.

The effect of cell debris and conditioned media interference on inactivation by the copper alloy was investigated. Results are presented in Figure 4.3 and titers were measured after a 24 hour exposure. For conditioned media, addition of cells significantly prevented inactivation of virus independent of the concentration ($p<0.001$ for both). However, for fresh media, only the addition of 10^6 sonicated cells was significantly different from no
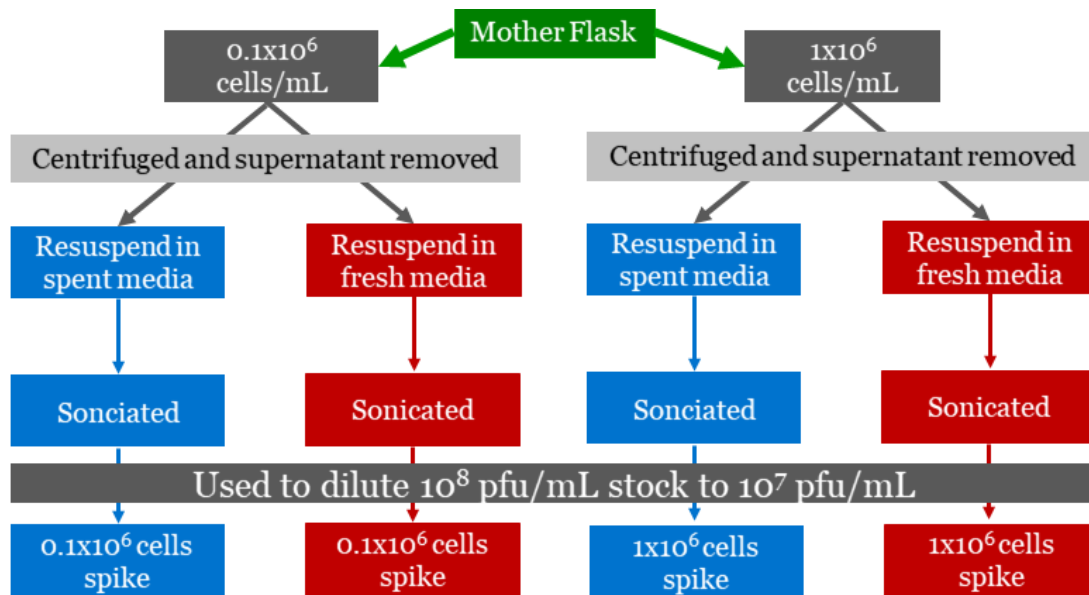
Figure 4.2: A diagram demonstrating the workflow for the experiment performed in Figure 4.3. Cells in the exponential growth phase (mother flask) were collected and resuspended in conditioned (spent) or fresh media. Conditioned media was made by clarifying media from the mother flask. These cells suspensions were then sonicated and used as the diluent to make the working virus solutions (10^7 PFU/mL).

cells ($p<0.05$). Comparing the media while keeping the cell spike constant showed that the inactivation by conditioned media was significantly lower than that of fresh media.

4.3.1.2 Conditioned Media as a Soiling Agent

The protective effects of conditioned medium was investigated and is shown in Figure 4.4. Here, virus inactivation was investigated for up to 24 hours. Virus was suspended in either conditioned or fresh media using two independently prepared batches of P2 (working) virus stock.

In Figure 4.4, points at or below the detection limit (denoted by an asterisk) were not
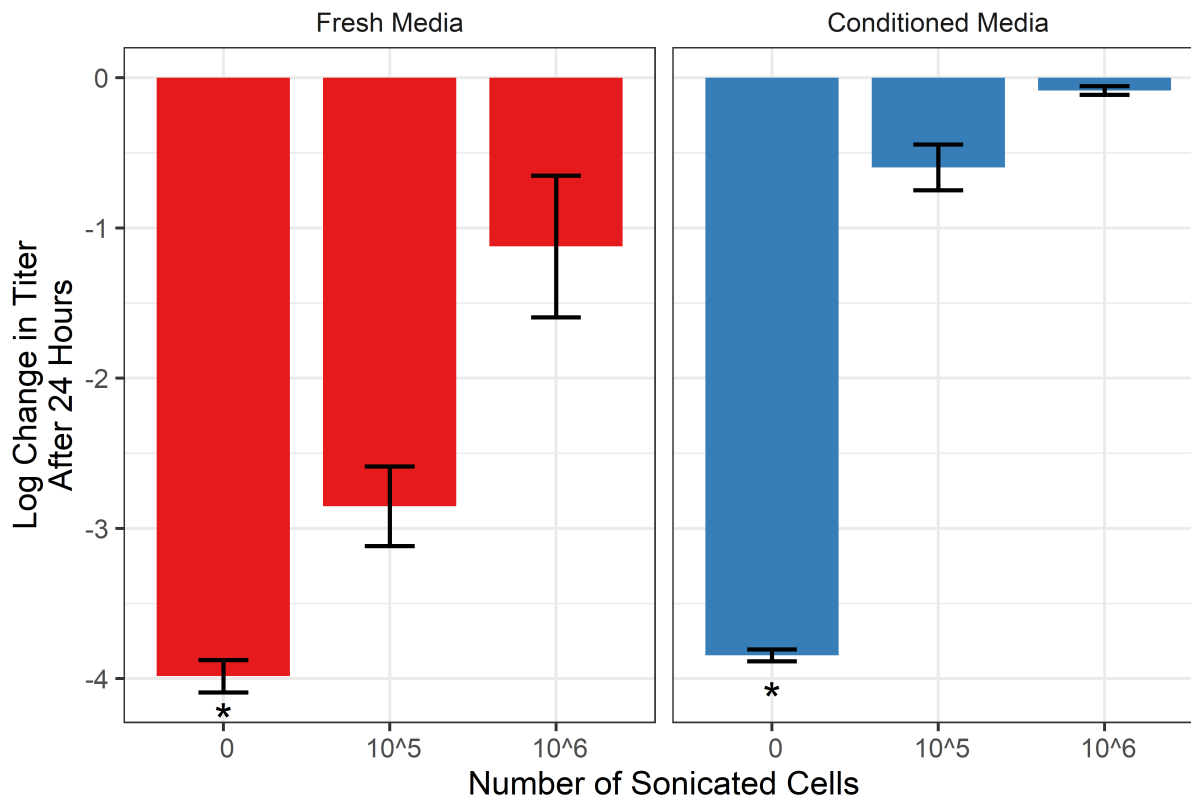
Figure 4.3: Inactivation of the virus solutions on copper alloy for 24 hours. Virus was diluted into either fresh (red bars) or conditioned media (blue bars) with cell debris in the spike concentrations (x-axis). Log difference represents the change from starting virus concentration to virus concentration after 24 hours. Each condition is the average of biological replicates (n=2). Error bars represent the range of the data. Asterisks indicate data with at least one replicate at or below the detection limit.

included in calculating the trend line. Model parameters were found to be significantly different between conditioned and fresh media ($p<0.05$), and are presented in Appendix B These results indicate that virus in conditioned media followed a significantly slower inactivation ($p<0.05$).

To determine if organic deposition from the soil was preventing ion leaching, ICP-OES was performed on these virus samples. These results are presented in Figure 4.5. Concentrations at each time point for copper, nickel, or zinc were not significantly different for soiling conditions ($p<0.05$), indicating that soiling did not prevent leaching of metal ions.

4.3.1.3 Soiling Agents Used in Standard Tests

In developing a standard protocol for measuring antimicrobial activity of copper surfaces, the EPA initially proposed a soil made of PBS, FBS, and TX-100 (denoted Old EPA Soil). However, during public consultation of the protocol, it was suggested that the soil be changed to follow the current ASTM standard (denoted New EPA Soil) of FBS, yeast extract/yeastolate, and bovine/porcine mucin. Therefore, the effect of different soiling agents (conditioned media, Old EPA, and New EPA) was investigated.

No significant difference was found between any soiling condition at any time point ($p>0.05$) as shown in Figure 4.6. Solid lines connect the time points, while dashed lines indicate the next point was below the detection limit and are meant to help guide the reader's eye. Points below the detection limit are indicated by an asterisk matching the sample colour.
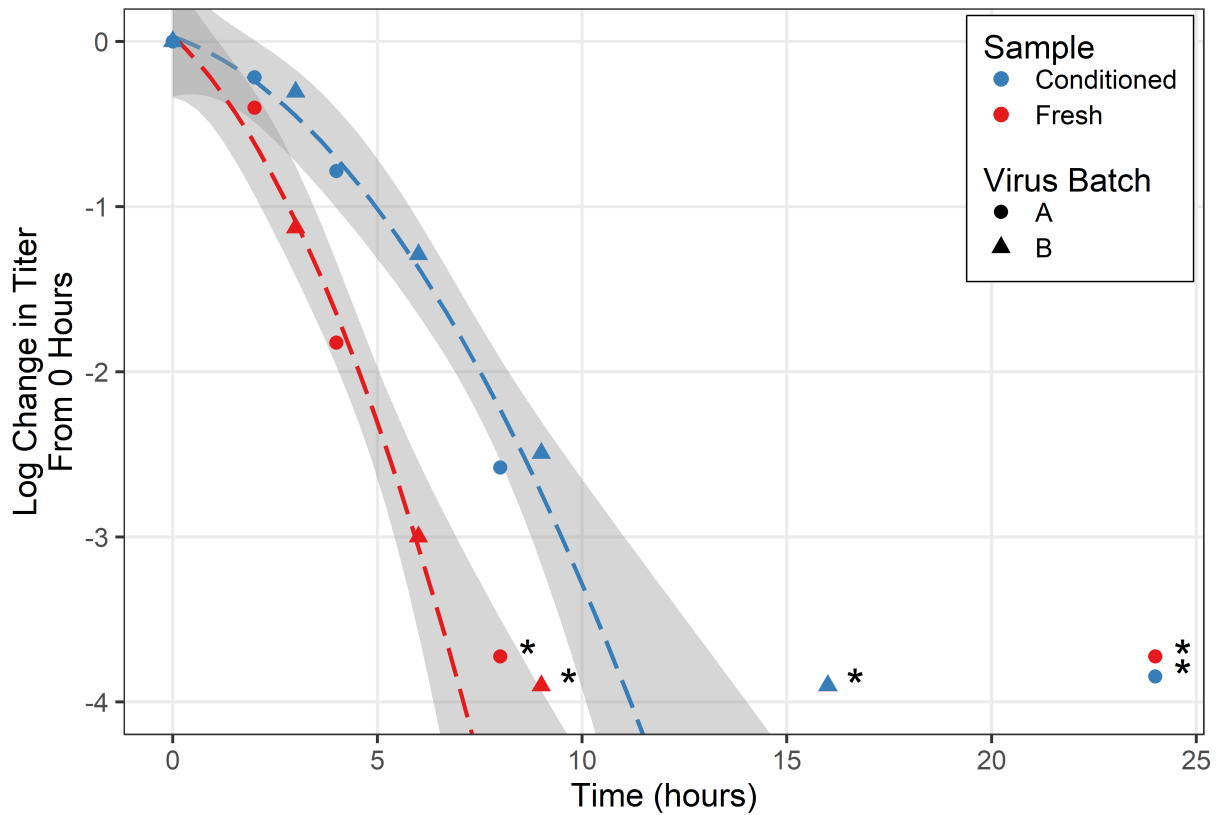
Figure 4.4: A time course analysis of virus inactivation in fresh or conditioned media. Trend lines (dashed) with standard deviation (grey area) were calculated only using data above the detection limit. Virus Batch A (circles) is the geometric mean of two technical replicates and two biological replicates (n=4). Virus Batch B (triangles) is the geometric mean of two technical replicates only (n=2). Asterisks indicate data with at least one replicate at or below the detection limit. Model parameters are presented in Appendix B.
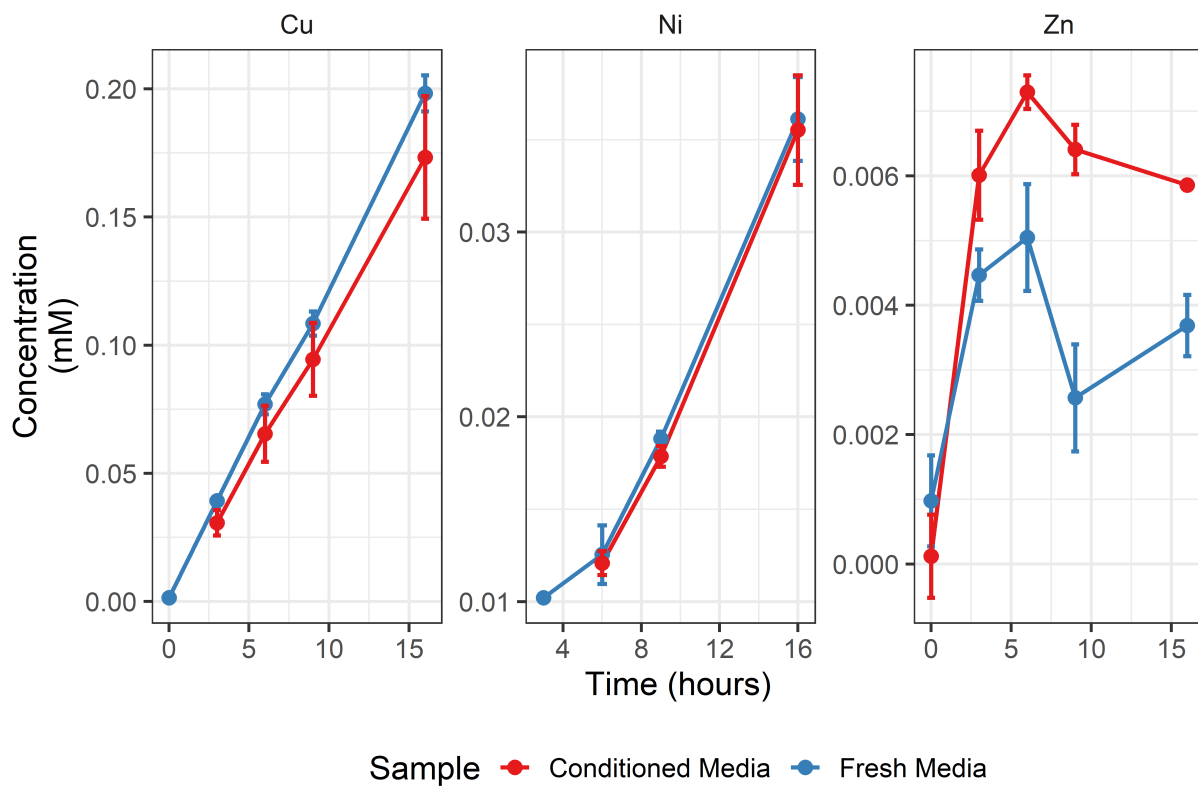
Figure 4.5: ICP-OES of leachates from two soiling conditions exposed to the alloy from 0 to 16 hours. Only the alloy's major constituents are presented. Each sample is the average of two technical replicates and two biological replicates (n=4). Error bars represent the standard deviation. Lines are not the result of any regression, but help to visualize trends in the data.
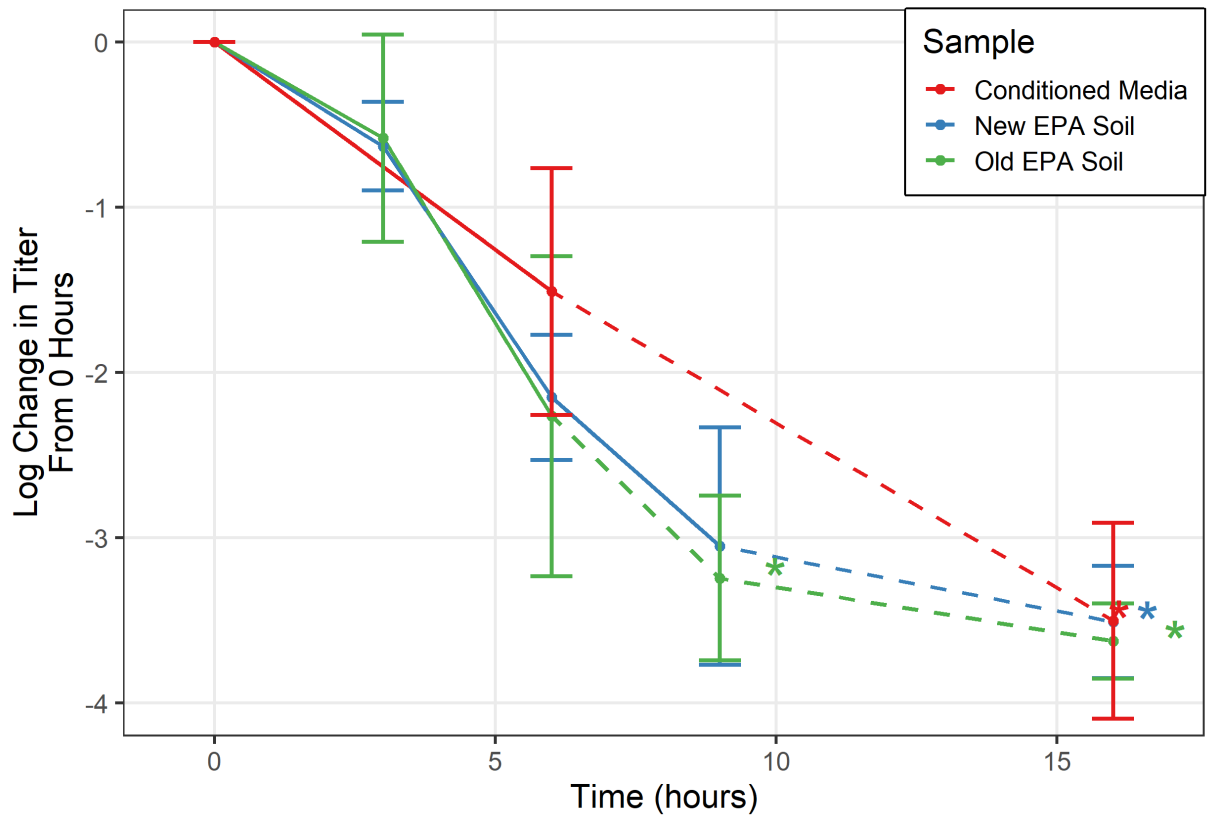
Figure 4.6: Titer of three different soiling conditions from 0 to 16 hours. Lines are not the result of any regression, but help to visualize trends in the data. Solid lines are between points in which all replicates were above the detection limit. Dashed lines indicate the next point had a replicate at or below the detection limit. Each point represents the geometric mean of two technical replicates and two biological replicates (n=4). Error bars represent the standard deviation. Asterisks indicate at least one replicate was at or below the detection limit.

4.3.1.4 Repeated Surface Exposures

This copper alloy surface is intended for a hospital setting where it is likely to become contaminated multiple times before being cleaned. A cursory investigation into the surface's ability to repeatedly inactivate virus was performed. Further, a time series was used to discern any changes in inactivation kinetics.

Using three biological replicates, it was found that the surface was unable to repeatably inactivate the virus. At 16 and 24 hours, the second exposure coupons had a significantly reduced inactivation of virus ($p<0.05$), as shown in Figure 4.7. Between exposures, the surface was cleaned using 70% ethanol and gentle scrubbing under running deionized water. Due to the drastic decrease in virucidal activity between the first and second exposure, a third exposure was not performed. ICP-OES of the repeated exposure showed that copper and nickel leaching decreased significantly ($p<0.05$) on the second exposure (Figure 4.8).
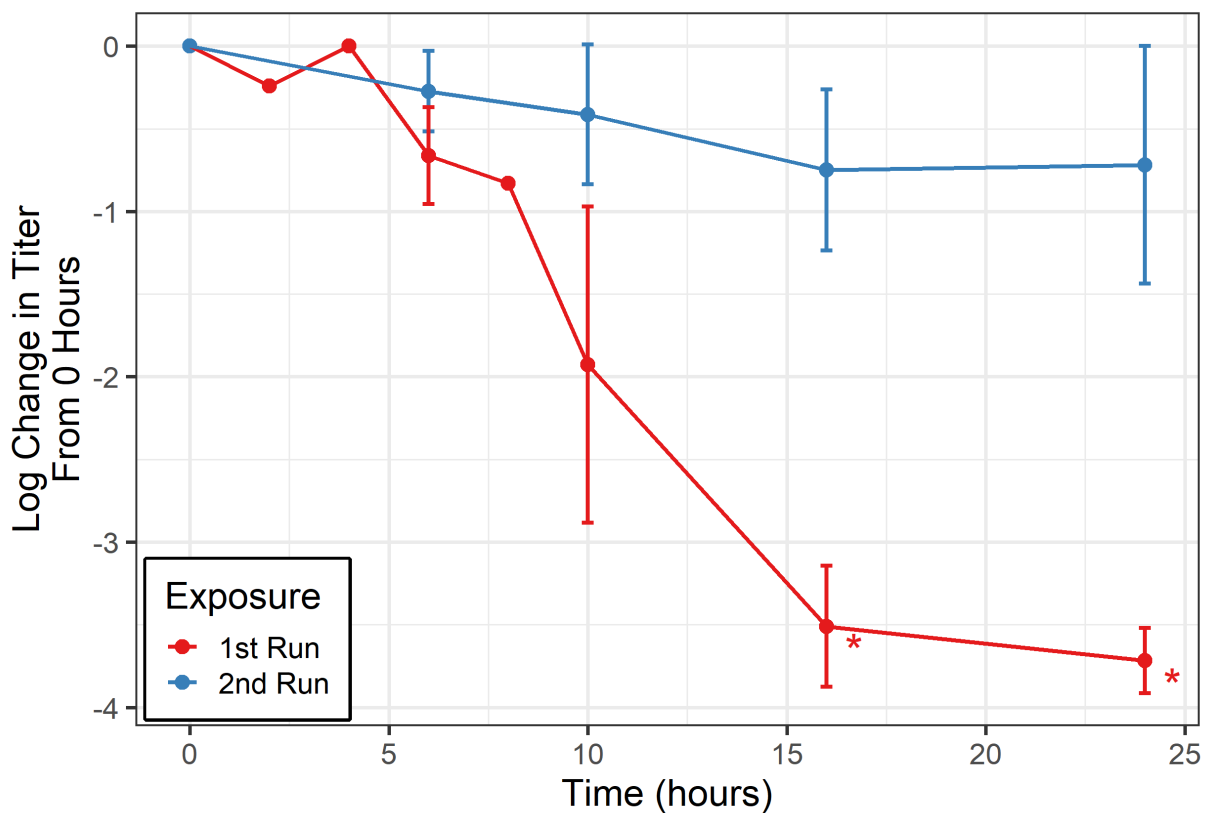
Figure 4.7: Copper alloy was subject to two rounds of virus exposure. The first exposure is presented in red points and lines, and the second exposure is presented in blue points and lines. Each point represents the geometric mean of three biological replicates (n=3). Error bars represent the standard deviation. Asterisks indicate at least one replicate was at or below the detection limit. Lines are not the result of any regression, but help to visualize trends in the data.
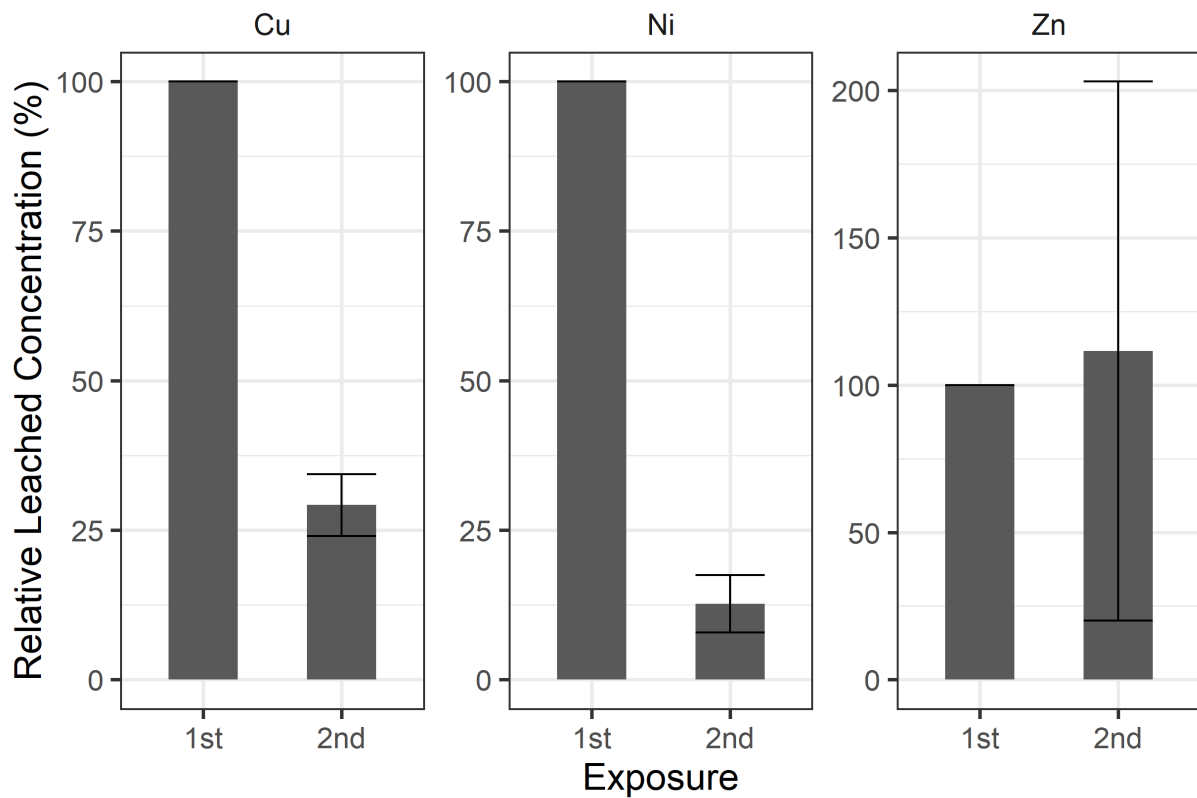
Figure 4.8: ICP-OES of the samples collected for titering in Figure 4.7. Only the alloy's major constituents are presented. Each bar represents the geometric mean of three biological replicates (n=3), and are normalized to concentrations from the first exposure. Error bars represent the standard deviation.

4.3.2 Dried Virus

In infected patients, expelled virus is typically in microliter volumes that dry fairly quickly in ambient humidities. The following tests evaluate the virucidal properties of the copper alloy surface using the methods described in Section 4.2.3.2.

4.3.2.1 Inactivation of Dried Virus

Inactivation of the dried virus was compared to stainless steel and the copper alloy. Drying of virus resulted in a 1-log loss ($\pm$ 0.28), as shown by recovery from stainless steel (Figure 4.9). Virus was judged to have dried after 45 minutes in a biosafety cabinet using visual inspection. Copper alloy achieved inactivation beyond the detection limit ($\geq$3.69-log) when in conditioned medium. A control for all ambient factors excluding humidity, virus stock, did not have a significant change (0.22-log $\pm$0.34).

4.3.2.2 Soiling Interference on Dried Virus Inactivation

Next, the effect of soiling on the copper alloy was determined. Virus was suspended in fresh media, conditioned media, or new EPA soil. No significant difference ($p>0.05$) was found between conditioned media, fresh media, or New EPA soil and is presented in Figure 4.10. Virus in conditioned media and fresh media were inactivated beyond the detection limit ($\geq$3.40-log $\pm$ 0.76 and $\geq$3.46-log $\pm$ 0.54, respectively), while the New EPA Soil samples still had detectable virus after drying ($\geq$2.8-log $\pm$ 1.2).
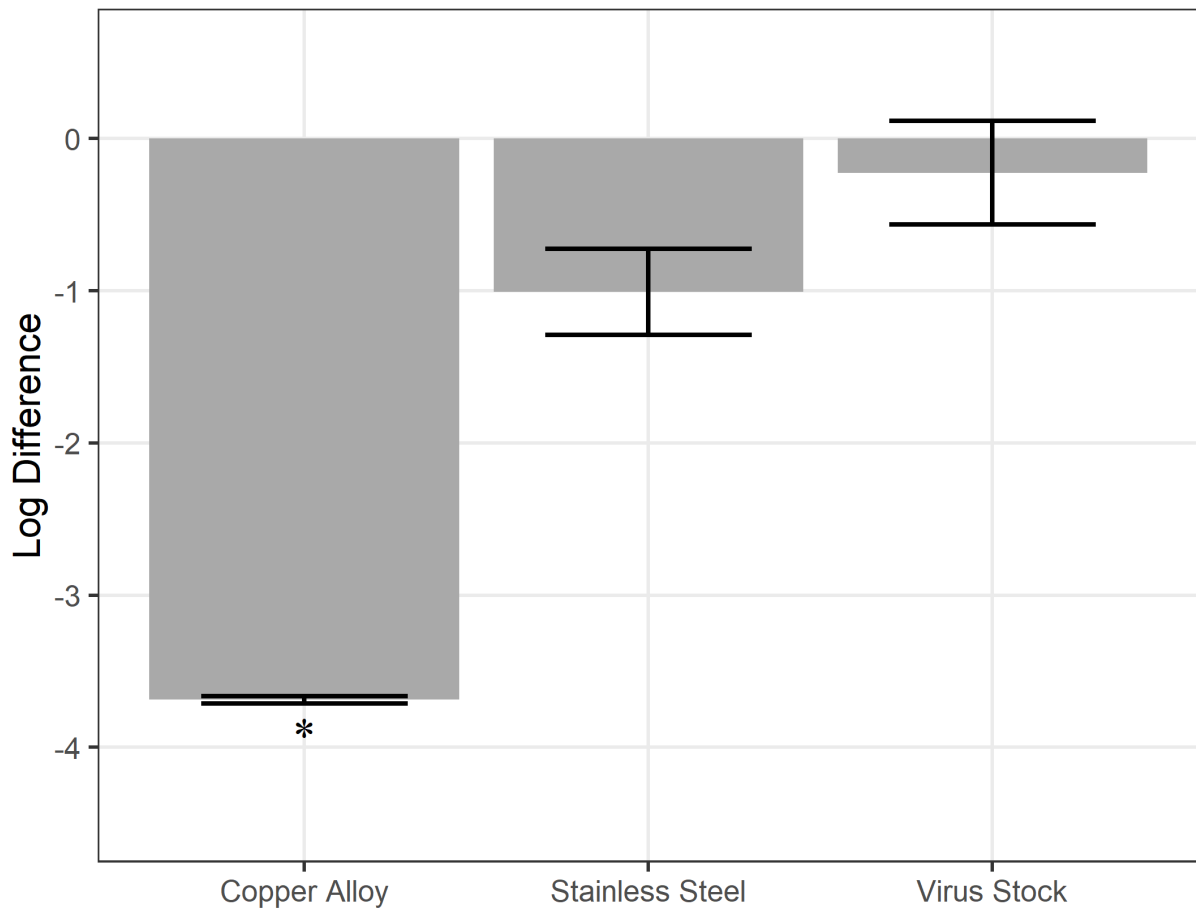
Figure 4.9: Virus was dried onto either copper alloy or stainless steel. Virus stock represents virus exposed to only the ambient temperature and light in a sealed micro-centrifuge tube. Each bar is the geometric mean of two technical replicates and two biological replicates (n=4). Error bars represent the standard deviation. Asterisk indicates at least one replicate was at or below the detection limit.
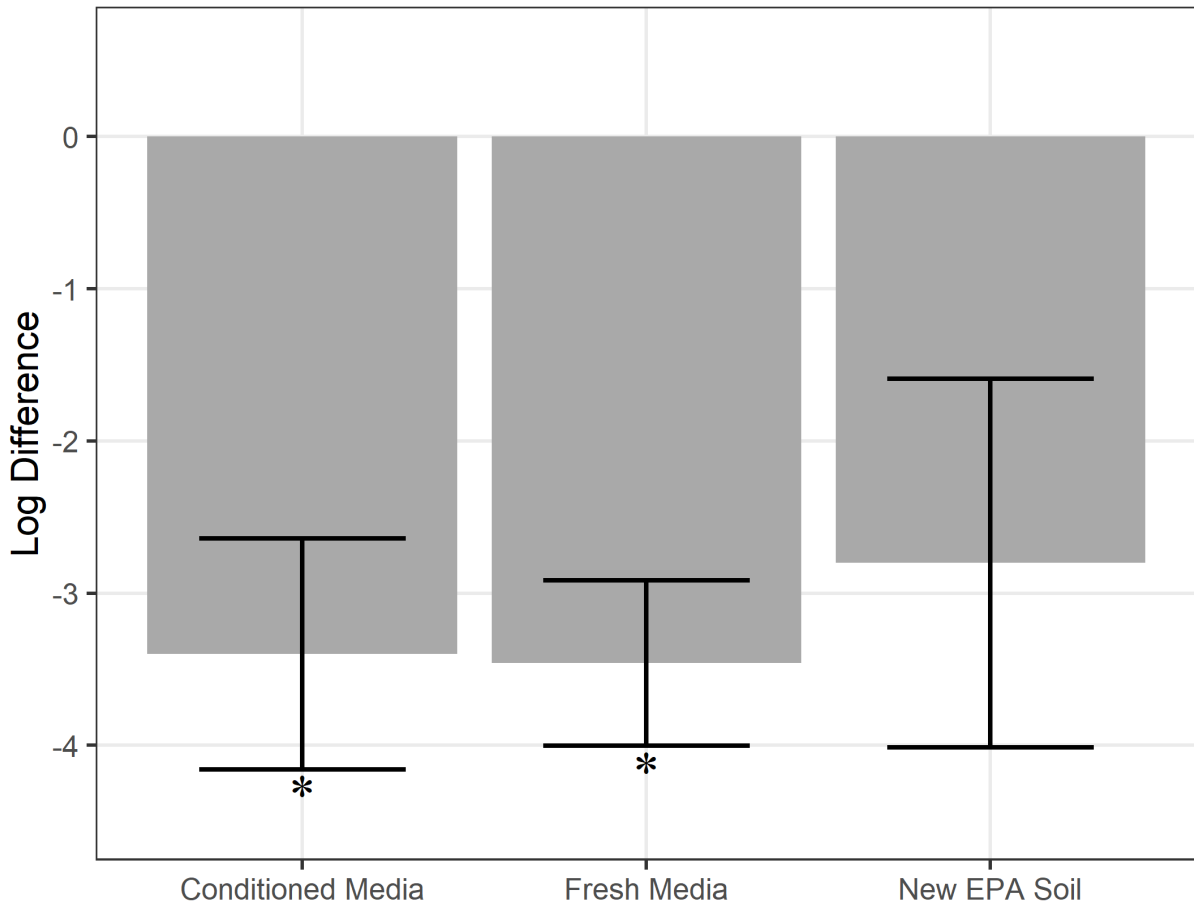
Figure 4.10: Virus suspended in three different matrices was dried onto the copper alloy. Each bar is the geometric mean of two technical replicates and two biological replicates (n=4). Error bars represent the standard deviation. Asterisks indicate at least one replicate was at or below the detection limit.

4.3.2.3   Repeated Surface Exposures

Finally, the ability to repeatedly inactivate virus was tested. The same copper alloy surface was subjected to three virus exposures under the same soiling conditions. Between each exposure, the surface was cleaned by gentle scrubbing with 70% ethanol followed by a rinse with deionized water. Before reuse, the coupons were sterilized by immersing in 70% ethanol for at least 5 minutes. No significant difference was found between each exposure or soiling condition ($p > 0.05$ for both), as shown in Figure 4.11. These results indicate that dried virus was continually inactivated by the surface.

Virus inactivation resembled that of ICP-OES shown in Figure 4.12. For clarity, only the copper ions leached from the surface are presented in the ICP-OES data collected for these samples. The copper ion concentrations were also representative of alloy ions leached, as the ions leached in proportional concentrations.
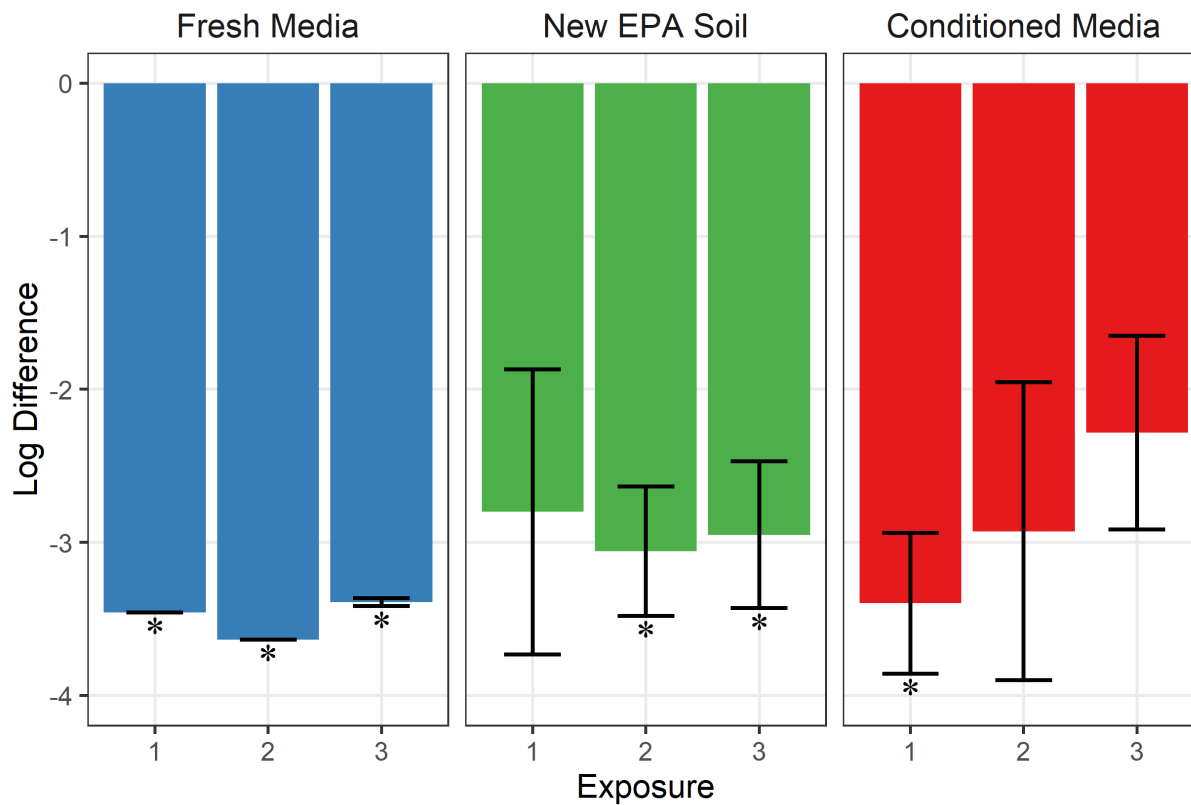
Figure 4.11: As in Figure 4.10, virus suspended in three different matrices was dried onto the copper alloy. The alloy was exposed to each virus suspension a total of three times. Each bar is the geometric mean of two technical replicates and two biological replicates (n=4). Error bars represent the standard deviation. Asterisks indicate at least one replicate was at or below the detection limit.
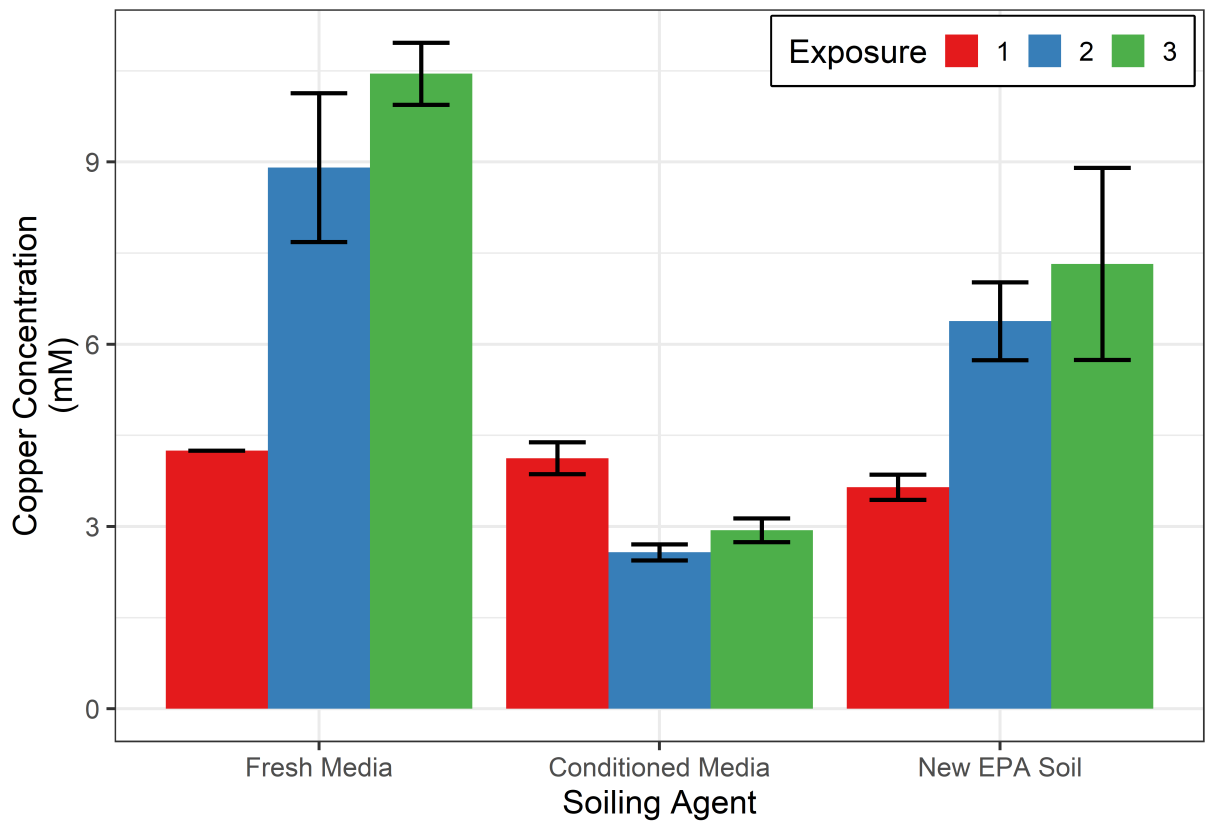
Figure 4.12: ICP-OES of samples collected in Figure 4.11. Only the copper ion concentrations are presented here for clarity. Error bars represent the standard deviation. Each colour corresponds to the exposure.

4.4 Discussion

The economic and physical burden of nosocomial infections have been well documented, with surface contamination identified as a significant source of these infections [Scott II, 2009]. Several stringent methods have been developed to reduce the impact of surface contamination on nosocomial infection spread [Scott II, 2009]. However, it appears that there are still significant issues with surface contamination persisting after thorough decontamination [Manian et al., 2011].

Research has shown both enveloped ([Warnes et al., 2015a], [Casanova et al., 2010]) and non-enveloped ([Warnes et al., 2015b]) viruses can persist on high-touch non-porous surfaces for many days. Further, a review by Kutter et al. [2018] found that the transmission routes for many enveloped viruses is complex but that the spread by foamites on hard surfaces was consistently implicated. To combat this, several self-sterilizing technologies have been developed.

Copper surfaces have been developed and tested for antimicrobial activity [Villapun et al., 2016], but there is a significant drop-off between lab-tested reductions and those seen in practice [Michels et al., 2015]. This shows that further standard method development is necessary, particularly in the preparation of tested viruses. Our goal was to first establish virucidal activity by a copper-nickel-zinc alloy and then explore potential sources of variability between the lab and practical application. This was achieved by investigating the virus matrix and variability in testing conditions.

When investigating virus droplet disinfection, our results showed that copper ion leaching was associated with the inactivation of virus. This has also been indicated in previous

studies [Warnes et al., 2015a,b; Noyce et al., 2006]. We have also showed that soiling under wet conditions will significantly decrease the virucidal activity of copper as a self-sterilizing surface, indicating that virus sample preparation may bias the inactivation properties of the alloy. This is particularly important because many virucidal surface tests in literature utilize an unknown soiling level [Warnes et al., 2015b,a; Noyce et al., 2007].

Both cell debris and conditioned media acted as a soiling agent, but conditioned media appeared to have a much stronger effect than cell debris alone. Cell debris and conditioned media combined (representing high soiling) showed almost complete protection of the virus from any virucidal activity by the surface. It is likely that soil load is high in shedded infectious material, especially with viral infections which require cell lysis as part of shedding.

Interestingly, the protective effects of soiling in wet conditions were not observed for dried virus. Since complete inactivation was achieved when the virus dried, developing a surface with faster drying time may be of interest. The inactivation of virus by copper alloys is consistent with literature data, as many of these studies were performed with small virus samples (<50 µL) dried onto the surface [Warnes et al., 2015a,b; Warnes and Keevil, 2013; Manuel et al., 2015].

To the authors' knowledge, this was the first time concentrations of leached metals from a surface were directly compared to virucidal activity, as opposed to indirectly (using copper-chelating molecules such as EDTA) [Warnes and Keevil, 2013] or for bacteria [Molteni et al., 2010; Santo et al., 2012]. Crucially, we showed soiling did not prevent virucidal activity by inhibiting leaching of metals, but rather, likely by sequestering of

metal ions. In our studies of soiling agents, both the current ASTM standard (New EPA Soil) and our conditioned media had similar protective effects against virucidal activity of the surface. However, the authors recommend that the ASTM soiling be employed in all future studies, as it provides a uniform and consistent source of soiling across virus and host combinations.

This work on the virus matrix extended on tests of copper leaching by different solutions Molteni et al. [2010]. In their work, Molteni et al. [2010] showed that a buffer known to dissolve copper (Tris-Cl) and conditioned medium were able to leach copper and completely kill *Enterococcus hirae* in 12 minutes. However, water and NaP_i buffer leached much lower copper concentrations and were not able to inactivate *E. hirae* for multiple hours [Molteni et al., 2010]. In a more focused approach, we tested only fresh and conditioned media for the matrix and showed that the level of soiling will further alter the alloy's virucidal properties.

Soiling has been recognized as an interfering factor for quite some time, and the use of standardized test protocols helps to normalize the variability from this factor [Steinmann, 2001]. Although there are no standard protocols in place for self-sterilizing virucidal surfaces, similar test conditions for disinfectants have good translatability (ie ASTM E2197 - 17e1, E1053 - 11). Only a few studies testing virucidal surfaces employed the soil loads specified in those standards. To shed some light on accurate soil loads, it would be beneficial to look at organic soil loads in virus samples, possibly by quantifying total protein, lipid, and nucleic acid content. Therefore, it is worth investigating these interfering factors to ameliorate the difference between lab and field tests.

This study did not investigate the effects of humidity as it has been shown that humidity does not impact the efficacy of copper's virucidal activity [Hodek et al., 2016]. Additionally, a study investigating the effect of humidity on Influenza A stability found that relative humidity had no significant impact on survivability [Kormuth et al., 2018]. Since Influenza A is an enveloped virus that is fairly prone to environmental stresses, it can be reasonably assumed that relative humidity will only impact the drying time for many viruses. However, if the evaporation rate of the virus is linked to the efficacy of the disinfectant (as with ethanol [Boyce, 2018] or silver surfaces [Hodek et al., 2016]), drying should be controlled to achieve a roughly standard drying time. When using 'ambient' humidity, it should still be recorded as the levels can change, with seasonal fluctuations ranging from 15% to 48% in one study [Ramos et al., 2015]. To this end, it would not only be beneficial to monitor the temperature and humidity in the lab, but also the tested clinical settings to help delineate ambient condition effects from other environmental factors in the clinical tests.

Some limitations of this study were the viral assay method and virus selection. Our experimental set-up prevented us from being able to detect complete inactivation of virus, with a detection limit of 2.13×10^3 PFU/mL. Additionally, the tested virus is not of clinical relevance. However, baculovirus is stable for many environmental conditions [Jarvis and Garcia, 1994] and may be considered a conservative surrogate for enveloped viruses. Further, ease of genetic manipulation allowed easier quantification and safe handling made baculovirus a good candidate for method development. In only testing an enveloped virus, we considered a scenario in which some soiling has occurred, but with a virus that was susceptible to environmental stresses. Non-enveloped viruses are less susceptible to environmental factors, although there is data to suggest that they will also be inactivated by

copper ions [Warnes and Keevil, 2013].

In conclusion, a copper-nickel-zinc alloy was shown to have strong virucidal effects for a conservative enveloped virus surrogate. Copper, nickel, and zinc ions were all shown to leach from the alloy surface and are the likely cause of virucidal activity by this surface. Virucidal activity was achieved under moderate soiling, but lost under high soiling generated by routine virus amplification procedures. The surface was able to repeatably inactivate dried virus droplets under moderate soiling conditions, but was able to do so for virus droplets kept wet under high humidity.

Chapter 5

Metal Ion Virucidal Activity

5.1 Introduction

Metal ions leached into solution are likely the source of virucidal effects seen in Chapter 4, and have been previously reported for virucidal [Warnes et al., 2015b] and bactericidal [Molteni et al., 2010] studies of copper. These ions interact with the outer surface of the pathogen first, leading to inactivation [Warnes et al., 2015b; Molteni et al., 2010; Horie et al., 2008].

The copper(II) ion (Cu^{2+}) is the most cytotoxic divalent ion among common heavy metals [Horie et al., 2008]. It is likely that Cu^{2+} is the dominant species in our tests, as the virus matrix exhibited a noticeable colour change after incubation on the copper-alloy surface (shown in Figure B.1). The distinctive blue colour, is similar to a phenomenon in copper water pipping leaching and is termed 'blue water corrosion' [Critchley et al.,

2004]. This colour change is indicative of copper complexing with organic matter and is associated with the presence of microorganisms [Tuck et al., 2010; Critchley et al., 2004]. ICP-OES confirmed copper leaching, and also identified nickel and zinc (the alloy's other constituent metals) leaching from the surface (Chapter 4); however, ICP-OES does not provide information on ion valence, only atomic concentration. The causative agent behind leaching is further explored in Chapter 6.

The goal of this chapter was to determine which divalent metal cation from the copper alloy has the strongest effect at inactivating virus. To achieve this, factorial experiments were performed using copper, nickel, and zinc solutions generated from sulphate salts. Additionally, the effect of soiling on the virucidal properties of these metal cations was investigated.

5.2   Materials and Methods

5.2.1   Metal Ion Solutions

Copper sulphate was supplied by EMD Chemicals (Ontario, CAN). Nickel and zinc sulphates were supplied by Sigma-Aldrich (Ontario, CAN). The metal ion solutions were created as 10X concentrations by dissolving the respective sulfate salt in deionized water (dH_2O) at neutral pH to generate divalent ionic species. All solutions were stored at 4 °C until use for the inactivation experiments.

5.2.2 Virus Production

Baculovirus (*Autographa californica* multiple nucleopolyhedrovirus) was engineered to express the fluorescent protein mKOκ under the late and strong viral p10 promoter using the Bac-to-Bac$^{\text{TM}}$Baculovirus Expression System (Fisher Scientific, Ontario, Canada). A passage 2 working stock of the virus was made by infecting $2\text{x}10^6$ cells/mL of exponentially growing Sf-9 cells with recombinant virus at a MOI of 0.01. Cell viability was measured daily until it was between 80 and 60% (72-96 h post-infection), after which virus supernatant was harvested, purified by low speed centrifugation (800 x g, 10 minutes) and filtration through a 0.2 µm filter. Purified virus was stored at 4°C until use.

The enveloped baculovirus was selected for it's safe handling and ease of manipulation. Using genetic manipulation to express a fluorescent protein allows a more consistent and accurate judgment over cytopathic effects when scoring end-point dilution assays. Further, red fluorescent protein was selected to avoid the auto-fluorescence of growth media when exciting green fluorescent protein. Auto-fluorescence can mask fluorescence from infected cells, thereby lowering accuracy of the end-point dilution assay.

5.2.3 End-Point Dilution Assay

Virus was titered as described in Chapter 4 and is repeated here. Sf-9 cells from an exponentially growing suspension culture were diluted to $2.0\text{x}10^5$ cells/mL, and 100 µL of this dilution was seeded into each well of a flat-bottom tissue-culture treated 96-well plate (Fisher Scientific, Ontario, Canada). The cells were given at least one hour to attach, while kept inside of a humidified box at 27°C. The virus was serially diluted in the range of

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10^{-2} to 10^{-8}, creating seven test dilutions and diluting metal ions to a non-cytotoxic level. After the cells attached, 10 µL of the serial dilutions was added to each column, creating 12 replicates in one plate. The diluent, media, was used as a negative control and also added in 12 replicates. One 96-well plate was created for each experimental condition. Plates were incubated for seven days at 27°C, after which they were checked for red fluorescence using a fluorescent microscope. As virus was scored with fluorescence, and interference of metal ions with cytopathic effect scoring is avoided. Results were reported as plaque forming units per mL (PFU/mL).

5.2.4 Virus Incubation with Metal Ions

In a 1.5 mL centrifuge tube, 700 µL of virus stock was incubated with 100 µL of each metal ion solution for a total volume of 1 mL. Conditions were tested in two 2^3 factorial design experiments with a range of metal concentrations. In the first iteration (Table 5.1), the high concentrations were Cu: 4mM | Ni: 0.65mM | Zn: 0.75mM, and low concentrations were ultra-pure water (UPW). In the second iteration (Table 5.2), the high concentrations were Cu: 12.1mM | Ni: 1.5mM | Zn: 0.5mM, and low concentrations were UPW. Center points were half of the high concentration values and created using 50 µL of each metal ion solution and 150 µL UPW, for a total volume of 1 mL.

5.3 Results

5.3.1 Initial Factorial Experiment

A 2^3 factorial design experiment was used to evaluate the vircudial activity of divalent ionic species of copper, nickel, and zinc. The high level concentrations were based on growth media leachates of a 24 hour exposure to the copper alloy; sterile deionized water was used as the low level (Table 5.1). However, since this experiment was only used as a preliminary screen, no replicates or statistical analysis was performed.

Metal ions tested in the combinations outlined in Table 5.1 along with the results. Percent and log difference were calculated as the change from 5 minute (t_i) to 24 hour (t_f) exposures. Results were also plotted in Figure 5.1 for easier interpretation. The control in Figure 5.1 represents the virus stock used to perform the experiment. Combination H should represent the virus stock after being diluted with UPW, but combinations D and F showed higher titers. This may be explained by the variability in the assay.

Taking samples at 5 minutes and 24 hours after incubation allowed to correct for the initial variability when determining the inactivation. The results of this analysis are shown in Table 5.1 for both the percent difference and the log difference. Combinations with copper (A, C, E, G) all had the largest decrease, indicating that it has the strongest effect.

Figure 5.1: A factorial designed experiment testing the effect of copper, nickel, and zinc on virus inactivation in solution. Combinations are as outlined in Table 5.1 and presented again below the figure. Red bars indicate titers after 5 minutes of exposure, and blue bars represent titers after 24 hours of exposure. Ctrl (control) is the titer of undiluted virus stock.

Table 5.1: Combinations of divalent metal cations in the first factorial design of experiment (Fig 5.1)

Combination	Copper	Zinc	Nickel	Initial Titer (t_i) (pfu/mL)	Final Titer (t_f) (pfu/mL)	Percent Difference (t_i to t_f)	Log Difference (t_i to t_f)
Virus Stock	–	–	–	2.18×10^8	2.18×10^8	–	–
A	High	High	High	1.83×10^7	2.16×10^5	98.8%	-1.93
B	Zero	High	High	8.36×10^7	6.15×10^6	92.6%	-1.13
C	High	Zero	High	1.37×10^7	5.54×10^5	95.9%	-1.39
D	Zero	Zero	High	2.48×10^8	1.75×10^7	92.9%	-1.15
E	High	High	Zero	7.17×10^6	3.50×10^5	95.1%	-1.31
F	Zero	High	Zero	1.75×10^8	1.12×10^8	31.0%	-0.16
G	High	Zero	Zero	3.50×10^7	6.40×10^5	98.2%	-1.74
H	Zero	Zero	Zero	5.54×10^7	6.40×10^7	-15.5%	0.06

5.3.2 Factorial Design with Variation in Virus Preparation

A more robust factorial experiment was performed using divalent cationic copper, nickel, and zinc concentrations based on a new batch of copper-alloy surfaces. This additional experiment allowed for the inclusion of a center-point and replicates to perform a statistical analysis. As before, the high ion concentrations were those determined from a leachate (Cu: 12.1mM | Ni: 1.5mM | Zn: 0.5mM), the center-points were half the concentration of the high level, and the low concentrations were of sterile deionized water. The virus sample was prepared in two different ways: as described in Section 5.2.2 (filtered) or by omitting the filtration through a 0.2 µm filter (unfiltered).

As determined in Chapter 4, the unfiltered virus sample contains a significant amount of organic debris and sequesters metal ions to yield lower virus inactivation. The corresponding surface experiments (Figure 4.2) had no virus inactivation. Copper and nickel

Table 5.2: Combinations of divalent metal cations in a second factorial design of experiment (Fig 5.2)

Combination	Copper	Zinc	Nickel	Log Difference	
				Unfiltered	Filtered
Center Point	Mid	Mid	Mid	-2.62	-3.76
A	High	High	High	-3.57	-4.72
B	Zero	High	High	-1.23	-1.24
C	High	Zero	High	-3.26	-4.79
D	Zero	Zero	High	-0.52	-1.23
E	High	High	Zero	-3.02	-4.81
F	Zero	High	Zero	-0.04	-0.01
G	High	Zero	Zero	-3.42	-4.78
H	Zero	Zero	Zero	0.00	-0.01

had a significant effect on reducing viral titer, and the interaction between copper and nickel was also significant for both methods of virus preparation as presented in Table 5.2 and Figure 5.2. A significant difference was found between the preparation method for the center-point and combinations A, C, E, and G. An ANOVA table of these results is presented in Appendix Tables D.1, D.2, and D.3. A further analysis on the unfiltered data showed no difference between any of the copper combinations. Interestingly, the reduction by combination B (high zinc and nickel) was significantly greater than that of combination D (high nickel only). This difference indicates that a synergistic effect between the two metals may be helping to further reduce the virus.
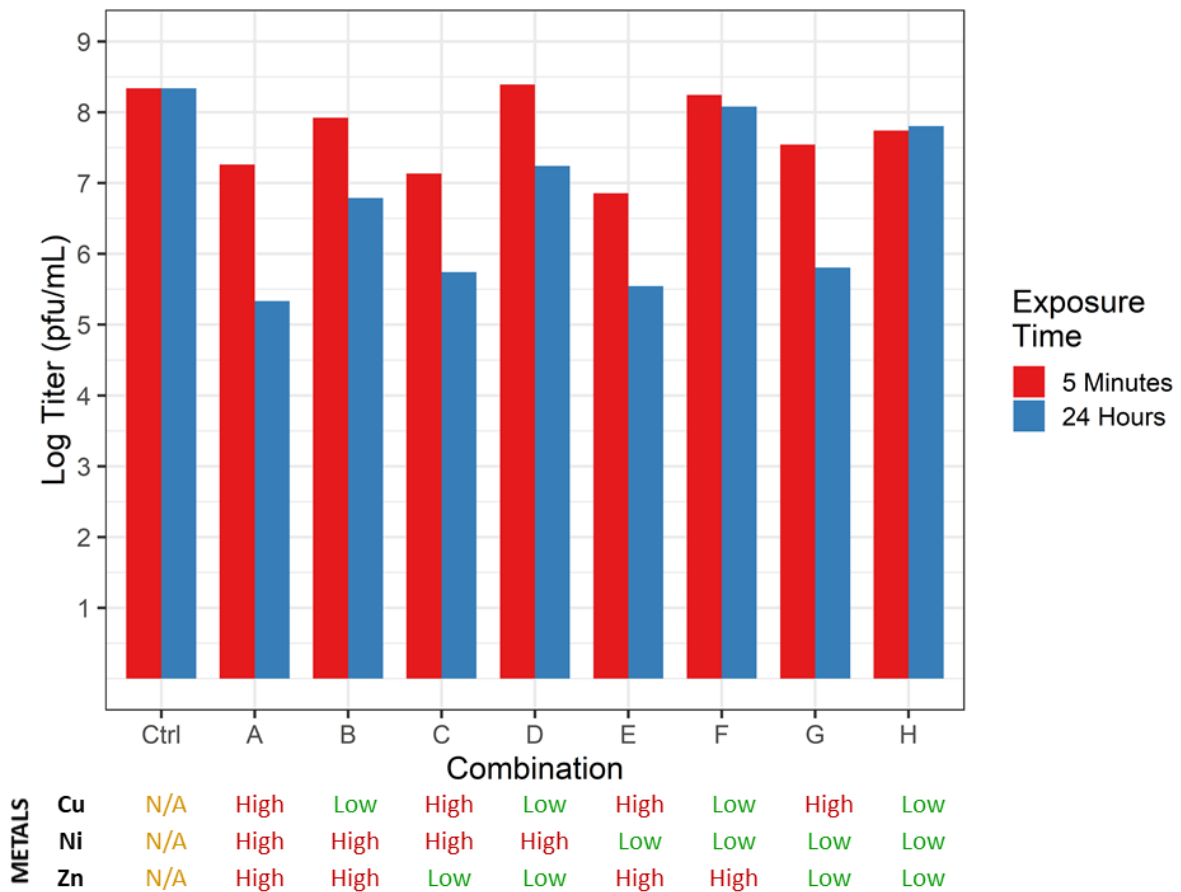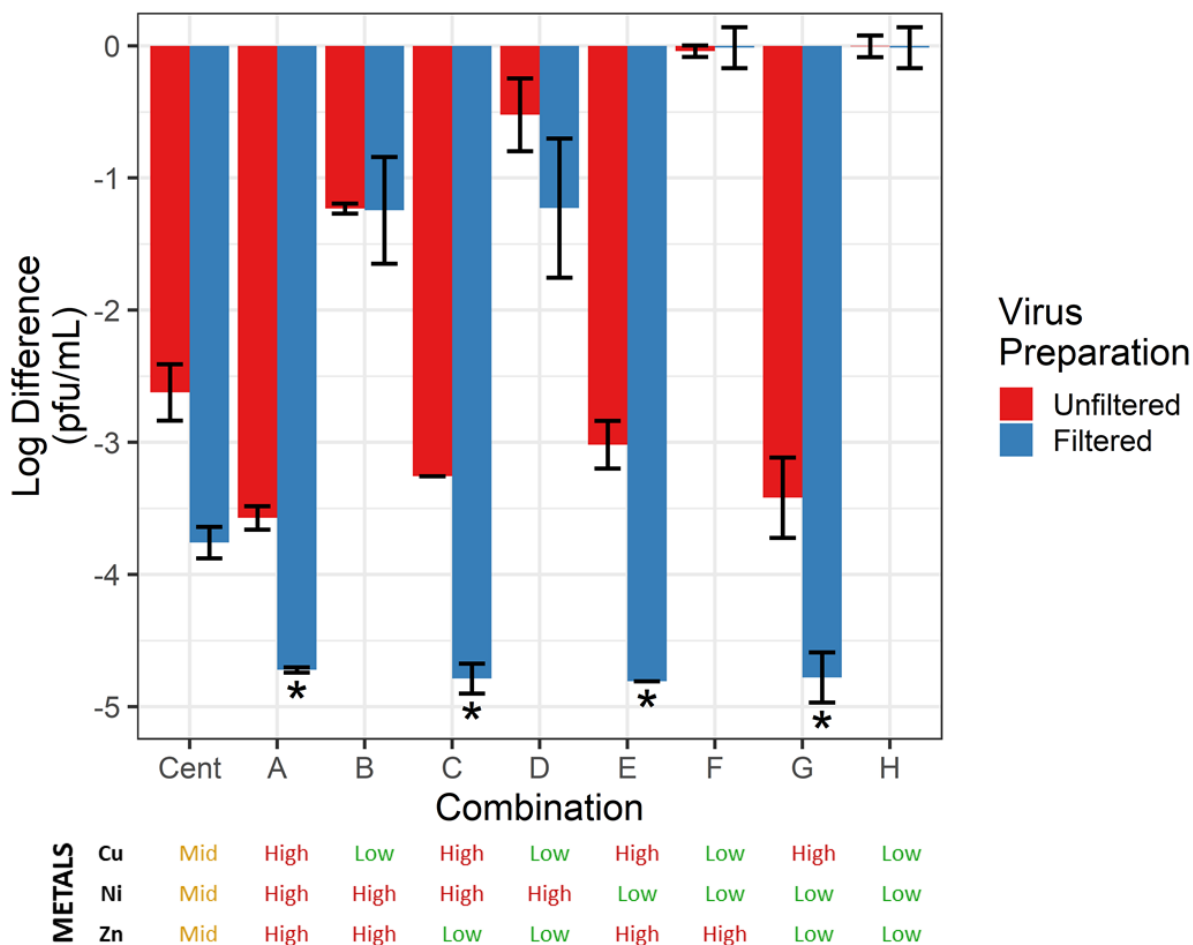
Figure 5.2: A factorial experiment testing the effect of copper, nickel, and zinc of virus inactivation in solution. Inactivation was tested using two different virus preparation procedures, unfiltered (red bars) and 0.2 μm filtered (blue bars). Combinations are as outlined in 5.2 and presented again below the figure. Each bar represents two technical replicates (n=2), except in the case of center-points which had four technical replicates (n=4). Asterisk indicates that at least one replicate was below the detection limit.

5.3.3 Copper Ion Concentration to Inactivate Virus

From the results of the factorial experiments, copper was identified as having the strongest virucidal effect. An exploration of the minimum required concentration of copper to inactivate filtered virus in conditioned media was performed and the results are presented in Figure 5.3. Results indicate that no significant inactivation occurs for concentrations at or below 1 mM of copper(II). However, between 1 and 2 mM the effect of copper(II) starts to significantly inactivate virus ($\geq$1-log). This value is expected to fluctuate dependent on the level of soiling.
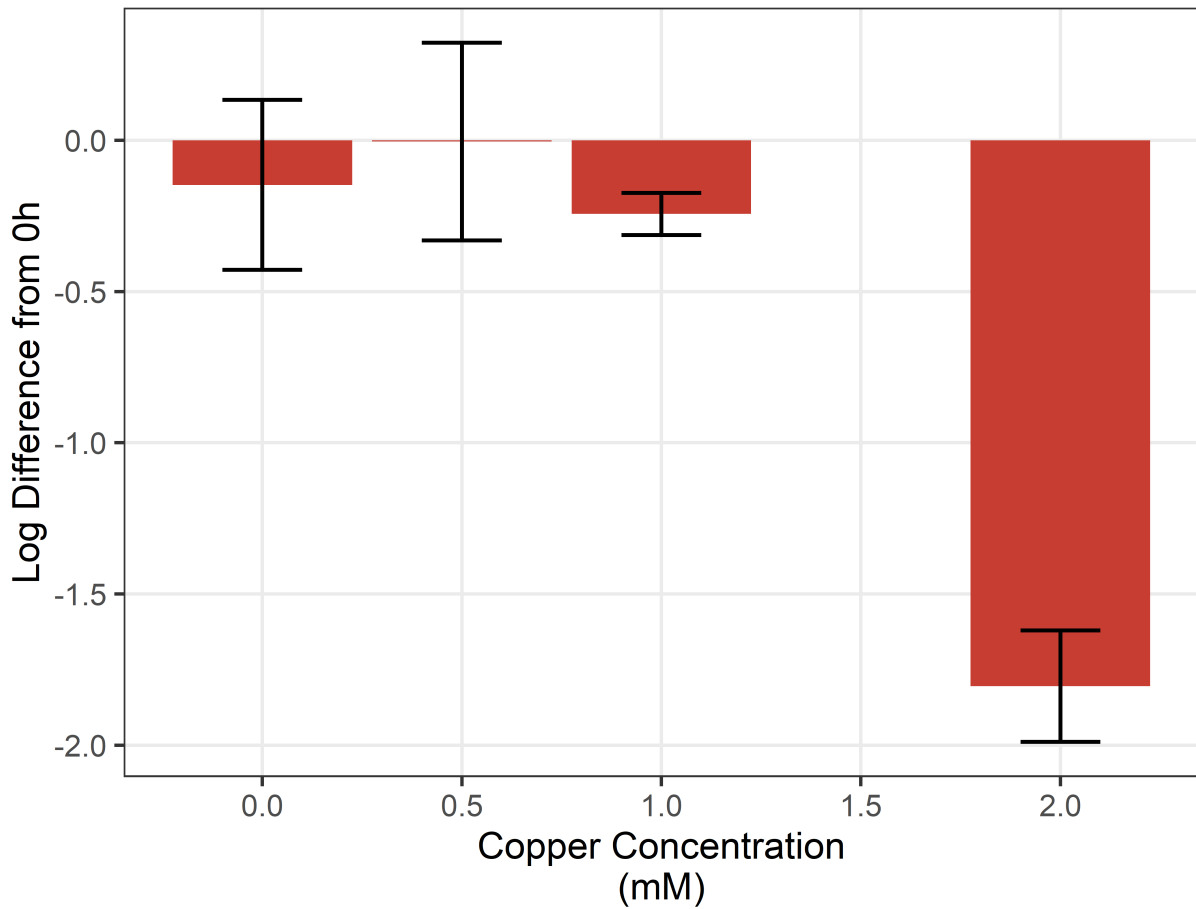
Figure 5.3: Inactivation of filtered virus in conditioned media by copper(II) ions in solution after 24 hours was measured. Four levels of copper were tested: 0, 0.5, 1, and 2 mM. Each bar represents the geometric mean of two biological replicates performed with two technical replicates (n=4). Log difference was determined as change from the immediate exposure to the 24 hour exposure of each replicate.

5.4 Discussion and Conclusions

In this work we selected metal-sulphate salts to generate the metal ion solutions. Previous work in our lab has shown that metal-sulphate salts have a lower cytotoxic effect than metal-chlorine salts (A. Tulahdar, unpublished). The cytotoxicity was confirmed in a study by Horie et al. [2008], but was only caused by zinc salts and not copper salts. Since these metal salts target the cell membrane [Horie et al., 2008; Molteni et al., 2010] and baculovirus uses the host cell membrane to obtain its envelop, it is likely that the baculovirus will be susceptible to the same membrane damage. To avoid potentially confounding metal-salt toxicity with the virucidal properties of these metals, the sulphate salts were selected.

Copper surfaces have been shown to have strong virucidal effects, likely due to copper ionic species leaching from the surface Warnes et al. [2015a]. For virucidal activity by a copper surface, there is emerging evidence that Cu^+ is more virucidal than Cu^{2+} [Warnes and Keevil, 2013]. However, our tests show divalent copper and nickel species are able to significantly inactivate virus. Virucidal activity has previously been confirmed for ionic copper at a similar concentration (15.75 mM) against filtered preparations of enveloped and non-enveloped viruses [Sagripanti et al., 1993]. Another enveloped virus, Influenza A H9N2, was inactivated by copper ions (as low as 2.5 µM) but not nickel (up to 250 µM) [Horie et al., 2008] in filtered preparations. The inability of nickel to inactivate Influenza A [Horie et al., 2008] is in contrast to our ion factorial tests, although our tests used concentrations 2-fold higher.

Our results indicate that there is a minimum threshold of copper ion concentration to achieve effective disinfection. However, these results are specific to the soiling conditions of

conditioned media and for a time of 24 hours. When compared to the copper concentration required to inactivate Influenza A, Horie et al. [2008] found a concentration 400-fold lower than our results indicate. This may be because they did not include soiling, or because Influenza is less resistant to environmental stresses [Patois et al., 2011].

Overall, these studies show that metal ions are likely the cause of virucidal activity by the alloy surface seen in Chapter 4. Cu^{2+} has the greatest virucidal effect, and there is some indication that a synergistic effect may be present between copper and nickel. Results also indicate that the copper alloy surface leaches a much higher concentration than necessary to inactivate the virus. However, the concentration of metal ions required for virucidal activity is likely dependent on soil concentration. Organic soils can act as quenchers of these metals, as metal ions are known to bind non-specifically to organic matter [Santo et al., 2012]. For pathogens, the binding results in destabilization of membranes and genomes, and is the key to inactivation [Santo et al., 2012].

Future work should test copper, nickel, and zinc ions at constant levels to delineate the overall increase in metal ion concentration from the effect of each ion. Further, tests on ionic species with different ionic charges may help to clarify some of the conflicting work in literature.

Chapter 6

Characterization of Alloy Leaching

6.1 Introduction

Studies have shown that the leaching of copper ions from a solid copper surface is responsible for the inactivation of virus [Warnes et al., 2015b] and bacteria [Molteni et al., 2010]. Further, copper leaching is an important factor in inactivating virus, as leached ions have been identified to be strongly virucidal (Chapter 5). Results from Chapter 4 indicate that the growth media is able to leach metal ions from the surface. However, as pathogens are excreted in biological matrices of varied compositions, it is important to elucidate the cause of leaching so that acutal-use efficacy can be predicted. Understanding leaching is also important for safety, as high concentrations of metals like copper and nickel can be toxic [Tchounwou et al., 2012].

Leaching of copper by bacteria (biocorrosion) is a common problem in copper pipes

[Vargas et al., 2017]. Biocorrosion occurs when biofouling leads to corrosion, and typically occurs in soft water with high organic content at ambient temperatures Tuck et al. [2010]. The precise mechanism by which the corrosion occurs is a complex phenomenon, but involves creation of microenvironments with various dissolved oxygen (DO) concentrations and oxidation-reduction conditions [Vargas et al., 2017] under biofilms. Biocorrosion is only possible in these high-flow environments due to biofilms that shield bacteria and allow capture of scarce nutrients [Vargas et al., 2017]. Interestingly, biofilm (made of extracellular polymeric substances, EPS) only initially protects copper from corrosion (2 hours), but after long term exposure (11 days) starts to exhibit corrosive behavior [Chen and Zhang, 2018]. In a similar vein to piping biocorrosion, implant corrosion is caused by biological molecules.

Some early work on implant corrosion has shown that protein is corrosive to copper, among other alloys [Clark and Williams, 1982]. It was proposed that disulfide bonds potentially acted as catalysts to help oxidize the surface and the resulting metal-protein complex resulting in dissolution of the metal [Clark and Williams, 1982]. However not all biological molecules are corrosive, as amino acids have been employed as very effective corrosion inhibitors [Hamadi et al., 2018].

The goal of this chapter was to elucidate the cause of leaching from the alloy. First, varying the virus medium was investigated. Then, the impact of osmolarity on leaching was tested using salt solutions. Finally, the effect of protein on leaching was explored.

6.2 Materials and Methods

6.2.1 Salt Solutions

Sodium chloride (NaCl) and calcium chloride (CaCl$_2$) salts (Millipore Sigma, Toronto, ON, Canada) were used to make solutions of known concentration and osmolarity using ultra-pure water (UPW). The pH of these solutions was not controlled, but all solutions had a pH between 6 and 8. Chlorine salt solutions were used to encourage corrosion, as opposed to sulphate salts used in Chapter 5.

6.2.2 Test Surface Preparation

Aereus Technologies provided copper-alloy (UNS C75200) coated stainless steel and stainless steel (UNS S30400) coupons (1 x 1 x 1/16 in). Alloy-plated coupons were created using a patented liquid-spray application process and had a semi-polished finish on both sides. The alloy-coating material is made from 65% copper, 18% nickel and 17% zinc. Before use, surfaces were cleaned using 70% ethanol followed by a soak in 70% ethanol for at least 5 minutes immediately before use.

6.2.3 Surface Exposed Samples

Coupons were placed in a 60 mm Petri dish and held at >90% humidity in a sealed container. 750µL of growth medium or salt solution was pipetted onto the coupons, creating a pool on top of the coupon. After 24 hours, the solution was collected and stored at 4°C

until wet ashing. All tests were performed in a biosafety cabinet in a temperature controlled room set to 21°C.

6.2.4 Wet Ashing

Surface exposed samples were diluted (0.5mL sample in 9.5mL UPW) and placed on a heating block set to 110 °C in a fume hood. 1 mL of aqua regia acid (3:1 molar ratio of hydrochloric acid to nitric acid) was slowly added and the mixture was allowed to reflux for 15 minutes. A further 2 mL of aqua regia was added slowly to the sample and refluxed for an additional 30 minutes. A digestion blank of 10mL UPW was performed along-side the digestion to match the sample preparation. After wet-ashing was complete, samples were cooled to room temperature and filtered.

6.2.5 Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES)

Samples were analyzed for 20 commonly occurring elements. Calibration curves with a range 0 to 100 ppm for each element allowed quantification. Samples were loaded in with an internal 10 ppm yttrium standard to adjust any changes in intensity cause by matrix effects using a Prodigy High Dispersion ICP (Teledyne Leeman Labs, New Hampshire, USA). ICP-OES yields molar concentration and is not capable of distinguishing ionic species.

6.3   Results

6.3.1   Effect of Virus Preparation on Leaching

Virus preparation has been previously established as an interfering factor of the copper alloy's virucidal properties (Chapter 4). Comparing the heavily-soiled unfiltered virus stock's ability to leach metal ions and a 1:10 dilution of the same matrix, it was found that heavy soiling hinders leaching. This is shown in Figure 6.1, with the effect most apparent in copper and nickel leaching. Due to the small concentrations of zinc leaching, it is difficult to discern the effect of virus preparation on this metal's leaching.

The impact of soiling was further investigated by ICP-OES of the soiling experiment performed in Figure 4.3. Virus solutions were prepared using combinations of sonicated cells and conditioned media as described in Chapter 4. The soiling combinations and resulting metal ion leaching are presented in Figure 6.2. Results indicate that under heavy soiling (10^6 sonicated cells and conditioned medium) leaching may be hindered; however, inactivation still occurs in all cases of moderate soiling (fresh medium) (Figure 4.3).
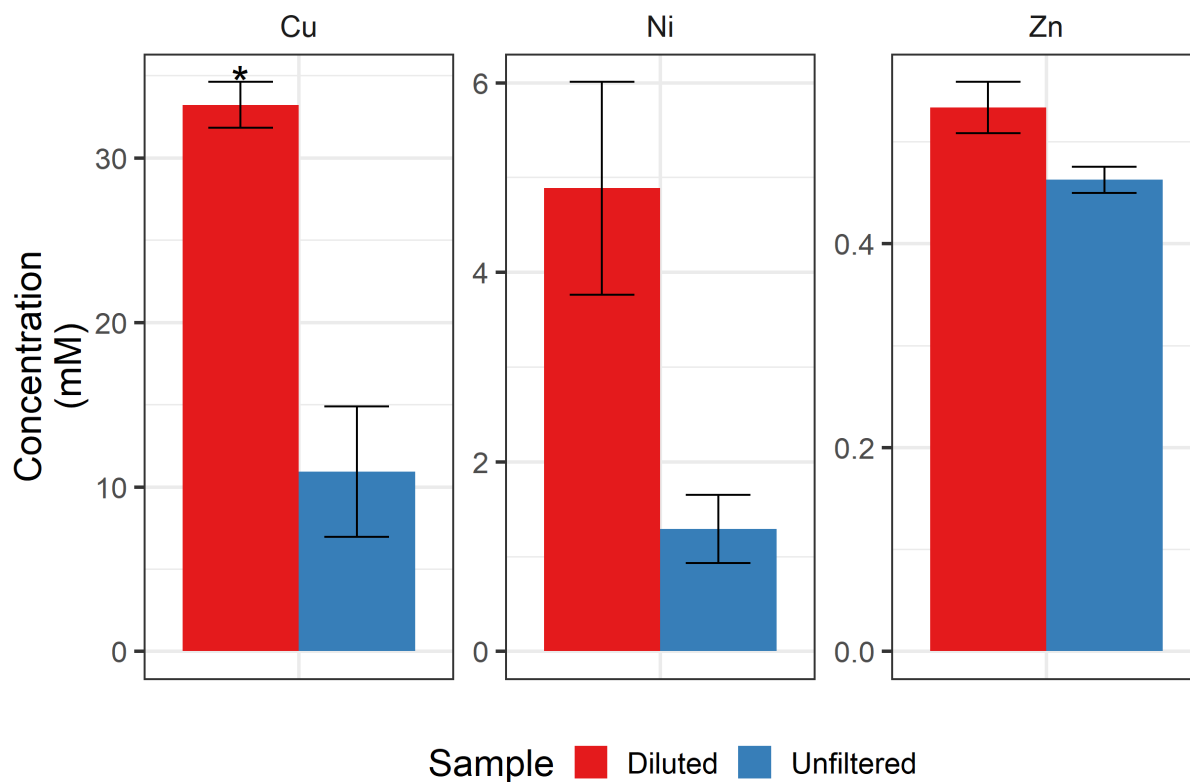
Figure 6.1: ICP-OES of samples exposed to the copper alloy surface for 24 hours, where virus was unfiltered (blue bars) or diluted 1:10 into fresh media (red bars). Each bar is the average of biological duplicates. Asterisks indicates the value was outside the calibration curve. Error bars represent the range of data.
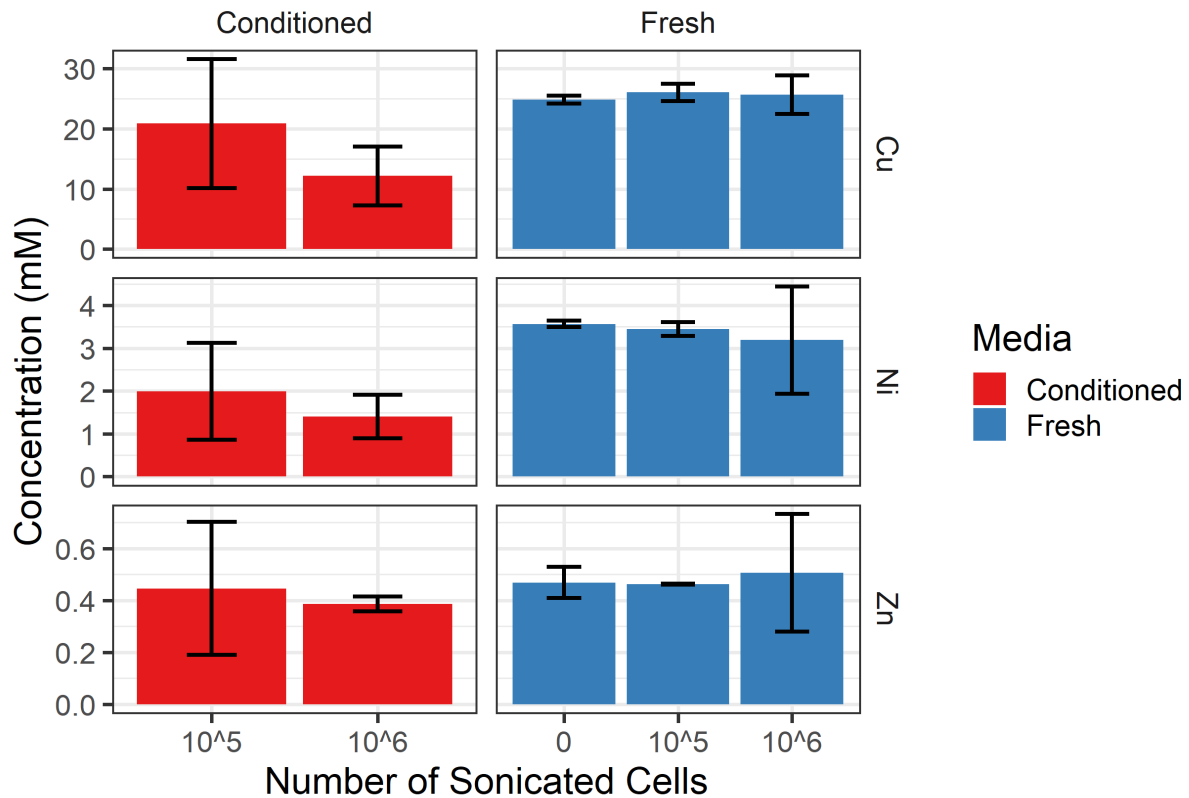
Figure 6.2: ICP-OES results of copper alloy leachates from Figure 4.3. Only the three constituent ions from the alloy are presented. Virus was diluted 1:10 with fresh or conditioned media spiked with three levels of cell debris: no cells (blue bars), 1×10^5 cells (red bars), or 1×10^6 cells (green bars). Each bar represents the average of two biological replicates (n=2). Error bars represent the range of data.

6.3.2 Virus Medium and Leaching

The change of copper leaching into the virus media (insect cell growth medium, Sf900 III) over a period of 24 hours is presented in Figure 6.3. Results show that in 6 hours, the copper concentration is higher than the level to achieve inactivation (Fig 5.3). Copper was present in the initial media in a µM concentration. The presented leaching concentrations only show the copper detected above those baseline amounts. This was not performed for nickel and zinc as their initial concentrations were below the detection limit.

Figure 6.4 is shown again as part of the investigation in leaching kinetics 4.5 (Figure . Here, a filtered virus stock is diluted into cell growth media that has (conditioned) or has not (fresh) been used for cell growth. The conditioned media was clarified with centrifugation only, whereas the fresh media does not require any clarification. Results show that neither copper nor nickel exhibited different leaching kinetics.
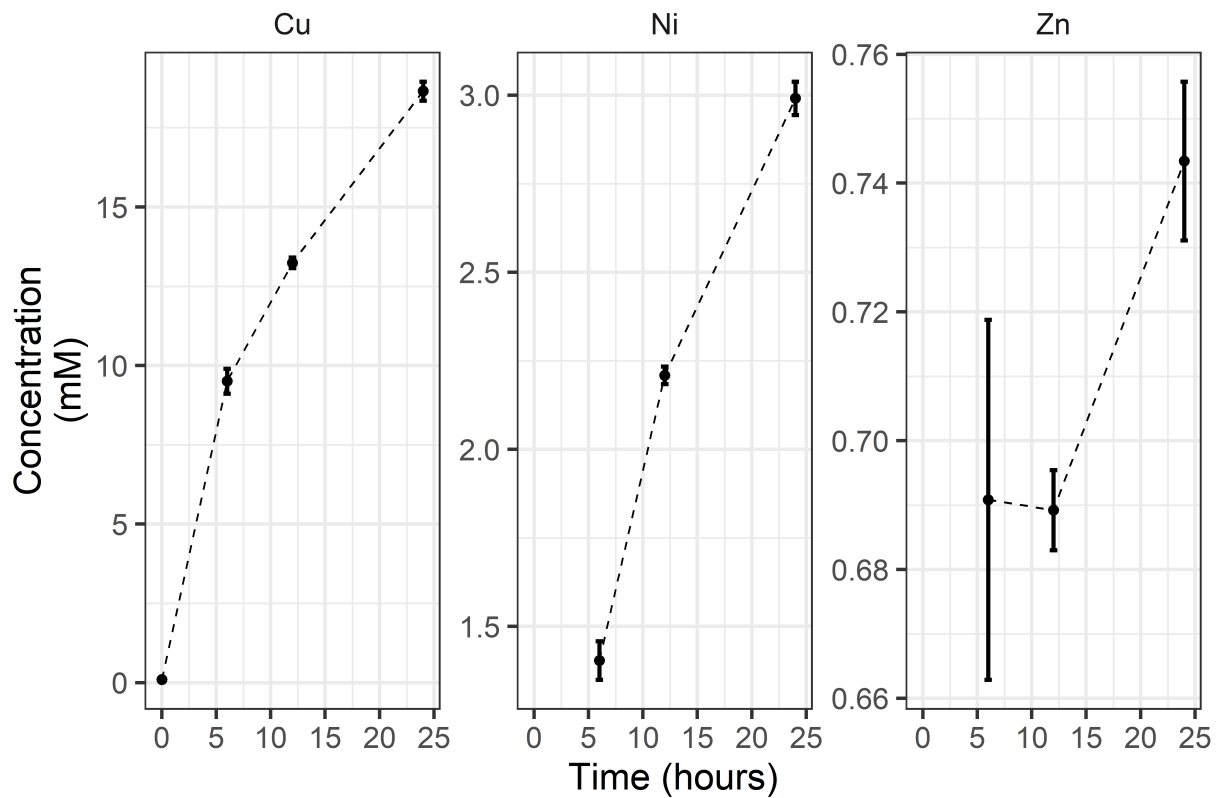
Figure 6.3: Time course of ions leaching from copper-alloy into Sf900 III media as determined by ICP-OES. Only the three metal ions that originate from the alloy are shown here. Each point represents the mean of two biological replicates. Error bars represent the range of data.
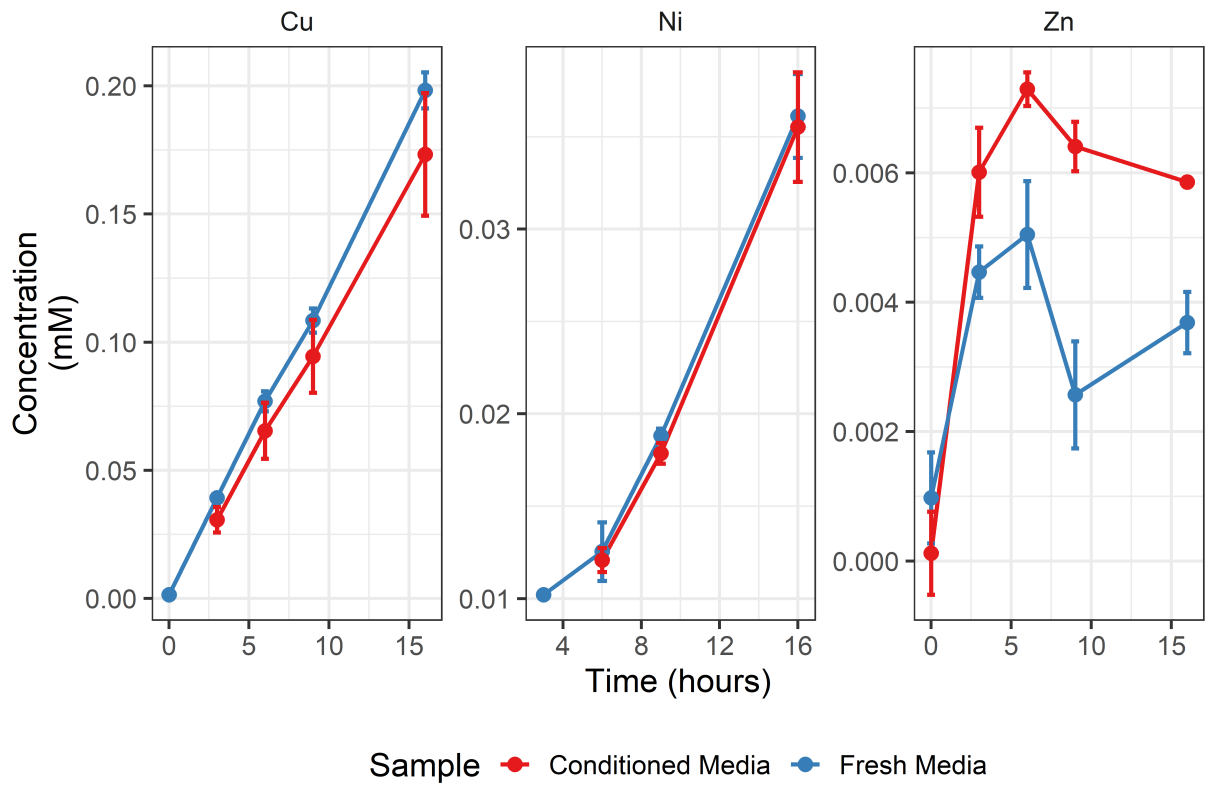
Figure 6.4: A time course of leachates from the copper alloy surface into conditioned (red) or fresh (blue) Sf900 III media. Only the three constituent ions from the alloy are presented. Each bar represents the average of two biological replicates with each of those having two technical replicates (n=4). Error bars represent the standard deviation. Lines are not the result of any regression, but help to visualize trends in the data.

6.3.3 Commercial Versus In-House Medium on Leaching

Next, the leaching ability of Sf900 III was compared to an in-house insect cell growth media, ALIM3 (Figure 6.5). Copper and nickel appear to be leached in higher concentrations for ALIM3, but Sf900 III leaches more zinc. Again, due to the low concentrations that zinc leaches, it is difficult to determine the validity of this result.
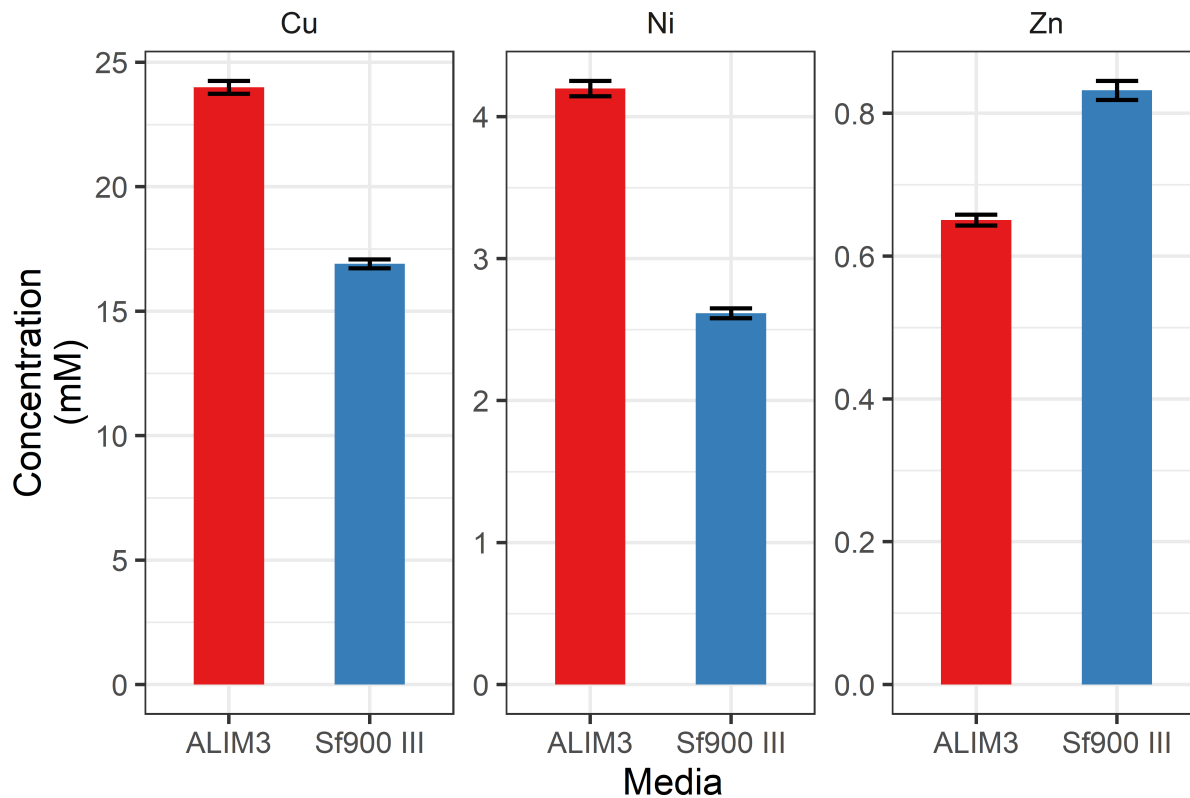
Figure 6.5: ICP-OES results of copper alloy leachates after 24 hours from two different cell growth medias is shown here, in-house growth media ALIM3 (red bars) and commercial Sf900 III (blue bars). Only the three constituent ions from the alloy are presented. Each bar represents the average of two biological replicates (n=2). Error bars represent the range of data.

6.3.4 Osmolarity of Salt Solutions

To investigate if osmolarity was the cause of leaching, $CaCl_2$ and NaCl salt solutions were used. $CaCl_2$ was tested at 50, 100, and 200 mM, which corresponds to 150, 300, and 600 mOsm/L. NaCl was tested at 100 and 200 mM, which corresponds to 200 and 400 mOsm/L. Results are presented in Figure 6.6, and indicate that osmolarity is not a driving factor for metal ion leaching.
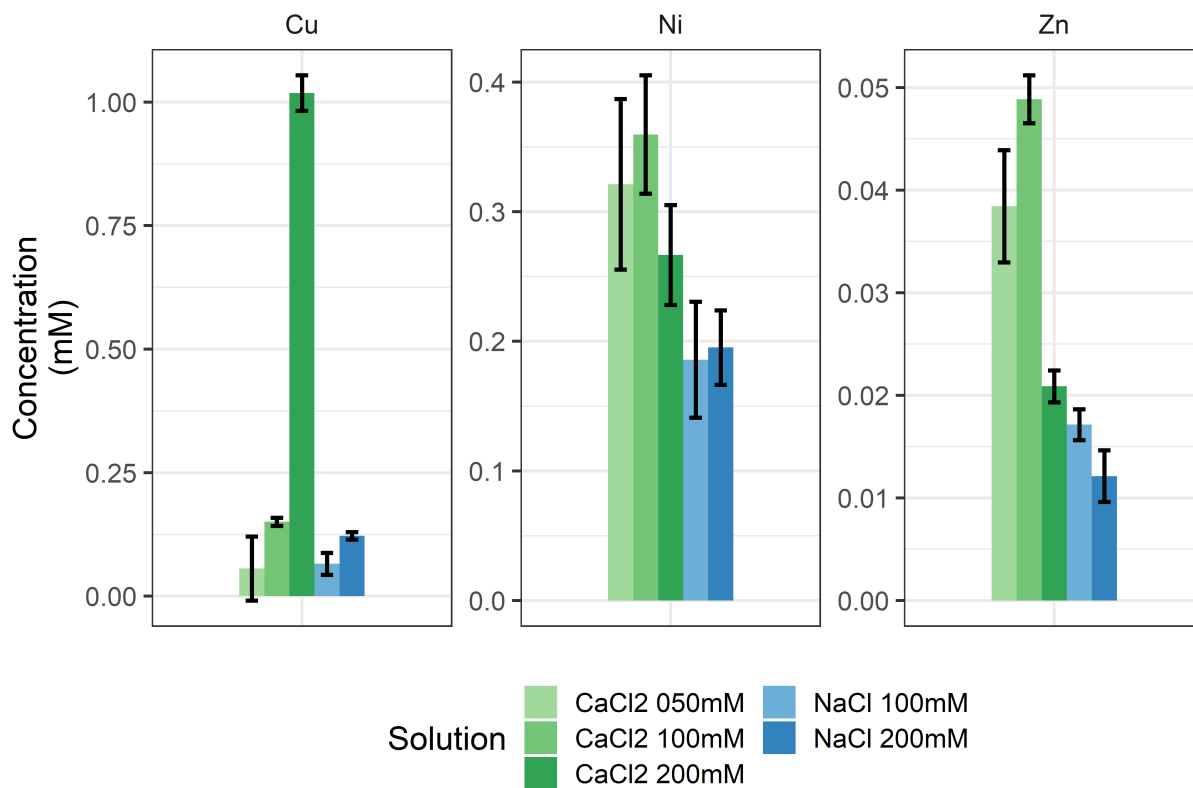
Figure 6.6: Leaching of metal ions from the copper alloy surface after 24 hours was investigated using NaCl (blue) and CaCl$_2$ salt solutions. Concentrations are outlined in the legend below the figure, and only the three metals making the alloy are presented. Bars represent two technical replicates (n=2). Error bars represent the range of data.

6.3.5   Effect of Protein on Leaching

To determine if proteins are encouraging leaching, the old EPA soiling agent (Chapter 4) was tested on the copper alloy. Old EPA soiling agent is comprised of PBS (phosphate buffered saline; a typical biological buffer), FBS (fetal bovine serum; a cell culture additive rich in proteins), and TX-100 (Triton X-100; a nonionic surfactant). The leaching of copper, nickel, and zinc by each of these components was tested in an additive manner and compared to conditioned media (Figure 6.7).

Results indicate that conditioned media leaches more copper than PBS, PBS & FBS, or PBS & FBS & TX-100. Figure 6.7 further indicates that osmolarity is not the primary cause of leaching. Sf900 III has an approximate osmolarity of 360mOsm/L, much lower than that for the 600 mOsm/L $CaCl_2$ solution tested.
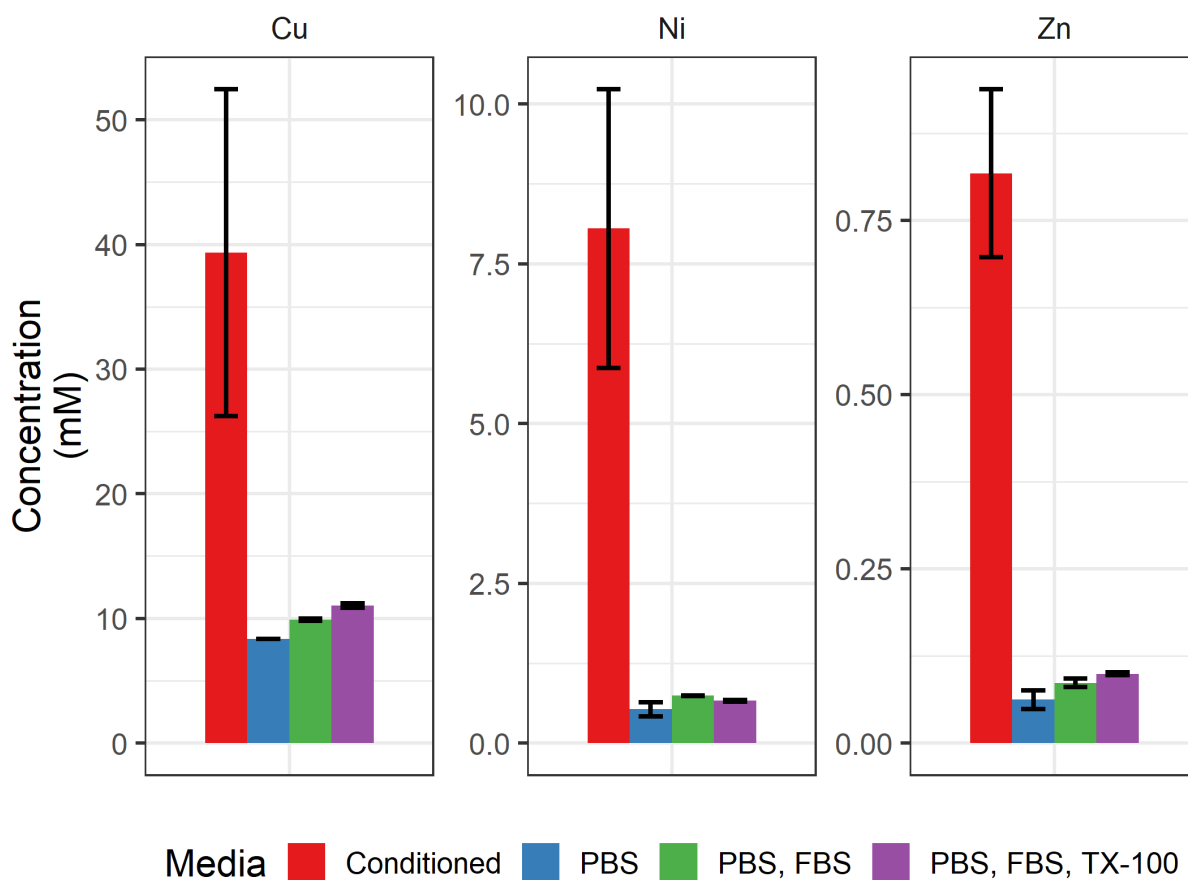
Figure 6.7: Leaching by the copper alloy after 24 hours into conditioned media (red) is compared to a common saline buffer used in biological work (PBS; blue), PBS with protein (PBS, FBS; green), and PBS with protein and a detergent (PBS, FBS, TX-100; purple). Only the three constituent ions from the alloy are presented. Each bar represents the average of two technical replicates (n=2). Error bars represent the range of data.

6.4   Discussion and Conclusions

Generally, chloride [Nam et al., 2016] and sulfide [Xie et al., 2007] are known corrosive agents to metal surfaces. Chlorine in the salt from sweaty hands has been known to corrode unprotected metal sheets handled by metal workers since the 1920's [Burton et al., 1976]. However, nickel silvers, like the alloy used in these experiments, are known to have good chlorine corrosion resistance and are used in seawater applications [Cohen, 2005]. It is known that biocorrosion is a problem for copper piping [Vargas et al., 2017] and proteins have been shown to corrode copper [Clark and Williams, 1982]. Curiously, amino acids, the building blocks of proteins, are effective corrosion inhibitors [Hamadi et al., 2018].

Organic soils are rich in biomolecules that could potentially be the cause of metal ion leaching from the surface. Unfiltered stock with heavy soiling appeared to prevent leaching of metal ions. This builds on work performed by Molteni et al. [2010], where they identified Tris-Cl (a known solid metal dissoluting buffer) and growth medium as able to leach significant concentrations of copper from a 99.9% copper surface (42 and 14 mM respectively) but water and NaP_i buffer were unable to leach appreciable concentrations (55 and 57 μM). Further results found that copper leachate concentrations in heavy soiling was higher than the minimum to inactivate virus (Chapter 5), but was unable to inactivate virus. These results indicate that biomolecules in heavy soiling could have quenched the virucidal metal ions.

At lower levels of soiling, both conditioned and fresh media leach metal ions at a comparable rate, and this leaching is comparable to fresh in-house growth medium. A common component of growth media is amino acids and is a potential source of surface

corrosion. However, several amino acids have been used as corrosion inhibitors [Amin and Khaled, 2010]. This includes sulfur-containing amino acids [Li et al., 2017] which could be considered corrosive due to the copper sensitivity to sulfides [Xie et al., 2007]. Therefore, it is unlikely that the leaching seen from conditioned media is due to the amino acids in growth media. It is possible that macromolecules with close functionality to ammonium are causing leaching, as ammonium is a strong corroder of nickel silvers [Cohen, 2005]. The in-house growth media, ALIM-3, can be used to help elucidate which of these macromolecules is causing corrosion as all the components are known.

The mode of corrosion from growth media is likely uniform surface corrosion, as evidenced by uniform surface discoloration after exposures. This discoloration persisted even after light scrubbing and small volume exposures (Chapter 5). This corrosion is likely caused by electrochemical interactions with the media [Cohen, 2005].

Chlorine is a known corrosive agent [Nam et al., 2016], and was also investigated using the salt solutions. However, the chlorine content in media is not the sole corrosive agent as the concentrations tested were greater and did not induce significant leaching. Another common corroding condition is high or low pH [Cohen, 2005]. All media tested was of roughly neutral pH and so this factor was not considered.

Overall, it was determined that heavy organic soiling can decrease the leaching of metals form the copper alloy. These concentrations, however, are still above the minimum amount required to inactivate virus but were unable to do so. Moderate soiling did not prevent surface leaching, with conditioned and fresh media leaching metals at similar rates over 24 hours. An in-house growth medium with known components showed comparable leaching

to commercial medium, and so can potentially be used to elucidate the corrosive agent. Osmolarity, chlorine, and protein did not show any appreciable leaching of metals.

Chapter 7

Conclusions and Recommendations

Unfortunately, testing is sporadic and inconsistent on the virucidal properties of self-sterilizing surfaces designed to reduce HAIs. There is a need to develop methods so that factors known to generally interfere with biocidal properties can be accurately investigated and accounted for. Furthermore, only BSL-2 viruses have been used to investigate virucidal activity, but they are not practical for method development. This thesis investigated the virucidal properties of a copper-alloy and factors that may mask the virucidal properties of a copper alloy when used in healthcare institutions. A BSL-1 enveloped virus, AcMNPV, was used to aid in the development of a method that accurately represents healthcare use conditions. The enveloped nature could allow this virus to be susceptible to the same enveloped-based inactivation mechanism for bacteria seen in literature. This potential susceptibility could allow easier to assessment virucidal activity by the surface. The key factors that interfered in the alloy's ability to inactivate the virus are soiling conditions, physical state of the virus (wet or dry), and leaching of metal ions as shown in Chapter

4. Additionally, copper and nickel were identified as the alloy constituents responsible for virus inactivation as shown in Chapter 5.

7.1 Virus Inactivation

The effects of virus droplet under high humidity (wet virus) inactivation were studied in the most depth. Wet virus is protected by organic soiling when exposed to the copper-alloy, and the extent of the protection is proportional to the soil load, as measured by cell debris and conditioned media. No soiling (fresh media) reached a > 4-log reduction after 7 hours, light soiling (conditioned media or EPA soiling) reached a > 4-log reduction after 11 hours, and heavy soiling (sonicated cell debris and conditioned media) had no significant reduction after 24 hours (<1-log). Using ICP-OES, results indicated that metal ion leaching kinetics were not significantly different between no soil and a light soil load (p>0.05). It was also demonstrated that the surface lost virucidal activity on a second exposure to soiled samples (<1-log reduction on second exposure). Conversely, protective effects from soiling were not seen for dried virus (retained >3-log reduction). Metal ion leaching was significantly reduced by soiling in dried virus, and the surface was able to repeatedly inactivate virus (>3-log reduction). The difference between wet and dry conditions are unexpected and warrant further investigation.

A few areas in which this work can be continued is testing inactivation of non-enveloped viruses, investigating interfering factors (such as soiling and humidity), and characterization of the alloy surface changes after exposure to samples. Non-enveloped viruses are an important area to explore as they are generally less susceptible to environmental factors,

and so may require longer exposure times and resistance to complete inactivation in dry conditions. It is conceivable that soiling will protect non-enveloped viruses from the greater concentration of metal ions required to inactivate them. Once the virus is dry, this mode of inactivation likely ceases. Using different soiling solutions would be a good way to elucidate the agent causing the levels of leaching seen in the cell growth media. It may be interesting to also investigate humidity, non-enveloped viruses, and other soiling solutions. Humidity is an interesting factor to explore because it may impact the rate of drying, leading to higher rates of metal ion leaching as the corrosive agent requires moisture to leach. The change in surface chemistry may help resolve the difference between wet and dried virus. Using energy-dispersive X-ray spectroscopy (EDS), the elemental surface composition can be studied to help elucidate differences of the alloy after repeated exposures to the wet or dried virus droplet.

7.2 Virucidal Properties of Metal Ions

Divalent metal ion solutions were used to determine which alloy components resulted in virus inactivation. A factorial design testing copper, nickel, and zinc found that a copper and nickel ions have a strong effect at reducing the virus ($Pr<0.001$), but zinc does not ($Pr>1$). This is likely because zinc is leached in concentrations at which the effect is masked. The interaction between copper and nickel was found to be significant, but the observed reduction was not greater than copper alone. It was also shown that, for conditioned media, there is a minimum copper ion concentration required for virus inactivation (>1-log reduction for >2 mM copper in conditioned media).

The potential for ion testing to rapidly design virucidal alloys should be explored. Further, these studies can be used to evaluate the synergistic combinations of metals to establish an optimally virucidal surface. The ion tests can also be used to further study the effect of soiling on virucidal properties to identify what organic soils have the strongest quenching of virucidal and biocidal metal ions. This information can be used to formulate cleaning agents to specifically remove the identified component of organic soils.

7.3   Leaching Mechanism

We were unable to establish the driving force behind metal ion leaching from the alloy. However, results indicated that sodium and calcium salt solutions of varying osmolarity did not a leach concentration of metal ions comparable to growth media. The tested salts also indicated that chloride is also not a significant factor. Using an in-house growth media, the leaching seen by commercial media was replicated (24 mM Cu and 17 mM Cu, respectively). It is conceivable that this media can be used to identify the factors involved in leaching.

Future work to investigate the leaching mechanism should be performed using the in-house growth medium. Since this medium is defined, each component can be tested using an experimental design to identify the key factor(s).

7.4   Recommendations

The following recommendations are based on the results of this thesis:

- Because we have shown the copper-nickel-zinc alloy to have virucidal properties, clinically relevant viruses should be investigated

- Because the dried virus droplet was able to repeatably inactivate virus and wet virus droplets were not, organic material deposition on the surface under these conditions should be investigated

- Because the ion solutions were shown to have virucidal properties similar to the alloy surface, ion solutions can be used to optimize copper, nickel, and zinc concentrations to rapidly test for virucidal activity

- Because the in-house growth medium is defined and was able to leach metal ions, the components of the media should be used to elucidate leaching agent(s)

7.5   Concluding Remarks

Broadly speaking, current literature does not test virucidal properties of self-sterilizing surfaces with enough caution. Many purification protocols have a rigorous procedure, whereby any potential soiling agent is removed and virus is resuspended into saline buffer. These procedures tend to overestimate the surface's performance. Alternatively, other procedures are not stringent enough and likely contain high levels of organic soiling generated from the virus amplification procedure, negating the effects of viable surfaces. In either case, the soiling level is unlikely to represent the intended conditions the surfaces has been designed for.

This work has shown that soiling can strongly reduce the ability of surfaces to inactivate virus. Further, the dynamic effect of the soiling was determined by testing various soiling formulations and concentrations of natural soiling generated by infections. Other parameters that decreased the surface's virucidal properties include the metal composition of the surface and showed copper was responsible for the virucidal properties of the alloy. Properties of the matrix itself are involved in the leaching of the surface, but a sole corrosive agent could not be determined.

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APPENDICES

Appendix A

Supplementary Tables for Literature Review Data

Table A.1: Table Illustrating Variability in Virucidal Disinfectant Tests

Condition	Reference																		
Virus	[Buhr et al., 2014]	[Cook et al., 2016]	[Cook et al., 2015]	[Cromeans et al., 2014]	[Dagher et al., 2017]	[Franz et al., 2018]	[Gallandat and Lantagne, 2017]	[Gallandat et al., 2017]	[Goyal et al., 2014]	[Julian et al., 2014]	[Manuel et al., 2017]	[Moorman et al., 2017]	[Nayak et al., 2018]	[Rabenau et al., 2014]	[Smither et al., 2018]	[Vimont et al., 2014]	[Zeitler and Rapp, 2014]	[Zonta et al., 2016a]	[Zonta et al., 2016b]
AiV				x															
Avian Influenza									x										
BPV														x					
CHIKV						x													
CPV														x					
EBOV-KIK		x																	
EBOV-MAK		x	x												x				
EBOV-MAY		x																	
FCV				x					x			x						x	x
HuAdV-1									x										
HuAdV-5														x					
HuNoV GI.5				x															
HuNoV GI.6											x								
HuNoV GII.13				x															
HuNoV GII.4											x	x							
InfA																	x		
M13							x												
MNM														x					
MNV-1									x							x			
MNV-1 CW1																		x	x
MNV-1 CW3				x															
MS2	x								x	x									
MVM																			

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	Condition	[Buhr et al., 2014]	[Cook et al., 2016]	[Cook et al., 2015]	[Cromeans et al., 2014]	[Dagher et al., 2017]	[Franz et al., 2018]	[Gallandat and Lantagne, 2017]	[Gallandat et al., 2017]	[Goyal et al., 2014]	[Julian et al., 2014]	[Manuel et al., 2017]	[Moorman et al., 2017]	[Nayak et al., 2018]	[Rabenau et al., 2014]	[Smither et al., 2018]	[Vimont et al., 2014]	[Zeitler and Rapp, 2014]	[Zonta et al., 2016a]	[Zonta et al., 2016b]
	PEC				x															
	PEDV					x														
	Phi6								x	x										
	PPV															x				
	PR772								x											
	SwIV										x									
	TGEV										x									
	TuV				x								x							
	VACV																		x	
Temperature	-	x																		
	20-22C														x					
	21.5 C			x																
	22 C																x			
	27 C		x																	
	Not Reported				x					x	x	x	x	x	x	x		x	x	x
	Room Temperature					x	x	x								x				
Humidity	-	x																		
	~10%RH							x												
	30% RH			x																
	Ambient					x										x				
	Not Reported		x		x		x		x	x	x	x	x	x	x		x	x	x	x
Soil Type	0.3%BSA					x									x			x	x	x
	80 mg/L humic acid									x										
	90% human whole blood															x				
	90% media															x				

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Condition	[Buhr et al., 2014]	[Cook et al., 2016]	[Cook et al., 2015]	[Cromeans et al., 2014]	[Dagher et al., 2017]	[Franz et al., 2018]	[Gallandat and Lantagne, 2017]	[Gallandat et al., 2017]	[Goyal et al., 2014]	[Julian et al., 2014]	[Manuel et al., 2017]	[Moorman et al., 2017]	[Nayak et al., 2018]	[Rabenau et al., 2014]	[Smither et al., 2018]	[Vimont et al., 2014]	[Zeitler and Rapp, 2014]	[Zonta et al., 2016a]	[Zonta et al., 2016b]
90% rat whole blood															x				
ASTM		x	x				x	x			x	x				x			
Med	x			x					x										
None													x						
Not Reported					x														
1-propanol														x					
2-propanol														x					
Alcocid (alcohol, biguanide)																		x	
Bacillol AF (commercial multi-alcohol)					x														
Bioxy (commecial PAA)					x														
CAP													x						
ECO										x									
EtOH		x	x											x				x	
Fresh NaOCl									x										
GDA														x					
Glucoprotamin																	x		
Hydrogen Peroxide Vapour									x										x
HTH									x										
Kenocept G (alcohol, biguanide)																		x	
Kenochlore (NaOCl)																		x	

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Condition	[Buhr et al., 2014]	[Cook et al., 2016]	[Cook et al., 2015]	[Cromeans et al., 2014]	[Dagher et al., 2017]	[Franz et al., 2018]	[Gallandat and Lantagne, 2017]	[Gallandat et al., 2017]	[Goyal et al., 2014]	[Julian et al., 2014]	[Manuel et al., 2017]	[Moorman et al., 2017]	[Nayak et al., 2018]	[Rabenau et al., 2014]	[Smither et al., 2018]	[Vimont et al., 2014]	[Zeitler and Rapp, 2014]	[Zonta et al., 2016a]	[Zonta et al., 2016b]
Kenocid 210 (QAC, alcohol, aldehyde)																		x	
Kenocid 2100-2 (PAA + H2O2)																		x	
NaDCC								x											
NaOCl		x	x	x				x	x		x				x				
NEW												x							
PAA														x	x	x			
Peform (oxidizing non-ionic surfactant)						x													
PES-Solid in DSS	x																		
PES-Solid in SDX-12	x																		
PES-Solid in water	x																		
PPA																x			
QAC						x													
SDC												x							
Virocid (QACs, alcohol, aldehyde)																		x	
Class																			
Alcohol		x	x											x				x	
Chlorine		x	x	x				x	x		x	x			x			x	
Other						x								x			x	x	
PAA	x				x									x	x	x			

Condition	Reference																		
	[Buhr et al., 2014]	[Cook et al., 2016]	[Cook et al., 2015]	[Cromeans et al., 2014]	[Dagher et al., 2017]	[Franz et al., 2018]	[Gallandat and Lantagne, 2017]	[Gallandat et al., 2017]	[Goyal et al., 2014]	[Julian et al., 2014]	[Manuel et al., 2017]	[Moorman et al., 2017]	[Nayak et al., 2018]	[Rabenau et al., 2014]	[Smither et al., 2018]	[Vimont et al., 2014]	[Zeitler and Rapp, 2014]	[Zonta et al., 2016a]	[Zonta et al., 2016b]
Plasma													x						
Silver											x								
Vapour									x										x

Table A.2: Table Illustrating Variability in Self-Sterilizing Virucidal Tests

	Condition	Reference					
		[Bleichert et al., 2014]	[Feuillolay et al., 2018]	[Manuel et al., 2015]	[Park et al., 2014]	[Warnes et al., 2015a]	[Warnes et al., 2015b]
Virus	HSV-1		x				
	HuCoV-229E					x	
	InfA		x				
	MNV + FCV				x		
	MNV-1						x
	MPXV	x					
	MS2				x		
	VACV	x					
	HuNoV GI.1				x		
	HuNoV GII.4			x	x		
	HuNoV GII.4 VLP			x			
Temperature	35 C		x				
	rt			x	x	x	
	22 C						x
	-	x					
Humidity	>90%RH		x				
	NR				x	x	x
	amb			x			
	-	x					
Soil Type	2%FBS in EMEM		x				
	NR	x				x	x
	None				x		
	media			x			
	-						
	0.125%BSA in EMEM		x				

Condition	Reference					
	[Bleichert et al., 2014]	[Feuillolay et al., 2018]	[Manuel et al., 2015]	[Park et al., 2014]	[Warnes et al., 2015a]	[Warnes et al., 2015b]
PP-metal_microsphere		x				
Copper (C11000)	x		x		x	x
Nickel (O2200)					x	
Zinc (Z13000)					x	
Copper-nickel (C75200)					x	
Bronze (C51000)			x		x	
Copper-nickel (C70600)			x		x	x
Copper-nickel (C72500)					x	x
Copper-nickel (C71000)					x	x
Copper-nickel (C71500)					x	x
Brass (C21000)					x	x
Brass (C22000)					x	
Brass (C23000)					x	x
Brass (C26000)			x		x	x
Brass (C28000)					x	x
Muntz metal (C28000)			x			
F-TiO2				x		

Appendix B

Supplementary Data and Analysis for Wet Virus Experiments

B.1 Model Parameters for Figure 4.4

Data was fit using the lm function and a second order polynomial in R. Subsequent polynomials were compared using a least-square means and a paired t-test. The result of this t-test was used to judge the significance of the difference between models.

Model for conditioned media is presented below and had an adjusted $R^2 = 0.9619$:

$$y = -0.9592 - 2.7196x - 0.5710x^2$$

Model for fresh media is presented below and had an adjusted $R^2 = 0.9799$:
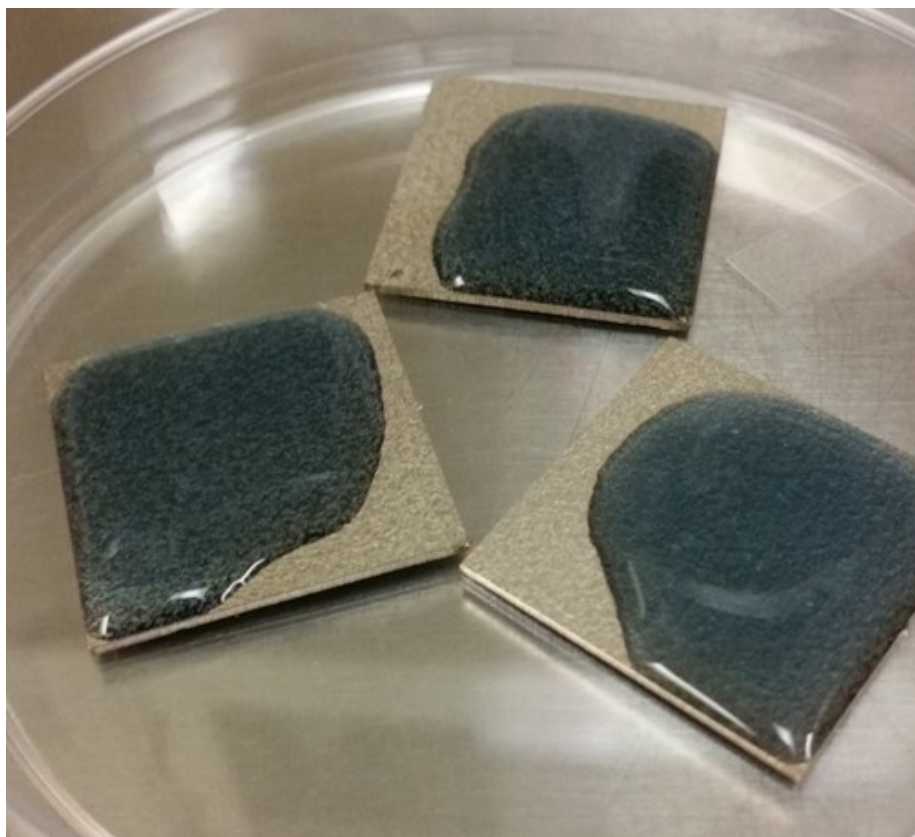
$$y = -1.0590 - 2.5929x - 0.4714x^2$$

Figure B.1: Insect cell media leaching metal ions after 24 hours of exposure. Media begins as a light tan shade and progresses to the deep blue pictured here. ICP results have indicated that the copper concentration is very high and is likely the reason for the colour change.
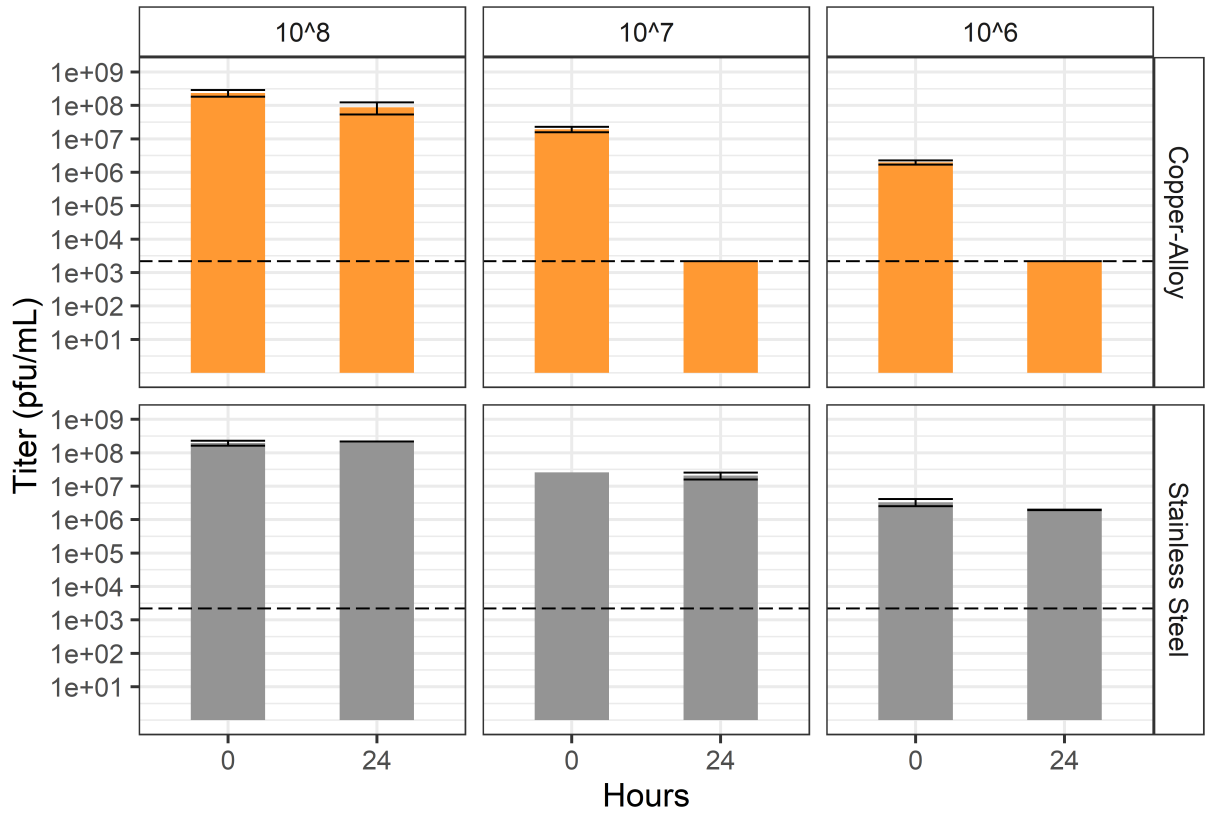
Figure B.2: Various concentrations of virus were exposed to copper-alloy (gold bars) and stainless steel (grey bars). Highest virus concentration (10^8 PFU/mL) was undiluted, while all other concentrations were diluted into fresh media. Dashed line shows the assay's detection limit, with bars at the line indicating concentrations were at or below the detectable threshold.

Appendix C

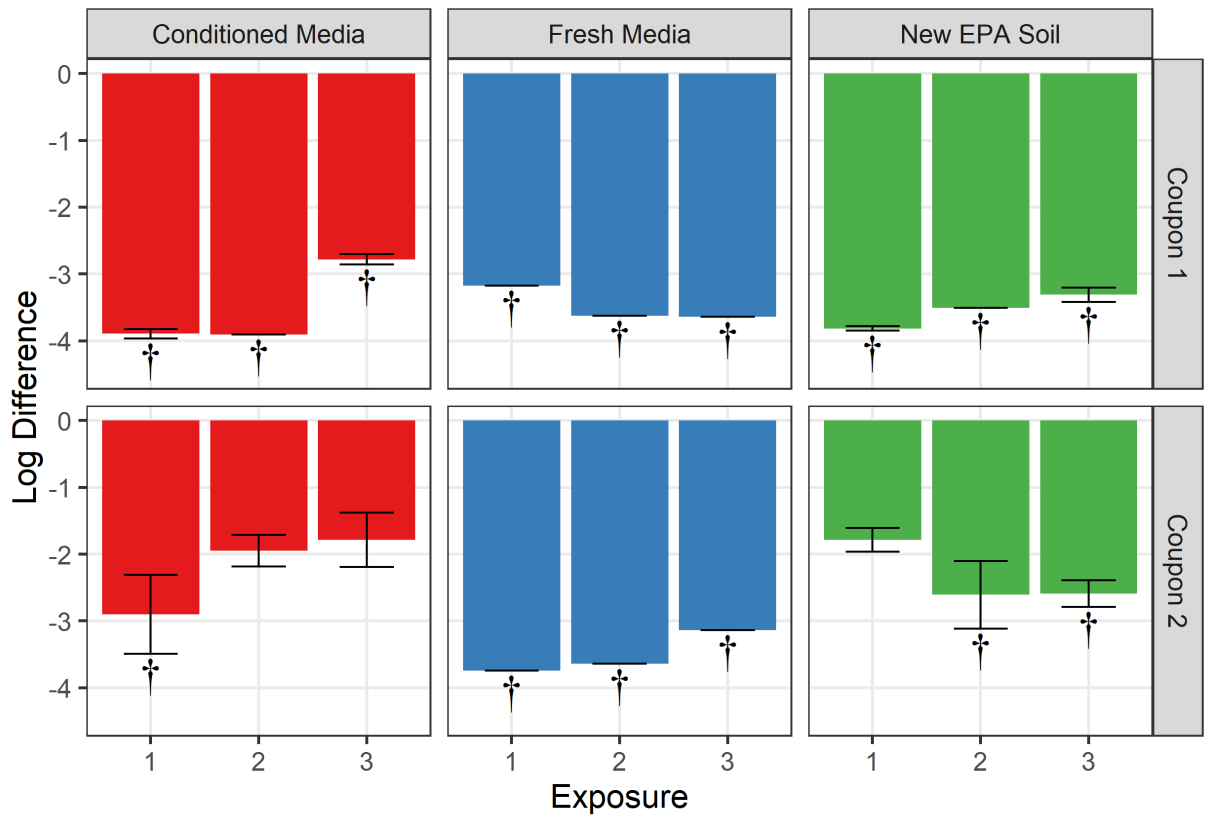# Supplementary Data and Analysis for Virus Drying Experiments

Figure C.1: Biological replicates of dried virus inactivation in various soiling conditions for multiple exposures.

Appendix D

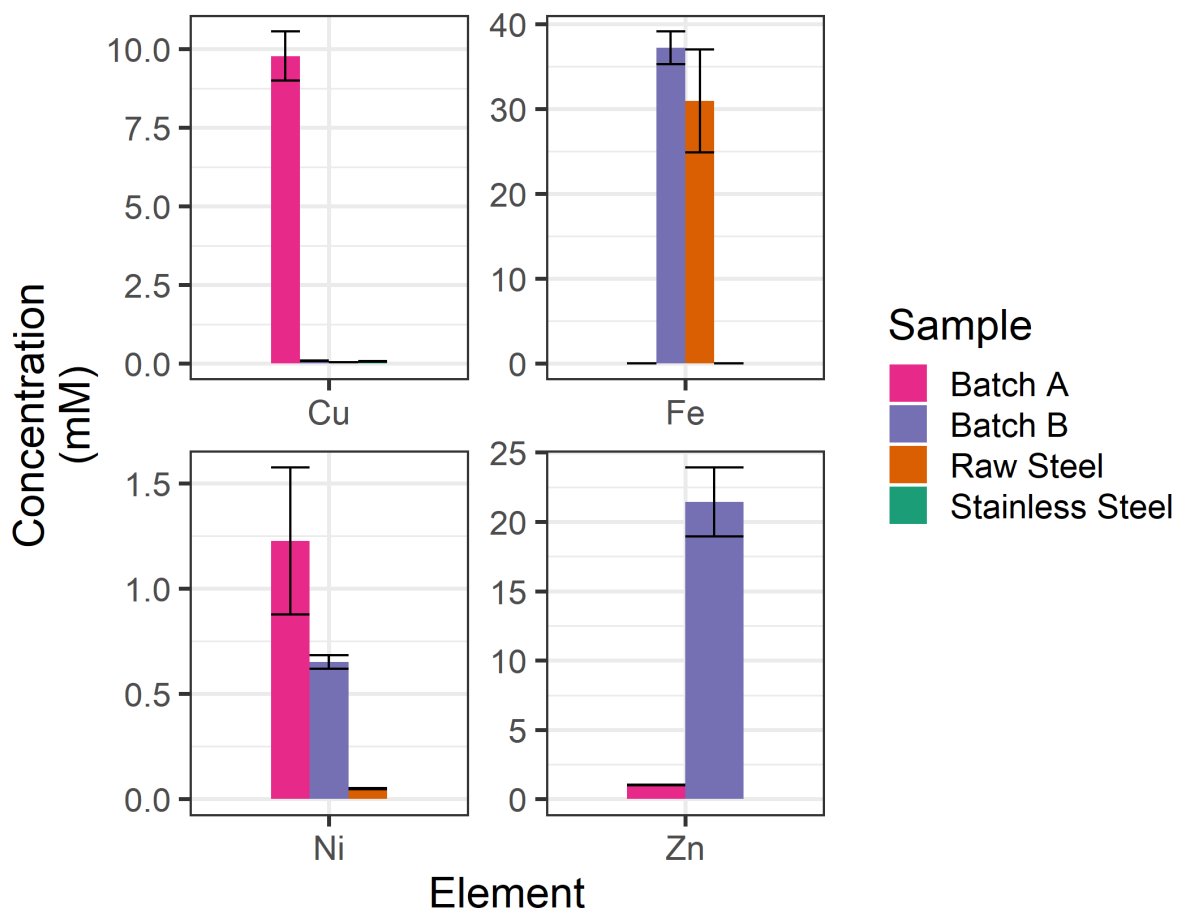# Supplementary Data and Analysis of Metal Ion Solution Data

Figure D.1: ICP-OES of tested surface from two separate batch preparations. Batch A and B represent two intermediate batches of copper alloy surface that were tested. Batch B utilized a thinner coating than Batch A causing the substrate material (raw steel) to leach through the coating.

Table D.1: ANOVA Table for DOE of Unfiltered Virus in Fig 5.2

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	Significance[1]
Cu	1	6.57E+16	6.57E+16	46.229	1.91E-05	***
Ni	1	2.88E+16	2.88E+16	20.243	0.000728	***
Zn	1	1.59E+15	1.59E+15	1.119	0.310965	
Cu:Ni	1	2.87E+16	2.87E+16	20.211	0.000732	***
Cu:Zn	1	1.60E+15	1.60E+15	1.123	0.310217	
Ni:Zn	1	4.41E+14	4.41E+14	0.31	0.587723	
Cu:Ni:Zn	1	4.33E+14	4.33E+14	0.305	0.591061	
Residuals	12	1.71E+16	1.42E+15			

[1] Signif. codes: Pr<0.001 "***" , Pr> 1 " "

Table D.2: ANOVA Table for DOE of Filtered Virus in Fig 5.2

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	Significance[1]
Cu	1	4.54E+16	4.54E+16	28.586	1.75E-04	***
Ni	1	3.35E+16	3.35E+16	21.078	0.00062	***
Zn	1	1.51E+12	1.51E+12	0.001	0.975884	
Cu:Ni	1	3.35E+16	3.35E+16	21.078	0.00062	***
Cu:Zn	1	1.51E+12	1.51E+12	0.001	0.975882	
Ni:Zn	1	1.51E+12	1.51E+12	0.001	0.975891	
Cu:Ni:Zn	1	1.51E+12	1.51E+12	0.001	0.975874	
Residuals	12	1.91E+16	1.59E+15			

[1] Signif. codes: Pr<0.001 "***" , Pr> 1 " "

Table D.3: Tukey's Test for Pairwise Difference of an ANOVA Comparing Processing for Each Combination Presented in Fig 5.2

Combination	Comparison	diff	lwr	upr	p.adj	Significance [1]
Cent	Filtered_Unfiltered	1.136651	0.565093	1.70821	1.04E-05	***
A	Filtered_Unfiltered	1.150538	0.342233	1.958844	0.001284	***
B	Filtered_Unfiltered	0.012989	-0.79532	0.821294	1	
C	Filtered_Unfiltered	1.529948	0.721643	2.338254	2.28E-05	***
D	Filtered_Unfiltered	0.707019	-0.10129	1.515324	0.13199	
E	Filtered_Unfiltered	1.789666	0.981361	2.597971	1.77E-06	***
F	Filtered_Unfiltered	-0.02615	-0.83445	0.782158	1	
G	Filtered_Unfiltered	1.360041	0.551736	2.168347	0.000134	***
H	Filtered_Unfiltered	0.009872	-0.79843	0.818178	1	

[1] Signif. codes: Pr<0.001 "***" , Pr> 1 " "