Mechanisms of virucidal action of alcohol and metallic ions against non-enveloped viruses

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by

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Summary

Studying the mechanism of action (MoA) of biocides against pathogenic microorganisms is crucial to understand their efficacy and limitations, and to develop more efficient microbicidal formulations. Combining alcohol and zinc has been reported to enhance microbicidal activity, but the reasons for such activity are unknown. This study focuses on the impact of combining ethanol and zinc salt at pH 10.5 against non-enveloped viruses.

The study is focused on three different aspects: i) virucidal activity screening of ethanol:zinc combinations against bacteriophages and human viruses; ii) impact of ethanol:zinc combinations on virus structure, particularly the viral capsid and nucleic acid, using Transmission Electron Microscopy (TEM); Atomic Force Microscopy (AFM) and agarose gel DNA electrophoresis and iii) chemical speciation and stability of ethanol:zinc combinations over time.

The combination of ethanol with zinc salt was found to be more effective against viruses than control formulations containing sole active ingredients and/or excipients only. Activity test of 40%(w/v) ethanol with 0.1% (w/v) zinc salt with excipients (RB-002 formulation) against F116 and adenovirus type 2 (AdV2) at 60 min contact time yielded 0.68 ± 0.02 and 5.26 ± 0.10 $\log_{10}$ reduction, respectively. In comparison, 0.1% (w/v) zinc salt only with excipient (RB-002G formulation) showed no virucidal activity against bacteriophage F116 (0.14 ± 0.02 $\log_{10}$ reduction) and AdV2 (0.80 ± 0.12 $\log_{10}$ reduction) in suspension. Differences between activities against bacteriophage MS2 and poliovirus type 1 were similar as the ones found between F116 and AdV2. Formulation containing 40%(w/v) ethanol with 0.1% (w/v) zinc salt produced a range of structural damage to F116 and attP AdV5 indicating possible capsid alteration. Effect of the combined formulation on viral capsid was confirmed with AFM with a possible decreased in virus capsid stiffness and significant virus capsid height reduction over 10 min contact time. F116 DNA damage was detected upon exposure to 40%(w/v) ethanol with 0.1% (w/v) zinc salt with excipients, but no damage was detected on AdV2 DNA through electrophoresis analysis.

The alcohol/zinc formulation system at pH 10.5 was shown to have promising virucidal activity against non-enveloped viruses at room temperature following an alteration of the viral capsid, and possible damage to the viral nucleic acid. This study also showed the limitations of using bacteriophage as surrogate for mammalian viruses.

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List of Abbreviations

1H-NMR	Proton Nuclear Magnetic Resonance
AdV2	adenovirus type 2
AdV5	adenovirus type 5
AFM	Atomic Force Microscopy
AFNOR	Association Française de Normalisation
ANOVA	analysis of variance
ASTM	American Society for Testing and Materials
attP AdV5	attP mutant adenovirus type 5
CEN	European Committee for Standardisation
CFU	Colony Forming Unit
DEFRA	Department of Environment, Food and Rural Affairs
DMEM	Dulbecco's Modified Eagle Medium (DMEM)
DNA	Deoxyribonucleic acid
dsDNA	double-stranded DNA
DVV	Deutsche Vereinigung zur Bekaempfung der Viruskrankheiten
EDAX	Energy dispersive Analysis of X-Ray
EDTA	Ethylenediaminetetraacetic acid
EPA	Environmental Protection Agency
EU BPR	The European Union Biocidal Product Regulation
FBS	Fetal Bovine Serum
FDA	U.S. Food and Drug Administration
FIFRA	Federal Insecticide, Fungicide, and Rodenticide Act
H0	null hypothesis
H1	alternative hypothesis
HAIs	Hospital-associated infections
HBS	HEPES Buffered Saline
HEK293	Human Embryonic Kidney cell 293
HeLa	Human cervical epithelioid carcinoma
HIV	Human Immunodeficiency Virus
ICP-MS	Inductively coupled plasma mass spectrometry
LC/MS	Liquid Chromatography - Mass Spectrometry
MEM	Minimum Essential Medium
MoA	Mechanism of Action
MOI	Multiplicity of infection

NMR	Nuclear Magnetic Resonance
NTA	Nitrilotriacetic acid
PBS	Phosphate Buffered Saline
PFU	Plaque Forming Unit
Pv1	poliovirus type 1 (LSc-1ab)
QACs	Quaternary Ammonium Compounds
RB	Reckitt Benckiser
REACH	Regulation for Registration, Evaluation, Authorisation and Restriction of Chemicals
RNA	Ribonucleic acid
ROS	Reactive Oxygen Species
SAXS	Small Angle X-Ray Scattering Spectroscopy
ssDNA	single-stranded DNA
TAE	Tris-acetate-EDTA
$TCID_{50}$	Tissue culture infectious dose
TEM	Transmission Electron Microscopy
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
VOCs	Volatile Organic Compounds
WHO	World Health Organization

1.Introduction

1.1 Biocide overview

1.1.1 Historical background and current state of biocidal products

The application of biocides as disinfectants, sterilants and preservatives go back to ancient times. Back then, the use of biocides was mainly based on empiric practices and a developed understanding on how it worked was non-existent. For instance, sulfur burned to sulfur oxide was used as a disinfectant for house fumigation approximately 800 BC (Price, 2001). Other illustrative examples include use of metal-based vessels (mainly copper and silver) as well as salts and spices to preserve fish and meat (Block, 2001; Patel, 2015). Louis Pasteur's achievements towards the creation of microbiology scientific field and Robert Koch's associations of diseases to microorganisms triggered a rapid development in the understanding of microbial diseases, preservation of food and disinfection practices (Block, 2001; Blevins and Bronze, 2010). At the end of nineteenth century, the use of iodine for wound disinfection, chlorine water in obstetrics and phenol (carbolic acid) for wound dressing and antiseptic surgery was reported (Russell, 2002). In the beginning of the twentieth century great advances in chemistry led to the introduction of novel biocides such as novel chlorine-releasing agents and quaternary ammonium compounds (QACs). Several agents introduced since 1945 including biguanides, cationic/anionic/non-ionic surfactants, bisphenols, aldehydes, iodine-releasing agents and peracetic acid are still in use nowadays (Ortega Morente et al., 2013). In recent years, a deeper understanding of disinfection practices and hygiene concepts as well as emergence of resistance among target organisms have been leading to a stronger need for biocide research and development (Cerf et al., 2010; Fraise, 2013; Jutkina et al., 2018). More focus has been given to agent chemical properties, mechanisms of action and synergy through combination of agents rather than discovery of novel active agents. Additionally, high costs associated to the registration of a novel active substance under REACH process in the European Union market and toxicology and environmental impact research costs are one of the main causes the number of available biocides is decreasing nowadays even though the need for biocides in the

market is increasing (Bruns et al., 2005; Ferrario and Rabbit, 2012; Gustavsson et al., 2017).

1.1.2 Biocide definition and market data

Biocidal products are chemical products based on inorganic or synthetic organic molecules (biocides) used for hampering spread and growth of harmful organisms and for preservation of materials from microbiological degradation (Chapman, 2003). In contrast to antibiotics, biocides employed to destroy bacteria (e.g. antiseptics and disinfectants) usually present a broad spectrum of action and possess several targets within the microbial cell (Maillard, 2002). Antibiotics tend to affect a specific microbial cell physiologic process, whereas biocides as disinfectants can interact with a wide range of cell components (e.g. cell wall, cytoplasmic membrane, DNA, RNA) trough physicochemical interactions or chemical reactions (McDonnell and Russel, 1999). Generally, biocide activity is highly influenced by many factors like chemical reactions of formulation components, presence of organic load, temperature, concentration, number of microorganisms, contact time, relative humidity, pH, type of surfaces and structure as well as genome content of the target organism (Russell, 2003).

Due to their broad spectrum of action, biocides are applied in various sectors ranging from disinfection of healthcare settings to industrial processes as wells as preservation of product quality (e.g. foods, beverage and pharmaceutical formulations). Despite the major source of exposure in homes due to use of wipes and cleaning products, it is known that biocides are employed as preservatives in many consumer products we have contact with on a daily basis (Wieck et al., 2017). Table 1.1 shows a list of common areas of application with respective active substances grouped in chemical groups:

Table 5.1 Main areas of application of biocides with respective most commonly applied active substances (adapted from von Barbro et al. 2003; SCENIHR, 2009 and Wieck et al., 2017).

Areas of application	Active substances (biocides)
Healthcare	Quaternary ammonium compounds (QACs); Biguanides; Phenols; Alcohols; Aldehydes
Household	QACs; Biguanides; Anionic agents; Isothiazolinones; Halogens
Food industry	QACs; Organic acids;
Pharmaceutical industry	QACs; Alcohols; Anionic agents; Organic acids; Metallic salts
Cosmetic industry	QACs; Aldehydes
Surface preservation	QACs; Phenols;
Veterinary hygiene	Anionic surfactant; Glutaraldehyde

Biocide addition to food and medicines is done mainly to avoid product degradation (e.g. malodours, changes of colour and organoleptics) (Paulus, 2004; Raczek, 2004; Condell et al., 2012) and spoilage, which could cause health risks to consumers, or affecting their perception of the product. Other examples of products in which biocides act as preservatives include paints, wood, textiles, coating films, leather and fuel (Kaehkoenen and Nordstroem, 2008; SCENHIR, 2009). In terms of disinfection, biocides are used for controlling of potential sources of contamination on inanimate and living surfaces. For example, in healthcare facilities biocides are used to decontaminate skin of patients and professionals and any instrument in contact with patients. It is assumed that proper use of biocide is essential for the success of programs associated to prevention and control of healthcare-associated infections (HAIs) (Maillard, 2005).

Sterilization and disinfection processes in industrial settings are also relevant as they are important to establish and maintain quality control of product batches (Holah, 2013). An example of application in industrial settings is that virus contamination of bioreactors associated to therapeutic drugs production line must be controlled; otherwise product availability and safety for consumer can be compromised (Hollark et al., 2010).

In terms of market value and potential, an industrial biocide market study carried out by Allied Market Research (2017) estimates that biocide total market was around US$ 6.5 billion in 2015 and it is forecasted to reach a market value of US$ 9.9 billion in 2022. The global market increase will be driven mainly by industrial and public water treatment, paints, coating and personal care products industries (Allied Market Research, 2017).

1.2 Viruses

1.2.1 Impact of viruses on humans

Viruses are one of the most common pathogens of humans, whose infections tend to cause substantial morbidity and mortality (Nwachuku and Gerba, 2006; Guidry et al. 2014). Despite major advances towards development of diagnostics and therapeutic methods, aspects associated to globalization like ease of world travel and interdependency of countries represent major challenges for infection contention. Emerging infectious diseases impose a major threat to human health and global instability (Dunn and Miller, 2014). There are three main factors associated to the emergence and re-emergence of infectious diseases: i) viral; ii) human and iii) ecological factors (Ka-Wai Hui, 2006). The first one is associated to natural genome recombination of viruses (Tumpey and Belser, 2009), while the latter ones are related to human population increase, social behaviour and climatic changes (Morens and Fauci, 2013). Furthermore, unhygienic conditions and unawareness of people regarding prevention methods and lack of proper healthcare infrastructure in some regions of the globe can contribute to pandemics emergence (Maillard et al., 2013; Zambon, 2014). Examples of such emerging diseases are the HIV/AIDS, pandemic (H1N1) Influenza A and most recently the Ebola virus disease in West Africa (Fauci and Morens, 2012; WHO, 2014), as well as Zika and Yellow Fever virus outbreaks in South America (Makhluf and Shresta, 2018). It is accounted that approximately 75% of emerging diseases are caused by RNA viruses, most of which are transmitted through mucosal and respiratory route (Zambon, 2014). Viral pandemics could have strong negative impacts on economy at global level and interfere strongly with normal life activities

leading to public fear and other negative outcomes like social unrest and high mortality (Morens and Fauci, 2013; Dunn and Miller, 2014).

1.2.2 Nosocomial infection and disinfection practices

Viruses can also be associated to nosocomial infections, which are acquired 48 hours after admission to hospital settings (Breathnach, 2013). Nosocomial infections can be endogenous, resulting from infectious agent within patient's body, or exogenous, transmitted from source within hospital (Aitken and Jeffries, 2001). This type of infection is still responsible for considerable illness, mortality and financial burden to hospitals, patients and society (Breathnach, 2013). To illustrate this, it is estimated that 5 to 10% of in-patients in British and Irish hospitals have a nosocomial infection, where higher prevalence is found in intensive care units and surgical wards. Infections acquired through invasive surgery present a mortality index as high as 20% (Huttunen et al., 2013). Financial costs in the UK in terms of increased hospital stay was estimated at £1000 million per year (Plowman et al., 2001). For instance, it was estimated that in the United States nosocomial infections increase the hospital care cost of a patient by US$10,375 and tends to increase length of stay by 3.30 days (Hassan et al., 2010). A summary of most common viruses related to nosocomial infections including its transmission route and control of spread guidelines is found in Table 1.2.

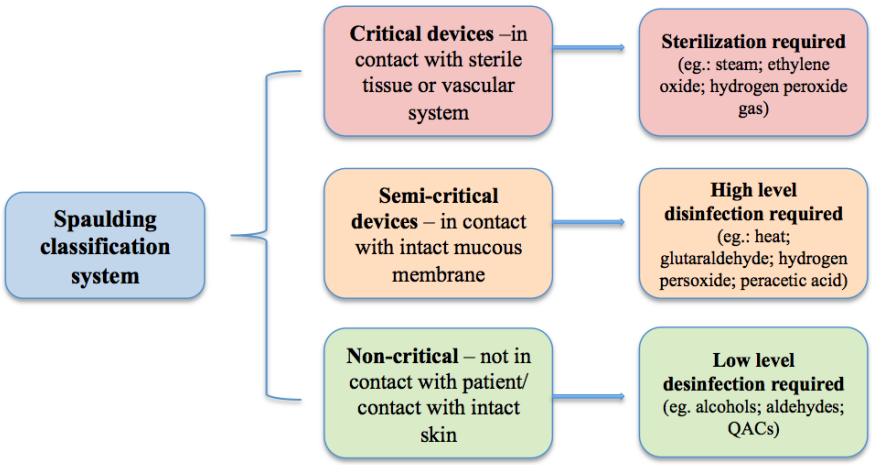
Table 1.6 Viral agents responsible for nosocomial infections including transmission route and control of spread in healthcare environment.

Route	Virus	Control of spread	Reference
Respiratory	Respiratory Syncytial Virus (RSV)	Rapid diagnosis; wearing of aprons and gloves; hand washing; source isolation; surface disinfection	Hall (1981); Aitken and Jeffries (2001)
	Influenza Viruses A and B	Similar procedures to control RSV; wearing of masks; source isolation in negative pressure room	Tablan et al. (1994)
	Parainfluenza viruses 1 to 3	Similar procedures to control RSV	Aitken and Jeffries (2001); Brady et al. (1990)
	Rhinovirus		
	Adenovirus	Emphasis on equipment and surface disinfection	
Fecal-oral	Rotavirus Enteroviruses	Patient isolation, rigorous hand washing with hand rubs	
Blood	Hepatitis B	Routine immunization of healthcare staff	Aitkens and Jeffries (2001)
	Human Immunodeficiency Virus Type 1	Infected health care workers excluded from exposure-prone procedures Good hygiene practices Antiretroviral drug for post-exposure prophylaxis	

Disinfection and good hygiene practices are essential to prevent and control infection spread because of the high infectivity associated to pathogenic viruses and the lack of effective vaccination against many viral infections. Disinfection practice is associated with reduction and inactivation of contaminating microorganisms. On the other hand, sterilisation involves the complete deactivation of viable organisms and pathogens from a surface or product. Disinfection can be carried out through thermal kill, physical removal or use of chemicals (biocides) (McDonnell and Burke, 2011). Biocides active against viruses are denominated virucides and their application is regarded in the literature as an effective measure to interrupt transmission of pathogenic viruses in fluid or dried on surfaces (Sakudo et al., 2010). Virucides have to be effective against a broad range of virus and their activity must be evaluated through standardised

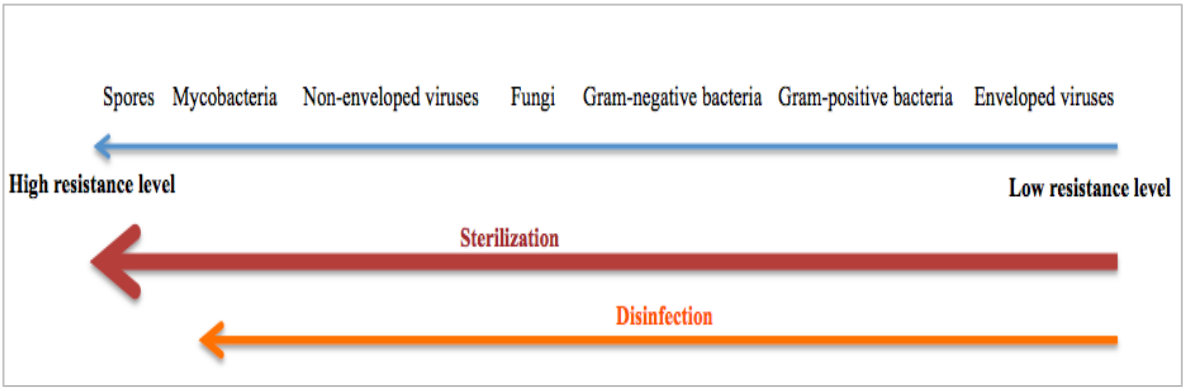
efficacy testing. Since virucidal activity is influenced by many parameters (e.g. pH, temperature, surface properties, etc.), standardised efficacy test procedures are complex. Hence, national recommendations for virucidal activity test are usually followed (Steinmann, 2001). Comparison of activities against other microorganisms like bacteria might not be valid as some viruses can be more resistant. In order to help healthcare workers to decide whether equipment sterilization is necessary and which disinfection procedure to be carried out, the Spaulding classification system was developed and has been used since 1957 (Spaulding, 1957). It defines minimum levels of disinfection in terms of infection risks associated to medical devices as shown in Figure 1.1.

Figure 3.1 The Spaulding classification system for surgical and medical devices (Spaulding, 1957; McDonnell and Burke, 2011).



According to the Spaulding system, enveloped viruses are efficiently eliminated by low to high disinfection and sterilisation practices, whereas non-enveloped viruses are only affected by high level disinfection and sterilisation practices. Figure 1.2 shows the revisited Spaulding classification, which includes decreasing level of resistance associated to different pathogens.

Figure 1.4: Revisited Spaulding classification system commonly used nowadays in healthcare settings including pathogens with distinct levels of resistance (adapted from McDonnell and Burke, 2011).



Furthermore, biocides must be safe for the users and be in compliance with environmental directives (European Commission, 2013). Despite being important for virus infection control, activity and mechanism of actions of biocides against viruses are not so well studied when compared to bacteria. The susceptibility of viruses to different biocides as well as potential damages is summarized in Table 1.3.

Table 1.7: List of common active substances and effect on different viruses reported in the literature.

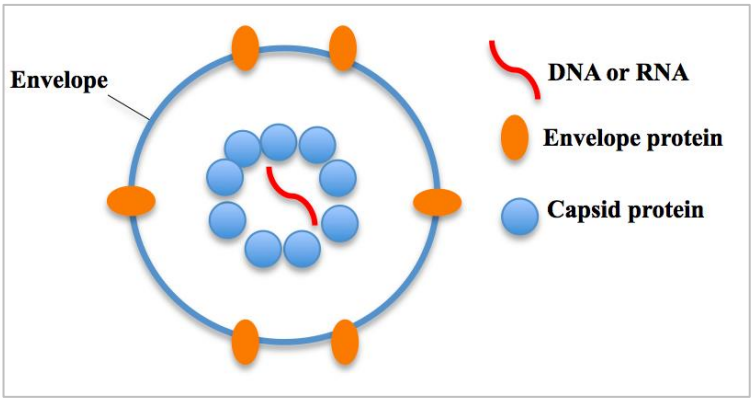
Active substances (biocides)	Damage on viruses	Affected viruses	References
Aldehydes (e.g. Glutaraldehyde)	Alteration of viral envelope; capsid markers and genome	Enveloped and large-enveloped viruses; small non-enveloped viruses (e.g. Human Rotaviruses; F116; poliovirus)	Springhorpe et al. (1986); Maillard et al. (1995)
Alcohols (e.g. ethanol and isopropanol)	Alteration of viral envelope; capsid and markers	Highly active against enveloped viruses (eg. Hepatitis B Virus); limited effect on non-enveloped viruses (e.g. Norovirus; F116; MS2)	Steinmann et al. (2013); Maillard et al. (1995)
Metallic salts (e.g.: Copper salts; Zinc salts and Silver salts)	Alteration of viral capsid and genome	Enveloped and non-enveloped viruses (e.g.HIV-1; R17 bacteriophage; poliovirus)	Sagripanti and Lightfoote (1996); Thurman et al. (1989)
Quaternary ammonium compounds (e.g. cetylpyridinium chloride; cetrimide)	Alteration of viral envelope and capsid	Highly active against enveloped viruses (e.g. coronavirus); limited efficacy on non-enveloped viruses (e.g. F116)	Rabenau et al. (2005); Maillard et al. (1995)
Phenols (e.g. essential oils)	Alteration of viral envelope and capsid	Highly active against enveloped viruses (e.g. Avian Influenza Virus); limited efficacy on non-enveloped virus (e.g. F116)	Wanaratana et al. (2010); Maillard et al. (1995)

1.2.3 Basic Viral Structure

In order to gather deeper knowledge interaction of biocides with viruses, it is important to define viral particles, structure and possible targets. In general terms, a virus is an obligate intracellular parasite that does not have any metabolic activity and cannot produce energy by itself (Knipe et al., 2006). By infecting a suitable host, the

viral genome is replicated with the help of host cellular machinery resulting in production of infectious progeny virions. Viral particles are relatively small (20-300 nm) compared to other organisms and by definition they contain RNA or DNA genome protected by a symmetric virus-coded protein coat (capsid). The coat proteins present helical or icosahedral symmetry, which form an open or closed structure, respectively (Sakudo et al., 2010). The nucleic acid-associated and capsid proteins along with the genome form the nucleocapsid, which is surrounded by a lipid bilayer for enveloped viruses (Gelderblom, 1996). The main function of the capsid is to protect the nucleic acid. The 2-dimensional basic structure of an enveloped virus (infectious unit) is depicted in Figure 1.3.

Figure 1.3: Basic mammalian enveloped virion structure (adapted from Sakudo et al. 2010).



Viruses are able to infect a wide range of organisms including bacteria, fungi, protozoa, animal and plant cells (Sakudo et al., 2010). Some viruses have hosts in more than one Kingdom (cross-Kingdom virus) and/or Phyla (cross-Phylum virus). For instance, reoviruses and bunyaviruses, possess animal and plant cells as hosts. The classification system based on the replication-expression strategies and the type of nucleic acid encapsulated was developed by Baltimore (1971) and revisited by Kooning (1991). It is shown in Table 1.4 as follows:

Table 1.8: Different classes of viruses according to their replication strategy (Baltimore, 1971; Kooning, 1991).

Class	Nucleic acid	Examples
I	Double-stranded DNA (dsDNA)	Adenoviruses, Poxvirus
II	Single-stranded DNA (ssDNA)	Parvoviruses
III	Double-stranded RNA (dsRNA)	Reoviruses
IV	Single-stranded positive sense RNA (+ssRNA)	Picornaviruses
V	Single-stranded negative sense RNA (-ssRNA)	Rhabdoviruses
VI	Reverse single-stranded positive sense RNA (+r ssRNA)	Retroviruses
VII	Reverse double-stranded DNA (r dsDNA)	Hepadnaviruses

The vast majority of viruses infecting prokaryotes like bacteria and archaea have dsDNA, +ssRNA and dsRNA, whereas eukaryotes host mostly RNA viruses and retroviruses. It was observed that diversity of RNA viruses as well as retroviruses infecting eukaryotes surpass the abundance of DNA viruses (Kooning, 2015). Throughout the present literature review, focus will be given to viruses responsible for infecting exclusively bacteria (bacteriophages) and mammalian cells (mammalian viruses) only.

1.2.3.1 Bacteriophages

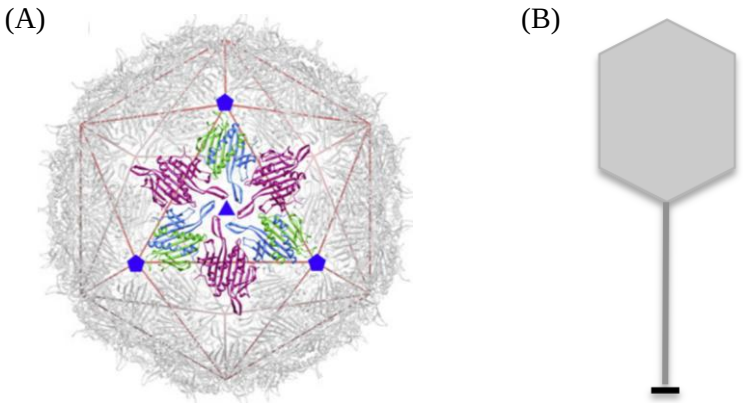
Bacteriophages are bacterial viruses, which are able to invade bacterial cells. They can be divided into two categories according to their infection cycle: lytic and lysogenic phages. Lytic phages are capable of lysing bacterial cell upon release. On the other hand, lysogenic phages integrate viral nucleic acid within the host chromosome/plasmid and persist indefinitely in the host cell. For lytic phages, the infection cycle starts with a highly specific attachment to host with subsequent transcription, replication and maturation of newly synthesized virions (Kutter et al., 2005; Pinto et al., 2012). Due to its high infectivity towards bacteria as well as ease of propagation, potential bacteriophages applications include alternative medical treatment for antibiotic resistant bacteria (Sulakvelidze et al., 2001; Hanlon, 2007; Beeton et al., 2015); biological control for plant pathogens in agriculture (Susianto et al., 2014); control of bacterial population during wastewater treatment (Withey et al., 2005) among

others. Moreover, some bacteriophages have been used as surrogates for mammalian viruses to validate virucidal efficacy testing (Jones et al., 1991; Shirasaki et al. 2009; Do-Kyun et al., 2017). Advantages of using bacteriophages as surrogates include ease of cultivation to high concentrations, easy detection by rapid and inexpensive assays and structural similarity to some mammalian viruses (Aranha-Creado and Brandwein, 1999; Hanlon, 2007; Magri et al., 2015).

1.2.3.1.1 MS2

The bacteriophage MS2 is 275 Å virus comprising a (+) single stranded RNA, the coat protein and the A protein. The MS2 capsid has an icosahedral shape formed by the arrangement of 90 coat protein homo-dimers (Caspar and Klug, 1962). The capsid also presents a single copy of the A protein, which is involved in RNA packing, host recognition, attachment and RNA transfer into the host (Stockley et al., 1994). MS2 genome encodes four proteins: coat protein, maturation/assembly protein, a replicase and a lyticase (Toropova et al., 2008). MS2 bacteriophage is widely employed as a convenient surrogate of non-enveloped human enteroviruses, like poliovirus type 1 and human norovirus in virucidal activity testing of chemical disinfectants (Macinga et al., 2008, Pinto et al., 2010). MS2 is depicted in Figure 1.4 A.

Figure 1.4: Distinct bacteriophage structures, where (A) represents bacteriophage MS2 (Toropova et al., 2001) and (B) bacteriophage F116 (adapted from Maillard et al., 1995).



1.2.3.1.2 F116

F116 is a pilus-specific *Pseudomonas* temperate phage comprising a linear genome characterized by 61.7 kb double stranded DNA. It has an icosahedral head with 70 nm in diameter and a 70-nm-long non-contractile rod-shaped tail (Byrne and Kropinski, 2005). F116 has been useful for studying the effects of biocides due to its large size, which allows investigation of possible structural damages through electron microscopy (Maillard et al., 1993; Maillard et al., 1995). Bacteriophage F116 is shown in Figure 1.4 B.

1.2.3.2 Mammalian viruses

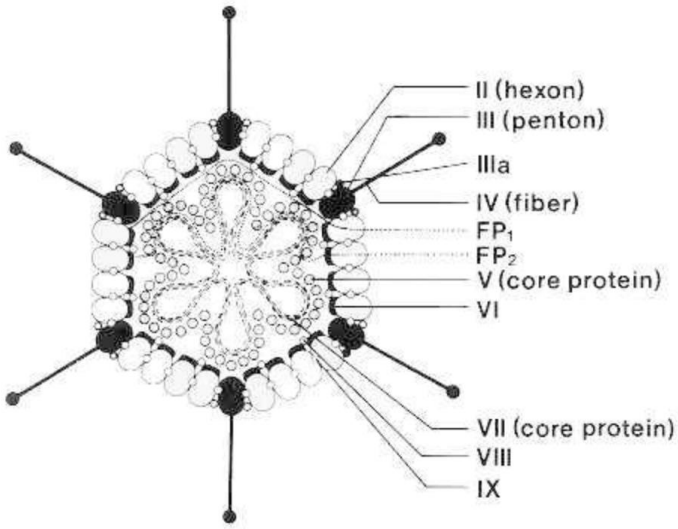
Mammalian viruses replicate by infecting eukaryotic cells, whose metabolic processes are organized in organelles, and membrane-trafficking is carried out through endocytosis as opposed to bacterial cells. Several differences can be found in terms of virus-host interaction and infectious cycle. For example, it has been reported that many RNA and some DNA viruses rely on the endosomal sorting complex required for transport (ESCRT) machinery for envelopment, maturation and egress from cells (Tandon et al., 2009). The host infection can present either cytopathic or non-cytopathic effects, where the first is associated to cell death and release of newly formed virions and the latter is associated to a persistent infection with generation of virions without cell death.

Regarding their resistance to chemical disinfectants, human viruses are divided into three groups according to the Klein-DeForest Scheme (DeForest, 1983) as follows: A) lipophilic viruses (enveloped viruses), B) viruses with intermediate solubility (large non-enveloped viruses) whose sensitivity is moderate and C) hydrophilic viruses (small non-enveloped viruses) whose sensitivity to chemical agents is usually low (Sauerbrei et al., 2004). According to this classification system, bacteriophages F116 and MS2 would belong to group B and C, respectively. Despite the classification still being valid and that non-enveloped viruses are generally more resistant, virus inactivation kinetics can still be different even for related viruses. For example, Sharp and Leong (1980) reported that poliovirus I (Brunhilde strain) was more resistant to free-chlorine than poliovirus I (Mahoney strain).

1.2.3.2.1 Adenovirus

Adenoviruses are non-enveloped viruses members of the virus family *Adenoviridae* with icosahedral capsid ranging from 70 to 100 nm in diameter. They present linear double-stranded DNA molecules with 26-45 kb in size, which makes them regarded to be medium-sized when compared with other DNA viruses (Davison et al., 2003). The characteristic icosahedral-shaped capsid architecture composed of four major proteins such as hexon (e.g. polypeptide II), penton (e.g. penton base), fiber (polypeptide IV) and minor proteins (polypetides IIIa and VI), are present in all adenoviruses identified (Doerfler, 1996; Rux and Burnett, 2004). The icosahedral shell is formed by a total of 240 trimeric hexons, 12 pentamers and 12 fibre trimers (Kay, 2011). Basic structure is depicted in Figure 1.5.

Figure 1.5: Structure of adenovirus virion with relative orientation of protein components including a double-stranded DNA in its core (adapted from Brown et al. (1975)).



More than 100 different adenovirus species have been identified in different vertebrates including mammals (mastadenoviruses), birds (aviadenoviruses) and reptiles. Human adenoviruses are involved in several infections in the respiratory tract, conjunctivitis as well as gastroenteritis. These viruses are classified into six subgroups (from A to F) and then subdivided into distinct serotypes according to the induced

immunological response. For instance, serotype 2 and 5 of subgroup C are the most extensively studied human adenoviruses (Rux and Burnett, 2004). Adenoviruses have been widely used as model systems for study of different phases of virus infection as well as potential vectors for gene therapy. This is mainly due to ability of infection of wide range of hosts, recovery of high titres in cell culture and high level of transgene expression without viral genome integration into host genome (Choi and Chang, 2013). Adenovirus type 2 (AdV2) and type 5 (AdV5) are both recognized by ASTM International as representative non-enveloped viruses to be used for assessment of virucidal activity of biocides in suspension or against viruses adhered to non-porous inanimate surface through standardised tests (ASTM, 2011a and ASTM, 2011b).

1.2.3.2.2 Poliovirus

The poliovirus is a positive-stranded RNA non-enveloped enterovirus belonging to *Picornaviridae* family and the causative agent of poliomyelitis (Ehrenfeld et al., 2010). The capsid diameter is around 30 nm and it presents an icosahedral geometry containing up to 60 copies of four structural proteins: VP1, VP2, VP3 and VP4 (Louten, 2016). The latter is located within the capsid and function has not been fully unraveled yet. Figure 1.6 shows the capsid framework and disposition of VP proteins on the outer part of viral capsid including symmetry axes.

Figure 1.6: Poliovirus capsid icosahedral framework with VP1, VP2 and VP3 proteins on the outer part of the capsid. Numbers represent the different symmetry axes of viral capsid. Adapted from Hogle (2002).

Polioviruses can be divided into 3 different serotypes (type 1, 2 and 3) with minor structural differences between each other and all of them can cause poliomyelitis. The serotypes differ in terms of the ability to induce protection against second paralytic attacks and virulence was found to be significantly higher for serotype 1 during peak of poliomyelitis incidence in the United States in the pre-vaccination era (Nathanson and Kew, 2010). All the three different serotypes initiate host cell attachment and entry by interaction with human cell receptor CD155, a membrane anchored glycoprotein with respective extracellular domains (He et al., 2003). Poliovirus type 1 is recommended as a target organism for assessing virucidal efficacy of biocides by standardized method according to the European norm (DIN, 2013).

1.3 Disinfection kinetics

The selection of a biocide and design of an efficacious disinfection system requires knowledge about the activity and limitations associated to the chemical agent and material to be disinfected as well as disinfection kinetics. Moreover, it is important to ensure safe application of the product and avoid health risks by minimizing exposure to product itself and possible disinfection by-products. In order to predict disinfection performance and evaluate certain disinfection design criteria, kinetic models have been developed throughout the years. The differential rate law from which several kinetic models have been developed is mathematically described in Equation 1.1 as follows:

$$\frac{dN}{dt} = -kmN^{x}C^{z}t^{m-1} \qquad \text{(Equation 1.1)}$$

Where $\frac{dN}{dt}$ represents the microorganism inactivation rate; k the reaction rate; N the number of survivors; C concentration of disinfectants; t the contact time and x, z and m a set of empirical constants (Gyurek and Finch, 1998). The assumptions underlying kinetic models are that mixing is adequate to disperse uniformly a disinfectant agent and a microorganism; operational parameters, such as temperature, pH and disinfectant concentration are constant throughout disinfection process (Somani and Ingole, 2010). The reaction rate is determined experimentally and according to Wigginton et al. (2012)

the rate constant associated to the loss of virus replication function ($k_{replication}$) is related to reaction rate (k) as described by Equation 1.2.

$$k_{replication} = k - k_{binding} - k_{injection} \quad \text{(Equation 1.2)}$$

Where $k_{binding}$ is the rate constant associated to virus-host binding loss and $k_{injection}$ is the rate constant for loss of virus genome injection (Wigginton et al., 2012). This way the authors computed the k value (inactivation rate constant) for several disinfectants by plotting Ln (Virus titre after exposure/Virus titre before exposure) *vs.* disinfectant dose (Wigginton et al., 2012) comparison of different disinfectants against MS2 could be performed.

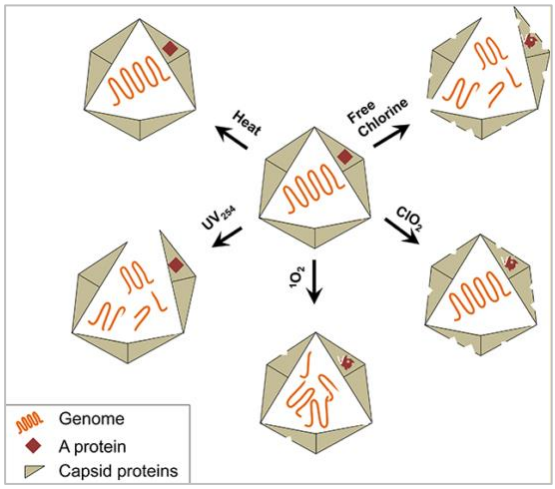
Similarly to other microorganisms, virus disinfection can present a first-order kinetics where an exponential kill dependent on the contact time, temperature and disinfectant concentration is observed, thus representing an ideal scenario for disinfection process design. Nevertheless, disinfection process in real life tends to deviate from a first-order kinetics due to variation of environmental parameters (e.g. pH, temperature, disinfectant availability, organic load) and virus intrinsic properties. The latter is mainly associated to the formation of virus aggregates, conformational shift in the virion capsid and virus surface charges, which may affect its sensitivity to disinfection (Young and Sharp, 1985).

1.4 Mechanisms of virucidal action

Biocides present a lack of selective toxicity and target specificity in contrast to antibiotics for example. Rational design and improvement of novel virucides have been carried out because of the need for reducing virucide concentration and efficacy maximisation. The study of mechanisms of virucides action has been essential for the development of novel formulations and for gathering deeper insight about the reasons underlying selectivity of virucidal activity. The mechanism of action consists on a systematic understanding of virus inactivation through association of formulation components to specific modifications and loss of virus functionality, which helps the comprehension about virucide limitations and virus adaptation to environmental stress

(Wigginton et al., 2012). Figure 1.7 depicts several modes of action associated to different virucidal agents against non-enveloped virus.

Figure 1.7: Distinct modes of action associated to virucidal agents against non-enveloped virus (taken from Wigginton et al., 2012).



Although the study of mechanisms of action is important for development and improvement of disinfection systems, there is still a lack of information regarding the mechanism of virucidal action against non-enveloped viruses. Several proposed mechanisms of action of virucides are based on activity against unrelated organisms like bacteria and yeast. Nevertheless, this approach may not be valid as those microorganisms present metabolic activity and consequently higher number of target sites when compared to viruses (Maillard et al., 2013).

Owing to their simpler and smaller structure, viruses present fewer target sites for virucidal activity when compared to bacteria. It is accepted that potential target sites can be the envelope (for enveloped viruses), capsid and viral genome. The viral envelope has a lipidic nature and can be disrupted via biocide interaction with lipids (e.g. alcohols and quaternary ammonium compounds). The capsid is constituted primarily by proteins and protects the viral nucleic acid from environmental stressors. Some chemical agents (e.g. glutaraldehyde, peracetic acid, copper ions) were shown to interact with capsid proteins resulting in conformational changes and denaturation (Chambon et al., 2005; Sauerbrei et al. 2007; Horie et al. 2008). The rupture of the viral capsid could lead to the release of potential infectious viral nucleic acid. Consequently,

this could give rise to a phenomenon called “multiplicity reactivation”, process whereby virions pool genetic information and generate an infectious unit by complementary reconstruction (Luria, 1947). In order for a treatment to be considered effective, biocides (virucidal and virustatic) must inactivate viruses to such an extent that virus infectivity and reproduction is hampered. Virucidal biocides refer to agents, which destroy completely the target virus, whereas virustatic biocides are associated to inhibition of virus proliferation by hampering interaction with the host. For instance, this may be achieved through virus immobilization (virustatic biocide) on a given surface, alteration of virus receptors involved in interactions with host cell and/or nucleic acid inactivation (virucidal biocide) (Thurman et al., 1989).

1.4.1 Alcohol as a virucidal agent

Several alcohols have been shown to be effective antimicrobials, thus they are widely employed in skin antisepsis products nowadays. Short-chain aliphatic alcohols such as ethanol, isopropanol and *n*-propanol are the most common used for alcohol-based biocides. The increase use of alcohols over other antimicrobials can be attributed to their rapid and broad-spectrum antimicrobial activity against bacteria, viruses and fungi (McDonnel and Russel, 1999; Guthery et al. 2005).

Little is known about the mechanism of action of alcohols, but studies on bacteria showed that ethanol and isopropanol are membrane active agents, which promote membrane disorganization leading to loss of cytoplasmic membrane function (Fizgerald et al., 1992; Ortega Morente et al., 2013). Alcohols show substantial virucidal activity against enveloped viruses as compared to non-enveloped ones, which suggests that the viral lipid envelope is a potential target site (van Engelenburg et al., 2002). The alcohols activity against non-enveloped viruses might be associated to alterations of structural proteins, which induces changes to the capsid structure and infectivity. Maillard et al. (1995) showed that F116 bacteriophage treated with ethanol and isopropanol presented fractured and folded capsids. Nath et al. (1982) studied the effects of different concentrations of ethanol on inactivation of DNA-polymerase associated with hepatitis B virus and they reported a ninety percent inactivation with 60% w/v ethanol. Moreover, it was suggested by Ito et al. (2002) that ethanol hampered

HBV binding to hepatocytes through HBV surface antigen protein complex degeneration.

In order to reduce the emission of volatile organic compounds (VOCs), reduce risks of tissue toxicity, efforts are being made to reduce alcohol content in some disinfectants (Kramer et al., 2006; Suchomel et al., 2009). Therefore, alcohols in synergistic combination with other antimicrobials (e.g. metals) are being studied not only to address those issues but to confer higher efficacy and persistence to biocidal products as well. For instance, chlorhexidine gluconate and quaternary ammonium compounds have been used as preservatives to alcohol-based skin antisepsis products in Europe and in the United States (Gaonkar et al., 2006).

1.4.2 Metal interaction with viruses

Metal ions present several roles in biological systems such as: transfer of electrons, mediator of interaction between proteins and ligands and nucleophilic catalyst. Most of those processes rely on protein-metal interactions, where metal ions (e.g. Cu^{2+} and Zn^{2+}) are critical for protein's stability and activity (Chatuverdi and Shrivastava, 2005). Since various structural and non-structural viral proteins require metal ions to be fully active, it can be said that metal ions are crucial for protection of viral genome, replication and pathogenesis. To illustrate this, it was shown that human immunodeficiency virus type 1 (HIV-1) nucleic acid annealing steps require the activity of the nucleocapsid protein, which contain several metal ion binding residues (Guo et al., 2000). Furthermore, it was shown that zinc ion is involved in the correct folding of NS3 protease related to hepatitis C virus maturation (Urbani et al., 1998). There is also evidence that viruses rely on the host intracellular store of zinc for their newly synthesized proteins. On the other hand, it was reported that host cells use metal ions to inhibit virus replication and alter genome of the virus (Chatuverdi et al., 2004). Despite the importance of metals to virus infectivity cycle, they were also found to present significant virucidal activity above certain concentration levels. For instance, silver nanoparticles were demonstrated to be highly active against influenza A virus, human immunodeficiency virus and adenovirus (Chen et al., 2013). Zinc and copper salts are also found in the literature to present virucidal activity and as such mechanisms of action have been proposed.

1.4.2.1 Zinc salts

Zinc is the most common metal to bind with virus proteins and possess an essential role in the folding and stability of important protein domains referred as zinc finger domains (Coleman, 1992). Despite its importance for virus viability, zinc has been shown in the literature to exert inhibitory effects against several viruses including gastroenteritis virus (Wei et al., 2012), rhinovirus (Korant and Butterworth, 1976), hepatitis C virus (Yuasa et al., 2006), respiratory syncytial virus (Suara and Crowe, 2004), coronavirus and arterivirus (te Velthuis et al., 2010) *in vitro*.

The specific mechanism of virucidal action associated to zinc ions is still unknown and several modes of action are suggested for different viruses. The modes of action proposed in the literature are: i) blockage of binding and virus-host interaction; ii) prevention of polypeptide breakdown during viral replication; iii) inhibition of transcriptase proteins and polymerases. Suara and Crowe (2004) studied the effect of zinc salts on respiratory syncytial virus replication and observed that zinc salts inhibited virus adsorption, penetration and egress. They postulated that virucidal activity of zinc was related to a decreased capacity of host to support virus replication rather than a direct effect on the virus. On the other hand, Kuemel et al. (1990) showed through electron microscopy studies that there is a massive deposition of zinc onto Herpes Simples Virus (HSV) virion components, which affects glycoprotein function involved in virus adsorption. Alternatively, Wei et al. (2012) affirmed that zinc activity against transmissible gastroenteririts virus *in* vitro is due to inhibition of virus penetration and egress instead of virus adsorption. Korant and Butterworth (1975) suggested that zinc complexes with rhinovirus coat proteins inhibiting the proteolytic processing of virus polypeptides during virus assembly. Te Velthuis (2012) reported that zinc (II) inhibited the activity of RNA polymerase during elongation phase of RNA synthesis, which was reversed by chelating the zinc ions. Yuasa et al. (2006) demonstrated that zinc inhibited replication of genome-length Hepatitis C RNA and it was hypothesised this could be caused by alteration of non-structural proteins functions involved in the viral genome replication. On the other hand, it was demonstrated recently that zinc salts are capable of blocking Hepatites E virus replication through direct inhibition of viral RNA-dependent RNA polymerase (Kaushik et al., 2017). Furthermore, interactions of metal

cations like zinc with DNA in solution were shown to alter its conformation and properties such as conductivity although deeper knowledge about the nature of those interactions and consequences for DNA integrity are still required (Lee et al. 1993; Labiuk, 2003). Thus, it is reasonable to include possible interaction of metal ion such as zinc with viral nucleic acid as a possible cause for zinc virucidal activity.

1.4.2.2 Copper salts

Copper salts have been used in disinfection processes for centuries due to its toxicity against several pathogenic microorganisms and relatively safety to humans below certain concentrations. Copper-based disinfectants present widespread application in the modern healthcare setting, where it is used in the control of pathogenic microorganisms in water distribution systems and for coating of medical devices (O'Gorman and Humphreys, 2012; Boyce, 2016). It is generally accepted that toxicity of copper to microorganisms might be related to its capacity to bind to thiol-containing groups and redox properties. The binding of copper to protein thiol groups can denature proteins and consequently hamper binding of other important trace elements. On the other hand, the redox cycling between cuprous (Cu(I)) and cupric (Cu(II)) catalyses the generation of reactive oxygen species like superoxide anions and hydroxyl radicals (Borkow and Gabbay, 2005). The formation of hydroxyl radicals is thought to occur via a Fenton reaction in the presence of hydrogen peroxide as follows (Urbanski and Beresewicz, 2000):

$$Cu^{+} + H_2O_2 \rightarrow Cu^{2+} + \ .OH\ (\text{free radical}) + OH^{-} \qquad \text{(Equation 1.3)}$$

Copper virucidal activity was first reported by Yamamoto et al. (1964). They observed that MS2 bacteriophages were fully inactivated when left on copper-based containers. Since then, copper either as metal complexes or salts were reported to be effective on inactivating viruses like HIV-1 (Sagripanti and Lightfoote, 1996), HSV (Sagripanti et al., 1997), avian influenza virus (Horie et al., 2008) and poliovirus (Yahya et al., 1992).

Despite wide spread use of copper for disinfection purposes, the exact mechanism of action by which copper promote viral inactivation has not been fully

understood yet. It is hypothesized that copper ions may possess a multifactorial mechanism of action in bacteria (Warnes and Keevil, 2011). The suggested mechanism of toxicity towards viruses associated to copper include alteration of protein complexes and function; denaturation of nucleic acid by site-specific Fenton reaction and/or chelation as well as free radical generation through coordination with other toxic molecules (Thurman et al., 1989; Carubelli et al., 1995).

Regarding denaturation of protein complexes, Karlstroem and Levine (1991) reported that HIV-1 protease, which is highly involved in virus replication, was inhibited by stoichiometric concentrations of copper ions. They also showed that protease inhibition by copper was concentration-dependent and relied on the presence of cysteine residues, which confirms protein inactivation involved binding of copper to thiol groups. In terms of nucleic acid denaturation, Wong et al. (1974) obtained a 6-7 $\log_{10}$ reduction of bacteriophage R17 due to cleavage of RNA by using vitamin C in combination with copper. These authors concluded that hydroxyl radicals generated from hydrogen peroxide through Fenton reaction were responsible for RNA cleavage. Sagripanti et al. (1997) reported HSV inactivation by copper ions and proposed that copper-mediated DNA damage involved binding of Cu (II) to nucleic acid, which produced oxidative base damage and breakage of DNA phosphodiester backbone. In general, nucleic acids offer several potential binding sites for metals with high proton affinity, which include nitrogen and oxygen groups associated to bases, hydroxyl groups on ribose and negatively charged O atoms in the phosphate residues. Factors like pH, competition with other ions (ionic strength of solution), ability to form chelation complexes and degree of base stacking strongly interfere with the interaction between nucleic acids and metal ions like copper (Martin and Mariam, 1979). The specific affinity of copper to G-C pairs of DNA results in crosslinking within and between strands, i.e. helical structure disorder. It is thought that subsequent DNA degradation steps are linked to generation of hydroxide radicals causing significant damages. Although nucleic acids are shown to be highly susceptible to copper, it is probably not the primary target, as nucleic acid cannot be readily accessed. The interaction of copper with other viral substructures and accessibility to viral nucleic acid will probably determine the overall virucidal activity. It was postulated that viruses with RNA genome and lipid envelope are highly susceptible to copper, whereas double-stranded DNA viruses tended to be more resilient except if they are enveloped (Li and Dennehy, 2011). It was also found that cupric ions were able to coordinate toxic molecules like

adriamycin and mitomycin C. Ueda et al. (1980) observed strand scission of bacteriophage phi X174 DNA in the presence of previously reduced mitomycin C with cupric ions. The latter was responsible for generation of mitomycin C semiquinone radical that promoted DNA degradation.

In terms of structural damage, it was observed through electron microscopic analysis that cupric ions induced morphological abnormalities on H9N2 avian influenza virus. This type of damage might be related to certain protein complexes denaturation. Furthermore, it was reported that copper was significantly more effective than zinc in virus inactivation (Horie et al., 2008).

1.4.3 Synergy

In contrast to additive effect, which takes place when overall biocide activity is the sum of activities of individual substances, synergy between biocides occur when the overall effect is greater than the sum of individual effects. Research into synergy between antimicrobials is increasing due to several factors such as, need for better biocide efficacy to avoid resistance and reduced costs associated to product development when compared to novel compounds (Lambert et al., 2003). One of the bases for antimicrobial synergy research is that synergy is more likely to occur when antimicrobials with distinct targets are mixed together, which must be confirmed through experimental verification (Hugbo, 1976).

Synergistic combinations of copper and zinc ions with other antimicrobial agents have been investigated. For example, zinc pyrithione formulations with alcohol have been used successfully in skin antisepsis products and are shown to have significant virucidal activity against human coronavirus and HIV-1 (Guthery and Anderson, 2005). Synergistic combination of copper with free chlorine against poliovirus was also reported with respective mechanism of action. Since chlorine is a halogen that can act in part as an oxidizing agent, it was proposed that chlorine causes severe damages to the protein capsid and consequently, it would facilitate interaction of copper with nucleic acid (Borkow and Gabbay, 2005). Moreover, combination of quaternary ammonium compounds and Cu (II) was reported to be an effective combination against complex and resilient microbial structures like biofilms, which demonstrates the potential role of metallic ions in increasing activity of a biocide

(Harrison et al., 2008). Novel microbicidal formulations containing reduced amount of alcohol with zinc or copper were shown to present synergistic virucidal activity against a wide range of viruses (Ijaz et al., 2012; Ijaz and Zhu, 2014a). However, underlying mechanism of action of those formulations remains to be studied.

1.5 Aim of the project, hypothesis and relevance

The main goals of this PhD project are (i) to understand the mechanism(s) by which the combination of alcohol and zinc ions, in particular low concentrations of ethanol (around 40% w/v) with zinc ions, exhibit virucidal effects against non-enveloped viruses and (ii) to elucidate the mechanisms of potentiation of virucidal activity through combinations of those actives compounds. The working hypotheses based in the literature review for this project were:

- Hypothesis 1: the combination of alcohol and zinc is more effective at inactivating non-enveloped viruses than zinc or alcohol as sole active ingredient
- Hypothesis 2: the action of combination alcohol/zinc could weaken the viral capsid and have nucleic acid as an additional target for virucidal action.

Furthermore, this work also aims to demonstrate the effect of zinc ions on formulation stability notably by understanding the chemical composition in such formulations over extended periods of time under specific storage conditions.

The aims of the project are planned to be achieved through the following steps: (i) investigation of the virucidal activity related to formulations with different ratios of zinc/ethanol at different pHs on surrogates (bacteriophage MS2 and F116) and mammalian viruses (adenovirus and poliovirus); (ii) investigation of formulations impact on bacteriophage structure, morphology and mechanical properties; (iii) investigation of formulations influence on physicochemical properties of the phages (surface charge, aggregation capacity, protein and nucleic acid damages); (iv) identification of chemical elements responsible for the virucidal action and formulation

stability studies and (v) investigation of formulation activity and mode of action on mammalian viruses (adenovirus type 2 and poliovirus LSc-1ab).

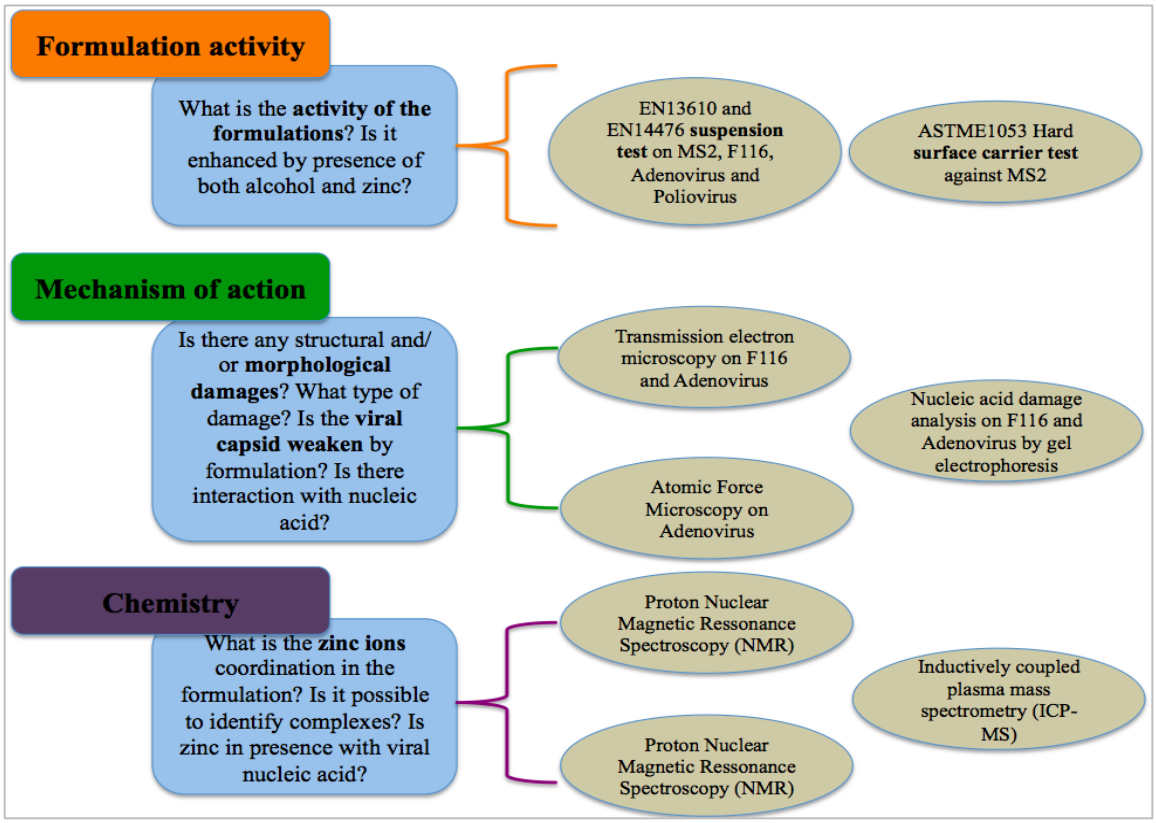
Given the aforementioned importance of biocides to control infection spreads in many different sectors, especially household and healthcare settings, it becomes evident the need for the development and improvement of biocides. In this context, the present project will also have an impact on development of novel biocidal products as gathering information about synergism between active compounds and their mechanisms of action is essential for the rational design and improvement of biocidal products with a virucidal activity.

2. General materials and methods

2.1 Rationality behind research methodology

The initial focus was given to formulations containing ethanol and zinc at a given pH. The goals of the study were therefore set as follows: (i) to confirm the virucidal activity of formulation containing ethanol and zinc at pH 10.5 (prepared according to Reckitt Benckiser (RB) instructions based on Patent WO 2014/006380) against bacteriophages and human viruses; (ii) to study the impact of the complex zinc/ethanol on virus capsid morphology and susceptibility to mechanical stress (e.g. spring constant and breaking force); (iii) to verify possible interactions of alcohol/zinc with viral nucleic acid and (iv) to evaluate the chemical entities present in the formulation at a given temperature and pH conditions and possibly relate them to virucidal activity. Those aspects were crucial for proposing a mechanism of virucidal action associated to the combination of alcohol and zinc as active ingredients. Figure 2.1 shows a summary of different experiments performed throughout the research project divided into three main aspects: general activity, mechanism of action and chemistry of formulations.

Figure 2.1: Methodology for the present study divided by topics and respective scientific questions.



2.1.1 Testing formulations

The initial testing formulations in the present project included distinct alcohol concentrations combined with a fixed zinc salt concentration as active ingredients plus a fixed amount of excipients at a given pH. This initial testing was performed in order to screen for virucidal activity at different ethanol concentrations with zinc salt and analyse virucidal activity patterns. At later stage, a formulation system comprised by a fixed amount of alcohol with zinc salt including excipients at fixed pH was selected based on its virucidal activity against bacteriophage MS2 for mechanism of action studies. The selection made it possible to analyse a formulation through different experiments and infer about the mechanism of action within a reasonable time frame for this project. Since the project was carried out in partnership with RB, an industrial partner, there was a growing interest to evaluate activity of alcohol and zinc in a formulation as close to the final biocidal product formulation as possible. Therefore, testing formulations included excipients at a given pH as described in Patent WO

2014/006380 rather than active ingredients alone (Ijaz and Zhu, 2014). Additionally, it was intended from the very beginning of the project that proposed mechanism of action was to be included in RB's intellectual property portfolio.

2.1.2 Virucidal activity of formulations

Virucidal activity of a biocide can be affected by several parameters such as contact time, pH, temperature, organic load, virus type and concentration of both virus and active ingredients (Maillard et al., 2013). Several standardised virucidal activity tests were develop at national scale to standardise the experimental methods and parameters which resemble as accurate as possible those found in actual practice (DVV, 2012; Rabenau et al., 2014). Therefore, standardised virucidal efficacy tests of formulations were performed throughout the present study not only to evaluate activity of testing formulations and establish comparisons amongst them but also to verify compliance with current national standards for virus disinfection efficiency.

Standardised virucidal efficacy tests of biocide in suspension (ASTM E1052-1: 2011; EN13610: 2013) or dried on an inanimate surface (ASTM E1053: 2011) were carried out to confirm the virucidal activity of formulations. The EN13610 was used as a screening test for initial virucidal activity assessment of formulations, whereas the ASTM E1053 was used to simulate formulation activity on surfaces and to compare with data produced by RB. The reasons for using bacteriophage MS2 for both tests were: (i) that it is a harmless organism, (ii) easy and fast to propagate and (iii) a well-known surrogate for non-enveloped viruses (Macinga et al., 2008; Sinclair et al., 2012; Gallandat and Lantagne, 2017). On the other hand, the virucidal suspension test on F116 aimed to evaluate the use of F116 as a model for structure damage study through transmission electron microscopy. It allowed for the selection of an appropriate contact time at which the virus would not be severely killed (i.e. <99% reduction in concentration) to allow observation of distinct virion structural changes induced by formulations. It was hypothesized that ethanol may allow better penetration of zinc ions into the virions through denaturation of capsid proteins, followed by zinc interaction with nucleic acid materials.

Standard activity tests (EN13610: 2013) in suspension were performed against both adenovirus and poliovirus in order to compare with data obtained by activity tests

against bacteriophages and evaluate surrogacy capacity as well as formulation efficacy against human pathogens.

2.1.3 Mechanisms of virucidal action of formulations and specific interactions with viral targets

The mechanism of action (MoA) of a given biocide consists about revealing possible target sites on viruses, which are responsible for the observed virucidal activity. As an example, it is generally accepted that infectivity of enteric viruses (e.g. MS2, Adenovirus, Poliovirus) relies heavily on functional integrity of the viral capsid as well as its genome (Fong and Lipp, 2005; Hijikata et al., 2016). The stability of the capsid is especially important as it is in involved in virus attachment and entry into host and protection of genome against degradation from surroundings (Pecson et al., 2009). Therefore, the focus was given on the study of the influence of testing formulations on virus capsid integrity and genome integrity, i.e. formulation potential to fully inactivate non-enveloped viruses.

Regarding the impact of testing formulations on viral capsid, nano-indentation studies and transmission electron microscopy imaging upon exposure of viruses to testing formulations were performed to analyse possible damage to the viral capsid integrity. Transmission electron microscopy (TEM) against F116 was shown to be effective at showing morphological changes induced by different biocides (Maillard et al., 1995; Pinto, 2010). F116 has been shown to be an interesting model because of its complex structure including capsid and tail. Nano-indentation studies on adenovirus capsid were shown to be effective methodology for analysis virus capsid mechanics after exposure to different environmental parameters (Ortega-Esteban et al. 2013; Marchetti et al., 2016). Therefore, the overall impact of testing formulations on virus capsid structure was analysed through aforementioned methodologies.

In terms of viral genome as potential target for alcohol/zinc virucidal action, the screening of possible adenovirus and F116 viral DNA damage upon exposure to testing formulation was analysed by gel electrophoresis and restriction enzyme digestion pattern analysis as described in the literature (Maillard et al., 1996; Sutherland et al., 2006 Pinto, 2010). The DNA damage analysis is also relevant as it was demonstrated by Marchetti et al. (2016) viral capsid structural changes are closely related to possible

genome conformational changes. Therefore, those studies were able to provide a full picture about what happens to viruses upon exposure to alcohol and zinc and possible relation to formulation virucidal activity.

2.1.4 Formulation composition and speciation

Regarding formulation chemistry analysis, Proton Nuclear Magnetic Resonance Spectroscopy (1H-NMR) was envisioned as a method to analyse zinc coordination and bioavailability in formulation by identification of distinct chemical elements, which could be responsible for the virucidal activity. The methodology was based on the study by 1H-NMR on zinc interactions with nucleic acid carried out by Aoki and Kimura (2004). In this project, a preliminary experiment was performed to evaluate the formation of zinc complexes with triethanolamine, a chemical compound present in the formulation. Additionally, techniques based on mass spectroscopy were employed to possibly identify complexes and other chemical entities as performed by Bester and Lamani et al. (2010). The main goal was to identify chemical entities, which could be associated with efficacy against non-enveloped viruses observed through activity tests.

2.2 Biocide formulation and preparation

Formulations with different ethanol concentrations and with same amount of zinc were prepared, in order to study the influence of different ethanol concentrations on virucidal activity and mechanism of action.

Formulation composition and buffering system at pH 10.5 was based on *Example 41* found in the Patent WO 2014/006380 (Ijaz and Zhu, 2014). The formulation protocol for the formulation was supplied by Reckitt Benckiser (Montvale, USA) and the main composition of the formulation is presented in Table 2.1.

Table 2.1: Formulation composition associated to biocide with 0.1% Zinc at pH 10.5 with varying ethanol concentrations based on the *Example 41* of Patent WO 2014/006380.

Chemicals	Concentration (% w/v)
Citric acid anhydrous (Acros Organic, UK)	0.12
Zinc sulphate heptahydrate (Sigma Aldrich, UK)	0.10
Ethanol (Fisher Chemical, UK)	25.0 to 80.0
Triethanolamine (Acros Organic, UK)	0.10
Ammonium hydroxide (Fisher, UK)	0.10
Monoethanolamine (Acros Organic, UK)	1.04

Formulations were prepared with deionized water and stored in glass bottles in dark environment for 4 months and the pH was measured on a weekly basis.

In addition to formulations prepared in Cardiff according to Table 2.1, Reckitt Benckiser sent two batches of formulations with similar chemicals in December 2014 and September 2015. Three sets of formulations were therefore studied throughout the present study and labelled as presented in Table 2.2.

Table 2.2: Different formulations analysed throughout experimental work.

Set	Formulation	Composition
Formulations prepared in Cardiff	CA001 (base formulation)_	40% (w/v) Ethanol + 0.1% (w/v) Zinc sulphate heptahydrate + excipients
	CA001-A	35% (w/v) Ethanol + 0.1% (w/v) Zinc sulphate heptahydrate + excipients
	CA001-B	33.5% (w/v) Ethanol + 0.1% (w/v) Zinc sulphate heptahydrate + excipients
	CA001-C	30% (w/v) Ethanol + 0.1% (w/v) Zinc sulphate heptahydrate + excipients
	CA001-D	27.5% (w/v) Ethanol + 0.1% (w/v) Zinc sulphate heptahydrate + excipients
	CA001-E	25% (w/v) Ethanol + 0.1% (w/v) Zinc sulphate heptahydrate + excipients
	CA001-F	80% (w/v) Ethanol + 0.1% (w/v) Zinc sulphate heptahydrate + excipients
	CA001-X	40% (w/v) Ethanol + excipients
	CA001-AX	35% (w/v) Ethanol + excipients
	CA001-BX	33.5% (w/v) Ethanol + excipients
	CA001-CX	30% (w/v) Ethanol + excipients
	CA001-DX	27.5% (w/v) Ethanol + excipients
	CA001-EX	25% (w/v) Ethanol + excipients
	CA001-FX	80% (w/v) Ethanol + excipients
	CA-001-G	0.1% (w/v) Zinc sulphate heptahydrate + excipients
	CA-001-EXCP	Excipients
Formulation sent by RB (December 2014)	RB-001 (base formulation)	40% (w/v) Ethanol + 0.1% (w/v) Zinc sulphate heptahydrate + excipients
	RB-001-A	35% (w/v) Ethanol + 0.1% (w/v) Zinc sulphate heptahydrate + excipients
	RB-001-B	33.5% (w/v) Ethanol + 0.1% (w/v) Zinc sulphate heptahydrate + excipients

	RB-001-C	30% (w/v) Ethanol + 0.1% (w/v) Zinc sulphate heptahydrate + excipients
	RB-001-D	27.5% (w/v) Ethanol + 0.1% (w/v) Zinc sulphate heptahydrate + excipients
	RB-001-E	25% (w/v) Ethanol + 0.1% (w/v) Zinc sulphate heptahydrate + excipients
	RB-001-F	80% (w/v) Ethanol + 0.1% (w/v) Zinc sulphate heptahydrate + excipients
	RB-001X	40% (w/v) Ethanol + excipients
	RB-001-AX	35% (w/v) Ethanol + excipients
	RB-001-BX	33.5% (w/v) Ethanol + excipients
	RB-001-CX	30% (w/v) Ethanol + excipients
	RB-001-DX	27.5% (w/v) Ethanol + excipients
	RB-001-EX	25% (w/v) Ethanol + excipients
	RB-001-FX	80% (w/v) Ethanol + excipients
	RB-001-G	0.1% (w/v) Zinc sulphate heptahydrate + excipients
	RB-001-EXCP	Excipients
Formulation sent by RB (September 2015)	RB-002 (base formulation)	40% (w/v) Ethanol + 0.1% (w/v) Zinc sulphate heptahydrate + excipients
	RB-002G	0.1% (w/v) Zinc sulphate heptahydrate + excipients
	RB-002EXCP	Excipients
Lysol formulation sent by RB	C0042-042	Lysol commercial formulation batch 1
	C042-084	Lysol commercial formulation batch 2

2.3 Neutraliser preparation

The neutraliser formulated to quench the formulations under study was composed by a mixture of L-Cysteine ($1g.L^{-1}$) (Sigma-Aldrich, UK) with sodium thiosulphate [0.1% w/v] (Sigma-Aldrich, UK) in distilled water. The neutraliser was prepared according to Allan et al. (2011), where heavy metal (Zinc) and alcohol activities are quenched by L-cysteine and dilution, respectively. Neutraliser quenching

efficacy was evaluated prior virucidal activity tests (see section 2.7). After preparation, neutraliser was sterilized by autoclaving at 121ºC for 15 min (Sanders, 2012).

2.4 Host propagation and enumeration

The hosts for bacteriophage MS2 and F116 were *Escherichia coli* (NCIMB 9481) and *Pseudomonas aeruginosa* PAO1 (NCIMB 10548), respectively. Both hosts were cultivated in 10 mL tryptic soy broth (Oxoid, Cambridge, UK) for 24 h at 37ºC and centrifuged at 3276g for 15 min (Primo R, Thermo Scientific). After supernatant removal, the bacterial pellet was resuspended in 5 mL of phosphate buffered saline (PBS) solution (Fisher Chemical, UK).

The bacterial enumeration was performed by drop counting method according to Miles and Misra (1938), where suspension is 10-fold serially diluted by adding 100 µL of suspension in 900 µL of PBS. Five drops of 10 µL of selected dilutions are plated in Tryptic Soy Agar (TSA) plates for a total of 3 biological replicates. Colonies were counted for each dilution after 24 h incubation at 37ºC.

In addition, several cryogenic vials were prepared for each cultivated host by mixing 900 µL of bacteria suspension with 100 µL glycerol (Fisher Scientific, UK) and stored at -80ºC. Each vial was used only once for bacterial propagation and discarded afterwards. The resulting bacterial suspension was kept at 4ºC and used for further bacterial propagations within the next 4 days.

2.5 Phage propagation, purification and enumeration

Bacteriophage MS2 (NCIMB 10108) and F116 (NCIMB 10882) were propagated according to Pinto et al. (2010). Phages were propagated in plate cultures, which consisted of agar supplemented with 0.5 mM and 1 mM calcium chloride (Fisher Chemical, UK) for MS2 and F116 propagation, respectively. Phages were harvested from plates presenting more than 30 plaques by adding 2 mL of SM Buffer (composed by 0.05 M Tris/HCl, 0.2% w/v $MgSO_4$ and 0.01% w/v fish-skin gelatin) to the 65% agar layer and removing it with a plastic L-shaped spreader. Layers were transferred to propylene centrifuge tubes and centrifuged at 11,000g (Rotor JA25.50, Beckman Coulter) for 15 min at 4ºC. Following that, the supernatant was filtered in a two-step

filtration process by using 0.45 μm and 0.20 μm membrane filters. Stocks were prepared adding 10% w/v glycerol to phage suspension and stored at -80ºC.

Phages were enumerated by Soft Overlayer Agar technique as described by Pinto et al. (2010). Ten-fold serial dilutions of phages were prepared by adding 100 μL of phage to 900 μL SM Buffer. Then, 100 μL of phages were mixed with 100 μL of host cells (10^8 colony forming unit (CFU) mL^{-1}) in 5 mL of 65% w/v molten agar and suspension was poured into TSA plates. After 24h incubation at 37ºC, plaques were counted and expressed as plaque forming units per mL (PFU mL^{-1}).

2.6 Poliovirus propagation, purification and enumeration

Mammalian viruses were propagated and purified according to Ichim and Wells (2011). To produce viruses, human cervical epithelioid carcinoma (HeLa) cells were grown in Dulbecco's Modified Eagle Medium (DMEM) with 10% FBS and penicillin/streptomycin (Growth media) (Life Technologies, UK) mix until 70 to 80% confluence was reached. HeLa cells were infected by adenovirus at multiplicity of infection (MOI) of 10 and viral supernatant was harvested 5 days post-infection. Viral supernatant was filtered by 0.45 μm pore size membrane filter and added to centrifuge tube, which was spun down at 49,000g (Rotor SW28, Beckman Coulter) for 90 min at 4ºC. The medium was decanted into bleach-filled container and final drops were carefully removed so tubes have similar volumes. Then, 30 mL of viral supernatant were loaded to the tubes for another round of centrifugation under same conditions described previously. In total, 4 rounds of centrifugation were performed and pellets resuspended by careful pipetting.

The viral pool was titrated according to the Spearman-Kaeber method (Flint and Enquist, 2008). HeLa cells were grown on a 24-well microplate with 2mL of growth media for 2 days at 37ºC under 5% carbon dioxide. Serial dilutions of virus pool until 10^{-8} infective units/mL were prepared with DMEM containing 2% FBS with penicillin/streptomycin mix (maintenance media). One hundred microliters of each virus dilution were added to each well and incubated for 1 h at 37ºC under 5% carbon dioxide. After short incubation, 2 ml of maintenance media was added to each well and incubation for 5 to 7 days at the same conditions previously described took place. Cytopathic effect was observed under microscope (objective 20X, Leica) and 50%

tissue culture infectious dose ($TCID_{50}$) was determined by the Spearman-Kaeber formula.

2.7 Purification of attP Adenovirus type 5 (attP AdV5) and Adenovirus type 2 (AdV2)

The attP Adenovirus type 5 and Adenovirus type 2 were propagated in HEK293 HeLA and HeLA cells respectively and highly purified by ultracentrifugation in caesium chloride (CsCl) gradient as described by Condezo et al. (2015). Viruses were harvested 3 days post-infection. Following that, they were purified by ultracentrifugation at 219,000g (LE-80K Ultracentrifuge, Beckman Coulter) for 90 min at 18ºC in 1.25 $g.mL^{-1}$ and 1.40 $g.mL^{-1}$ CsCl step gradient. TD1X Buffer (137 mM NaCl, 5.1 mM KCl, 700 mM $Na_2.HPO_4.7H_2O$ and 25 mM Tris base at pH 7.4) was used to prepare CsCl gradients. Following the first centrifugation, viral bands were extracted and centrifuged in 1.31 $g.mL^{-1}$ CsCl step gradient for 18 hours at 219,000g. Viral bands obtained were desalted through column chromatography (Bio-Rad 10 DC, UK), eluted with HBS Buffer and stored in aliquots with 10% glycerol at -80ºC. The viral titre was quantified as determined by Spearman-Kaeber method for 6^{th} and 7^{th} day post-infection as described by Flint and Enquist (2008).

2.8 Statistical analysis

All the experiments were repeated three times independently, with exception of activity test against poliovirus where one single independent sample was attained. The Wicoxon-Mann-Whitney test was the statistical test applied throughout the study of formulations virucidal activity to evaluate the statistical significance associated to differences between two independent samples. It is a non-parametric test, which is commonly applied as an alternative to t-Student for samples with small dimension, heterogeneous variances and non-normal distributions. The statistical hypothesis associated to the bilateral test are described as follows: i) null hypothesis (H0): differences between two independent samples are not significant ($p>0,05$) and ii) alternative hypothesis (H1): differences between two independent samples are significant ($p<0,05$). On the other hand, the one-way ANOVA parametric test was used

for result differences found during nano-indentation studies and virucidal activity tests when normal distribution was observed by Kolmogorov Smirnov test (Zar, 1999; Wessa, 2013).

The Chi squared test was used to analyse statistically significant differences in proportions among independent groups comprised by each type of damage observed through transmission electron microscopy. The null hypothesis (H0) corresponds to no effect of formulation on observed damage pattern, whereas alternative hypothesis (H1) states there is an effect of formulation on damage pattern.

The software used for statistical analysis was GraphPad Prism 6.0 for Macintosh (GraphPad software, USA).

3. Virucidal activity screening of formulations containing ethanol and zinc with controls against bacteriophages and mammalian viruses

3.1 Introduction

Biocides play a significant role on disinfection procedures and prevention and spread of infectious agents. Since the virucidal activity associated to active ingredients cannot be based exclusively on chemical composition or comparison with activity against microorganisms like fungi and bacteria, it becomes essential to evaluate virucidal activity through standardised activity tests. Authorisation of novel biocidal products is regulated by the EU Biocides Regulation 528/2012 (EU BPR) in the European Union. Guidelines for assessment of product activity for disinfection of viruses were elaborated by AFNOR (Association Française de Normalisation) in France, DEFRA (Department of Environment, Food and Rural Affairs) in the United Kingdom, DVV (Deutsche Verinigung zur Bekaempfung der Viruskrankheiten) in Germany (Maillard, 2013). This led to the development of the EN13610 and EN14476 standard quantitative suspension tests for assessing virucidal activity against bacteriophages of chemical disinfectants used in food and industrial areas in Europe by CEN (European committee for Standardisation) (CEN, 2003; prEN14476). In the United States, the ASTM (American Society for Testing and Materials) guideline is followed and application for registration of a novel biocidal product needs to be in compliance with requirements imposed by EPA (Environmental Protection Agency) under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (EPA, 2012). For instance, the ASTM guideline requires a minimum 3 $\log_{10}$ reduction to consider biocide as active against viruses (ASTM International, 2011), whereas European standards require a minimum of 4 $\log_{10}$ reduction at 5 min contact time (prEN 14476, 2011). Some of the different standard tests for virucidal efficacy against viruses of biocidal products are summarised in Table 3.1.

Table 3.1: Details of different standard tests for quantification of disinfectants activity against viruses.

Standardisation organisation	Standard of virucidal activity	Standard title	Test conditions	Reference
ASTM	ASTM E1052-11	Standard Test Method to Assess the Activity of Microbicides against Viruses in Suspension	One part of virus suspension is added to 9 parts of test substance; 5 or 60 min contact time depending on regulatory agency; room temperature	ASTM (2011a)
	ASTM E1053-11	Standard Test method to Assess Virucidal Activity of Chemicals Intended for Disinfection of Inanimate, Nonporous Environmental Surfaces	2.0 mL of test substance is added to for each dried virus film carrier covering entire surface; contact time depending on regulatory agency; room temperature	ASTM (2011b)
	ASTM E2011-13	Standard Test Method for Evaluation of Hygienic Handwash and Handrub Formulations for Virus-Eliminating Activity Using the Entire Hand	Virus suspension is spread on palm of the hand for 180s and amount of test substance is added according to manufacturer; 20s contact time; Temperature at 35±2^{o}C	ASTM (2013)
CEN	EN 14476	Quantitative suspension test for the evaluation of virucidal activity of chemical	One part of virus suspension is added to 8 parts of test substance+1 part of interfering substance; no	CEN (2011)

		disinfectants and antiseptics in human medicine	longer than 60 min contact time; 4 log_{10} virus inactivation must be achieved for validation; 20^{o}C temperature for surface disinfection	
	EN13610	Quantitative suspension test for the evaluation of virucidal activity against bacteriophages of chemical disinfectants used in food and industrial areas – test methods and requirements (phase 2, step 1)	One part of bacteriophages suspension is added to 9 parts of test substance; at least 15 min contact time, 4 log_{10} virus inactivation for validation; 20±1^{o}C temperature	CEN (2003)

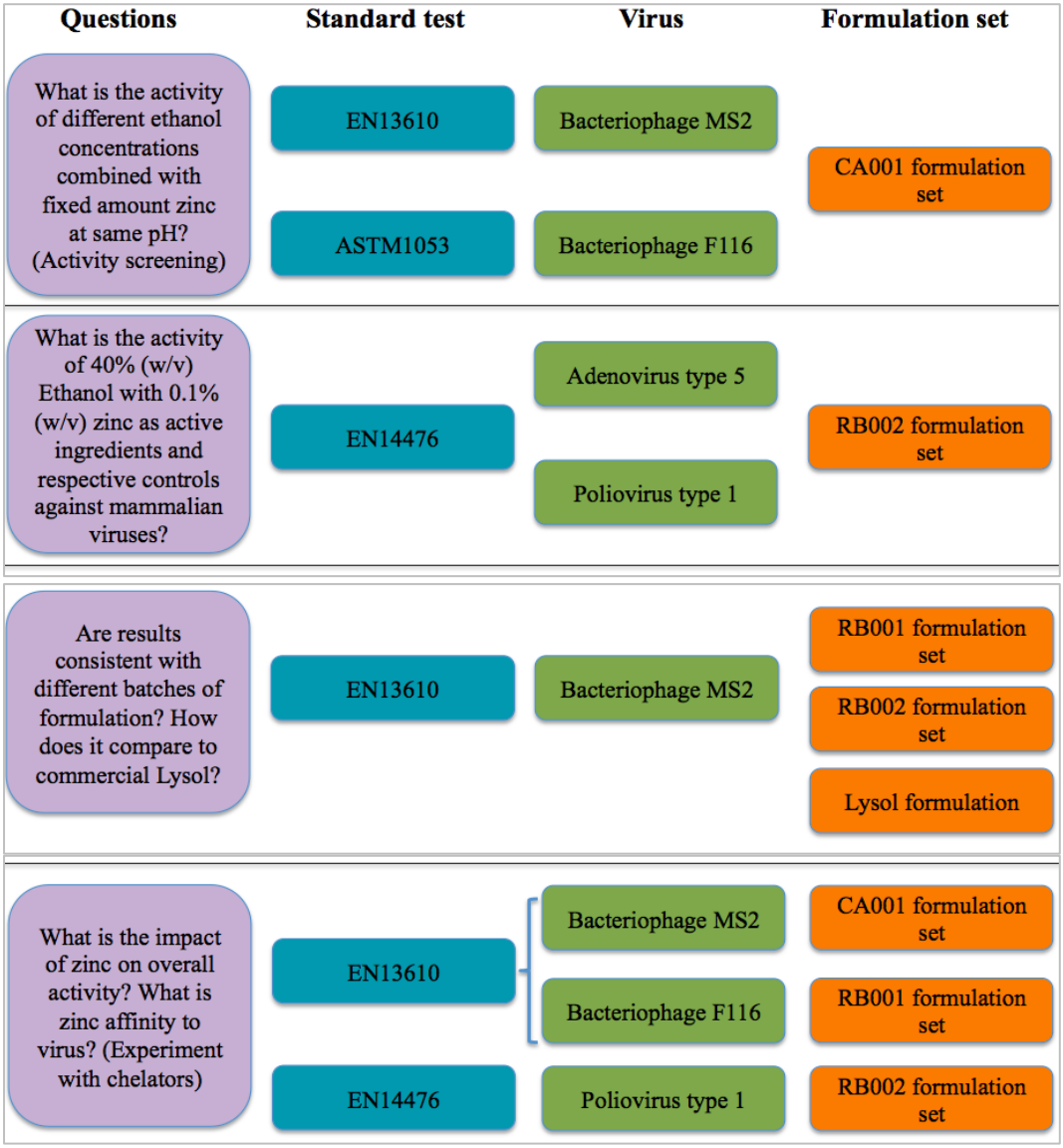
The aim of this section was to quantify virucidal efficacy of formulations with different ethanol concentrations containing zinc sulphate heptahydrate at pH 10.5 against MS2 by suspension (EN13610) and carrier tests (ASTM E1053). Virucidal activity test (EN13610) against F116 in suspension was also carried out to evaluate the capability of using the referred phage for mechanism of action studies by TEM imaging. In addition, two batches of formulations with similar composition were prepared by RB and their virucidal activity was evaluated and compared with the ones presented by formulations prepared in Cardiff. This was carried out in order to verify formulation protocol consistency between Cardiff University and Reckitt Benckiser. Moreover, the active role of zinc on the overall activity of formulation as well as its affinity towards virus was investigated through suspension tests with formulation comprising both ethanol and zinc as active ingredients in presence of different chelating agents. The activity against commonly used mammalian viruses, adenovirus type 2 (AdV2) and poliovirus type 1 (Pv1), through EN14476 activity standard test was carried out in order to evaluate efficacy of formulations for inactivation of human pathogens and surrogacy

capacity by bacteriophages under test. In this context, this section will elucidate the overall activity of aforementioned combination and it represents a first step at describing the mechanism of action associated to the combination of alcohol with zinc against non-enveloped viruses.

3.2 Materials and Methods

The different formulations tested in the present study and respective compositions as wells as the rationale behind experimental design are shown in Table 2.2 (see Chapter 2) and Figure 3.1, respectively.

Figure 3.1: Summary of all the standard activity tests performed in this section with corresponding formulation set under analysis and scientific questions.



3.2.1 Neutraliser efficacy test of virucidal activity test against bacteriophages

Neutralisers were used for quenching test substance activity at specific time points in order to evaluate efficacy of test substance at different contact times.

The neutraliser comprised a mixture of L-cysteine ($1 g.L^{-1}$) (Sigma-Aldrich, UK) with sodium thiosulphate [0.1% w/v] (Sigma-Aldrich, UK) in distilled water. The neutraliser efficacy for quenching biocidal activity was evaluated for both the host and the phage according to Maillard *et al.* (1998). Initially formulation and neutraliser were mixed at a ratio 1:8 and 1 mL of bacterial suspension (10^7 CFU mL^{-1}) was added to the mixture with 10 min contact time in order to evaluate cytotoxicity of neutraliser towards bacteriophage host. Control was performed by adding 1 mL of bacterial suspension (10^7 CFU mL^{-1}) to the mixture of formulation with sterile water (1:8 ratio). Bacteria were enumerated by drop count method as described previously.

A similar procedure was carried out for phages (10^8 PFU mL^{-1}) and they were enumerated by soft overlayer agar technique. The viral titre reduction (R) associated to the neutraliser was computed through Equation 3.1 as follows:

$$R = \log(X_o) - \log(X) \qquad \text{(Eq. 3.1)}$$

where X_o represents initial concentration of bacteria (CFU mL^{-1}) or phage (PFU mL^{-1}) before exposure and X the concentration after exposure (CFU mL^{-1} or PFU mL^{-1}).

3.2.2 Neutraliser efficacy test of virucidal activity test against mammalian viruses

The neutraliser efficacy for quenching test substance activity against mammalian viruses was adapted from standard EN14476 (CEN, 2011). Dilution in ice-cold medium was performed by adding 0.1 mL of virus suspension (10^8 PFU/mL for adenovirus; 10^5 PFU/mL for poliovirus) to a mix comprising 9 mL ice-cold Minimum Essential Medium (MEM) (Sigma, UK) with 2% fetal bovine serum, 0.1 mL sterile distilled water and 0.8 mL test substance (formulation containing ethanol and zinc as actives). After 30 min contact time in ice bath, 0.1mL aliquot was added to a MicroSpin S400 chromatography column (GE, UK) and eluate was serially diluted. Every dilution factor was added separately to wells with 70-80% confluence Hela cells monolayer. Virus titre was determined by Spearman-Kaeber method 6 to 7 days post-infection. Neutralisation

efficacy is attained when difference of virus titre reduction with control test substance (PBS) is equal or less than 0.5 log reduction.

3.2.3 Virucidal suspension test against bacteriophages MS2 and F116

The virucidal suspension test against MS2 and F116 phages was performed to evaluate the virucidal activity of the formulations under study. The protocol was an adaptation of the BS EN 13610:2002 quantitative suspension test (CEN, 2003). Formulations were tested at room temperatures at three distinct contact times (5, 30 and 60 min) against MS2 without any interfering substance. The contact times above 30 min were chosen in order to evaluate formulation efficacy throughout time and to verify at which contact time activity values were comparable to the ones found by Ijaz and Zhu (2014). The contact times chosen for suspension test against F116 were 5, 10 and 15 min.

One mL of phage suspension (10^9 PFU mL^{-1}) was mixed with 9 mL of a formulation and after respective contact time, one mL of phage/formulation mixture was added to 9 mL of neutraliser. Subsequently, 10-fold serial dilution was carried out and phages from two different dilution factors were enumerated. Three biological replicates with 4 technical replicates were performed. A biological replicate refers to repeating the activity test with an independent phage samples with the same formulations, whereas technical replicates comprise test repetition on a given phage suspension sample. Controls were attained by exposing phages i) to formulations with similar composition without zinc to verify the direct effect of zinc and alcohol (CA001X and RB001X, see Table 2.2) and ii) to formulation without active compounds (ethanol and zinc) to test the effect of other formulation components (CA001EXCP, RB001EXCP, RB002EXCP, see Table 2.2) and to SM buffer (negative control). The viral titre reduction was calculated with equation 3.1. The biocide was regarded to be active against the phage when a decrease in phage concentration higher than 4 Log_{10} was achieved (CEN, 2003).

3.2.4 Virucidal suspension test in presence of chelating agents

This experiment was designed i) to estimate the complex stability constant of zinc ions present in formulation with phage particles and ii) to evaluate how strongly zinc present in formulation comprising alcohol and zinc as active ingredients (RB001 and RB002, see Table 2.2) tends to complex with the phage. Phages were exposed to formulation in presence of chelators with distinct complex stability constants with zinc. The concentration of each chelating agent to be added to formulation before activity test was estimated according to equation 3.2:

$$k_s = \frac{[ML]}{[M][L]} \quad \text{(Eq. 3.2)}$$

Where k_s is the complex stability constant; [ML] is the concentration of the metal complex in equilibrium; [M] is the concentration of metal ion and [L] the concentration of ligand. The chelating agents added to the formulation RB-001 as well as respective concentration and complex stability constant are presented in table 3.2.

Table 3.2: Different chelating agents used throughout virucidal suspension test in presence of chelating agents.

Chelating agent	Concentration (% w/v)	Complex stability constant with Zinc (k_s)
Ethylenediaminetetraacetic acid (EDTA)(Fisher Chemical, UK)	0.06	16.50
Nitrilotriacetic acid (NTA) (Acros Organics, UK)	0.09	10.66
Bicine (Sigma, UK)	0.15	6.50

Chelating agent was added to phage suspension (10^9 PFU.mL^{-1}) and activity test was carried out as described in section 3.2.3.

3.2.5 Hard-surface carrier test against bacteriophage MS2

The quantitative virucidal carrier test carried out was adapted to bacteriophages from the standard test ASTM E1053-1 (ASTM, 2011). It was performed to quantify the virucidal activity the different formulations against MS2 in the presence of 5% fetal bovine serum (FBS) (Sigma, UK) at room temperature at two distinct contact times (5 and 10 min). The main goal of this experiment was to simulate the activity of the biocidal product (formulation) prepared in Cardiff on a contaminated surface. That also enables comparison with virucidal activity reported in the patent for the same biocidal product described by Ijaz and Zhu (2014).

Four hundred µL of phage suspension (10^9 PFU mL^{-1}) containing 5% FBS was poured into glass petri dishes and spread all over the surface. Bacteriophages were allowed to dry under ambient conditions for 60 min and 2 mL of biocide was added to glass surface covered with bacteriophage layer. After an appropriate contact time, 2 mL of neutraliser was added and phages scraped off the glass surface with a L-shaped spreader. One mL was aliquoted from resulting suspension of phages. The latter were enumerated as previously described in section 3.2.3. Controls corresponded to exposure of phages to i) formulations with similar composition without zinc (CA001X, see Table 2.2); ii) formulations without active compounds (ethanol and zinc) (CA-EXCP, see Table 2.2) and iii) PBS solution (negative control). Possible loss of phage titre due to the drying process was investigated by comparison between enumerations of phage in suspension with the enumeration of phage dried on glass surface treated with PBS.

3.2.6 Virucidal suspension test against adenovirus/poliovirus

The quantitative virucidal suspension test carried out against adenovirus type 2 (AdV2) and poliovirus type 1 (Pv1) was based on the standard test EN 14476-11 (CEN, 2011). Five hundred microliters of inoculum were prepared by adding 340 µL of adenovirus type 3 stock suspension (10^9 infectious units/mL) to 160 µL of distilled water. One hundred microliter of inoculum was added to 900 µL of formulation to be tested. Upon completion of contact time, 100 µL was added to Microspin S-200 HR size-exclusion chromatography column in order to quench formulation activity and avoid cytotoxicity by formulation towards host cells. One hundred microliters of

mixture were serially diluted and added to wells with 70-80% confluence HeLa cells monolayer. Virus titre was determined by Spearman-Kaeber method as previously described for 6^{th} and 7^{th} day post-infection (Flint and Enquist, 2008).

3.2.7 Statistical analysis

All activity tests against bacteriophages were carried out with three biological replicates with at least 3 technical replicates each. The activity tests against AdV2 were performed with three biological replicates with 6 technical replicates each, whereas only one biological replicate was carried out for activity against Pv1.

The statistical significance of virucidal activity data was determined by a two-tailed Wicoxon-Mann-Whitney non-parametric test with 95% confidence interval (see Appendix A). All statistical analyses were carried out by Prism 6 software (version 6.0f, Graphpad Software).

3.3 Results

3.3.1 Neutraliser toxicity and efficacy test results – activity test against bacteriophages

Prior to virucidal activity tests, neutraliser efficiency for quenching the virucidal activity of formulation containing both alcohol and zinc as active ingredients (CA001-F, see Table 2.2), and neutraliser toxicity against MS2 and F116 were investigated. The formulation CA001-F (80% (w/v) ethanol with 0.1% Zn as actives) was selected for neutralizer efficiency as it contains the highest amount of ethanol in presence of zinc and could theoretically present higher virucidal activity. Results are shown in Tables 3.3 and 3.4. Negative control was performed by adding bacteriophage suspension to sterile water as described in section 3.2.1.

Table 3.3: Neutraliser efficiency test of formulation CA001 against MS2 and F116. Results are expressed in Log_{10}.

Bacteriophage	Log_{10} before treatment (PFU mL^{-1})	Log_{10} after treatment (PFU mL^{-1})	Log_{10} Reduction
MS2	7.10 (± 0.10)	6.90 (± 0.20)	0.26 (± 0.16)
F116	7.87 (± 0.05)	7.86 (± 0.07)	0.04 (± 0.05)

Table 3.4: Neutraliser toxicity test against MS2 and F116. Results are expressed in Log_{10}.

Bacteriophage	Log_{10} before treatment (PFU mL^{-1})	Log_{10} after treatment (PFU mL^{-1})	Log_{10} Reduction
MS2	6.44 (± 0.04)	6.30 (± 0.09)	0.14 (± 0.12)
F116	7.90 (± 0.03)	7.86 (±0.04)	0.05 (±0.03)

It was observed that the neutraliser was suitable for quenching possible virucidal activity of formulation. Differences in virus concentration before and after treatment were not significant for both MS2 and F116 (Mann-Whitney, $p>0.05$). Moreover, the neutraliser presented no significant toxicity against bacteriophages (Mann-Whitney, $p>0.05$).

Neutraliser efficiency for quenching activity of formulation containing 40% (w/v) ethanol with 0.1% zinc as active ingredients (RB002, see Table 2.2) was also carried out in presence of mammalian viruses as described in section 3.2.2. The formulation RB002 was selected for neutraliser efficiency test since it presents highest concentration of ethanol amongst formulation set to be tested against mammalian viruses. Results are shown in Table 3.5.

Table 3.5: Neutraliser efficiency test of formulation RB002 against adenovirus type 2 (AdV2) and poliovirus type 1 (Pv1). Results are shown in Log_{10}.

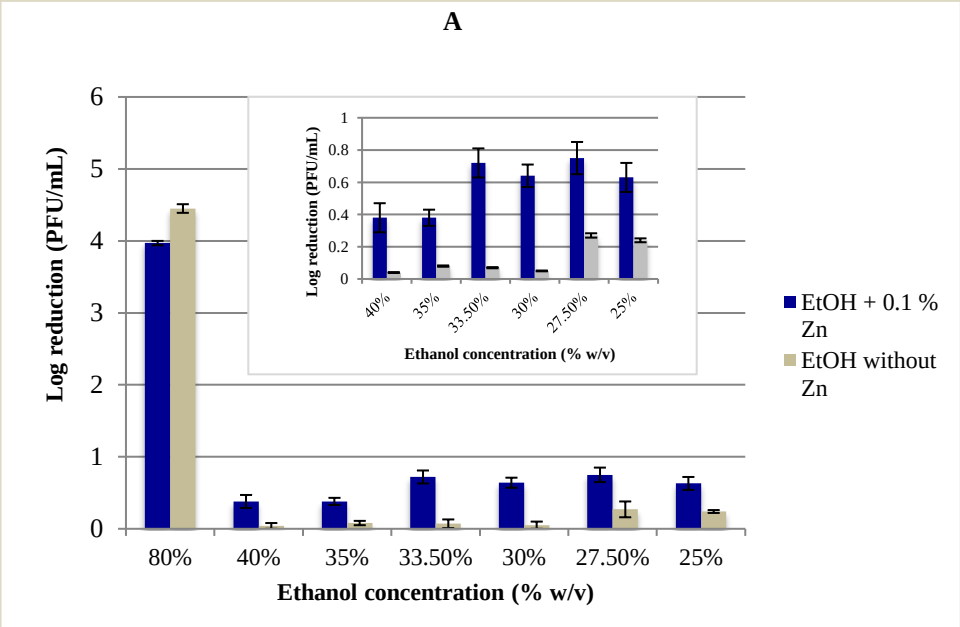
Mammalian virus	Log_{10} before treatment (PFU mL^{-1})	Log_{10} after treatment (PFU mL^{-1})	Log_{10} Reduction
Adenovirus type 2	7.34	7.20	0.14
Poliovirus type 1	6.18	5.90	0.28

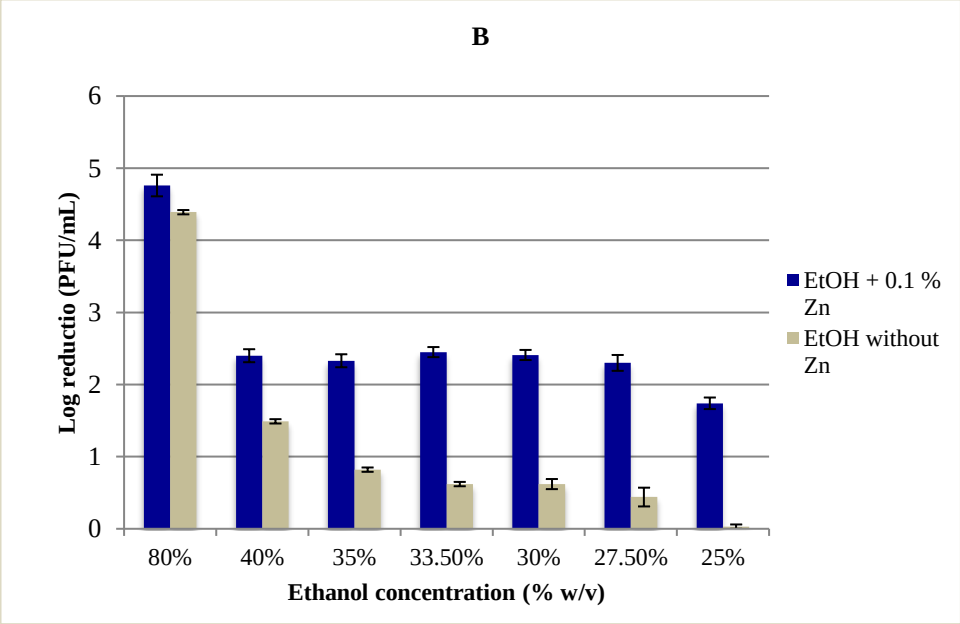
According to EN 14476 standard test for virucidal activity in suspension, neutraliser efficiency is verified when log_{10} reduction is equal or lower than 0.5. In this case, neutraliser was efficient at quenching formulation RB-002 activity against both viruses.

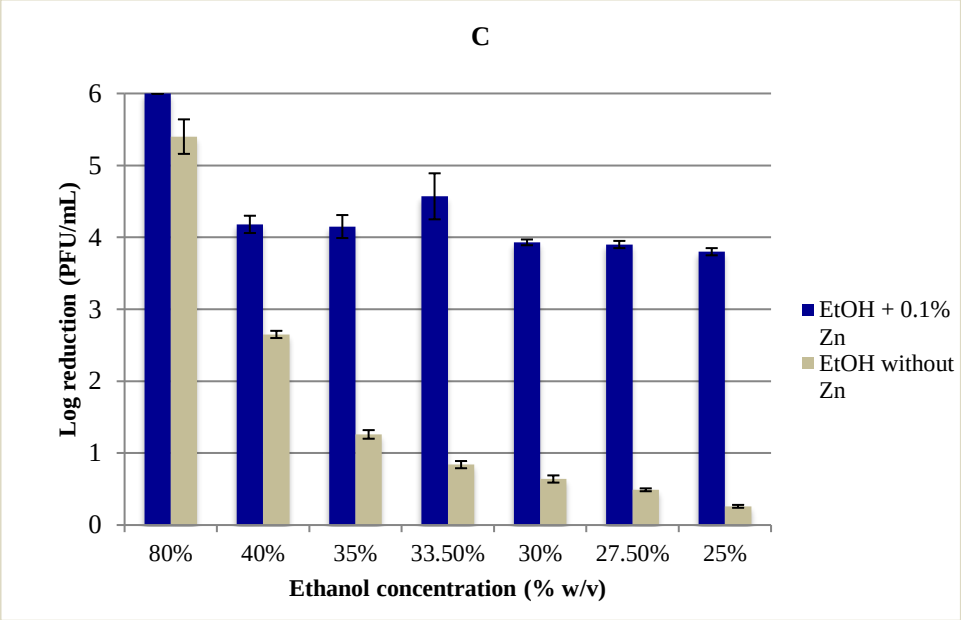
3.3.2 Virucidal suspension test against MS2

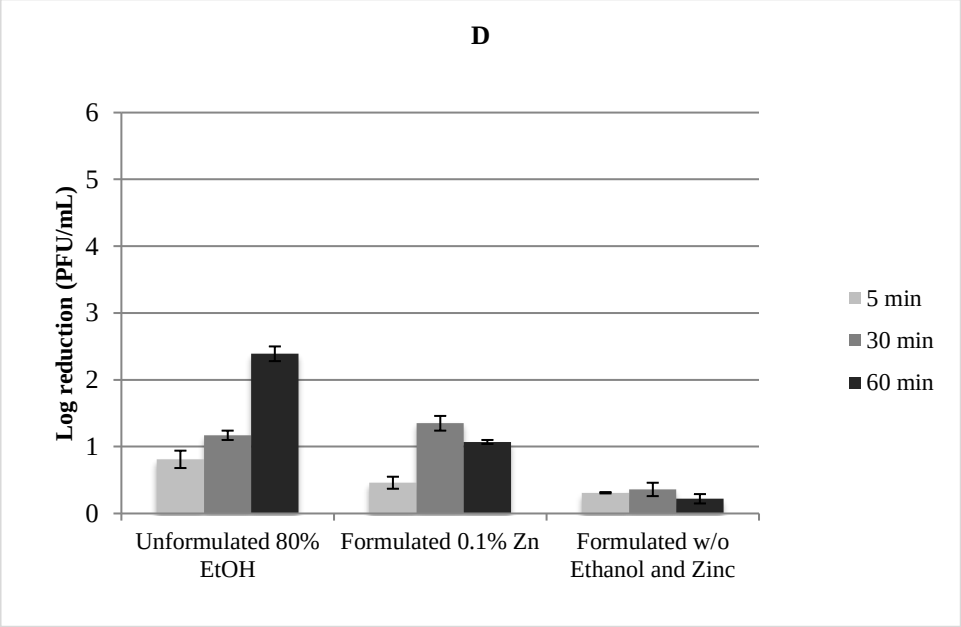
A virucidal suspension test based on the EN13610 Standard was carried out in order to quantify the virucidal activity of formulations against bacteriophage MS2. Three distinct contact times (5, 30 and 60 min) were used and results are presented in Figure 3.2.

Figure 3.2: Virucidal activity of formulations CA001 (CA001A to CA001F with different ethanol concentrations ranging from 80% to 40% (w/v) Ethanol with 0.1% (w/v) zinc sulphate heptahydrate at pH 10.5; CA-001-G, CA-001-EXCP and formulations CA001 with different ethanol concentrations without zinc (formulation CA001X to CA001FX) against MS2 in suspension at three contact times. A) 5 min; B) 30 min; C) 60 min; D) control formulations unformulated 80% (w/v) ethanol without zinc; CA001G (0.1% zinc + excipients, see Table 2.2) and CA001EXCP (excipients only, see Table 2.2) at distinct contact times. Three biological replicates with 4 technical replicates were conducted. (Note: Insert graphs show the results at reduced scale).









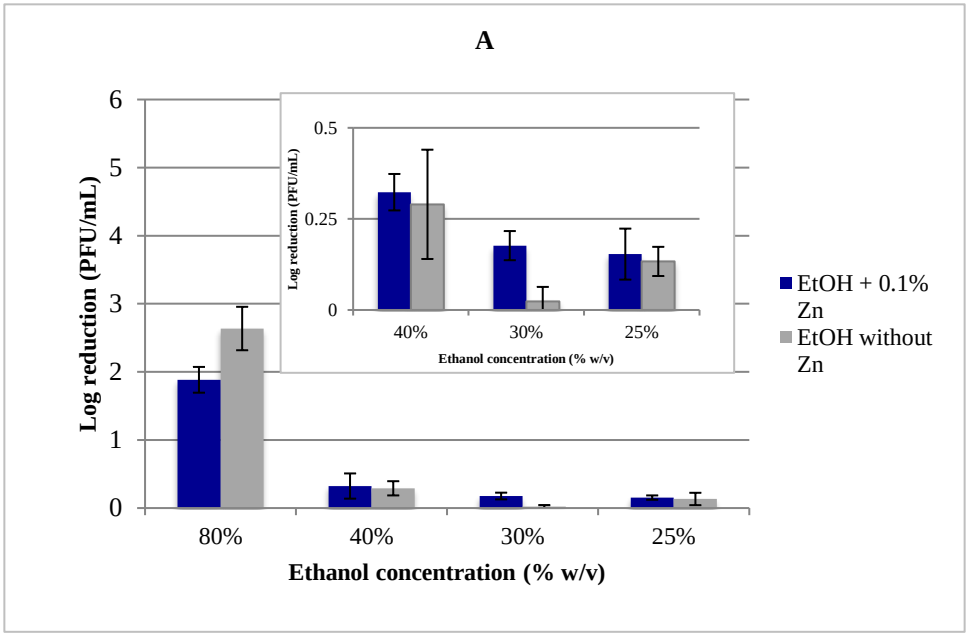
The virucidal activities associated to formulations with ethanol concentration between 40% and 25% w/v (CA001, CA001A to CA001F, see Table 2.2) were less than 1 $\log_{10}$ reduction at 5 min contact time (Fig. 3.2A). Virucidal activity at 5 min contact time of formulations CA001A to CA001F (containing alcohol and zinc as actives, see Table 2.2) was enhanced when compared to their counterparts CA001X, CA001AX to C001FX (formulations with different ethanol concentrations as sole active ingredient without zinc, see Table 2.2). There was however no significant difference (Mann-

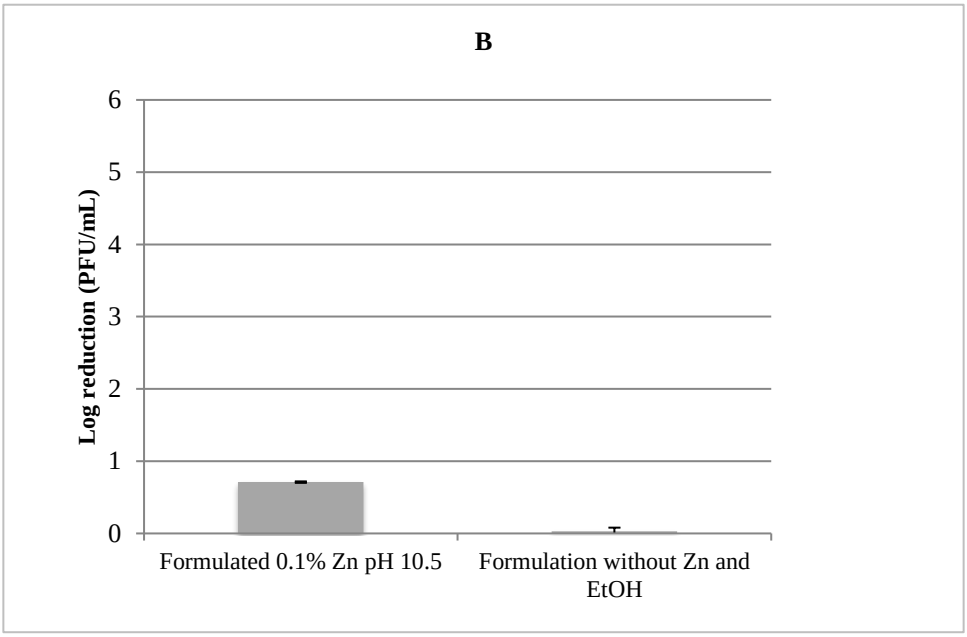
Whitney, $p>0.05$) in activity for ethanol concentration ranging from 40% (w/v) to 25% (w/v) with zinc (CA001, CA001A to CA001F, see Table 2.2). As contact time increases to 30 and 60 min, the reduction in virus concentration increased concomitantly and activity of formulation containing both ethanol and zinc (CA001) compared to counterparts without zinc (CA001X) becomes significant ($p<0.05$) for the ethanol concentration ranging from 40% (w/v) to 25% (w/v) (Fig. 3.2B and C); a 2.5 and 4 $\log_{10}$ reductions in virus concentration were achieved, although these reductions in concentration were significantly (Mann-Whitney, $p<0.05$) lower that formulation comprising 80% ethanol with zinc. It can also be observed that increased virucidal activity by the addition of zinc to the formulation is more noticeable at lower ethanol concentrations.

Controls at different contact times (80% (w/v) ethanol; CA001G and CA001EXCP) were performed to evaluate the virucidal activity associated to zinc and to other components of the formulation. By comparing results obtained at 5 min contact time, it was observed that the activity of ethanol ranging from 40% (w/v) to 25 % (w/v) with zinc (Fig. 3.2D) was comparable to the control formulation containing only zinc (Mann-Whitney, $p>0.05$). This demonstrates that the observed increased virucidal activity might be exclusively due to zinc ions. Formulation CA001EXCP (excipients only, see Table 2.2) show that excipients present no significant activity over time ranging from 5 to 60 min against bacteriophage MS2 as expected. Differences between formulations comprising ethanol and zinc compared to control CA001G (containing only 0.1% zinc as active, see Table 2.2) are significant at 30 and 60 min contact time (Mann-Whitney, $p<0.05$) (see Appendix A).

Similar formulations as the ones prepared in Cardiff were made by RB in different batches (formulation sets RB001 and RB002, see Table 2.2). The virucidal activities of formulations RB001 were evaluated and the results are shown in Figure 3.3.

Figure 3.3: Virucidal activity of RB-001, RB-001A to RB-001F (distinct ethanol concentrations with 0.1% (w/v) zinc sulphate heptahydrate as active, see Table 2.2); formulations RB001X, RB001AX to RB001FX (distinct ethanol concentrations without zinc, see Table 2.2); RB-001G (zinc as sole active ingredient) and RB-001-EXCP (excipients only) against MS2 in suspension at 5 min contact time. A) Activity test of formulations RB-001 (40% ethanol with zinc), RB-001A to RB-001F (from 35% (w/v) to 80% (w/v) ethanol respectively with 0.1% (w/v) zinc sulphate heptahydrate) and RB-001AX to RB001FX (formulation counterparts without zinc and alcohol as sole active) with different concentrations of ethanol; B) Control formulations RB-001G (0.1% (w/v) zinc as sole active) and RB-001-EXCP (excipients only). Three biological replicates with four technical replicates were performed. (Note: Insert graph shows the results at reduced scale).

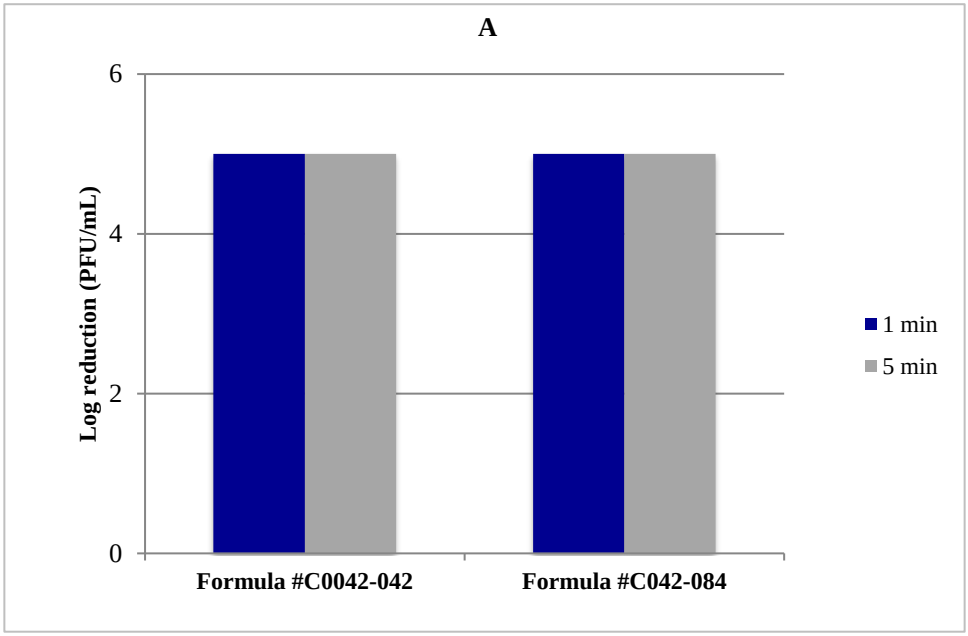


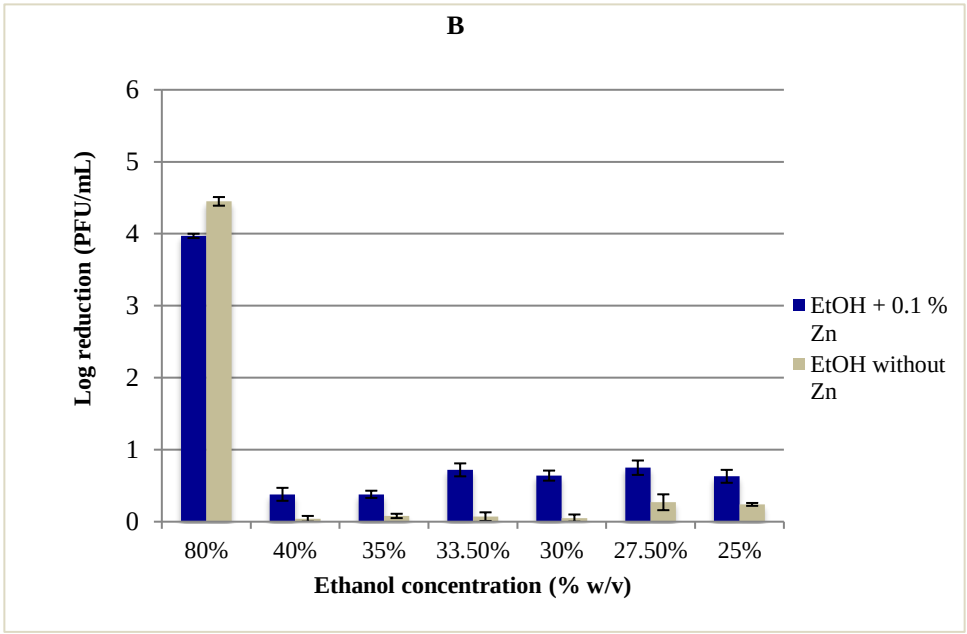


The virucidal activities were relatively low (less than 1 $\log_{10}$) for RB-001, RB-001A to RB-001E formulations with ethanol concentration ranging from 40% to 25% (w/v) (Fig 3.3A). The virucidal activities of RB-001 (40% (w/v) ethanol with 0.1% (w/v) zinc sulphate heptahydrate, see Table 2.2) were similar (Mann-Whitney, $p>0.05$) to CA-001, which attest for method consistency. Significant increased activity following the addition of zinc was only observed for formulation RB001C (containing 30% ethanol with zinc as actives) (Fig 3.3A). By comparison with control formulation RB001G (comprising only zinc as the active ingredient) (Fig. 3.3B), the activity of RB-001 with ethanol concentration range between 40 to 25% (w/v) ethanol may be exclusively due to zinc. Significant activity difference between CA001F and RB001F was found (Mann-Whitney, $p<0.05$). After investigation of formulation ingredients from suppliers between Cardiff and Reckitt Benckiser, the difference in activity observed can be attributed to the usage of industrial denaturated ethanol by Reckitt Benckiser. In Cardiff ethanol grade 99.8% (Fisher Chemicals, UK) was used.

Additional suspension tests against bacteriophage MS2 were performed at different contact times using commercial Lysol formulation at distinct contact times. The results are shown in Figure 3.4.

Figure 3.4: Virucidal suspension activity test against MS2 by exposure to different Lysol formulations at different contact times and comparison with CA001 formulations. A) Activity test on Lysol formulations (limit of detection was reached); B) Activity test on CA001 formulations. Three biological replicates with four technical replicates were performed.

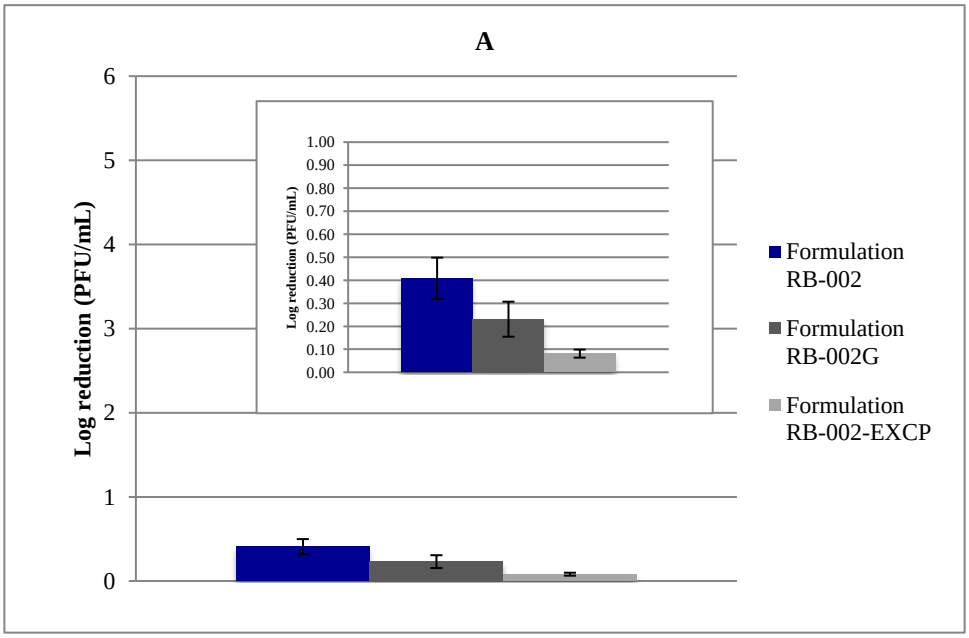


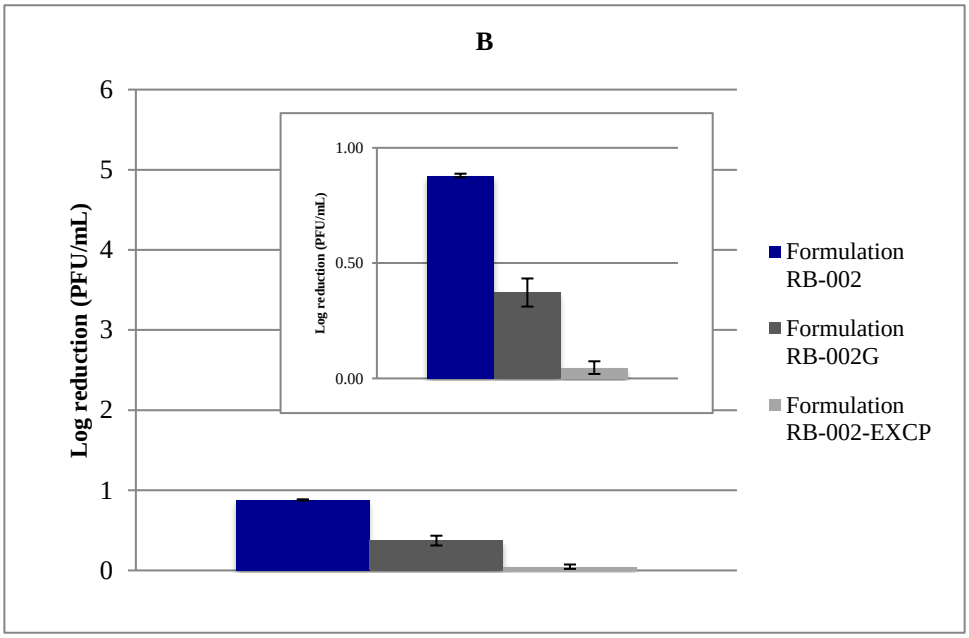


Both Lysol formulations C0042-042 and C042-084 (Fig. 3.4A) were highly effective against MS2 (more than 5 $\log_{10}$ reduction) in the first minutes of exposure when compared to formulations comprising zinc and ethanol (CA001, CA001A to CA001F) as sole active ingredients.

Formulation RB002 represented a newer batch of RB-001 for 40% (w/v) ethanol and 0.1% (w/v) zinc sulphate combination and respective control formulations (RB001G and RB001EXCP, see Table 2.2). Activity tests of formulations RB002 and derivatives were performed against MS2 at 5 and 60 min contact time and results are shown in Figure 3.5.

Figure 3.5: Virucidal activity of RB-002 formulation (40% (w/v) ethanol with 0.1% (w/v) zinc sulphate heptahydrate as actives), RB-002G (0.1% (w/v) zinc sulphate heptahydrate as sole active ingredient), RB-002EXCP (excipients only) at 5 and 60 min contact time against bacteriophage MS2. A) 5 min contact time; B) 60 min contact time. (Note: Insert graphs show the results at reduced scale).





The activity test at 5 min contact time allows a direct comparison with formulation RB-001, a previous batch of the formulation. The virucidal activities were relatively low (below 1 $\log_{10}$) at both contact times. These activities are comparable to the ones obtained for RB-001 (Mann-Whitney, $p>0.05$) and controls (RB-001G and RB-001EXCP) at 5 min contact time, which highlights the similarities between formulations and possible comparable mode of action against bacteriophage MS2 (see Figure 3.3A *vs.* Figure 3.5A).

At 60 min contact time, formulation RB-002 were the only ones presenting a significantly higher activity than at 5 min contact time. RB-002G containing zinc as sole active ingredient presented a higher activity than excipients demonstrating an increased efficacy by the addition of zinc. Overall the activity against MS2 was significantly higher when both ethanol and zinc were present (Mann-Whitney, $p<0.05$) than zinc as sole active ingredient.

3.3.3 Virucidal suspension test against bacteriophage F116

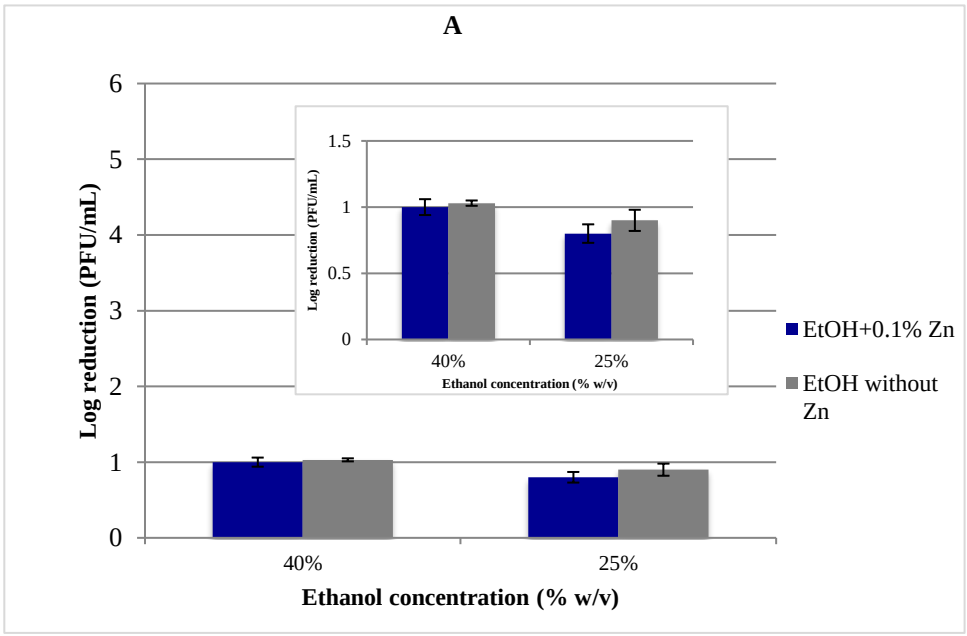
The virucidal suspension test based on EN13610 standard was used to evaluate the virucidal activity of formulations CA001 against bacteriophage F116. The results were used to define the contact time appropriate for TEM imaging studies; i.e. contact time that produced 1-$\log_{10}$ reduction in virus concentration. The results for two different contact times (5 and 10 min) are presented in Figure 3.6.

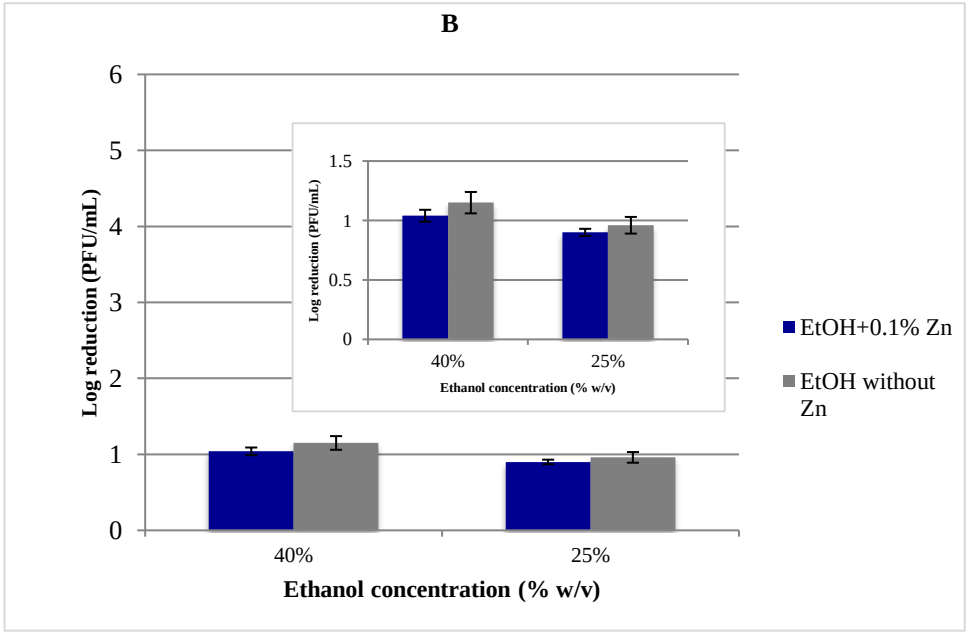
The virucidal activity associated to CA001 with 40% (w/v) ethanol (1 $\log_{10}$ at 5 and 10 min contact time) was only slightly higher compared to formulations with 25% (w/v) ethanol (0.8 $\log_{10}$ and 0.9 $\log_{10}$ for 5 and 10 min contact time, respectively). However, the difference in activity was not significant ($p>0.05$) (Fig. 3.6A). There was no significant ($p<0.05$) activity enhancement at 5 min contact time. At 10 min contact time, the virucidal activity of CA-001 containing 40% (w/v) ethanol with zinc was significantly higher ($p<0.05$) than with CA-001 with 40% ethanol only and 25% (w/v) ethanol with zinc (Fig. 3.6B). It was also observed that the differences between virucidal activity of CA001 was comparable to the one obtained for the CA001G comprising only 0.1% (w/v) zinc at 5 min, which showed that only zinc seems to contribute to virucidal activity of CA001 against F116. On the other hand, the virucidal activities for CA001 with both ethanol and zinc were significantly ($p<0.05$) higher than activity of CA-001G containing 0.1% zinc only for 10 min exposure time.

Difference between the virucidal activities against MS2 and F116 were significant ($p<0.05$), indicating that F116 was more susceptible to the formulations under study.

The virucidal activity of RB-002 formulations and derivatives were performed against bacteriophage F116 at 60 min contact time. Results are presented in Figure 3.7.

Figure 3.6: Virucidal activity of CA001 (40% (w/v) ethanol with 0.1% (w/v) zinc sulphate heptahydrate) and CA001E (25% (w/v) ethanol with 0.1% (w/v) zinc sulphate heptahydrate) against F116 at two contact times. A) 5 min contact time; B) 10 min contact time; C) control formulations CA001-G (0.1% (w/v) zinc sulphate heptahydrate as sole active) and CA001-EXCP (excipients only). Three biological replicates with four technical replicates were performed. (Note: Insert graphs show the results at reduced scale).





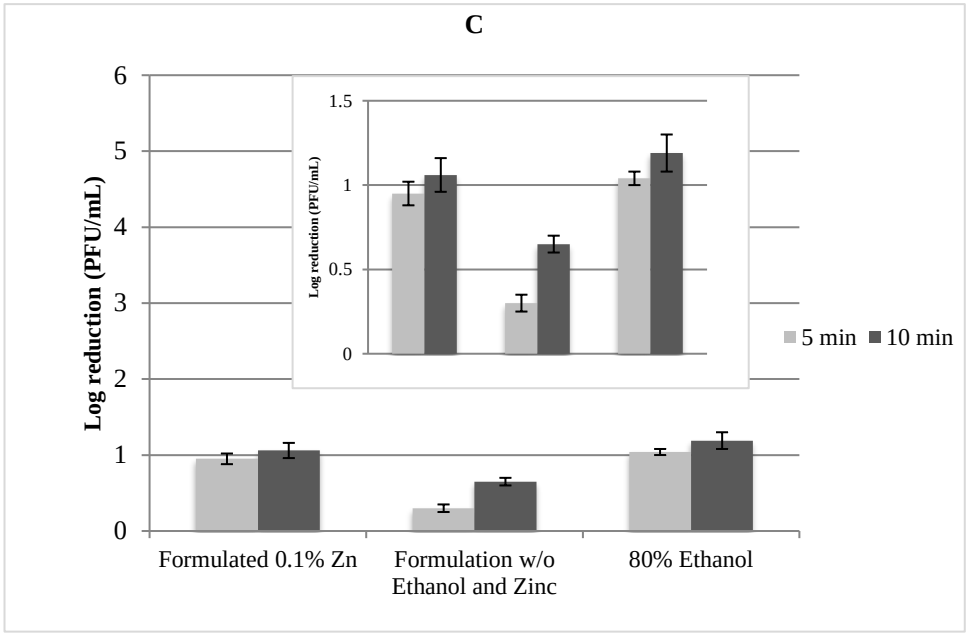
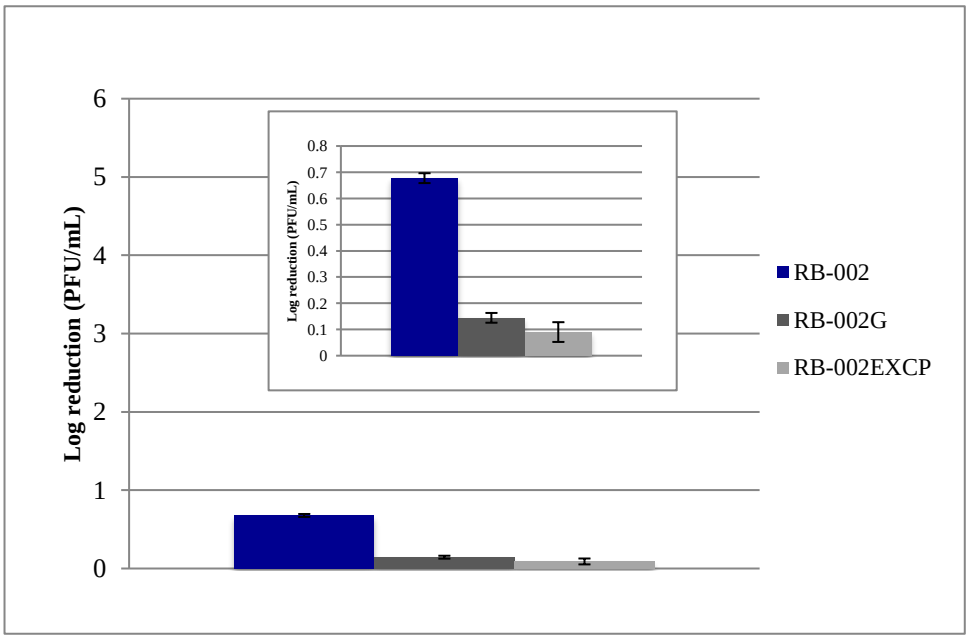


Figure 3.7: Virucidal activity of RB-002 formulation and derivatives at 60 min contact time against bacteriophage F116. RB-002 has 40% (w/v) Ethanol with 0.1% (w/v) $ZnSO_4.7H_2O$ as active ingredient; RB-002G 0.1% (w/v) $ZnSO_4.7H_2O$ and RB-002-EXCP is a negative control with formulation excipients only. (Note: Insert graph shows the results at reduced scale).

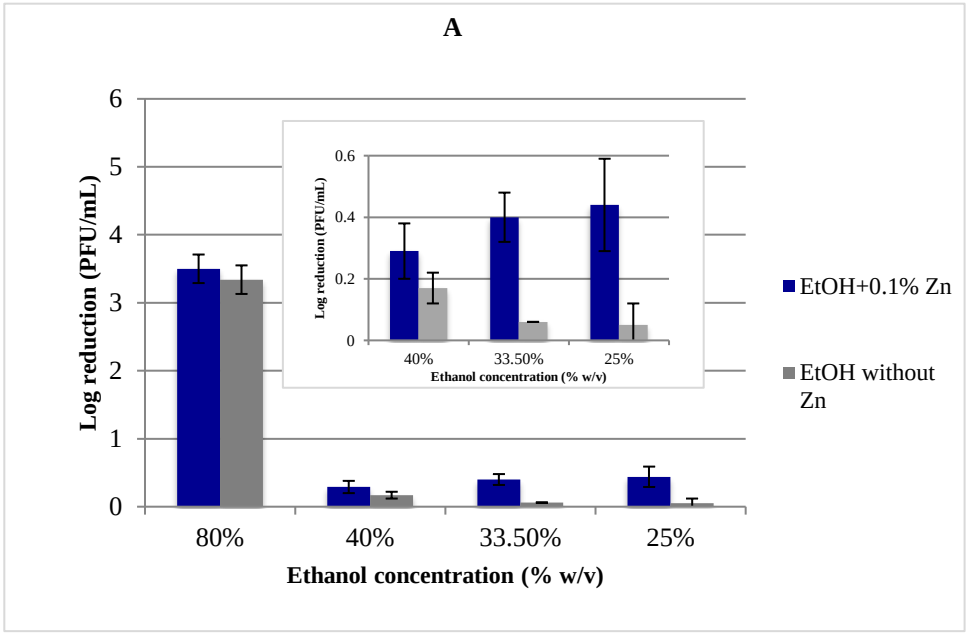


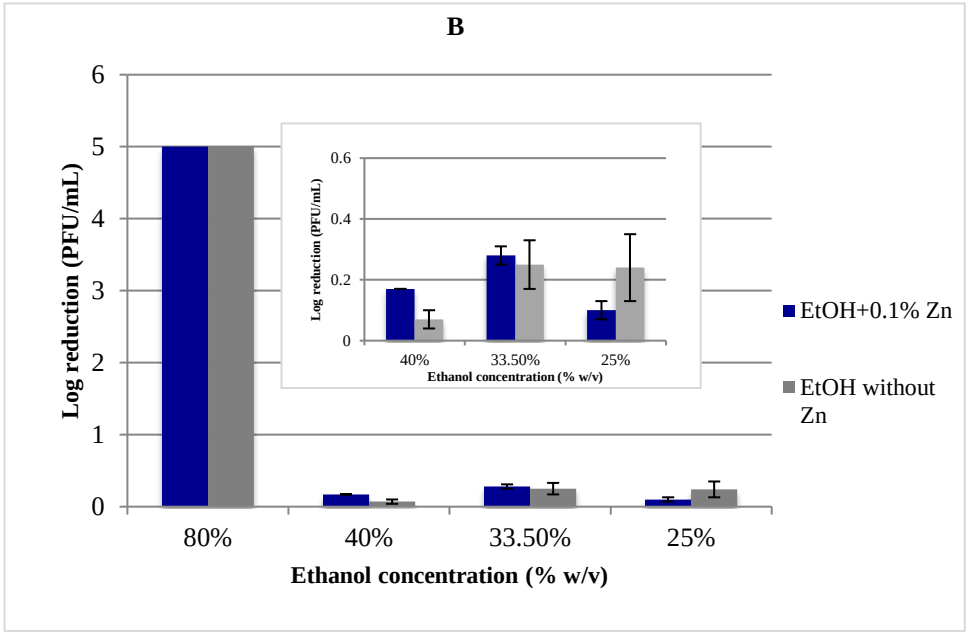
Similarly to the activity test against MS2, RB-002 formulations yielded relatively low efficacy against F116 (lower than 1 $\log_{10}$). Efficacy against F116 was comparable to MS2 ($p<0.05$) and this highlights bacteriophages insusceptibility to the formulations. Although the formulation RB-002G with zinc as sole active ingredient also presented higher efficacy than excipients, the difference was not significant ($p>0.05$), similarly to the result obtained against MS2.

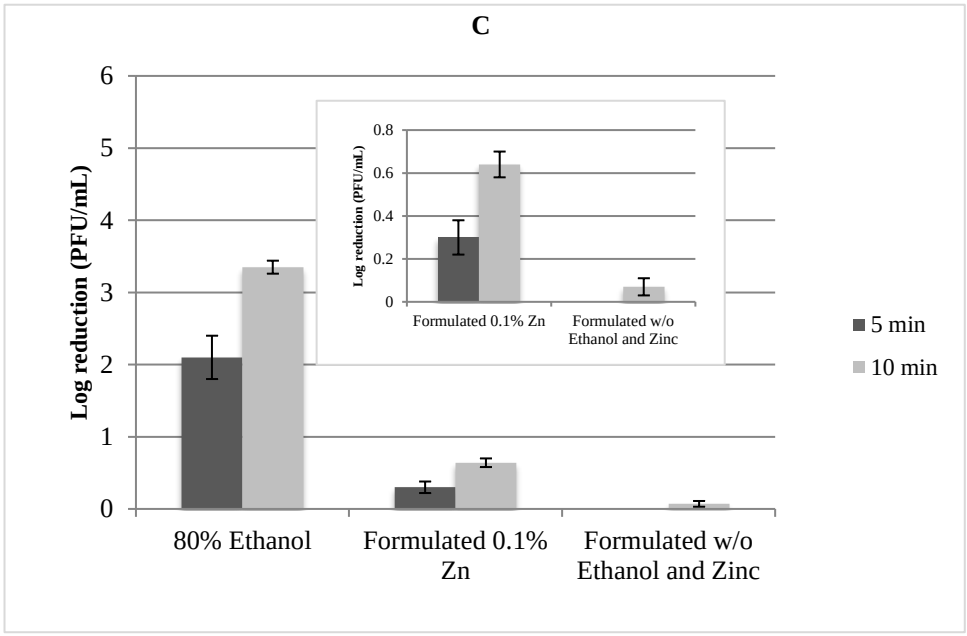
3.3.4 Hard surface carrier virucidal activity test against MS2

The hard surface carrier test was based on the ASTM 1053 standard and adapted for bacteriophages. The experiment was carried out to quantify the virucidal activity of formulations CA001 against bacteriophage MS2 dried on an inanimate glass surface in presence of soil load (5% fetal bovine serum) at different contact times. Figure 3.8 shows the results obtained for the formulations and controls at 5 and 10 min contact time.

Figure 3.8: Virucidal activity of CA001 (40% (w/v) ethanol with 0.1% (w/v) zinc), CA001B (33.5% (w/v) ethanol with 0.1% (w/v) zinc), CA001E (25% (w/v) ethanol with 0.1% (w/v) zinc) and CA001F (80% (w/v) ethanol with 0.1% (w/v) zinc) and counterparts without zinc (CA001X; CA001BX; C001EX; CA001FX formulations) against MS2 dried on an inanimate glass surface at two contact times. A) 5 min; B) 10 min; C) Control formulations unformulated 80% (w/v) ethanol CA001G (0.1% (w/v) zinc as sole active) and CA001EXCP (excipients only) at distinct contact times. Three biological replicates with four technical replicates were performed. (Note: Insert graphs show the results at reduced scale).



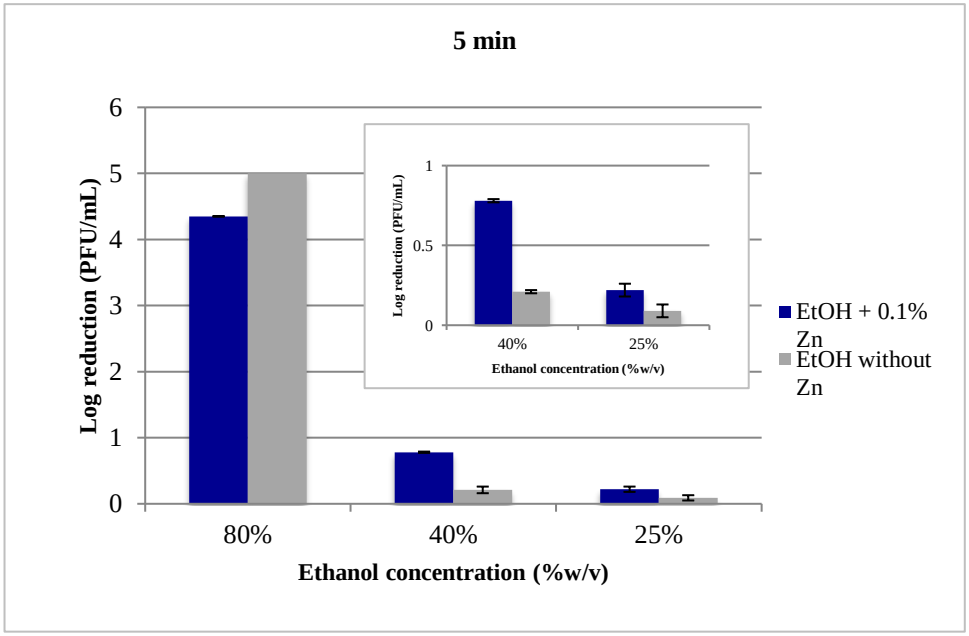




CA001F containing 80% (w/v) ethanol with zinc presented higher activity than the control formulation CA001FX comprising only 80% ethanol (w/v) (Fig. 3.8A). An increase in virucidal activity when zinc is present was observed for lower ethanol concentrations similarly to the activity tests of bacteriophage MS2 in suspension (see Fig. 3.2 *vs.* Fig. 3.8). At 5 min contact time, significant differences ($p<0.05$) in activity were found between CA001BX containing 33.5% (w/v) and CA001EX with 25% (w/v) ethanol only and their respective counterparts containing ethanol and zinc (Fig 3.8A). Nevertheless, there was no evidence (Mann-Whitney, $p<0.05$) of increased activities for the formulations containing 25% (w/v) to 40% (w/v) ethanol with zinc at 10 min contact time (Fig. 3.7B). The control formulation CA001G containing only 0.1% (w/v) zinc sulphate showed a virucidal activity similar to the test formulations within 5 min exposure time (0.30 $\log_{10}$) (Fig. 3.7C). This result shows that the activity may be exclusively due to zinc ions, which improves overall activity of formulations CA001 within the first 5 minutes of contact. On the other hand, significant activity differences (Mann-Whitney, $p<0.05$) were obtained between 25 to 33.5% ethanol with zinc and control formulation containing 0.1% zinc only as the active ingredient at 10 min contact time. It is noteworthy that results obtained for the hard surface test at 5 min contact time are comparable to the ones attained with the EN13610 virucidal suspension test in terms of activity enhancement by addition of zinc.

The virucidal efficacy of RB-001 formulations was also evaluated with a hard surface test and results are presented in Figure 3.9.

Figure 3.9: Virucidal activity of RB-001 (40% (w/v) ethanol with 0.1% (w/v) zinc), RB-001E (25% (w/v) ethanol with 0.1% zinc) and RB-001F (80% (w/v) ethanol with 0.1% zinc); RB-001X (40% (w/v) ethanol as sole active), RB-001EX (25% (w/v) ethanol) and RB-001FX (80% (w/v) ethanol) against MS2 dried on an inanimate glass surface at 5 min contact time. Three biological replicates and two technical replicates were performed. Limit of detection was reached for RB-001F, hence no error bar is shown. (Note: Insert graphs show the results at reduced scale).

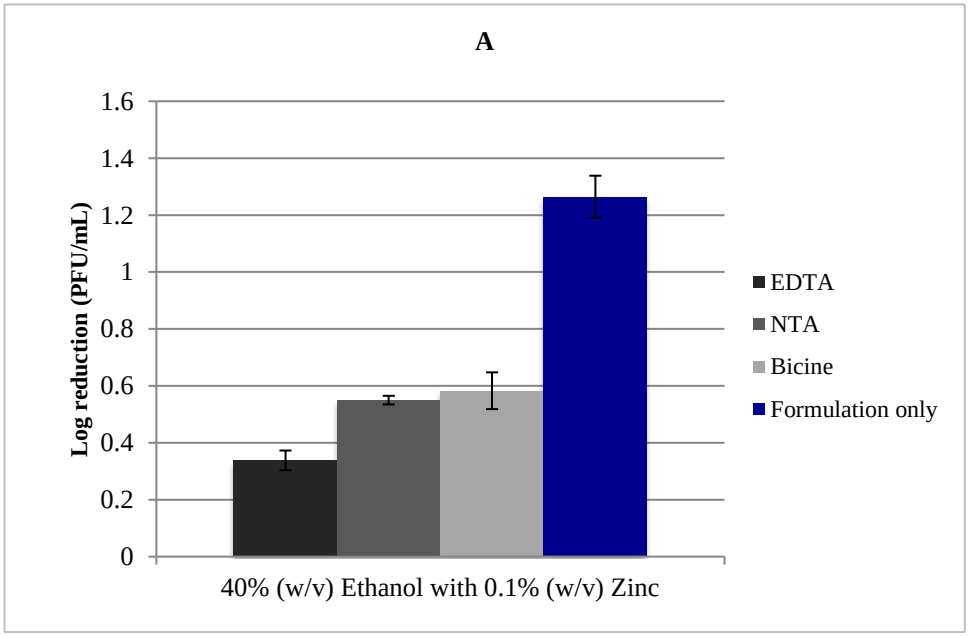


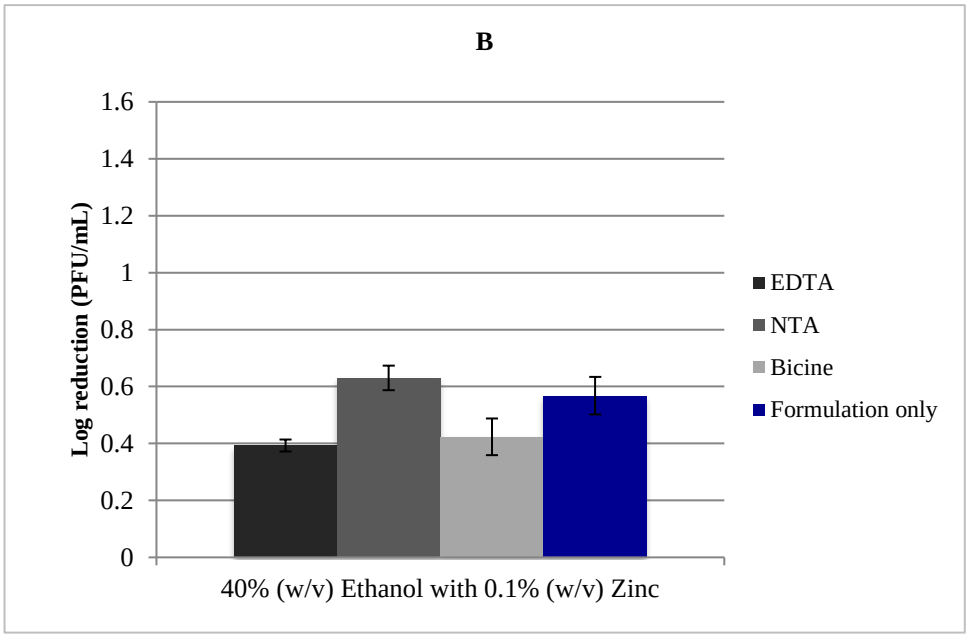
Significant (Mann-Whitney, $p<0.05$) increased in activity by addition of zinc was found for formulations containing 40 and 25% ethanol (w/v), although overall activities attained were low (< 1 $\log_{10}$). Reductions in virus concentration were comparable to the ones obtained with CA001 formulations (Fig 3.8), with the exception of the formulation RB-001FX comprising 80% ethanol.

3.3.5 Virucidal suspension test against MS2 in presence of chelators

The virucidal suspension test based on EN13610 standardised test in presence of chelating agents with different degrees of complex stability with zinc was carried out separately with CA001 and RB-001 at 60 min contact time. Ethylenediamine tetraacetic acid (EDTA) was the strongest chelator, nitrilotriacetic acid (NTA) an intermediary and bicine the weakest chelator of zinc ions used in the present study. The formulation activity against bacteriophage MS2 is shown in Figure 3.10.

Figure 3.10: Virucidal suspension test with CA001 and RB-001 formulations in presence of different chelators at 60 min contact time against bacteriophage MS2. A) Activity test on formulation RB-001; B): Activity test on formulation CA001. Three biological replicates with four technical replicates were performed.

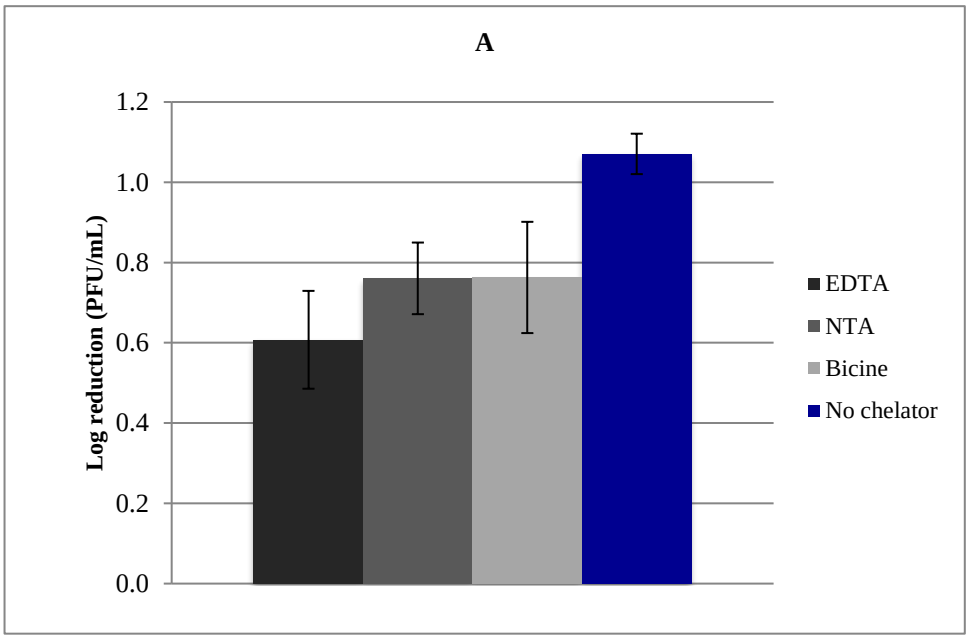


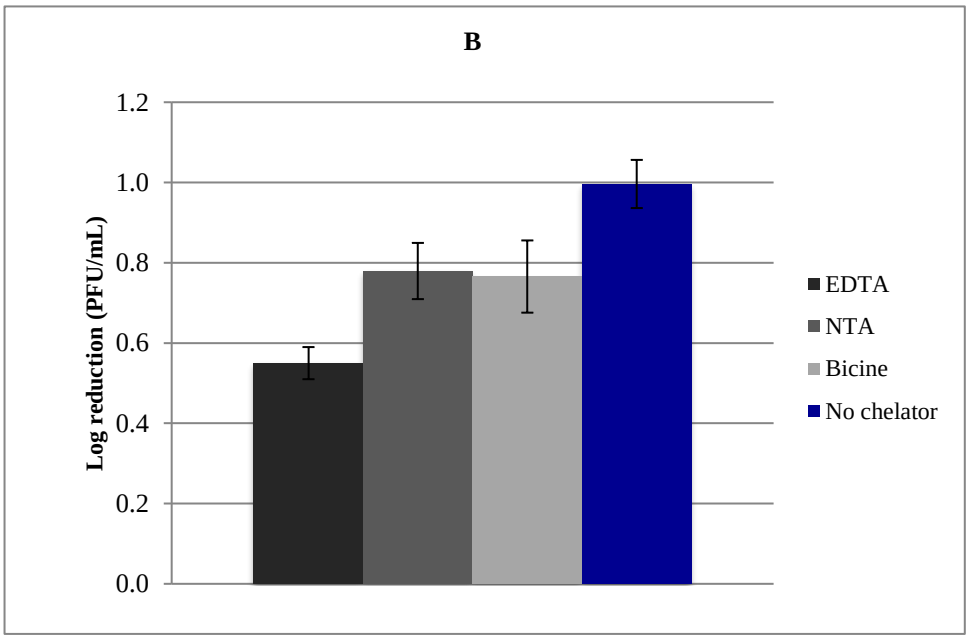


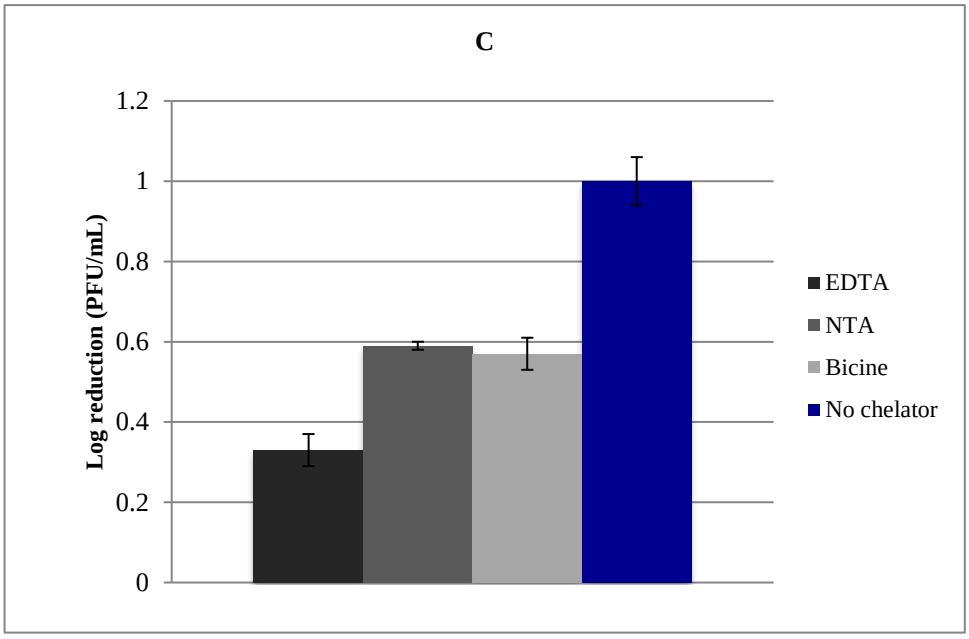
The activity of formulation RB-001 against MS2 was more affected by addition of chelating agents than CA001 (Fig. 3.9). EDTA was the most efficient chelator ($p<0.05$) to decrease virucidal activity. The significant reduction in virucidal activity following the use of the different chelators informs that zinc ions present in RB-001 bind weakly to bacteriophage MS2, with a complex stability lower than 6.50. On the other hand, CA001 was not strongly affected ($p>0.05$) by the addition of chelators, indicating that there may be a difference in terms free zinc ions between formulations CA001 and RB-001.

The same test in presence of chelating agents was carried out for RB-002 formulation and its activity was monitored over time by repeating the same activity test at different time points. That was performed at distinct time points in order to verify chemical stability of formulation system containing alcohol and zinc over time. The results are presented in Figure 3.11.

Figure 3.11: Virucidal suspension test of RB-002 formulation (40% (w/v) ethanol with 0.1% (w/v) zinc sulphate heptahydrate as actives) against MS2 in presence of different chelating agents at different times. A) Activity test performed 1 month after formulation preparation; B) 6 months after formulation preparation; C) 12 months after preparation.







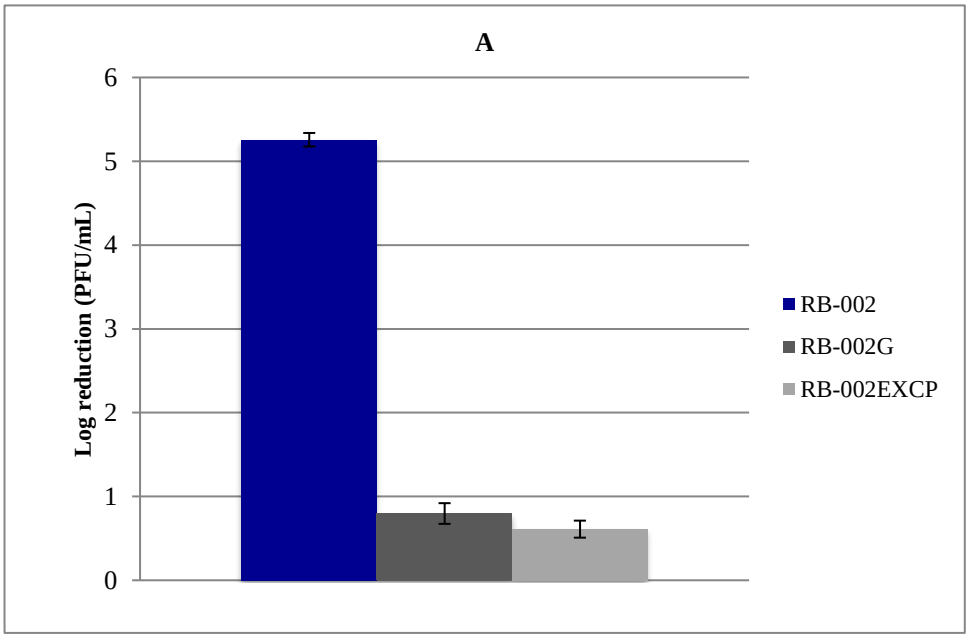
Although the activity of formulation RB-002 against MS2 remained the same (around 1 log_{10}) in absence of chelating agents after 12 months, it is noteworthy that impact of chelators is more pronounced after 12 months of preparation. The most significant (Mann-Whitney, $p<0.05$) reduction in activity was achieved in presence of EDTA (see Fig. 3.11 C) as expected. Bicine and NTA presented similar impact on formulation activity over the first 6 months of preparation (around 0.77 and 0.79 $log_{10,}$ respectively) and led to lower activity after 12 months (around 0.56 and 0.59 log_{10} respectively). This may be an indication that quantity of free zinc may change along time in formulation RB-002, hence there might be a distinct zinc coordination over time. This could possibly affect formulation stability. Similarly to formulation RB-001, zinc tends to bind weakly to bacteriophage and binds preferentially to the chelating agents as overall activity against MS2 was significantly reduced (Mann-Whitney, $p<0.05$).

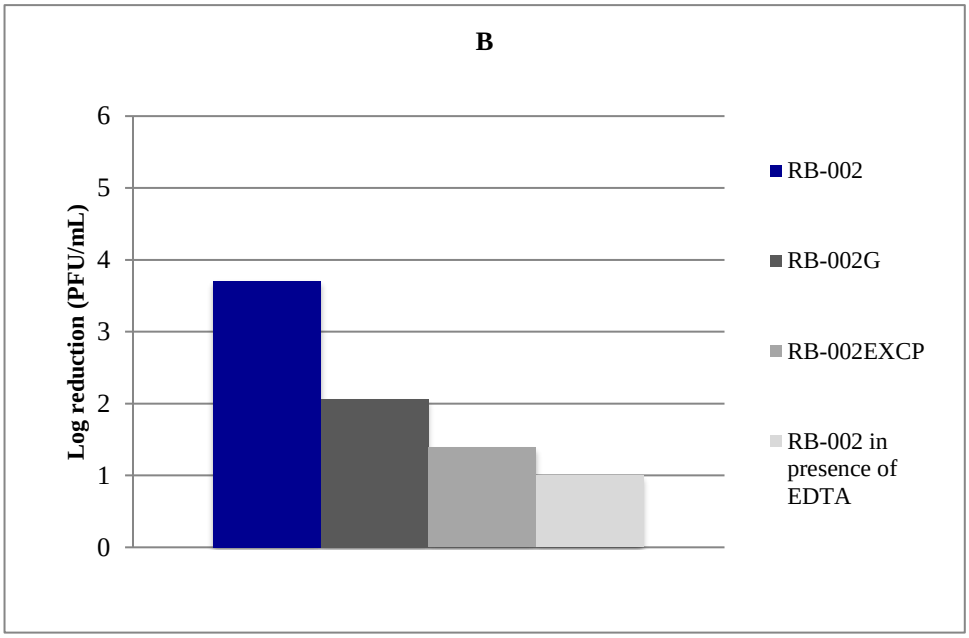
3.3.6 Virucidal suspension test against Adenovirus type 2 (AdV2) and Poliovirus type 1 (Pv1)

The virucidal suspension test of formulation RB-002 and derivatives was performed against AdV2 and Pv1 according to the EN14476 standard in clean conditions, i.e. with sterile hard water as the only interfering substance without organic

load. The main purpose was i) to evaluate formulation system activity against human pathogens like adenovirus and poliovirus and ii) to compare the virucidal activities against bacteriophage MS2 and F116 with mammalian viruses. Due to relatively low activity of the RB-002 formulations against bacteriophages, activity tests against mammalian viruses were performed only at 60 min contact time. The formulations activity against aforementioned mammalian viruses is presented in Figure 3.12.

Figure 3.12: Virucidal suspension test of RB-002 (40% (w/v) ethanol with 0.1% (w/v) zinc sulphate heptahydrate as actives); RB-002X (40% (w/v) with QAC as actives); RB-002G (0.1% zinc sulphate heptahydrate as sole active) and RB-002EXCP (excipients only) formulations against mammalian viruses at 60 min contact time in clean conditions. A) Activity test against Adenovirus type 2 (AdV2); B) Activity test against Poliovirus type 1 LSc-2ab (Pv1). Three independent biological replicates with 6 technical replicates were performed for activity against AdV2, whereas only one biological replicate with 6 technical replicates was attained for activity against Pv1.





The virucidal activities of RB-002 formulation containing 40% ethanol and 0.1% zinc sulphate as active ingredients against AdV2 and Pv1 were 5.2 and 3.7 $\log_{10}$ at 60 min contact time respectively (see Figure 3.12 A and B). The activities were significantly higher when compared to formulation RB-002G ($p<0.05$) counterpart containing zinc sulphate as sole active ingredient. For instance, RB-002G activities against AdV2 and Pv1 were 0.8 and 2.1 $\log_{10,}$ respectively. However, it can be noted that activity of RB-002G against Pv1 was much higher when compared to AdV2 pointing out that zinc may have a more active role against Pv1 when compared with AdV2. The excipients as sole components represented by formulation RB-002EXCP presented residual activity against both AdV2 and Pv1. The activity of RB-002EXCP (excipients only) against Pv1 was higher indicating higher susceptibility of the virus to the formulation excipients. On the other hand, AdV2 was shown to be more susceptible than Pv1 to formulation RB-002). It is important to stress out that activity against Pv1 was carried out with only one biological replicate, hence more replicates are necessary to verify the impact of formulations RB-002 against Pv1 and consequently evaluate if there are significant differences in susceptibilities between AdV2 and Pv1. Although standard activity test are not identical, the overall activities of RB-002 and RB-002G against mammalian viruses were significantly higher ($p<0.05$) than their activities against bacteriophages MS2 and F116 under same conditions, i.e. clean conditions with exposure to formulation for 60 min in suspension. Therefore, bacteriophages F116 and MS2 may not be suitable surrogates for mammalian viruses AdV2 and Pv1 for testing

these types of formulations. However, it is noteworthy that trends observed amongst formulations are similar between bacteriophages and mammalian viruses. For instance, the activity differences between RB-002 and RB-002G against F116 are similar to the ones presented against AdV2. Finally, RB-002 activity against Pv1 was reduced approximately 4 times when EDTA chelating agent was present.

3.4 Discussion

The virucidal activity of formulations containing different concentrations of ethanol with 0.1% zinc sulphate heptahydrate at pH 10.5 against MS2 were assessed with different virucidal standard protocols. A summary with the main results obtained through different activity standard tests of several formulations against non-enveloped viruses is presented in Table 3.6.

Table 3.6: Summary of the main results of different formulations virucidal activities against non-enveloped viruses in the present study.

Formulation set	Formulation	Test	Contact time	Virus	Viral titre reduction (log_{10})
Formulation prepared in Cardiff	CA001 (base formulation)	EN13610	60 min	MS2	4.18±0.12
			5 min	F116	0.98±0.06
		ASTM1053	5 min	MS2	0.29±0.09
	CA001A	EN13610	60 min	MS2	4.15±0.16
	CA001B	EN13610	60 min	MS2	4.57±0.32
		ASTM1053	5 min		0.40±0.08
	CA001C	EN13610	60 min	MS2	3.93±0.04
	CA001D				3.90±0.05
	CA001E	EN13610	60 min	MS2	3.80±0.05
			5 min	F116	0.85±0.07
		ASTM1053	5 min	MS2	0.44±0.15
	CA001F	EN13610	60 min	MS2	>6
		ASTM1053	5 min	MS2	3.5±0.20
	CA001X	EN13610	60 min	MS2	2.65±0.09
			5 min	F116	1.03±0.03
		ASTM1053	5 min	MS2	0.17±0.05
	CA001AX	EN13610	60 min	MS2	1.26±0.06
	CA001BX	EN13610	60 min	MS2	0.84±0.05
		ASTM1053	5 min		0.06±0.00
	CA001CX	EN13610	60 min	MS2	0.64±0.07
	CA001DX				0.49±0.05
	CA001EX	EN13610	60 min	MS2	0.26±0.04
			5 min	F116	0.94±0.08
		ASTM1053	5 min	MS2	0.05±0.07
	CA001FX	EN13610	60 min	MS2	5.40±0.24
		ASTM1053	5 min		3.34±0.20
	CA001G	EN13610	60 min	MS2	1.07±0.03

			5 min	F116	0.95±0.07
		ASTM1053	5 min	MS2	0.30±0.08
		EN13610	60 min	MS2	0.22±0.07
	CA001EXCP		5 min	F116	0.31±0.05
		ASTM1053	5 min	MS2	0
Formulations sent by RB (December 2014)	RB-001	EN13610	60 min	MS2	1.26±0.07
			5 min		0.32±0.17
		ASTM1053			0.78±0.08
	RB-001C	EN13610			0.18±0.05
	RB-001E	EN13610			0.15±0.03
		ASTM1053			0.22±0.05
	RB-001F	EN13610			1.88±0.19
		ASTM1053			4.35±0.09
	RB-001X	EN13610	5 min		0.29±0.10
		ASTM1053			0.21±0.05
	RB-001CX	EN13610			0.02±0.02
	RB-001EX	EN13610			0.13±0.09
		ASTM1053			0.09±0.04
	RB-001FX	EN13610			2.64±0.32
		ASTM1053			>5
Formulations sent by RB (September 2015)	RB-002	EN13610	60 min	MS2	0.88±0.04
				F116	0.68±0.02
		EN14476		AdV2	5.26±0.10
				Pv1	3.71
	RB-002G	EN13610		MS2	0.37±0.06
				F116	0.14±0.02
		EN14476		AdV2	0.80±0.12
				Pv1	2.06
	RB-002EXCP	EN13610		MS2	0.05±0.03
				F116	0.09±0.04
		EN14476		AdV2	0.61±0.10
				Pv1	1.39
Lysol	#C0042-42	EN13610	5 min	MS2	>5

formulations	#C042-084	5 min	>5

Increased activity linked to the presence of zinc was only observed after 30 min and 60 min contact time. Ijaz and Zu (2014) evaluated the virucidal activity of a similar formulation to CA001 and RB-001 against poliovirus-type 1 with the ASTM 1052 suspension test and obtained an activity greater than 6 $\log_{10}$ virus titre reduction after 5 min contact time. MS2 possesses similar properties (size, capsid geometry, nucleic acid) to poliovirus-type 1 (Macinga *et al*., 2008) and that the test methods (suspension test) were similar, the difference between results obtained here and the literature can be attributed to: i) mass transfer resistance caused by an improper mixing of active compounds (mass transfer resistance); ii) by the need of multiple targets on MS2 or iii) by differences in formulation chemistry. The mass transfer resistance hypothesis should be taken into account since virucidal activity of CA001 increased over time ranging from 5 to 30 and 60 min contact time. This might be associated to specific mixing times of the active ingredients and interaction with the target. For instance, MS2 bacteriophage is known to be a non-enveloped hydrophobic phage (Farkas *et al.*, 2014).
Formulations containing 80% (w/v) ethanol were used as positive control to compare with results from the literature, i.e. those formulations were used to verify that the standard test was suitable for analysis of virucidal activity. Jones *et al.* (1991) obtained a 0.70 MS2 $\log_{10}$ titre reduction after 2 min contact time with 70% ethanol, which is comparable to the results obtained in the present study. Maillard *et al.* (1994) demonstrated a 3.675 $\log_{10}$ reduction in MS2 titre with 70% ethanol for 20 min. The Lysol formulations (#C0042-42 and #C042-084) were also important for evaluating methodology in terms of virucidal efficacy where activity against MS2 were higher than 5 $\log_{10}$. Higher activity of Lysol formulations can be attributed to presence of additional active ingredients such as quaternary ammonium compounds (QACs), biguanides and synergy between ingredients. For instance, Pinto et al. (2010) obtained more than 5 $\log_{10}$ reduction by exposing MS2 to formulation with polyhexamethylene biguanide as active ingredient. The addition of QAC (Onyxide 3300) may have an impact on observed virucidal activity by #C0042-42 and #C042-084 against MS2 despite QAC being known to present relatively lower activities against non-enveloped virus (Feliciano *et al.*, 2012; Whitehead and McCue, 2010; McDonnell and Russel, 1999; Maris, 1995; Rutala *et al.*, 2006). For instance, Solomon *et al*. (2009) observed a QAC (Sentramax) yielded around 0.63 $\log_{10}$ reduction activity against MS2 in 10 min with a

modified carrier test. These results are in agreement with the literature where Su and D'Souza (2012) found out high initial titre of MS2 was reduced by an average 2 $\log_{10}$ regardless of QAC concentration at 2 hours contact time. Therefore, it is possible that observed activity increase for Lysol formulations against MS2 may be a purely additive effect of ethanol, QAC activity, metal ion and biguanides against the bacteriophage.

By comparing the results at 5 min contact time, the activity of ethanol with zinc system in formulations CA001 and RB-001 is comparable to the control formulations (CA001-G and RB-001G) containing only 0.1% (w/v) zinc. This demonstrated that activity might be exclusively due to zinc ions as excipients as sole ingredients (CA001EXCP; RB-001EXCP and RB-002EXCP) present activities lower than formulations containing zinc over time.

Virucidal efficacies lower than 1 $\log_{10}$ were obtained when formulations were tested against MS2 dried on hard surface. The control formulation CA001G containing 0.1 % (w/v) zinc sulphate presented comparable activity at the same contact times to formulations CA-001 containing both active ingredients indicating that the formulation activity might be exclusively due to zinc ions in the first minutes of exposure. It is also possible that MS2 might not be a suitable human virus surrogate for evaluating the virucidal activity associated to these specific formulations when tested with surface test.

Virucidal activity of formulations was also tested against bacteriophage F116. No evident increase in virucidal activity was observed in the presence of ethanol and zinc within 10 min contact times. Activity for control formulation CA001-FX composed by 80% (w/v) ethanol was similar to the one reported in a previous study (Maillard *et al.*, 1993). Although low activities (approximately 1 $\log_{10}$ reduction) were observed at 5 min contact time against MS2 and F116, it was observed that F116 was significantly more susceptible to the formulations comprising ethanol and zinc. Despite not being regarded as an accurate model for assessing biocides virucidal activity, F116 is a suitable model for studying the impact of formulations on virus structure (Maillard *et al.*, 1995)

Similar virucidal activity tests were performed against MS2 with RB-001 and RB-002 formulations to evaluate methodology consistency between laboratories and formulation chemistry differences. RB-001 results were comparable to CA001, except for formulations containing 80% ethanol. This difference was associated to the presence of industrial denaturated ethanol in RB-001F for instance. Denaturated ethanol is known to present other alcohol molecular entities such as methanol, which may influence

overall formulations RB-001F and RB-001FX activities (Noda *et al.*, 1981). Consequently, activity enhancement by addition of zinc at the ethanol concentration ranging from 33.5% (w/v) to 25% (w/v) could also be due to presence of denaturated ethanol. The formulation RB-002 presented similar activities to RB-001 against MS2 since they are the same formulations from different batches.

From the virucidal suspension test in presence of chelators, it was verified that zinc definitely plays a role in the activity of the formulation RB-001 and RB-002 through interactions with the bacteriophage MS2. Moreover, it also points that zinc availability in the formulation CA001 may be reduced when compared to formulations RB-001 an RB-002 as the former was not significantly affected by addition of chelating agents. This may be an indication that zinc is already complexed with other formulation excipients and not available for chelation or binding to virions. EDTA was the most efficient ($p<0.05$) to decrease the virucidal activity due to its strong chelating capacity with zinc (complex stability with zinc of 16.50) (Flora and Pachauri, 2010). Additionally, it is reported in the literature that EDTA binds tightly with zinc ions and it competes with proteins containing zinc-binding motifs (Michael *et al*., 1992; Nyborg and Peersen, 2004). Regarding formulation stability in terms of virucidal activity exclusively, it was shown that the impact of chelating agents such as EDTA was more pronounced 12 months after formulation RB-002 preparation. This may indicate a change of zinc coordination along time, which could possibly yield a different activity in the future.

Suspension tests against mammalian viruses were also carried out in order to evaluate the activity of formulations RB-002. Mammalian viruses are more suitable target organisms than bacteriophages due to their pathogenicity towards humans. Besides, it is important to analyse the use of bacteriophages MS2 and F116 as surrogates for these formulations activities against non-enveloped viruses. AdV2 was shown to be more susceptible to RB-002 (ethanol and zinc as active ingredients) than Pv1.

The observed activity difference between adenovirus and poliovirus may be related to activity of ethanol. Several studies in the literature have reported higher susceptibility of adenoviruses to alcohol, where results were shown to be variable across different alcohol aliphatic chains (Tyler et al., 1990; Kampf *et al*., 2013; Steinmann *et al*., 2013; Kampf, 2016).

Moreover, the distinct susceptibilities between AdV2 and Pv1 could be related to differences in terms of capsid surface charges and hydrophobicity nature where Pv1 was reported to be more hydrophilic than AdV2 (Bales and Li, 1993; Ionidis et al., 2016). On the other hand, Pv1 was demonstrated to be more susceptible than AdV2 to zinc as sole active ingredients and excipients (RB-002G and RB-002EXCP), where RB-002G was more effective against both viruses compared to RB-002EXCP.

Geist *et al*. (1987) also showed relatively low activity of zinc gluconate against non-enveloped rhinovirus and concluded activity depends on zinc salt concentration. Relatively low activities of formulation with zinc as sole active ingredient may be also related to low zinc concentration (around 6 mM). Korant and Butterworth (1976) described a stage of virion maturation where zinc was found to complex with rhinovirus capsid protein altering its structure and hampering subsequent cleavage reactions for virion formation. That illustrates the capacity of zinc to bind virus capsid proteins and possibly alter structure and the role of it in virus maturation and infectivity. Wei et al. (2012) hypothesized that the mechanism of action of zinc salts against transmissible gastroenteritis was similar to the one observed for non-enveloped rhinovirus. This was due to zinc binding to capsid allowing virus to bind host cell but hampering penetration and consequently virus infectivity. Kuemel et al. (1990) noticed that inactivation of HSV was caused by alteration of virus glycoproteins leading to inhibition of virus penetration in host cell. Thus, it is possible that activity of zinc against AdV2 and Pv1 was due to alteration on capsid protein resulting in structural changes which hampered virus penetration to some extent. AdV2 and Pv1 are known to require protein conformation (VP proteins for poliovirus, EA proteins for adenovirus) and an adequate capsid mechanical strength for infecting cells (Hogle, 2002). The mechanism of action associated to RB-002 against AdV2 may be strictly exclusively related to damage at the capsid level instead of nucleic acid damage.

Surprisingly activities by RB-002 against AdV2 and Pv1 were significantly higher than activities against F116 and MS2 (see Table 3.4). Although those bacteriophages are known to be suitable surrogates for mammalian virus treated with various formulation systems, differences in terms of isoelectric point and surface charges, capsid proteins and zinc finger domains and infection pathways could be responsible for observed disparities in terms of activity. Some of the differences between bacteriophage MS2 and mammalian viruses are highlighted in Table 3.7.

Table 3.7: Differences between MS2 and mammalian viruses (Pv1 and AdV2) in terms of physicochemical properties and susceptibility to formulation RB-002.

Virus	Isoelectric point (pI)	Capsid proteins	Viral titre reduction upon exposure to RB-002
Bacteriophage MS2	3.9 (You *et al.*, 2011)	Protein homo-dimers; single copy of protein A (Kuzmanovic *et al.*, 2003)	0.88±0.04
Adenovirus type 5 and 2	4.5 (Trilisky and Lenhoff, 2007)	Hexon; Penton Base (polypeptide III); fiber (polypeptide IV); polypeptide VI; polypeptides VIII and IX (San Martin, 2012)	5.26±0.10
Poliovirus type 1	6.6 (You *et al.*, 2011)	VP1, VP2, VP3 and VP4 (Minor, 1996)	3.71

This highlights possible distinct targets of alcohol/zinc against tested bacteriophages and mammalian viruses. For instance, Bales and Li (1993) discovered that MS2 was not a proper surrogate for poliovirus in filtration with silica beads as distinct isoelectric points between viruses contributed to different attachment rates at pH 7. The differences of isoelectric point (pI) between MS2 (pI 3.9) and Pv1 (pI 6.6) could alter the electrostatic barrier between virions and zinc ions resulting in distinct interactions and consequently overall activities at pH 10.5 (You et al., 2011).

The activity test of formulations containing only excipients (CA001EXCP; RB-001EXCP and RB-002EXCP) was important to verify the influence of excipients and pH on formulation overall activity. Those formulations yielded residual activities when compared to formulations containing both alcohol and zinc as active ingredients against bacteriophages and mammalian viruses. The pH was kept constant at 10.5 across all the formulations tested in this study. The pH may influence the overall activity of alcoholic solutions and could explain why relatively low activities were attained for both bacteriophages and higher activities were attained against mammalian viruses. For

instance, it was shown in the literature that activity of 70% ethanol against non-enveloped feline calicivirus went from 2.6 to 4.4 $\log_{10}$ reduction when pH was lowered from 7.4 to 3.0 (Park et al., 2010). The pH amongst ionic strength, temperature and hydrostatic pressure are environmental factors that have an impact on virus behaviour in suspension and capsid protein conformation, stability and virus aggregation (Furiga *et al.*, 2011). It has been reported in the literature that pH solutions lower than virus pI lead to virus aggregation and consequently lower penetration and virucidal efficacy of the active ingredient (Pinto, 2010). However, that should not be an issue impairing activity of the formulations containing both alcohol and zinc as active ingredients as formulation pH of 10.5 is much higher than pI of viruses under study. On the other hand, alkaline pH of 11.8 at was reported to degrade MS2 RNA through phosphate hydrolysis and affinity reduction for the capsid by deprotonation of lysine side chains over a 4 h contact time (Hooker *et al.*, 2003). It has also been shown that capsid stability of Adv5 (Rexroad *et al.*, 2006) and Pv1 (Wet and Kucinski, 1991) decreases as pH increases under different ionic strengths at 2 h or more contact time. Therefore, it is possible that the action of pH against MS2 at some extent may be due to formation of empty capsids, whereas against AdV2 and Pv1 capsid stability may be more predominant factor. The differences of RB-002EXCP activities against AdV2 and Pv1 may be mostly related to differences of physicochemical properties and proteins at the capsid level.

The reduction of RB-002 activity against Pv1 of approximately 4 times in presence of EDTA (see Figure 3.12B) was more pronounced than the one obtained under same conditions against MS2 (see Figure 3.11C). Date (1979) discovered divalent cations such as $Mg^{2+}$and Zn^{2+} are essential for binding of MS2 to the host (*E. coli* F-pilus) forming a highly stable complex important for infectious pathway. Hence, it is possible that differences in term of viral titre reduction by RB-002 between MS2 and Pv1 might be caused by possible higher attachment of MS2 to host and hampering of Pv1 host cell penetration. In other words, overall activities observed for RB-002 against bacteriophage and mammalian viruses may be due to virion structural damage but also the impact of monovalent and divalent cation present alters the dynamics between virus and hosts in a different manner.

In this context, the tested combinations of 40% ethanol with 0.1% zinc sulphate heptahydrate at pH 10.5 yielded viral inactivation to some extent where bacteriophages MS2 and F116 had around 1 $\log_{10}$ reduction (around 90% viral population) with RB-

001 and RB-002 at 60 min contact time for instance. The degree of virus inactivation by RB-002 against both AdV2 and Pv1 was even higher than the ones attained against bacteriophages. The data acquired throughout the present study is valuable as it shows the limitations of a novel formulation system. It also allowed inferring about the formulations stability over time and the role of free zinc ions in solution. The data is helpful to develop and design a potential new biocidal product with relatively low ethanol concentration. It was demonstrated the activity against non-enveloped viruses could be further enhanced by the addition of active ingredients like biguanides and/or QACs. Finally, the activity screening provided important data to discuss about the mechanism of action of alcohol and zinc against non-enveloped. It is possible that the activity of alcohol with zinc may be associated to capsid alteration and interaction with nucleic acid. Moreover, viral penetration to host caused by zinc may also play an important role in overall formulation efficiency against non-enveloped viruses. Further analysis of formulation activity containing alcohol and zinc against bacteriophage and adenovirus will be carried out in order to verify whether the hypothesis of having different mechanisms of action of alcohol and zinc combination against F116 and adenovirus is valid.

4. Mechanism of action of ethanol-zinc formulations against bacteriophage F116 and Human Adenovirus (AdV2 and attP AdV5)

4.1 Introduction

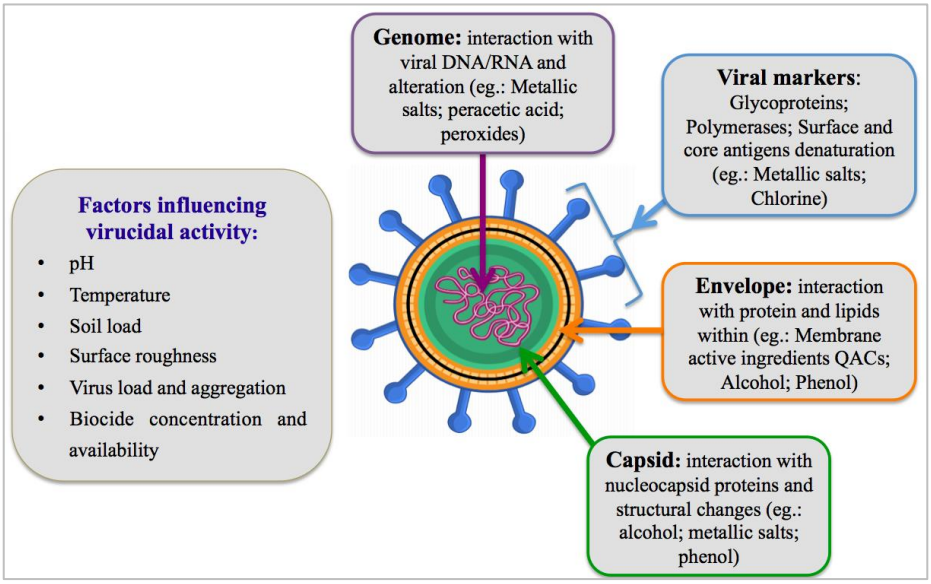
Understanding the mode of action of a biocidal product against viruses has been shown to be relevant for establishing scientific principles and for improvement of active ingredients (Myers, 2008). However, there is still relatively little information about the mechanism by which biocides interact with specific target sites leading to virus inactivation. Viruses are known to present less target sites for biocidal action than bacteria and fungi as they do not possess metabolic activity (e.g. transmembrane proton-motive force, efflux pumps and respiratory chain). Since biocides are multi-target antimicrobial agents with broader spectrum of action than more specific antimicrobials such as antibiotics, understanding interactions between biocides with pathogens (targets) will increase knowledge of mechanisms of viral inactivation, viral resistance mechanisms, and consequently it will improve efficacy of usage of biocides in practice (Russel, 2003).

In general, enveloped viruses are reported to be more susceptible to biocides than non-enveloped ones as the latter do not possess an envelope, which is a lipidic structure susceptible to membrane active agents action. Non-enveloped viruses still offer targets for virucidal action including the capsid, viral markers and genome (Maris, 1995; Maillard, 2001). However, mechanism of virucidal action against non-enveloped virions still remains largely unknown. Yoon et al. (2011) showed that inactivation of Adenovirus type 5 by sulfonic acid is mainly due to capsid protein conformational changes promoted by selective oxidation of sulphur-containing aminoacids, which leads to loss of viral infectivity. Lara et al. (2010) demonstrated that silver nanoparticles inhibit HIV-1 infectivity by interactions with glycoprotein 120, a viral marker, hampering virus binding to host cells. Yeap et al. (2015) showed that chlorine dioxide is a multi-target biocide against Murine Norovirus 1 as viral inactivation was caused by degradation of both viral capsid protein and viral RNA.

The resilience of pathogens towards a given biocide and associated inactivation kinetics are not only dependent on targets for virucidal action but also on many environmental parameters like temperature, pH, concentration of the active agent,

surface roughness and application techniques (Gerba, 2015). For instance, it was observed that quaternary ammonium compounds (QACs), known to be highly effective against enveloped viruses mainly due to disruption of lipid envelope, could also inactivate human enteric viruses (non-enveloped viruses) and more effectively than chlorine at higher concentrations in a given biocidal product formulation applied to a contaminated surface with hydraulic spraying (Bolton et al., 2013). QACs also were shown to have the potential to bind and alter conformation of DNA (Zinchenko et al., 2004). Thus, knowledge of potential targets of action of a biocide against microorganisms, effect of environmental parameters as well as effective application techniques are crucial information for the selection of the most suitable biocide for an all-round effective disinfection practice. Figure 4.1 summarises the main targets on a virus with envelope and examples of active ingredients, which were shown to inactivate viruses in the literature.

Figure 4.1: Factors influencing overall virucidal activity of biocides including potential interaction sites with viruses (targets) leading to virus inactivation. Each target depicted is followed by a brief description and examples of active agents known to cause virus inactivation through interaction with that particular viral component (Adapted from Russel (2003); Maillard et al. (2013) and Maris (1995)).



In this context, the main goal of this chapter is to analyse the capacity of ethanol/zinc system to promote structural changes on non-enveloped viruses and

identification of possible targets of action leading to a better understanding of the modes of action. Initially the impact of testing formulations on bacteriophage F116 morphology was assessed through Transmission Electron Microscopy studies. Energy dispersive analysis of X-rays (EDAX) study of the distribution of zinc atoms on bacteriophage F116 was performed to evaluate possible zinc deposition and localisation on damaged bacteriophages. Moreover, the influence of formulations on mammalian virus (adenovirus) capsid susceptibility to mechanical stress and virus integrity was verified through nano-indentation studies by Atomic Force Microscopy (AFM). Furthermore, viral purification and subsequent DNA extraction after exposure of viruses to formulations was performed in order to verify whether viral nucleic acid is a potential target for the virucidal formulation system under study.

4.2 Materials and methods

4.2.1 Transmission electron microscopy (TEM) Imaging on bacteriophage F116 and attP Adenovirus type 5 (attP AdV5)

4.2.1.1 Bacteriophage F116

TEM Imaging was performed to evaluate the impact of CA-001 and RB-002 formulations on structure and morphology of bacteriophage F116 and adenovirus type 2 according to Maillard et al. (1995).

The experimental procedure consisted in mixing 100 µL of F116 phage stock (10^{11} PFU.mL^{-1}) with 900 µL of the biocide for the appropriate contact time. The selected contact times are the ones that produce a 1 Log_{10} reduction in F116 (see section 3.3.3). Negative control with SM buffer solution was carried out for both formulation sets at 60 min contact time. After exposure, 20 µL of sample was aliquoted and mixed with 20 µL of 1% methylamine tungstate (Agar Scientific, UK) for 5 min. Copper-coated carbon grid (Agar Scientific, UK) was put in contact with negatively stained phage for 2 min and excess liquid was dried for 5 min on filter paper. Grids were left drying for 20 min. The imaging of samples was made through a JEOL 2100 electron microscope operating at 80 kV accelerating voltage with magnifications ranging from x10000 to 60000x.

4.2.1.2 attP Adenovirus type 5

TEM was performed to verify structural integrity of attP adenovirus upon exposure to formulations RB-001 or RB-002 diluted 1:1 in HBS buffer (20 mM Hepes, 0.15 M NaCl, pH 7.8) and compared with results obtained from the AFM study. HBS Buffer was used as negative control.

Carbon coated grid (Agar Scientific, UK) was glow discharged for 1 min and 5 μL virus droplet (10^{11}PFU.mL^{-1}) was added to the grid and incubated for 5 min. Following blotting with Whatmann paper, grid with adenovirus on the surface was put in contact with 45 μL of RB-001 or RB-002 diluted 1:1 for 1 min. Then, 500 μL HBS buffer was added and sample was stained with 1% Uranyl acetate. The imaging of samples was obtained through JOEL JEM 1230 transmission electron microscope at 100 kV.

The formulations, controls and contact times for each TEM analysis are shown in Table 4.1.

Table 4.1: Formulation sets under TEM analysis with respective controls and contact times.

Formulation set	Formulations	Composition	Contact time (min)	Virus
Formulations prepared in Cardiff	CA001	40% (w/v) Ethanol + 0.1% (w/v) Zinc sulphate heptahydrate + excipients	5	Bacteriophage F116
	CA001E	25% (w/v) Ethanol + 0.1% (w/v) Zinc sulphate heptahydrate + excipients	5	
	CA001X	40% (w/v) Ethanol + excipients	5	
	CA001EX	25% (w/v) Ethanol + excipients	5	
	CA001FX	80% (w/v) Ethanol + excipients	5	
	CA001G	0.1% (w/v) Zinc sulphate heptahydrate + excipients	15	
	CA001EXCP	Excipients	60	
Formulations prepared by RB (September	RB-002	40% (w/v) Ethanol + 0.1% (w/v) Zinc	1	attP Adenovirus type 5

2015)		sulphate heptahydrate + excipients	
	RB-002X	40% (w/v) Ethanol + 0.3% Zinc sulphate heptahydrate + QAC + excipients	1
	RB-002G	0.1% Zinc sulphate heptahydrate + excipients	1
	RB-002EXCP	Excipients	1

4.2.2 Energy dispersive X-ray Spectrometry (EDAX) on bacteriophage F116 after exposure to CA001 formulations

Sample preparation was based on EDAX studies carried out by Maillard et al. (1995b). Phages were exposed to CA-001 for the 5 min contact time, concentrated by centrifugation at 12000×g for 20 min and washed in 500 µL ultrapure water (Sigma, UK). This procedure was repeated twice. Samples were stained as described earlier in 4.2.1 and placed in contact with a nickel-based carbon grid (Agar scientific, UK). The EDAX analysis was performed by an energy dispersive analyser (X-MaxN 80T, Oxford Instruments) coupled to a JEOL 2100 electron microscope. The beam with accelerating voltage of 200kV was focused on an individual phage with a given structural damage for 5 min and data was analysed by the latest version of AZtecTEM software (Oxford Instruments).

4.2.3 Purification of attP Adenovirus type 5 (attP AdV5) and Adenovirus type 2 (AdV2)

The attP Adenovirus type 5 (attP AdV5) and Adenovirus type 2 (AdV2) were propagated in HEK293 HeLA and HeLA cells respectively and highly purified by

ultracentrifugation in caesium chloride (CsCl) gradient as described by Condezo et al. (2015). Viruses were harvested 3 days post-infection. Following that, they were purified by ultracentrifugation at 219,000g (LE-80K Ultracentrifuge, Beckman Coulter) for 90 min at 18ºC in 1.25 $g.mL^{-1}$ and 1.40 $g.mL^{-1}$ CsCl step gradient. TD1X Buffer (137 mM NaCl, 5.1 mM KCl, 700 mM $Na_2.HPO_4.7H_2O$ and 25 mM Tris base at pH 7.4) was used to prepare CsCl gradients. Following the first centrifugation, viral bands were extracted and centrifuged in 1.31 $g.mL^{-1}$ CsCl step gradient for 18 hours at 219,000g. Viral bands obtained were desalted through column chromatography (Bio-Rad 10 DC, UK), eluted with HBS Buffer and stored in aliquots with 10% glycerol at -80ºC. The viral titre was quantified as determined by Spearman-Kaeber method for 6^{th} and 7^{th} day post-infection as described by Flint and Enquist (2008).

4.2.4 Nanoindentation on attP Adenovirus type 5 through Atomic Force Microscopy

This experiment aimed to investigate the influence of RB-002 formulations on capsid mechanical properties of a mutant adenovirus type 5 (attP AdV5). The adenovirus mutant contains two insertions downstream the packaging domain and two deletions (E1 and E3), where the insertions correspond to one recombinase target sequence (attP) from bacteriophage phi and the other one to green fluorescent protein (Alba et al., 2011). The virus was obtained from Carmen San Martin's research group at Centro Nacional de Biotecnologia (Madrid, Spain). This type of mutant adenovirus was used due to lack of pathogenicity towards humans and the fact it has been extensively studied through nano-indentation in the literature (Alba et al., 2011; Alba et al., 2007). The mechanical stability was assessed in terms of viral capsid spring constant by applying a force to virus without structural damages according to the Hooke's Law spring constant equation (Equation 4.1) as follows:

$$F = -k \times d \quad \text{(Equation 4.1)}$$

Where F stands for applied force (nN); k the spring constant (nN/nm) and d is the total deformation (nm).

The experimental procedure was based on mechanical stability studies carried out by Herando-Perez et al. (2014). Virus fixation was achieved by leaving 20 μL of attP Ad5 viral particles stored in HBS Buffer (20 mM HEPES and 0.15 M NaCl, pH

7.8) on a muscovite mica surface (Agar Scientific, Spain) disk for 20 min at 4ºC. Viruses attached to the surface were washed five times with HBS buffer to remove viral particles not associated with the surface. Prior to virus exposure to formulation, virus adhered to mica surface are visualised by AFM as a control for virus structural integrity and stability on the aforementioned surface. Then, 180 µL of formulation RB-002 under analysis and diluted 1:1 with distilled water was put in contact with viruses on mica. After incubation time ranging from 30 seconds to 10 min, 500 µL of HBS buffer was added at the same time formulation is removed. The tip (Olympus, Japan) was pre-wetted with 20 µL of HBS Buffer and AFM equipment (Nanotec Electronica S.L., Spain) run in jumping mode with a rectangular cantilever (RC800PSA, Olympus, Japan) with nominal spring constant of 0.05 $N.m^{-1}$. The force *vs* distance curve was obtained by stopping lateral piezo scan when the tip was on non-structurally damaged virus centre point of symmetry and elongating z-piezo until the tip established mechanical contact with a virus and indented viral particle once at high enough force (200 nN) to cause virus capsid deformation. The linear deformation observed provided the spring constant *k* according to the Equation 4.1.

Additionally, breaking force was calculated as the force necessary to displace viral capsomers and cause capsid rupture. Capsid morphology was analysed by measuring capsid height (h) during AFM imaging. The viral capsid brittleness was assessed by determining the yield strength ratio (Y) according to Equation 4.2.

$$K\ (\%) = \frac{\delta_{critical}}{h} \times 100 \text{ (Equation 4.2)}$$

Where $\delta_{critical}$ stands for capsid deformation (nm) at which it breaks and h is capsid height (nm).

For imaging, 128 x 128 point images of viral particles were recorded by using the lowest force to reach high resolution of virus structure (100 pN) in jumping mode. All data processing was performed by using WSxM software (WSxM solutions, Spain). Statistical analysis was performed using ANOVA test for three independent samples when number of viruses analysed was higher than 24.

4.2.5 Effect of formulations on viral nucleic acid - Viral DNA extraction, quantification, analysis of DNA damage by biocides and presence of zinc in viral DNA

The following procedures were carried out in order to evaluate whether viral DNA damage is induced by formulations. This data will be very important to have a clear picture about the mechanism of action including viral DNA as a potential target for the combination of alcohol with zinc as active ingredients. The methodology was based on the one presented by Maillard (1994).

4.2.5.1 Bacteriophage F116

Bacteriophage F116 was propagated in *P. aeruginosa* PAO1 (ATCC BAA-47) and harvested as described in Chapter 2 with final concentration of 10^{11} PFU/mL.

4.2.5.1.1 Bacteriophage F116 exposure to biocide, nucleic acid extraction, purification and quantification

After bacteriophage harvesting, 200 μL of F116 suspension (10^{11}PFU/mL) was mixed with 800 μL of each formulation (see Table 4.1) for 2h contact time at 25ºC. Controls consisted of performing the same nucleic acid extraction from 1 mL F116 suspension and F116 suspension mixed with ultrapure water (Sigma, UK). F116 nucleic acid was extracted by a Phage DNA Isolation Kit (Norgen Biotek, Canada) with DNase treatment to digest possible DNA from the host bacteria prior viral DNA extraction. Five μL of RNase-Free DNase I (Norgen Biotek, Canada) was added to 1mL of phage/formulation mix and incubated for 15 min followed by DNase inactivation at 75ºC for 5 min. Then 500 μL of Lysis Buffer B (Norgen Biotek, Canada) was added to phage suspension, incubated at 65ºC for 20 min and added to binding column. The latter was centrifuged for 3 min at 6,000g, washed with 400 μL Wash Solution A (Norgen Biotek, Canada) twice with flow-through being discarded afterwards. The elution of viral DNA occurred by adding 50 μL of Elution Buffer B (Norgen Biotek, Canada) and centrifugation at 6,000g for 1 min. Purified dsDNA was quantified through fluorometric quantitation (Quibit 3.0 Fluorometer, ThermoFischer) and stored at -20ºC.

4.2.5.1.2 F116 DNA gel electrophoresis and DNA digestion by selected restriction enzymes

Analysis of bacteriophage dsDNA integrity after exposure to formulations and purification steps was performed. Three hundred and fifty ng of purified viral dsDNA was mixed with 5 μL gel loading buffer (ThermoFischer, United Kindgom) and loaded on 1.2% (w/v) agarose gel in TAE buffer (Sigma-Aldrich, United Kingdom) with SYBR Safe DNA gel stain (ThermoFischer, United Kingdom). The lambda DNA/HindIII Marker size profile and running buffer composition used for this study are presented in Table 4.2.

Table 4.2: Marker size profile bands and running buffer composition.

Marker size profile (bp)	Running buffer
23130 9146 6557 2322 2027 564 125	0.1% bromophenol blue 50% (v/v) glycerol 100 mM EDTA 0.5% sodium dodecyl sulphate

Electrophoresis was performed at 80V for 45 min and nucleic acid bands were visualised and photographed in a transilluminator (BioRad Gel Doc XR System, Germany).

In order to assess possible specific nucleic acid damage caused by alcohol/zinc combination, viral dsDNA purified after exposure to each formulation was digested by restriction enzyme Sau3AI (Thermo Scientific, Germany) as described in Table 4.3 prior to gel loading. The samples were loaded into 2% (w/v) agarose gel in TAE Buffer and electrophoresis was performed at 90 V for 1 h and 30min. Analysis was performed in triplicate with independent biological samples.

Table 4.3: Restriction enzymes used to digest F116 and AdV2 dsDNA extracted after exposure to formulations.

Viral dsDNA	Restriction enzyme	Recognition sequence	Digestion prediction	Reaction mix
F116	Sau3AI	↓GATC	1733 bp; 915 bp; 850 bp; 799 bp; 789 bp; 719 bp; 662 bp; 648 bp; 646 bp; 645 bp; 624 bp; 622 bp; 579 bp; 527	25-30 µL DNA; 1 µL enzyme; 2 µL 10X Buffer; 12-17 µL nuclease free water. Incubation at 37°C for 1h
AdV2	SmaI	CCC↓GGG	6923 bp; 6480 bp; 5522 bp; 4457 bp; 2925 bp; 2463 bp; 2269; 1503 bp; 1403 bp; 1008 bp; 575 bp; 230 bp; 179 bp	15-20 µL DNA; 1 µL enzyme; 2 µL Fast Digest buffer; 3-8 µL nuclease free water. Incubation at 37 °C for 5 min
	AatII	GACGT↓C	13273 bp; 10744 bp; 9975 bp; 1945 bp	

4.2.5.2 Adenovirus type 2 (AdV2)

AdV2 was propagated in HeLa cells and highly purified as described in section 4.2.4. Aliquots with viral concentration of 10^9 PFU/mL determined by viral titration were used for subsequent DNA damage analysis.

4.2.5.2.1 Adenovirus type 2 exposure to biocide, nucleic acid extraction, purification and quantification

Twenty µL of AdV2 suspension was put in contact with 80 µL of each formulation (see Table 4.1) for 2 h at 25°C. Control consisted of mixing virus suspension with PBS at the same ratio. AdV2 DNA was extracted and purified by a High Pure Viral Nucleic Acid Kit (Roche, Switzerland). Two hundred µL of Binding Buffer supplemented with 50 µL Proteinase K and Poly (A)(Roche, Switzerland) was added to 100 µL of virus mixed with formulation and mixture was incubated at 72°C for 15 min. Following that, mixture was added to a high pure filter column and centrifuged three times at 8,000g for 1 min with flow-through being discarded after each centrifugation cycle. Then, column was washed twice with 450 µL Wash Buffer. Elution of AdV2 dsDNA occurred by adding 40 µL of elution buffer and centrifugation at 13,000g for 1 min. Viral DNA was stored -20°C for further analysis.

4.2.5.2.2 Adenovirus type 2 DNA gel electrophoresis and DNA digestion by selected restriction enzymes

AdV2 nucleic acid integrity resulting from aforementioned purification step was analysed by electrophoresis. One hundred and fifty ng of purified viral dsDNA was mixed with 5 µL of gel loading buffer and loaded on 1.2% (w/v) Agarose gel in TBE buffer with SYBR Safe DNA gel stain (ThermoFischer, United Kingdom). The lambda DNA/HindIII Marker size profile and running buffer composition were used as described earlier. Electrophoresis was performed at 100 V for 45 min and bands were visualised as described earlier.

Similarly to the analysis on F116 DNA damage, purified AdV2 dsDNA was digested separately by SmaI and AatII restriction enzymes (Thermo Scientific, Germany) in order to evaluate more specifically potential damage to viral nucleic acid caused by formulations (see Table 4.3 for more details). After digestion, electrophoresis was performed on a 2% (w/v) agarose gel in TBE buffer at 100 V for 1 h and 30 min. Viral nucleic acid bands were visualised as mentioned earlier. Analysis was performed with three biological independent samples.

4.2.5.3 Quantification of zinc in DNA samples after purification by inductively coupled plasma-mass spectrometry (ICP-MS)

The analysis of zinc presence in purified viral DNA samples upon exposure to formulations RB-002 and RB-002G was performed by inductively coupled plasma-mass spectrometry (ICP-MS) according to Li et al. (2012). Viral nucleic acid samples were diluted 1:20 and analysed by standard addition method. The latter consists of 100 µL of viral nucleic acid sample mixed separately with 4, 5, 6 and 7 µL of standard solution. The method of internal standard addition was performed in order to reduce matrix effects where zinc absorbance could be interfered by other components and to enhance overall plasma stability. Samples were analysed by ICP-MS (Agilent 7900 ICP-MS, United States) with operational conditions described in Table 4.4.

Table 4.4: Operation parameters associated to the ICP-MS performed for zinc quantification.

Operational Parameters	Agilent ICP-MS
Laser ablation system	
Wavelength of Nd:YAG laser (nm)	266
Laser energy per pulse (mJ)	5
Laser power density (W cm^{-2})	$5x10^8$
Nebuliser type	
Spray chamber	Desolvator
RF Power (W)	1250
Cooling gas flow rate ($L.min^{-1}$)	18
Auxiliary gas flow rate ($L.min^{-1}$)	1.1
Nebuliser (carrier) gas flow rate ($L.min^{-1}$)	1.32
Solution uptake rate ($mL.min^{-1}$)	2
Ion extraction lens voltage (V)	2000
Mass resolution ($m/\Delta m$)	4400
Analysis time (min)	5
Number of runs	6
Number of blocks of runs	5

4.2.6 Statistical analysis

All the experiments in this chapter were performed as four independent biological replicates. For TEM studies on attP AdV5 and F116, statistical significance in terms of different types of damage observed was analysed by two-tailed Chi squared test with 95% confidence interval. One-way ANOVA was performed throughout nano-indentation studies to validate statistical significant after empirical normal distribution was confirmed by Kolmogorov-Smirnov test. For non-normal distribution, non-parametric analysis by Wilcoxon-Mann-Whitney statistical test was carried out.

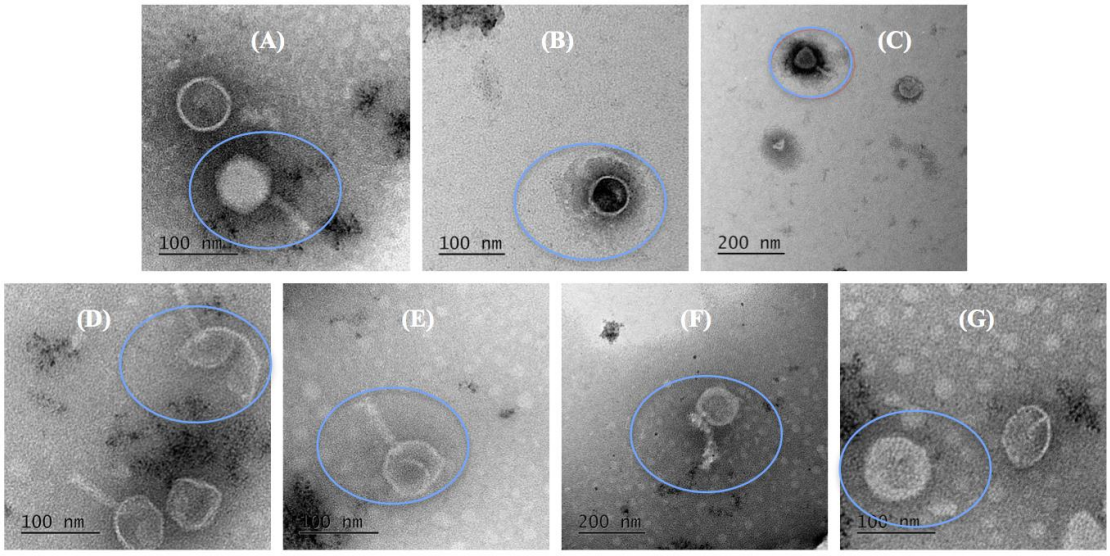
All statistical analyses were carried out by GraphPad Prism 6 software (version 6.0f, Graphpad Software).

4.3 Results

4.3.1 Structural and morphological changes induced in F116 by formulations prepared in Cardiff

TEM imaging of bacteriophage F116 was carried out following exposure of bacteriophage F116 to CA001 with different ethanol concentrations. A total of 1360 single phages were observed. Initially different types of damages were observed and divided into different groups: (i) intact phage, (ii) head or tail damage, (iii) head with no tail and (iv) tail with no head. Damages were then subdivided into different groups associated to structural changes such as: i) intact phage; ii) concentrated material inside head; iii) expanded material inside head; iv) folded head; v) folded head; vi) fractured head; vii) empty head; viii) damaged tail and ix) tail only. Figure 4.2 presents images collected throughout the present analysis for each type of structural changes.

Figure 4.2: Representative F116 structural damages identified throughout TEM analysis. Structures are highlighted by a blue circle. A) Intact F116 (x 60000); B) F116 head with concentrated material inside (x 50000); C) F116 empty head (x 25000); D) F116 fractured head (x 60000); E) F116 folded head (x 60000); F) F116 with damaged tail (x 25000) and G) F116 head with expanded material inside.



Morphological changes induced by different formulations were observed for a given number of phages and results are shown in Table 4.5. Significant morphological damage differences were found between formulation CA001 containing 40% (w/v) ethanol with zinc and formulation CA001-FX containing 80% (w/v) ethanol (Chi-Square $p<0.05$). Formulations CA001 and CA001-E comprising both ethanol and zinc yielded significant different morphological changes compared to formulation CA001-EXP without the mentioned active ingredients (Chi-Square $p<0.05$). Furthermore, formulations with and without both active ingredients yielded higher number of phages without tail when compared with phages treated with SM Buffer (negative control). It was also observed that formulations CA001 containing both ethanol and zinc produced a higher level of morphological damages, where formulation CA001 comprising 40% (w/v) ethanol with 0.1% (w/v) zinc was the most effective as lowest percentage of intact phages was observed. By comparing formulations CA001-EX with 25 % ethanol and its counterparts containing zinc sulphate heptahydrate, damages to the phage head and tail were higher (Chi-Square $p<0.05$) for the latter, however head or tail damage percentage are comparable between 40% with and without zinc sulphate.

Regarding structural changes, results are summarised in Table 4.6. Significant structural differences ($p<0.05$; see Appendix A) were found by comparison of formulation CA001-EX containing 25% (w/v) ethanol with zinc with formulation CA001-FX 80% ethanol and formulation CA001-G comprising zinc as the only active ingredient. Formulation CA001 comprising 40% (w/v) ethanol with zinc presented a significant ($p<0.05$) higher percentage of phages with concentrated material inside head compared to its counterpart without zinc and formulation CA001-G comprising 0.1% (w/v) zinc sulphate only. Moreover, formulations comprising ethanol and zinc led to significantly higher percentage ($p<0.05$) of phages with expanded and concentrated material inside head and capsid denaturation (head with folded structure), which may suggest that the formulation interact with phage nucleic acid. By taking into account only head with concentrated, head with expanded material inside and folded head, significant ($p<0.05$) differences are found by comparison of formulations CA001 and CA001-E containing both ethanol and zinc with formulation CA001-FX comprising 80% ethanol, indicating a distinct mode of action between the mentioned formulations. Formulation CA001-E containing 25% ethanol with zinc yielded higher damages to the virus capsid than 40% ethanol with zinc as higher percentage of phages with folded and fractured heads was observed. However, no significant difference (Chi-Square $p>0.05$) was found at this point between formulations CA001 and CA001-E in terms of overall structural damage pattern. Formulation excipients seemed to have an impact of virus structure as shown by structural changes induced by formulation CA001-EXCP without ethanol and zinc, more specifically in terms of capsid damage. Nevertheless, damage pattern observed for formulation CA001-EXCP was significantly ($p<0.05$) different compared to formulations CA001 and CA001-E with both active ingredients.

Table 4.5: Percentage of morphological changes induced by different formulations CA001 prepared in Cardiff and respective controls. Numbers in bold show statistical significant differences when compared to other formulations.

Formulation	Composition	Percentage of phage (%)				Total number of phage observed
		Intact phage	Head or tail damage	Head with no tail	Tail with no head	
CA001 (Base)	40% Ethanol + 0.1 % Zn	**13.5**	**36.7**	49.8	5.3	207
CA001-E	25% Ethanol + 0.1% Zn	25.7	**37.4**	36.9	4.9	206
CA001-G	0.1 % Zn	14.2	31.3	54.5	3.7	134
CA001X	40% Ethanol	32.1	38.9	33.3	3.1	162
CA001-EX	25% Ethanol	33.6	13.3	51.7	4.9	143
CA001-EXCP	Formulated w/o Ethanol and Zinc	22.9	24.7	50.6	7.8	166
CA001-FX	80% Ethanol	27.9	17.7	54.4	5.4	147
Negative control	SM Buffer	**50.3**	**7.9**	38.7	12	191

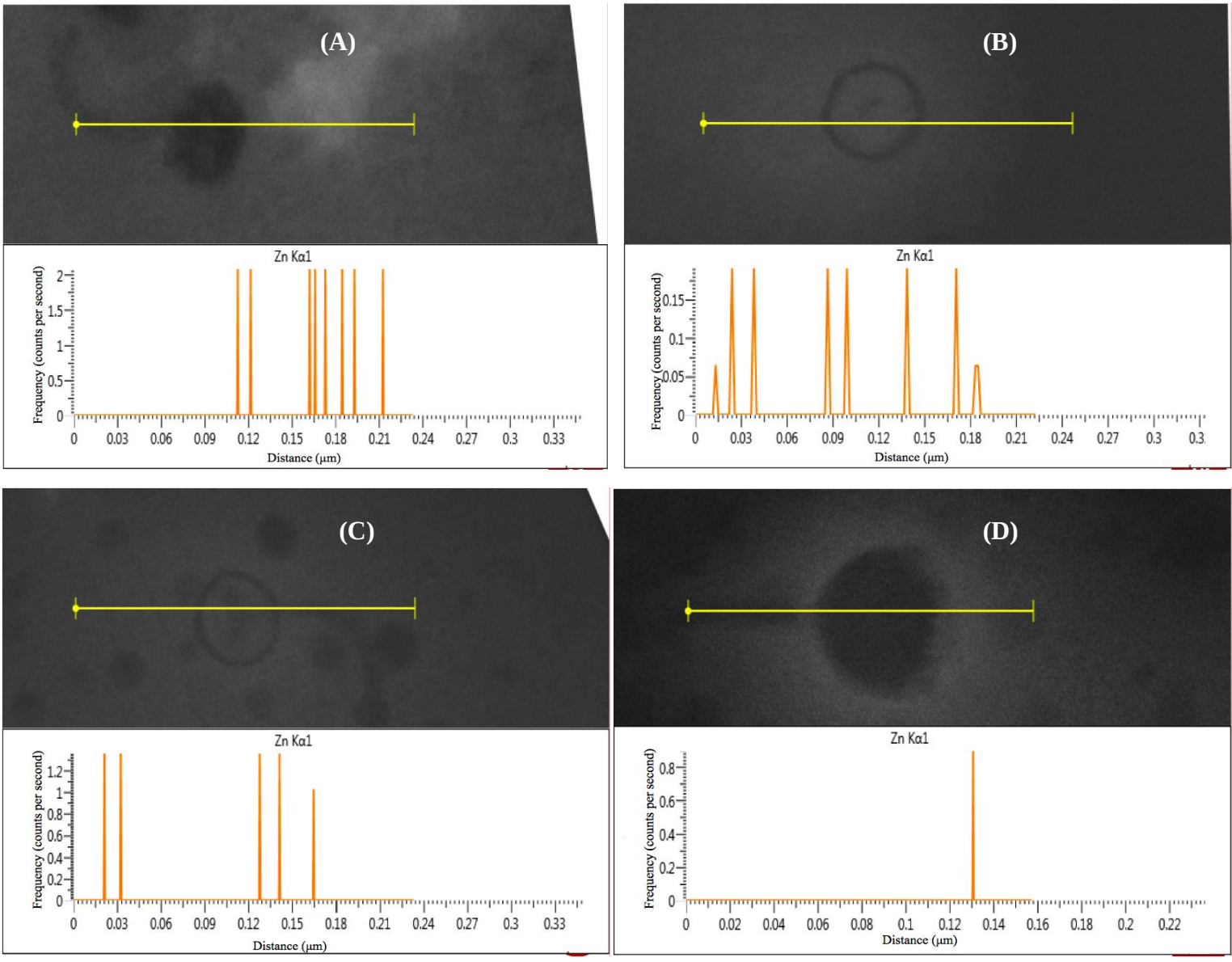
Table 4.6: Percentage of structural changes induced by different formulations CA001 prepared in Cardiff and respective controls (1360 phages were observed in total). Numbers in bold show statistical significant differences when compared to other formulations.

Formulation	Composition	Percentage of phage (%)							
		Intact phage	Head with concentrated material inside	Head with expanded material inside	Folded head	Fractured head	Empty head	Damaged tail	Intact tail
CA001 (Base)	40% Ethanol + 0.1% Zn	**13.5**	**31.4**	23.2	11.1	9.7	13.5	9.2	3.4
CA001-E	25% Ethanol + 0.1% Zn	25.7	**24.8**	14.1	14.1	11.2	**7.8**	9.2	2.9
CA001-G	0.1 % Zn	**14.2**	14.9	**29.9**	15.7	11.9	11.2	11.2	3.7
CA001X	40% Ethanol	31.5	12.3	9.9	16.0	16.0	19.1	6.8	1.9
CA001-EX	25% Ethanol	33.6	11.2	12.6	11.2	14.0	17.5	4.2	4.9
CA001-EXCP	Formulated w/o Ethanol and Zinc	22.9	5.4	6.6	17.5	25.9	23.5	**3.6**	3.0
CA001-FX	80% Ethanol	27.9	12.9	17.7	8.2	12.2	15	6.1	5.4
Negative control	SM Buffer	**50.3**	3.1	8.4	**5.8**	7.9	11.0	**2.1**	**11.5**

4.3.2 Energy-dispersive X-ray (EDAX) for detection of zinc associated to F116 virions exposed to formulations prepared in Cardiff

With a preliminary EDAX analysis, a line scanning of zinc was carried out for several phages exposed to distinct formulations. The results are shown in Figure 4.3.

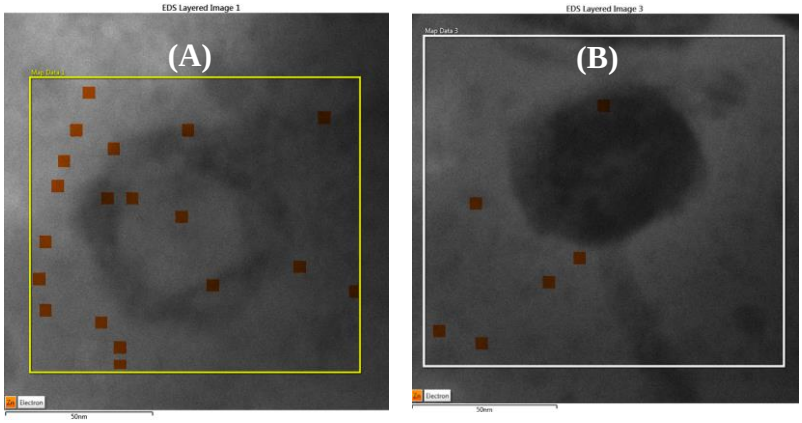
Figure 4.3: Counts per second of zinc atoms across phages with distinct damages after exposure to formulation CA001 containing 40% Ethanol with 0.1% Zinc and SM Buffer (negative control) with respective scanning transmission electron microscope (STEM) images. A) Intact F116 (x60000); B) F116 head with concentrated material inside (x60000); C) F116 with empty head (x60000) and D) Intact F116 after exposure to SM Buffer (x60000).



Damaged phages presented higher number of zinc counts around the phage particle when compared to intact one (Fig. 4.3 B and C), indicating that zinc may be involved with phage head damage. Zinc atoms strongly bound the phages and their immediate surrounding as samples were washed with ultrapure water after exposure to formulation. In addition, zinc frequency obtained for phage with concentrated material inside head was higher than the one attained for phage with empty head, suggesting a higher dispersion of zinc associated to the head of damaged phages. The control consisting of phage exposed to SM Buffer showed that trace amounts of zinc in the background can be detected, but it was not comparable to the amount associated with phages damaged by the complete formulation CA001.

In order to have a better insight on the dispersion of zinc across phage structures, the mapping of zinc atoms across phages with distinct structural damages exposed to formulation comprising 40% ethanol with 0.1% zinc was also performed by EDAX analysis. The preliminary result is presented in figure 4.4.

Figure 4.4: Zinc atoms (red points) detected by Energy Dispersive X-ray on bacteriophage F116 after exposure to formulation prepared in Cardiff comprising 40%(w/v) Ethanol with 0.1%(w/v) Zn. A) Head with expanded material inside head (x1000000); B) Intact phage (x1000000).



More zinc hits were observed all over the phage presenting material expanded within the capsid comparing to intact phages (Fig. 4.4 A). The higher number of zinc hits is an indication that zinc may be involved in structural damage. However, more

replicates and controls should be performed in order to analyse possible background noise and statistical significance of this experiment.

4.3.3 Nanoindentation study of attP Ad5 upon exposure to old and new formulations prepared by RB

In order to investigate the mechanical strength of adenovirus capsid following exposure to RB-002 formulations, atomic force microscopy (AFM) in jumping mode was carried out. Firstly, images of virions were obtained in order to determine virion symmetry and target for nano-indentation experiments. Images recorded before and after nano-indentation procedure are presented in Figure 4.5.

Figure 4.5: Selected individual frames of attP AdV5 before and after nano indentation upon exposure to RB-001 and RB-002. A) Virions at 3-fold symmetry before nano-indentation exposed to formulation RB-001; B) Virions at 3-fold symmetry after nano-indentation exposed to formulation RB-001; C) Virion at 3- fold symmetry nano-indentation exposed to formulation RB-002 and (D) Virion after nano-indentation exposed to formulation RB-002.

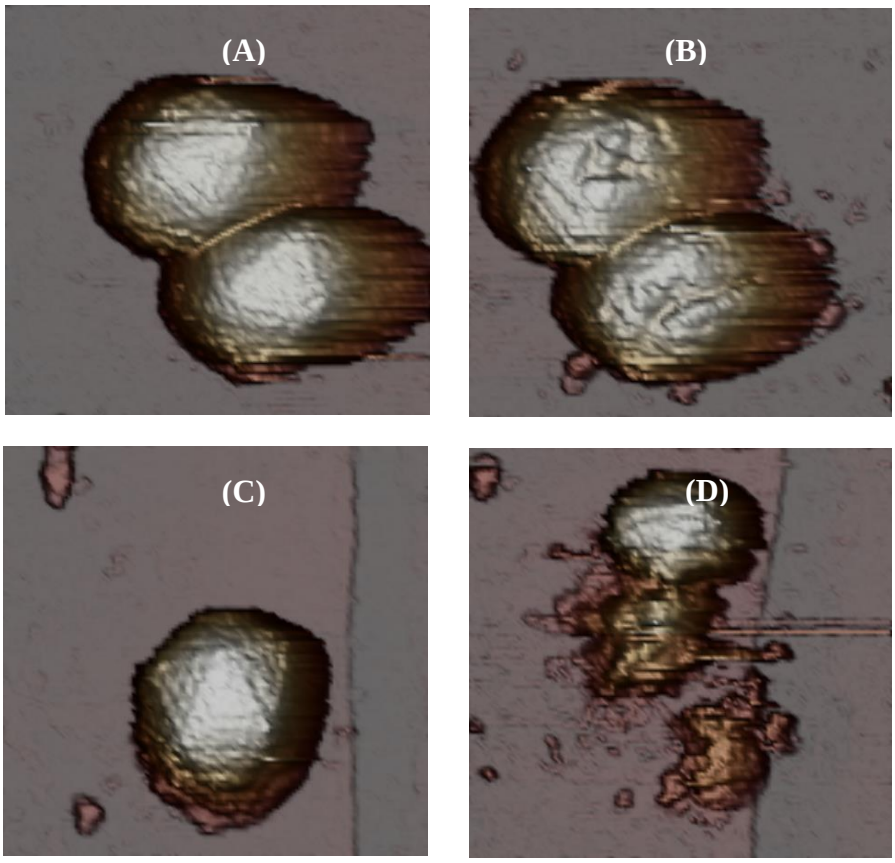


Figure 4.5 showed that misplacement of attP AdV5 capsomers (Fig. 4.5 B) and eventual viral disassembly (Fig 4.5 D) occurred throughout nano-indentation studies. The results for mechanical properties of virus capsid after exposure to RB-002 formulations are shown in table 4.7.

Table 4.7: Spring constants (k) of attP AdV5 adenovirus at symmetry 3 before exposure and after exposure to different formulations within 1 min contact time.

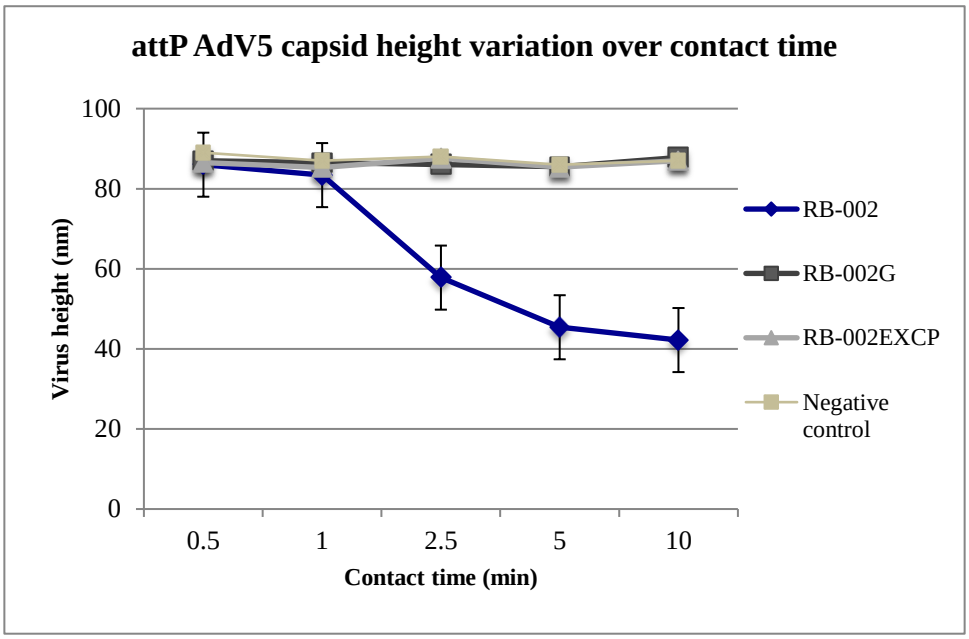
Formulation	Number of viruses	Symmetry	Spring constant (k) (nN/nm)	Breaking force (nN)	Critical strain (%)
RB-002	38	3	0.40 (±0.02)	4.2 (±0.1)	18.8 (±7.8)
RB-002G	37	3	0.38 (±0.02)	3.9 (±0.2)	19.8 (±7.7)
RB-002EXCP	31	3	0.36 (±0.02)	4.3 (±0.1)	18.7 (±5.0)
HEPES (negative control)	130	3	0.55 (±0.01)	4.7 (±0.1)	17.4 (±7.5)

Exposure to all the formulations reduced the spring constant of the virus in comparison with the negative control, suggesting capsid weakening induced at some degree by the formulations. Significant spring constant variation (ANOVA, $p<0.05$) was found between formulations containing both alcohol and zinc (RB-002) with formulation comprising excipients only (RB-002EXCP) and negative control (HEPES). However, no significant change was found between formulations RB-002 and RB-002G at 95% confidence interval. The results suggest excipients have the capacity to influence virus spring constant at some level.

Regarding the amount of deformation a virus capsid withstand before rupture evaluated through calculation of the critical strain, no significant difference was found between virus capsid exposed to different formulations (ANOVA, $p<0.05$). Nevertheless, exposure to formulations promoted minor increase in the critical strain of the virus, i.e. less virus brittleness.

Topography of viruses attached to mica surface and exposed to different RB-002 formulations prepared by RB were characterized in terms of height and results are shown in figure 4.6.

Figure 4.6: Topography and capsid height analysis of attP adenovirus type 5 exposed to different formulations at different contact times.

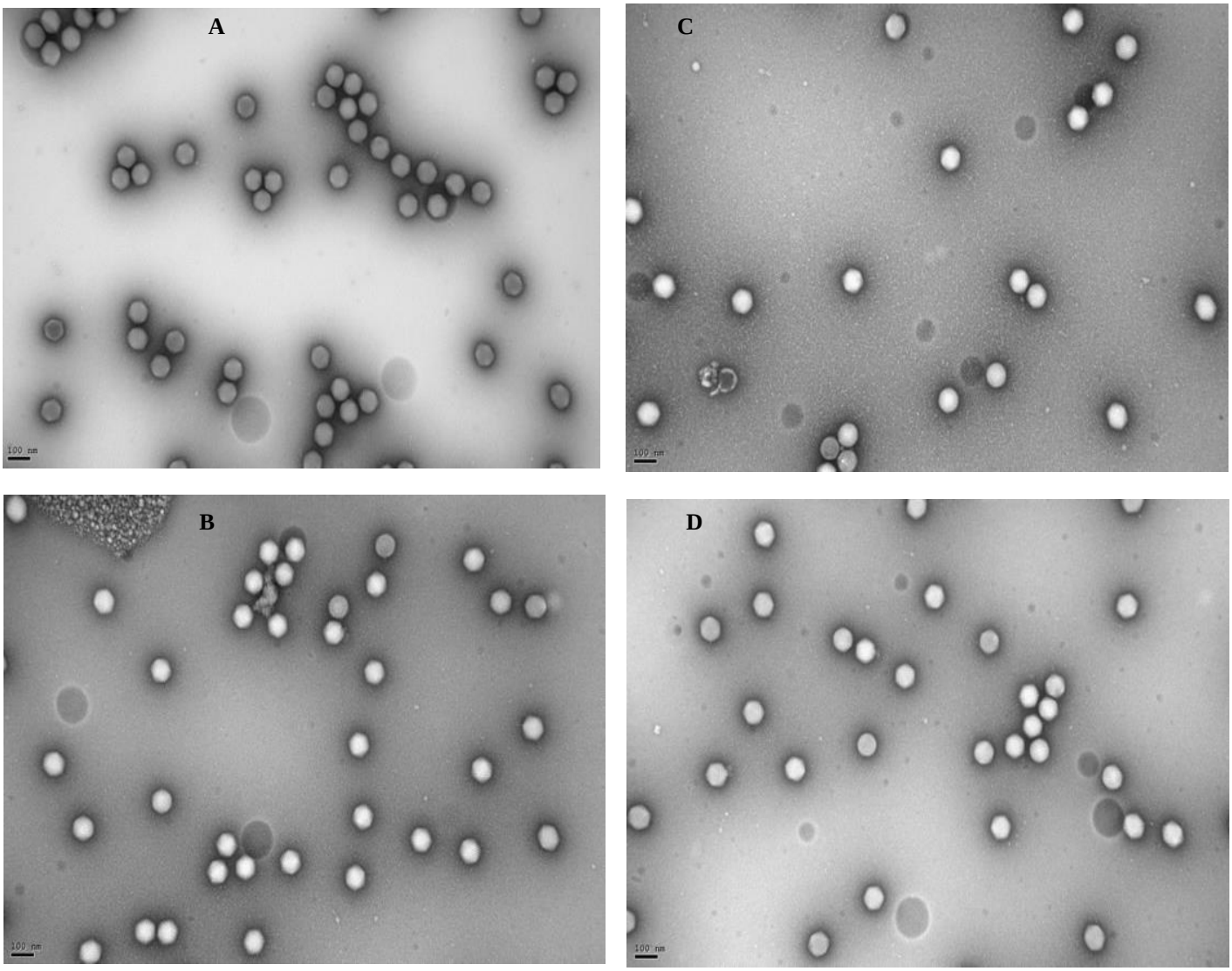


There was no significant virus topography variation in terms of viral height within the first minutes of contact between the different formulations. As contact time increases, the capsid height variation induced by formulation RB-002 is significantly higher (ANOVA, $p<0.05$), which shows the combination of both alcohol and zinc have an impact on overall virus topography. This suggests a distinct mode of action by RB-002 when compared to other formulations and may be caused by possible interactions of alcohol and zinc with virus capsid.

4.3.4 Transmission electron microscopy (TEM) imaging of attP Ad5 exposed to new formulations prepared by RB

TEM analysis was carried out in order to evaluate structural integrity of attP adenovirus after exposure RB-002 formulations. Micrographs are presented in Figure 4.7.

Figure 4.7: TEM images of aTTP adenovirus after exposure to distinct formulations. A) Virus exposed to RB-002 (x 75000); B) Virus exposed to formulation RB-002-G; C) Virus exposed to formulation RB-002-EXCP; D) Virus exposed to HBS Buffer as negative control (x 75000).



The structural appearance of viruses exposed to formulation RB-002G and RB-002EXCP (Fig. 4.7 B and 4.7 C, respectively) is comparable to the ones in the negative control (exposed only to HBS buffer) (Fig. 4.7 D). Viral particles exposed to formulations RB-002 (see Fig. 4.7 A) presented a different staining than remaining formulations. Staining of the samples was performed by addition of uranyl acetate, a well-known negative staining agent, which interacts with phosphates groups of DNA (Pandithage, 2013). It is possible that differences of staining may be due to structural damage to virus capsomers allowing higher penetration of uranyl acetate, which indicates possible structural damage at the capsid level. The quantification of damage and number of virus with distinct staining compared to other in the same sample was carried out and results are shown in table 4.8.

Table 4.8: Total number of viruses as well as percentage of virus with affected staining upon exposure to new formulations prepared by RB diluted twice with 30s contact time.

Formulation	**Composition**	**Percentage of virus with affected staining (%)**	**Percentage of broken capsids (%)**	**Total number of virus**
RB-002	40% (w/v) Ethanol + 0.1% (w/v) Zn + excipients	9.6	2.2	230
RB-002-G	0.1% (w/v) Zn + excipients	1.7	0.85	118
RB-002-EXCP	Excipients only	4.4	0.74	135
Negative control	HBS Buffer	2.6	2.6	116

Taking into account mechanical properties results through AFM nano-indentation, relatively low percentage of damaged virus after exposure to formulations RB-002 diluted 1:1. It suggests that despite formulations having an impact on virus spring constant, their structure and architecture seems to be preserved for the vast majority of viral population at 30s contact time. However, the percentage of virus with affected staining after exposure to RB-002 is significantly higher than ones obtained

after exposure to control formulations. Once more, it could indicate possible damages at capsid level (capsomeres displacement for instance) caused by the combination of alcohol and zinc as active ingredients.

4.3.5 DNA damage analysis – Is it an actual target for the formulation under study?

Previously TEM studies on the mode of action of alcohol/zinc combination against F116 showed this combination yields higher percentage of phages with expanded and concentrated material inside phage head. From there, it was hypothesized that the viral nucleic acid is a potential target for the virucidal combination under study. Therefore, AdV2 and F116 DNA were extracted after exposure to formulations and analysed by electrophoresis and restriction enzyme digestion in order to assess presence of DNA damage.

4.3.5.1 Viral DNA extraction – F116 and AdV2 DNA extraction after exposure to formulations

Initially viruses were separately exposed to different formulations and dsDNA was extracted after a given contact time with an appropriate extraction kit as described earlier. The concentration of viral dsDNA obtained is presented in table 4.9.

Table 4.9: Bacteriophage F116 and Adenovirus type 2 dsDNA extracted after exposure of virions to different formulations with 2 h contact time at room temperature.

Virus	Extraction kit	Formulation	dsDNA concentration (ng/µl)
Bacteriophage F116	Phage DNA isolation kit (Norgen Biotek, Canada)	RB-002	156 (±17)
		RB-002G	86 (±13)
		PBS (negative control)	46 (±13)
Adenovirus type 2 (AdV2)	High Pure Viral Nucleic Acid Kit (Roche, Germany)	RB-002	9.9 (±1.3)
		RB-002G	7.9 (±0.3)
		RB-002EXCP	6.4 (±0.9)
		HEPES (negative control)	7.3 (±1.6)

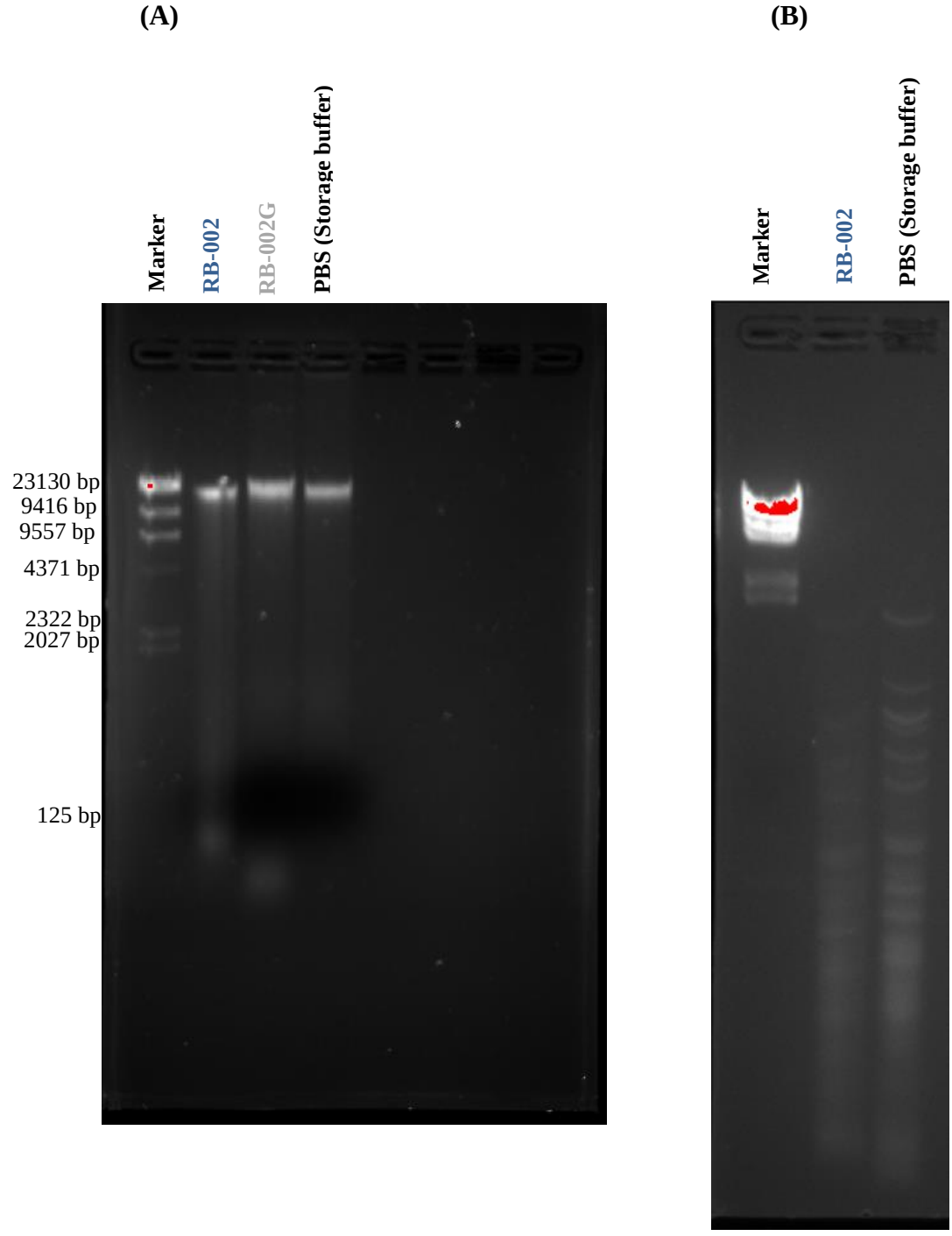
The concentration of DNA obtained after exposure of both viruses to formulation containing an alcohol/zinc combination (RB-002) was significantly higher (ANOVA, $p<0.05$) when compared to other formulations under study. It is possible that combination of alcohol and zinc enhances DNA extraction procedure due to damages caused to virus capsid. On the other hand, negative controls (PBS and HEPES buffer solutions) and RB-002EXCP yielded lower dsDNA concentration, which could be related to lower damage extent to virus capsids and subsequent less effective DNA extraction. In terms of AdV2 dsDNA extraction, dsDNA concentration obtained was significantly higher upon exposure to formulation containing zinc as sole active ingredient (RB-002G) when compared to counterparts containing formulation excipients only (RB-002EXCP). Hence, it seems zinc as sole active ingredients may have some impact on virus integrity which leads to more effective dsDNA extraction when compared to excipients only based formulation.

4.3.5.2 DNA damage analysis – is F116 DNA damaged to some extent?

Subsequently to extraction, F116 dsDNA integrity was analysed qualitatively by agarose gel electrophoresis before and after digestion by restriction enzymes for more

detailed DNA damage analysis. The DNA bands obtained after exposure to formulations as well as the ones obtained after restriction enzyme digestion are shown in Figure 4.8.

Figure 4.8: Bacteriophage F116 DNA damage analysis after exposure to formulations by agarose gel electrophoresis running with TBE buffer. A) F116 DNA bands extracted after exposure to different formulations in 1% agarose; B) F116 DNA bands obtained after exposure to formulations and digested by Sau3AI in 2% agarose gel.

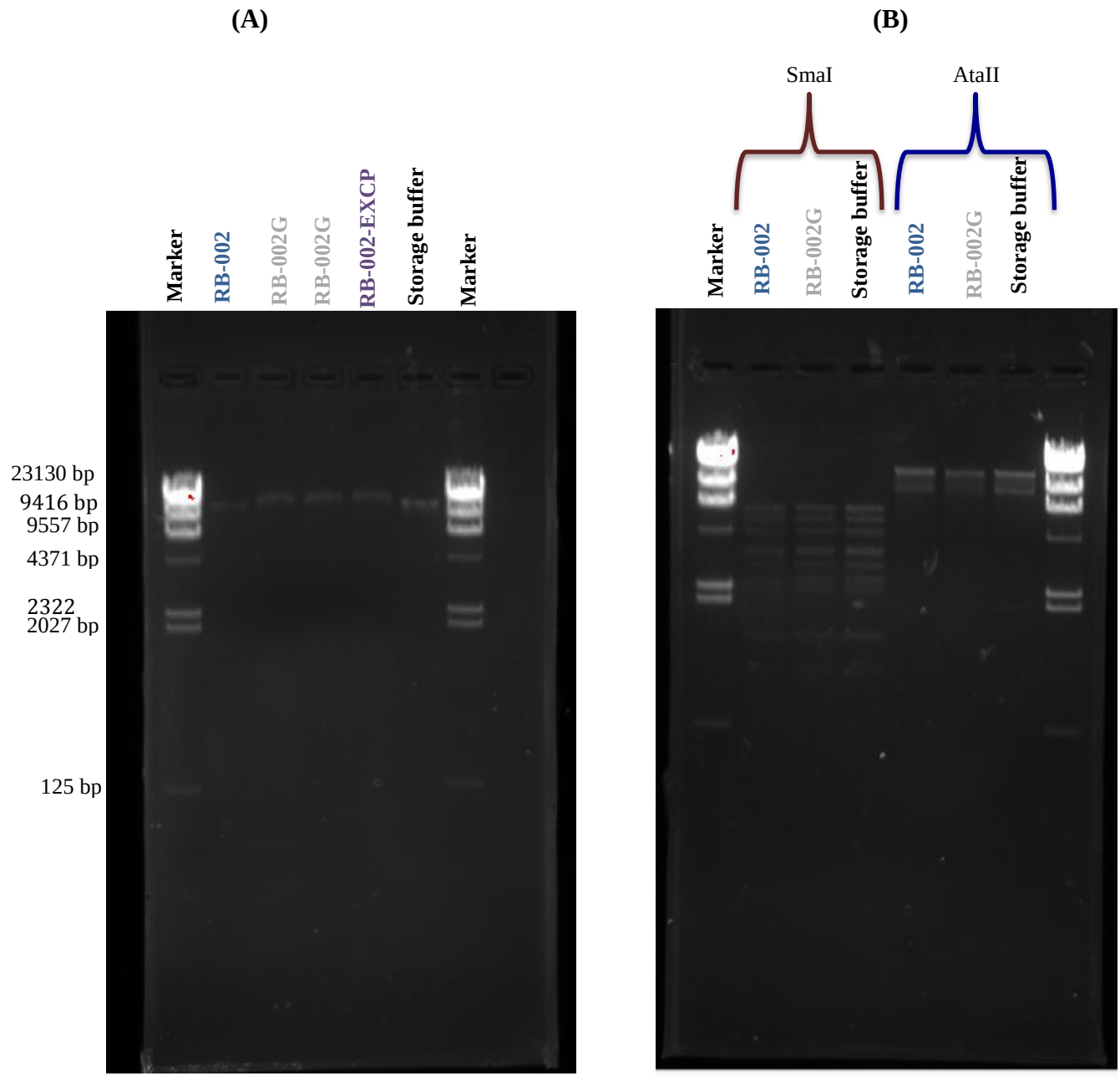


A smear can be observed in the lanes corresponding to DNA extracted upon exposure to formulations containing zinc (RB-002 and RB-002G) when compared to DNA extracted from virus exposed to storage buffer (negative control) (see Figure 4.8 A). A more detailed analysis of possible DNA damage induced by RB-002 was carried out by digestion of F116 DNA with Sau3AI restriction enzyme. Figure 4.8 B shows that DNA extracted after exposure to RB002 presents a slightly different profile containing bands with different migration distances when compared to negative control. This result suggests there may be minor damages to F116 DNA induced by formulation RB002 at the contact time under study.

4.3.5.3 DNA damage analysis – is Adenovirus DNA damaged to some extent?

Similarly to the F116 DNA damage analysis, the possibility of formulation induced AdV2 DNA damage was investigated by similar methodology. The AdV2 DNA bands obtained after exposure to formulations and extraction procedure are shown in Figure 4.9

Figure 4.9: Adenovirus type 2 (AdV2) DNA damage analysis after exposure to formulations by agarose gel electrophoresis running with TAE buffer. A) AdV2 DNA bands extracted after exposure to different formulations in 1% agarose; B) AdV2 DNA bands obtained after exposure to formulations and digested separately by SmaI and AatII restriction enzymes in 1% agarose gel.



Results show no significant migration distance differences between formulation RB-002 and RB-002G and no smear (see Figure 4.9 A). This indicates DNA samples are pure and that no apparent DNA damage is observed after exposure to different formulations. The difference in terms of migration between RB-002 and RB-002G may be related to the presence of ethanol in the sample, hence an artefact rather than actual nucleic acid damage. A more detailed analysis through digestion with two different restriction enzymes show no migration or band pattern difference between DNA extracted from AdV2 exposed to formulations and virus exposed to storage buffer. Those results show there is no apparent DNA damage when AdV2 is exposed to formulations for 2 h at room temperature.

4.3.5.4 DNA damage – zinc presence in extracted F116 DNA samples

In order to verify a possible link between zinc and F116 DNA damage, a preliminary quantitative analysis through ICP-MS was carried out in terms of zinc concentration present in aliquots of extracted F116 DNA. The zinc content found in DNA solutions extracted after exposure of F116 to different formulations is presented in table 4.10.

Table 4.10: Zinc content in samples of F116 DNA extracted after exposure of virus to different formulations through ICP-MS.

Formulation	Zinc content detected (in ppm)
RB-002	0.798 (±0.025)
RB-002G	1.192 (±0.027)
RB-002 in presence with EDTA	0.355 (±0.0347)
70% (w/v) Ethanol	0.299 (±0.053)
Negative control (PBS)	0.337 (±0.051)

As expected, higher amount of zinc was detected in F116 DNA samples extracted from bacteriophage F116 exposed to formulations containing zinc as an active ingredient. Nevertheless, the amount of zinc detected reduced drastically when the same

formulation was in presence of EDTA chelating agent, which may indicate higher affinity of zinc to the chelating agent instead of virus or its genome. The zinc content found for the F116 DNA exposed formulation RB-002 in presence of chelating agent was comparable to negative controls such as 70% (w/v) ethanol and storage buffer. The negative control point to minimal zinc content possibly already present in those formulations and/or add during the DNA extraction process. Difference between RB-002 and RB-002G were significant (ANOVA, $p<0.05$) which may indicate zinc is possibly coordinated to other ingredients of formulation when alcohol is present. The preliminary data shows higher zinc concentration in F116 DNA samples extracted after virus exposure to formulations with zinc as active ingredient which could be associated with viral DNA. However, zinc coordination and metal binding to viral nucleic acid could not be observed yet.

4.4 Discussion

TEM imaging was crucial to have a deeper insight on the effect of the formulations on virus structure. The present study revealed a damage pattern associated to the formulations comprising zinc and ethanol as they induced higher percentage of morphological changes to the head and the tail of F116 bacteriophages. This confirmed that the formulation can structurally damage viruses. In terms of structural damage, formulations CA001 and CA001-E containing both ethanol and zinc ingredients led to higher number of phages with expanded and concentrated material inside head, and folded head, which suggest an interaction of the referred formulations with virus genetic material. Zinc ions may be responsible for such effects. Kuemel et al. (1990) observed that interaction of zinc with herpes simplex virus not only lead to virus aggregation, but also to zinc deposition in virion components, resulting in a loss of infectivity. In order to confirm the zinc presence onto F116 capsid, an energy-dispersive X-ray (EDAX) spectroscopy was carried out and results show that phage with condensed material inside head present higher extent of zinc across its structure when compared to intact phage or phage with an empty head although a higher number of phages would be necessary to attest for statistical significance for each type of damage observed.

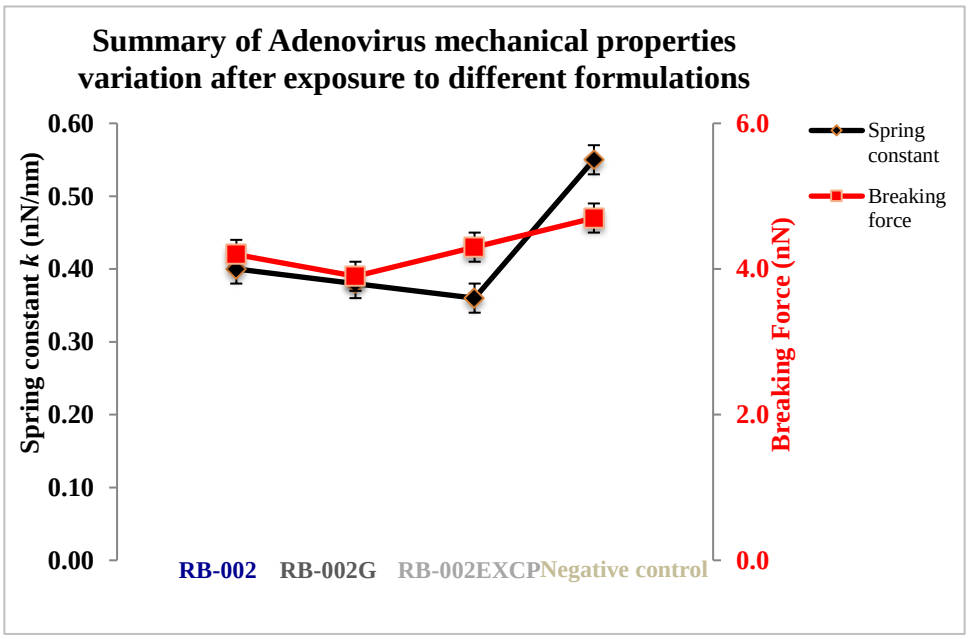
One of the aspects of zinc toxicity is related to oxidative stress by generation of reactive oxygen species (ROS) (Morina et al., 2010). Those species were shown to

induce several damages to proteins, nucleic acids and cell membranes through extensive oxidation (Wu et al., 2015). ROS were shown to cause structural alterations in DNA through base pair mutation, rearrangements, deletions and species such as singlet oxygen and hydroxyl radical have been reported in the literature to be virucidal against HIV and Hepatitis B viruses (Wiseman and Halliwell, 1996; Baugh, 2000).

Ethanol was reported in the literature to confer internal structure changes on non-enveloped virus (Maillard, 2001). By comparing 80% (w/v) ethanol solution with the formulations containing ethanol and zinc, it was shown that damage pattern was more diverse for the latter, which may result from the combination of action of both ethanol and zinc against different targets such as capsid proteins and nucleic acid, respectively. Formulation CA001 containing 40% (w/v) ethanol with 0.1% (w/v) zinc sulphate heptahydrate led to highest damage extension among other samples.

The AFM nano-indentation studies on attP AdV5 allowed the evaluation of the effects of formulations RB-002 on virus mechanical properties and morphology. The summary of the results about the influence of different formulations on the virus mechanical properties analysed through nano-indentation studies is shown in Figure 4.10 as follows

Figure 4.10: attP Adenovirus type 5 (attP AdV5) mechanical properties variation upon exposure to formulation RB-002 and respective controls. Red squares represent data series related to breaking force; black squares are related to virus spring constant.



A lower spring constant of the virus exposed to the formulation was observed when compared to HBS storage buffer (negative control). This would make the virus more susceptible to environment chemical and mechanical stress (Hernando-Perez et al., 2014b). Significant spring constant variation was found between formulations RB-002, RB-002G and RB-002EXCP, where the latter surprisingly yielded the lowest virus spring constant. This suggests a distinct mode of action when zinc as an active ingredient is present in the formulation as this is the only ingredient that differentiates RB-002 and RB-002G from RB-002EXCP. On the other hand, virions exposed to RB-002EXCP could withstand higher breaking force highlighting formulations containing zinc as active ingredient tends to weaken the virus capsid.

It is known that mechanical properties of viruses such as stiffness and capacity to withstand certain pressures such as DNA packing, cell entry process and extracellular conditions like osmotic pressure water activity and pH plays a significant role for their viability (Carrasco et al., 2011; Greber, 2016). In this case, virions exposed to formulations RB-002 and RB-002G presented a higher susceptibility to mechanical stress, which could hamper virus infectivity. The fragility rather than virus stiffness was verified to be more important in terms of capacity virus capsid withstand certain mechanical stress (Hernando-Perez et al., 2014a). Additionally, Llauro et al. (2015) demonstrated calcium ions could alter the mechanics of tomato bushy stunt virus capsid through calcium ion sequestration from intra-capsid binding sites reducing virus rigidity and resilience to mechanical stress and making it more susceptible also to internal pressure. Hence, it is possible that zinc may interact with the viral capsid in a similar way altering capsid mechanical stability.

Although RB-002 and RB-002G seemed to yield comparable changes to virion mechanical properties, it was verified that RB-002 led to significant viral capsid height reduction as contact time increases, i.e. viral capsid has shrunk with time. This indicates a distinct mode of action between RB-002 and RB-002G where the presence of both alcohol and zinc as active ingredients induced higher extent of structural damage to the adenovirus capsid. Ivanovska et al. (2007) reported that there was a direct relation between viral genome size including conformational changes with overall capsid mechanical stability as lambda phages capsid with wild-type genome could withstand twice force applied to lambda phages with DNA 78% shorter. It is possible that formulation containing both ethanol and zinc may induce certain capsid protein and DNA conformational changes. This can be involved in reduction of adenovirus capsid

stability not only by capsid structural changes but also through alteration of viral nucleic acid packing within capsid and consequently internal pressure variation. Kennedy and Parks (2009) hypothesized that tight packaging of unmodified DNA within adenovirus capsid may be important to exert a force on adenosome, i.e. virus capsid pentons present in the vertex region to be reoriented in a position, which favours capsid mechanical stability.

Moreover, TEM analysis of attP AdV5 upon exposure to formulations RB-002 prepared by RB diluted 1:1 show that virions did not present any significant structural damage even tough formulations RB-002 showed a different staining compared to formulations without ethanol or zinc. This may be associated to the presence of ethanol in the formulations RB-002, which can cause substructure damages on the capsid of non-enveloped viruses as reported in the literature (Macinga et al., 2008; Liu et al., 2010). The presence of alcohol as an active ingredient in RB-002 may be the reason that formulation had higher impact on virus topography as compared to RB-002G containing zinc as sole active ingredient.

Finally, the hypothesis of viral DNA damage induced by the combination of alcohol and zinc formulation was studied through a qualitative methodology based on the gel electrophoresis of DNA extracted after exposure of viruses to different formulations. The DNA bands extracted from F116 and AdV2 exposed to storage buffer negative control (see figure 4.8A and 4.9 A, respectively) were comparable to the ones found in the literature showing there is no apparent DNA damage caused by PBS and HBS storage buffers (Maillard, 1994; Elnifro et al., 2000). It was shown that presence of zinc in the formulation could lead to minor damage to F116 DNA as evidenced by presence of a smear and distinct restriction pattern obtained by DNA digestion by Sau3AI restriction enzyme. This result agrees with the ones obtained through TEM where higher percentage of phages heads with concentrated and expanded material inside was found for formulations containing zinc. On the other hand, no apparent DNA damage was observed under same conditions for AdV2. The difference of results between F116 and AdV2 may be related to distinct mechanism of action by alcohol/zinc combination and/or limitations of the technique to assess overall DNA damage. Damages observed against F116 DNA is most likely due to the presence of zinc in formulations as it was shown in the literature viral DNA is not a target for the action of alcohol (McDonnell and Russell, 1999; Pfaender et al., 2014).

It is known that free zinc ions (Zn^{2+}) have the potential to bind to DNA and consequently change its secondary structure mainly through interactions with DNA phosphate sugar backbone as well as guanine and cytosines by imino protons replacement (Aich et al., 1999). Furthermore, it was discovered that a high ratio of Zn^{2+} to DNA has the capacity of destabilizing the DNA double helix decreasing melting temperatures of DNA molecules (Souza et al., 2000; Labiuk, 2003). Thus, it is possible that zinc in formulations may bind to F116 DNA inducing conformational changes and double helix destabilization. This can make nucleic acid more prone to damages caused by chemical environment such as oxidative stress, cleavage by zinc complexes at alkaline pH (Yuan et al., 2006) and DNA crosslinking in presence of zinc (Labiuk et al., 2001). The preliminary quantitative analysis of zinc pool in solutions of F116 DNA by ICP-MS after extraction showed there were higher zinc concentrations in DNA samples from F116 exposed to formulations RB-002 and RB-002G as expected. Formulation RB-002 in presence of EDTA chelating agent yielded similar result as negative control (PBS), which indicate higher zinc concentration observed in DNA samples are due to exposure of viruses to formulations containing zinc. However, it was not possible to verify whether zinc pool was directly bound to extracted DNA.

The combination of alcohol and zinc showed to induced structural damages to both bacteriophage F116 and Adenovirus (AdV2 and attPAdV5) which could explain the virucidal activity of the formulation system under study. It is proposed that the viral nucleic acid is a potential target for the formulation against bacteriophage F116 where DNA conformation and potential damage was confirmed through TEM and gel electrophoresis analysis. On the other hand, AdV2 did not present significant DNA damage, which may be explained by a distinct mode of action and/or limitations of DNA damage analysis by electrophoresis. In order to verify the presence of DNA damage in adenovirus, it is recommended the quantification of lesions per kb by real-time polymerase chain reaction. Nevertheless, the presence of zinc in formulation had a significant impact on overall attPAdV5 mechanical properties where virions became less stiff and more prone to capsomere displacement by normal force (mechanical stress). The presence of alcohol in combination with zinc in formulation RB-002 led to profound viral topography changes like capsid height reduction, which could severely affect virus infectivity.

5. Formulation chemistry

5.1 Introduction

Biocidal products usually comprise not only the active molecules, but also other ingredients important for formulation stability such as co-solvents, pH buffers and anti-corrosive agents. For instance, the potential for triethanolamine present in the formulations under study to form complexes with metal ions such cadmium and zinc is well known (Shaban et al., 2003). Proton NMR spectroscopy was performed aiming to analyse potential complex formation of zinc and triethanolamine in the formulation.

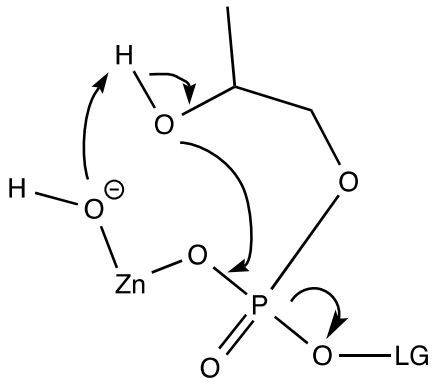
According to basic zinc speciation diagrams, zincate ($[Zn(OH)_4]^{2-}$) is the most common species found under alkaline solutions (pH 10-11) in aqueous system (Winand, 2010). Zincate salts have been reported in the literature to present antifungal activity, but no virucidal activity has been demonstrated yet (Amim et al., 2011). The Henderson-Hasselbach equation is presented below:

$$pH = pK_a + \log_{10}\left(\frac{[A^-]}{[HA]}\right) \quad \text{(Equation 5.1)}$$

Where $[A^-]$ is the molar concentration of acid's conjugate base; [HA] is the molar concentration of the undissociated acid and pK_a is the $-\log_{10}$ of the acid dissociation constant. Assuming that the pK_a of the zinc complex with water molecules is around 9.5 (Gilson and Durrant, 2009) and by taking this equation into account, it is possible to affirm that at pH of 10.5 concentration of the zinc complex in its hydroxide form is ten times higher than undissociated zinc complex in formulation. The Lewis acidic zinc complex with water in its base form is capable of promoting solvolysis cleavage of phosphate diesters of RNA through general-base-catalysed mechanism as depicted in Figure 5.1 (Zhang et al., 2014).

In this chapter, the main goal is to observe formulation chemistry in terms of zinc availability and possible identification of zinc species as well as possible complexation with formulation excipients.

Figure 5.1: General-base catalysed mechanism of solvolysis cleavage of phosphodiester by mononuclear zinc complex. LG stands for leaving group.



5.2 Materials and Methods

5.2.1 Proton Nuclear Nuclear Magnetic Resonance (1H-NMR) Spectroscopy

The NMR spectroscopy has been used to analyse the chemical content and structure of chemical and biological samples. The technique is based on the fact nuclei in different chemical environments will resonate at different frequencies due to electron shell differences culminating into a chemical shift (Chatham and Blackband, 2001). In the present study, NMR spectroscopy was carried out in order to analyse the chemical environment (chemical species and structures) present in the formulation under study, more specifically the possible complexation of triethanolamine with zinc, which could be responsible for lower virucidal activity observed by previous experiments.

Six mg of triethanolamine were mixed with 2 mg zinc sulphate heptahydrate using 40% (v/v) deuterated ethanol (D6-Ethanol) with 60 % (v/v) deuterated water (D_2O) as solvent. Solution was prepared in a 5mm NMR tube (Norell, US) and Proton NMR spectroscopy was performed by using a 400 MHz instrument. Controls consisted of triethanolamine and zinc sulphate heptahydrate mixed separately with the aforementioned solvent. Data was analysed through MestReNova software (v9.1.0, Mestrelab Research).

5.2.2 Liquid chromatograph mass spectrometry (LC/MS) analysis of RB002 formulations

In order to analyse the different RB002 formulations and possibly identify chemical species, which could be related to observed virucidal activity samples were analysed by LC/MS. The method was carried out according to Oestman et al. (2017).

Initially samples were diluted ten times with ultrapure water (MilliQ, UK) and 10 μL injected to liquid chromatography (Dionex UltiMate 3000 Thermofisher, UK) using acetonitrile (Fisher, UK) and ultrapure water as solvent A and B, respectively. The gradient program started at 3% (v/v) with solvent A ramping to 97% (v/v) at a flow rate of 0.3 mL/min through a C18 column at 40^{o}C. Following that, sample with eluent was submitted to a mass spectroscopy (amaZon SL Bruker, USA) module including an ion trap. The MS detector performed scans from 100 to 1000 amu/sec in positive mode. Syringe used for injection of samples as well as LC-MS system was cleaned with ultrapure water two times in between analyses of different formulations. The product ion mass spectra for each formulation was obtained and mass-to-charge ratio (m/z) was used to identify possible chemical species through MassBank software (MassBank Project, version 1.8).

5.2.3 Small angle X-ray scattering spectroscopy (SAXS) on bacteriophage MS2

The SAXS was carried out in order to characterize bacteriophage MS2 capsid conformational changes and RNA core during exposure to RB-001 and to highlight possible chemical interactions between formulation active ingredients and virus capsid proteins along prolonged contact times. The experimental procedure was based on the one described by Nguyen et al. (2011), except for bacteriophage purification where they were prepared by soft overlayer agar technique and dialysed with SM buffer. Samples of bacteriophage MS2 (10^{11}PFU.mL^{-1}) in contact with RB-001 were sealed in 1.5 mm diameter quartz glass capillaries and SAXS was performed at 10keV on beamline 21 in a synchrotron based in Diamond Light Source facility (Oxford, UK). The radiation scattered by sample under analysis was collected by a MarCCD 165 camera (Rayonix, USA) with pixel size 73.2 × 73.2 μm^2. The data was analysed was performed through ScAtter in BiosIsis software package (BioIsis, UK).

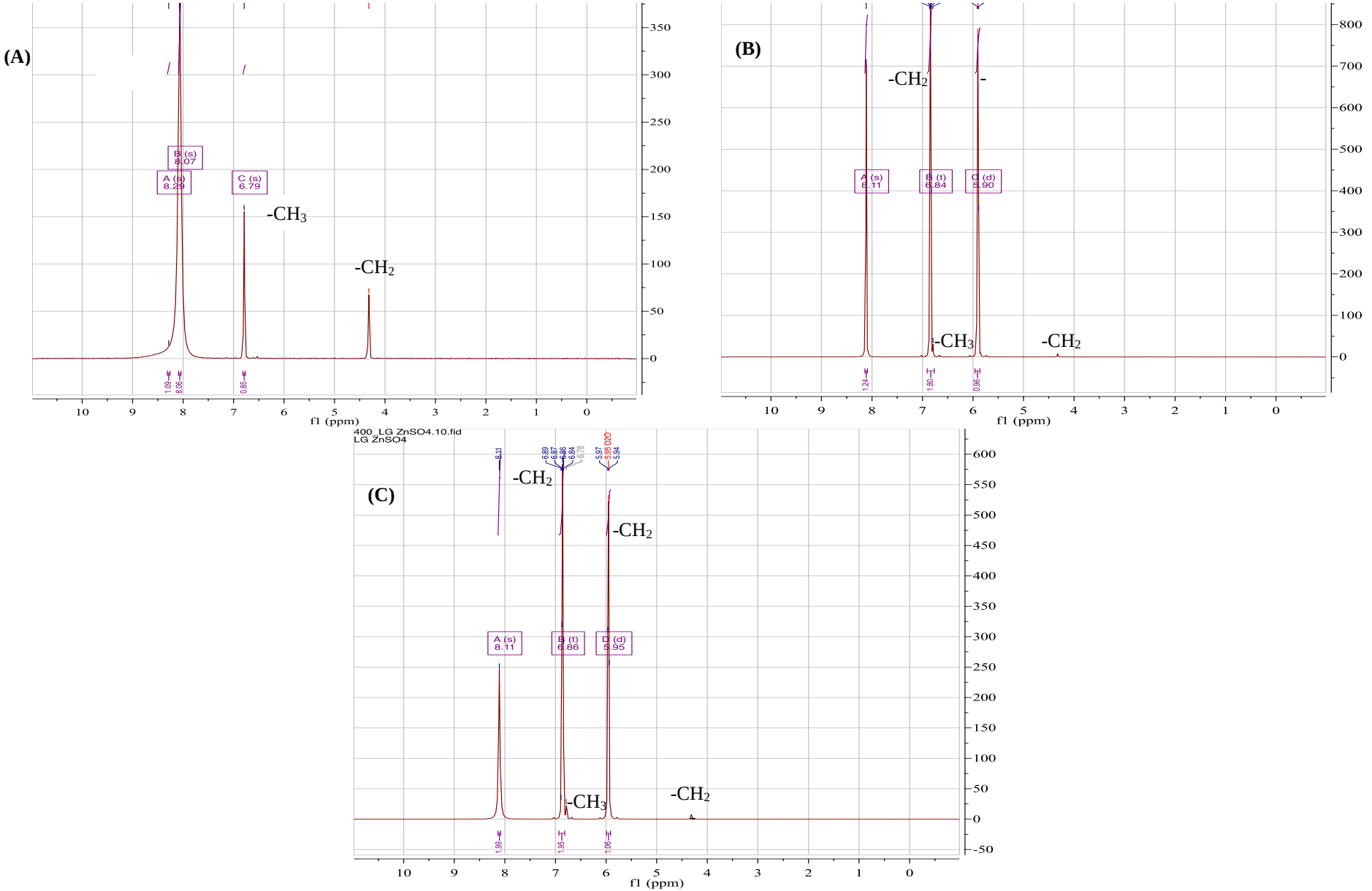
5.3 Results

5.3.1 Proton NMR Spectroscopy

Proton NMR spectroscopy was carried out in order to analyse the formulation chemistry and verify possible coordination of zinc ions to other formulation components at around pH 8.0.

The potential for triethanolamine to form complexes with metal ions such cadmium and zinc is well known (Shaban et al., 2003). Proton NMR spectroscopy was performed aiming to analyse potential complex formation of zinc and triethanolamine in the formulation. Spectra obtained for the solvent (60% D2O with 40% D6-Ethanol), triethanolamine and mixture of triethanolamine and zinc sulphate heptahydrate is shown in Figure 5.2.

Figure 5.2: Proton NMR at 400 MHz spectra of different solutions: (A) solvent only (60% D20 + 40% D6 Ethanol); (B) Triethanolamine in solvent and (C) Triethanolamine + Zinc sulphate heptahydrate in solvent. Peaks correspond to methyl (6.79 ppm in D_2O) and methylene groups of ethanol (4.32 ppm in D_2O).

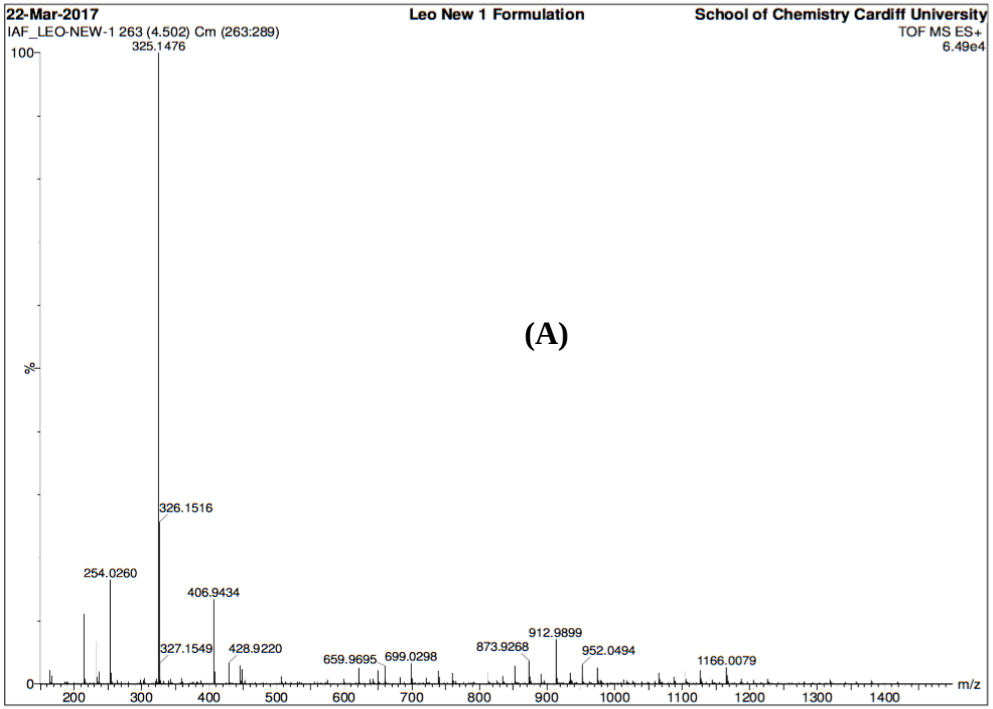


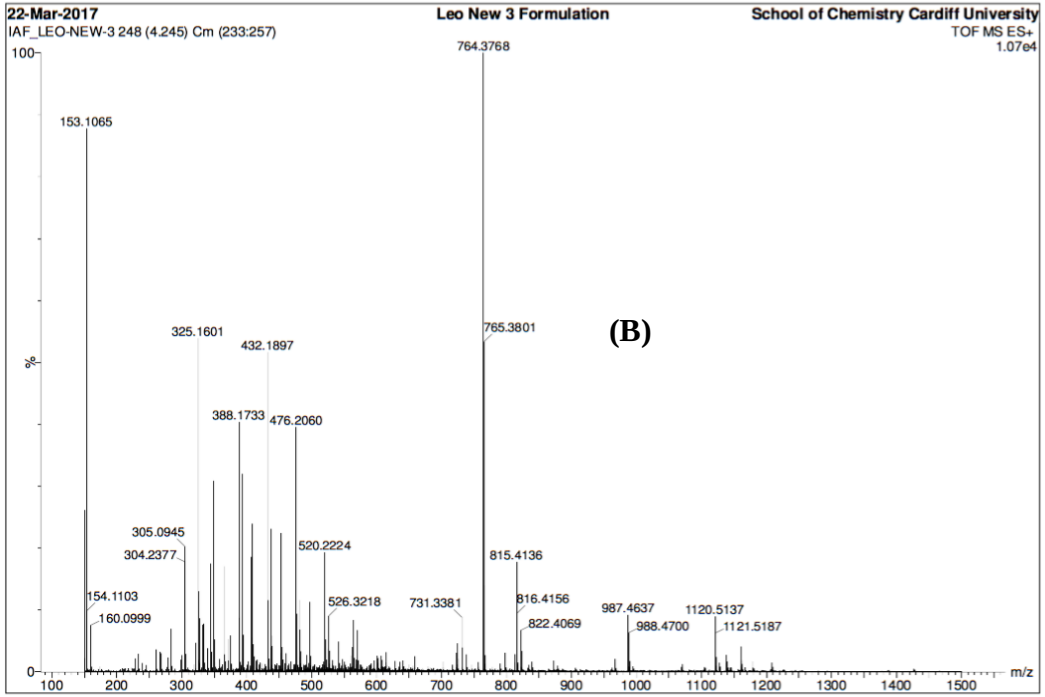
The solvent spectrum shows three main peaks. From left to right, first peak corresponds to non-deuterated water molecules, the second peak is related to methyl groups (6.79 ppm in D_2O) and the third one to methylene groups of ethanol (4.32 ppm in D_2O). In Figure 5.2 B, peaks corresponding to methylene groups of triethanolamine (6.84 ppm and 5.90 ppm) are more intense than the ones related to ethanol from the solvent. By comparing comparison with Figure 5.2 C, it was found that presence of zinc complexes with triethanolamine is not significant as chemical shifts obtained for solutions containing both zinc and triethanolamine were similar to the ones obtained with triethanolamine only. Further analysis with other sample components at a distinct operation frequency is necessary to validate the method.

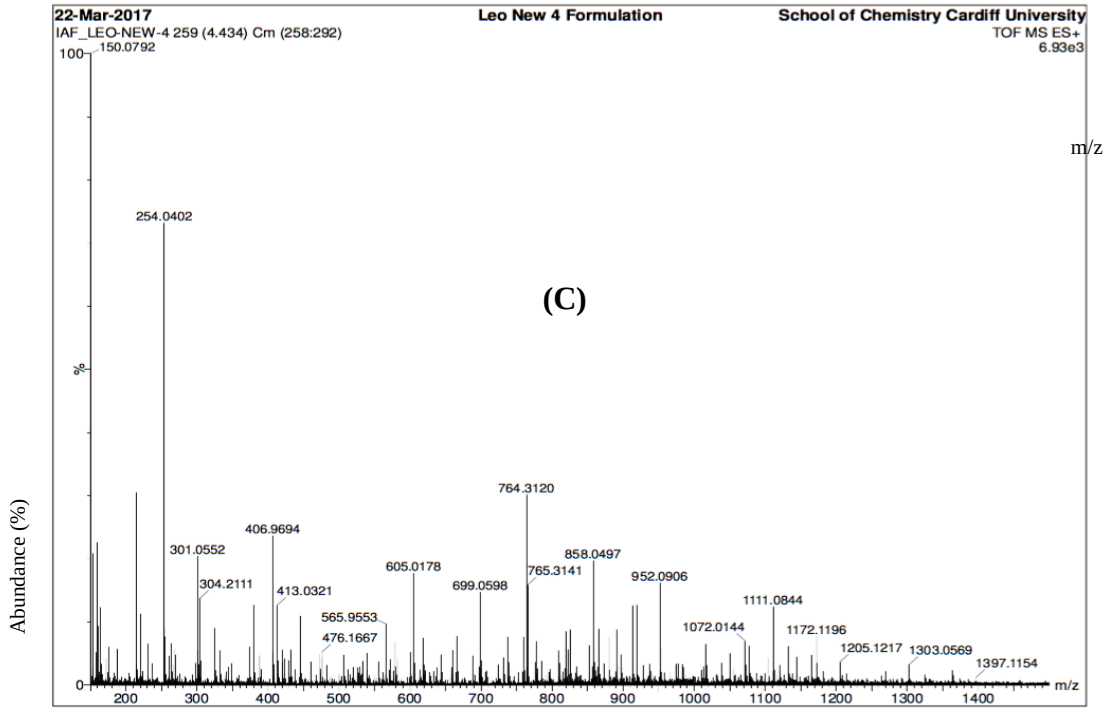
5.3.2 Analysis of RB-002 formulation chemistry by LC-MS – *was it possible to identify active compounds?*

As previously described, RB-002 formulation chemistry was further analysed by LC-MS as an attempt to verify and identify possible chemical entities responsible for activity against viruses. The results for the RB-002 formulation set are presented in figure 5.3.

Figure 5.3: Product ion mass spectra obtained by LC-MS analysis of different RB-002 formulations. (A) RB-002 – 40% (w/v) ethanol + 0.1 (w/v) zinc + excipients; (B) RB-002G 0.1% (w/v) zinc + excipients and (C) RB-002EXCP excipients only at pH 10.5.





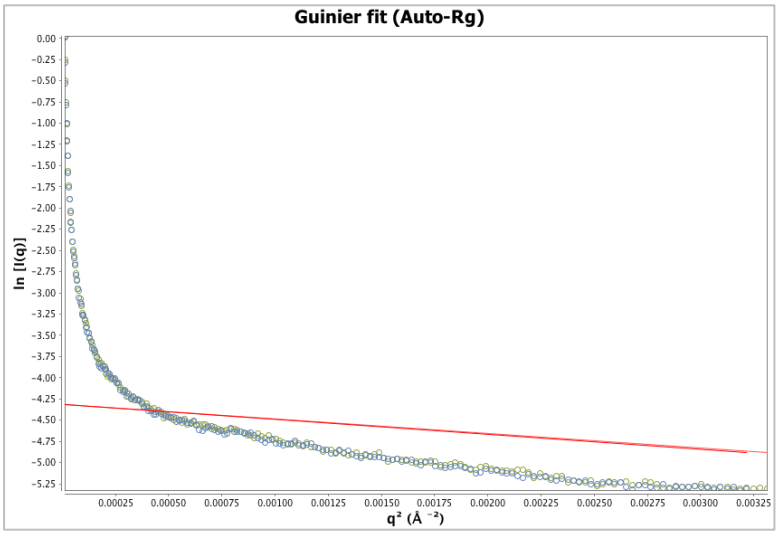


As expected, different m/z diagram profiles for different formulations presented distinct distributions of ions and abundance thereof. Formulation RB-002EXCP containing excipients only presented higher noise and extent of ionisation when compared to formulations RB-002 and RB-002. It was not possible to find hits for the obtained m/z peaks in positive mode by MassBank chemical database, thus identification of chemical species in the formulations was not possible.

5.3.3 Small-angle X-ray scattering spectroscopy on MS2 upon exposure to formulation RB001 (40% (w/v Ethanol+0.1(w/v) zinc sulphate heptahydrate + excipients)

SAXS analysis aims to evaluate the influence of formulations on bacteriophage MS2 overall size and structure of the protein shell and conformational changes as well as the amount of encapsulated RNA. In the present study, purified bacteriophage MS2 in presence of formulation CA001 was analysed. After obtaining the first set of data, Guinier analysis was performed in order to estimate particle radius of gyration and aggregation and obtained Guinier fit is shown in figure 5.4 as follows:

Figure 5.4: Guinier fit of sample composed of bacteriophage MS2 exposed to formulation CA001, where I(q) represents the scattering intensity resulting from the subtraction of the buffer from the sample and q represents the momentum transfer.



There was a non-linear dependence between Ln [I(q)] and q^2, especially for lower values of the latter (Fig. 5.4). The observed non-linear relation indicated the presence of aggregates in suspension, which strongly influenced data analysis and compromised its reliability (Putnam et al., 2007). Therefore, sample analysed was not suitable for verifying MS2 structure and conformational changes through SAXS.

5.4 Discussion

Since virucidal activities obtained in the present study was not comparable to the ones found in the literature (Ijaz and Zu, 2014), it was hypothesised that zinc was possibly coordinated to other formulation ingredients capable of forming complexes with metal ions, more specifically triethanolamine. Proton NMR spectroscopy at 400 MHz was performed to analyse formation of complexes between zinc ions and triethanolamine. The presence of zinc complexes with triethanolamine was not significant as chemical shifts obtained for solutions containing both zinc and triethanolamine were similar to the ones obtained with triethanolamine only (Fig. 5.2B). Nevertheless, it is suggested that further analysis with other sample components at a distinct operation frequency is necessary to validate the method and gather deeper understanding about the zinc coordination in the formulations and chemical environment (Skinner and Laurence, 2008). High presence of base form of zinc complexes with water are expected to be present in solution at that pH according to equation 5.1. However, it is essential to determine speciation diagram of zinc sulphate heptahydrate in formulation at different pHs. In this study, LC/MS of formulation RB-002 was carried out in order to verify whether it was possible to identify possible zinc species, which could be responsible for virucidal activity. Unfortunately, analysis of possible species from the given formulation did not reveal discrete Zn-ethanolamine adducts or any other zinc-related adduct. A more comprehensive chemical analysis of formulation set RB-002 through direct injection LC-MS/MS (tandem MS) analysis of solutions comprising one of RB-002 pure ingredient mixed with ultra pure water should be carried out in the future in order to reduce possible interferences from formulation excipients as well as matrix effects (Andersson et al., 2008). After obtaining LC-MS diagrams of each formulation ingredient in solution at pH 10.5, active ingredients like

ethanol and zinc sulphate heptahydrate could be added in order to possibly identify formation of active chemical species (Yamaguchi et al., 2007).

Regarding the analysis of viral capsid protein conformational changes and MS2 RNA structural alterations by SAXS, it is recommended that additional purification steps such as 2-step centrifugation in caesium chloride gradient and diafiltration will be performed to increase sample purity. In addition, PIPES (piperazine-*N, N'*-bis (2-ethanesulfonic acid)) buffer at pH 6.5 could be used in order to avoid virus aggregation and lead to initial viral titres up to 10^{15}PFU/mL as described by Nguyen et al. (2011).

Chapter 6 – General Discussion

6.1 Introduction

6.1.1 Biocides, viruses and public health

Biocides are synthetic or semi-synthetic chemicals, which can control growth and/or eliminate microorganisms associated to inanimate objects, solutions, surfaces or intact skin. Biocides can either be classified as sterilants, disinfectants or preservatives depending on whether they destroy all microbial life, eliminate infectious pathogenic microorganisms or hamper further growth of microbial population, respectively (SCENIHR, 2009). Biocidal products containing biocides are employed in a wide range of industrial, healthcare and household settings in which they play a crucial role in effective cleaning and disinfection procedures (Bloomfield, 2002; SCENIHR, 2009; WTO, 2014).

Mammalian viruses are obligate parasites grouped in different families and classified according to shape, size, envelope or lack thereof and genome differences (Regenmortel et al., 2000). Those viruses may represent serious threat to public health and are often associated to nosocomial infections in healthcare settings (Maillard, 2005). It is estimated that viruses alone account roughly 5% of approximately 7 million hospital acquired infections worldwide although it is thought to be an underestimated percentage (Aitken and Jeffries, 2001; Wright and Bieluch, 1993; Borkow and Monk, 2012). Carducci et al. (2011) discovered that 16.7% of all surface samples in healthcare settings were contaminated with viral nucleic acid. This impact is mainly due to the ease of viral transmission to hosts via contaminated surfaces like hospital floors, bedrails, doorknobs, medical devices and contaminated textiles which may even be source of aerosol contamination or directly contaminate patients and hospital personnel (Borkow and Monk, 2012). Besides, some viral characteristics contribute to ease of viral spread such as: i) high infectivity efficiency and ii) resilience for a long time when dried on inanimate surfaces in presence of organic load depending on environmental pH, temperature, level of viral aggregation and moisture (Kocwa-Haluch, 2001; Firquet et al., 2015). Hence, medical and scientific communities have been showing increased concern about control and prevention of pathogen outbreaks mainly in hospital settings. Disinfection and sterilisation practices have been incorporated as general measures to

prevent spread of pathogens (Rutala et al., 2017). The choice of a suitable disinfectant (biocidal product) with broad range of action and disinfection protocols is crucial to hamper virus spread and possible outbreaks.

Viruses present variable physicochemical properties, genome and structure, and as such distinct targets for virucidal action. This accounts for variability in susceptibility profiles to different biocides (Sauerbrei et al., 2004; Eterpi et al., 2009). In this context, the type of virus, biocide and its mechanism of action as well as environmental conditions should be taken into account in order to elaborate an effective disinfection protocol. For instance, non-enveloped viruses are known to be more resistant to certain biocides due to the lack of a lipid membrane (viral envelope) (Maillard, 2001). Table 6.1 shows clinically relevant non-enveloped viruses nowadays as well as effective biocides found in the literature.

Non-enveloped viruses present higher resilience to certain biocides (e.g. membrane active ingredients) and persistence for longer periods of time than enveloped counterparts on inanimate surfaces. Therefore, it is advisable to develop a disinfectant highly active against non-enveloped viruses and with a rapid and broad antiviral spectrum. Novel active ingredients and biocidal products are being developed not only to improve disinfection practices in different settings by higher bactericidal, fungicidal and virucidal efficacy but also to comply with toxicity and environmental guidelines. In addition, the possible link between co-selection and resistance of bacteria to certain biocides including excessive commonly use of the same active ingredient at sub-lethal concentrations highlight the need for the development of novel biocidal products with different combination of active ingredients (Wales and Davies, 2015). Biocidal products are an integral part of multi-barrier strategy to prevent spread of viruses and good hygiene practices as a whole.

Table 6.1. Most relevant human pathogenic non-enveloped viruses nowadays and respective pathogenesis.

Family	Viruses	Genome	Pathology	References
Adenoviridae	Adenovirus type 2, 5, 8, 40 and 41	dsDNA	Upper tract respiratory infection; keratoconjuctivitis; pneumonia	Kojaoghlanian et al. (2003); Rutala et al. (2006); Sauerbrei et al. (2004)
Caliciviridae	Astrovirus, Caliciviruses, Norovirus	+ssRNA	Epidemic gastroenteritis	Magulski et al. (2009)
Picornaviridae	Cocksackievirus, Rhinovirus Echovirus, Hepatitis A, Poliovirus type 1	+ssRNA	Common cold syndrome; Hepatitis; Poliomyelites	Ionidis et al. (2016); Wilton et al. (2014)
Reoviridae	Rotavirus and Reovirus	dsRNA	Severe diahrreia in infants	Sattar et al. (1994)
Mimiviridae	*Acanthamoeba* polyphaga mimivirus	dsDNA	Pneumonia	Campos et al. (2012)

6.1.2 Alcohol and zinc as active ingredients

Alcohols represent a group of active ingredients including mainly ethanol, *n*-propanol and isopropanol, which are present in low to mid-level disinfectants (WHO, 2014). This group of active ingredients tends to present a fast and wide spectrum of action, i.e. fungicidal, bactericidal and virucidal activity to some extent. The mode of action is mainly due to protein denaturation in presence of water. Higher activity against enveloped viruses when compared to non-enveloped ones suggests that viral envelope is also potential target for virucidal action (Soulet et al., 1998; Ionidis et al., 2016). However, the exact mechanism of virucidal action by alcohols against non-enveloped

viruses little is not entirely known yet. Furthermore, alcohols are supplemented with other active ingredients in order to increase the efficacy against wide range of pathogens. For instance, alcohol-based hand-rub sanitisers supplemented with hydrogen peroxide are formulations recommended by the World Health Organization (WHO) for hand disinfection in healthcare settings when commercial hand rubs are too expensive or out of reach (WHO, 2010).

Metallic ions such as copper, silver and zinc and respective salts were also shown as group of active ingredients effective against pathogenic microorganisms and are often used in conjunction with alcohols. For example, zinc pyrithione has been used as a preservative system in combination with alcohol in various skin antisepsis and cosmetic products (Crutchfield et al., 1997; Gutery et al., 2005). Although zinc salts are known in the literature to play a vital role in viral infective pathways and virus-host interactions, inhibitory effects against both enveloped and non-enveloped viruses have been also reported (Chatuverdi and Shrivastava, 2005; Husliz, 2003). Nevertheless, the mechanism of virucidal action associated to zinc salts is still largely unknown.

6.1.3 Objectives of the project

The main goal of the present project was to gather deeper insight into the mechanisms of virucidal action associated to formulations comprising the combination of ethanol and zinc sulphate as active ingredients against non-enveloped viruses. Initially, the virucidal efficacy tests of zinc/alcohol combinations against model virus (bacteriophages MS2 and F116) were performed for activity screening and structural studies. Subsequently, the efficacy of those formulations against human non-enveloped viruses (Adenovirus type 5 and Poliovirus type 1) was performed and structural studies allowed assessing potential viral targets for zinc/alcohol combination.

6.2 General considerations about the virucidal activity and mechanism of action associated to combination of ethanol and zinc as active ingredients against non-enveloped viruses

6.2.1 Impact of zinc and alcohol combination against non-enveloped viruses. What is the real impact?

At a first stage, the virucidal activity of formulations prepared in our laboratory containing a fixed amount of zinc sulphate heptahydrate with different ethanol concentrations against model viruses (bacteriophage MS2 and F116) was evaluated. The bacteriophage MS2 was firstly used for the following reasons: i) it is generally recognized to be a suitable surrogate for certain mammalian viruses like poliovirus type 1, norovirus and adenovirus**)** (Dawson et al., 2005); ii) it is safer to handle and iii) its propagation and quantification of plaque forming units is faster than with mammalian viruses allowing rapid screening of virucidal efficacy against non-enveloped viruses (Bae and Schwab, 2008). The formulation system containing 40% (w/v) ethanol with 0.1% (w/v) zinc sulphate hydrate was chosen for further investigation as it yielded higher viral $\log_{10}$ reductions for relatively low ethanol concentrations. Hence, that formulation system seemed to encompass the most beneficial trade off in terms of alcohol concentration reduction and virucidal activity at 60 min contact time. Table 6.2 shows a summary of the main virucidal efficacy tests of the aforementioned formulation system at pH 10.5 and respective controls against both bacteriophages and mammalian viruses at 1h contact time. Table 6.3 shows the virucidal efficacy tests of CA001 and RB-002 performed after different storage periods at room temperature and dark environment.

The virucidal activity of formulations containing both alcohol and zinc as active ingredients was predominantly higher than activity of formulation containing zinc as sole active ingredient against different non-enveloped viruses across different formulation batches. Furthermore, significant differences between virucidal activity of formulation containing zinc as sole active ingredient and formulations with excipients only, demonstrates the impact of zinc on the viability of non-enveloped viruses. It was noteworthy that formulation prepared in Cardiff (CA001) presented much higher activity than formulations prepared by RB (RB-001 and RB-002 series) (Table 6.2).

This could be due to differences in terms of ingredient purity and usage of denatured ethanol by RB as one of the active ingredients.

Table 6.2: Summary of the virucidal efficacy test results of different batches of the formulation system comprising 40% (w/v) ethanol with 0.1% (w/v) zinc sulphate at pH 10.5 against non-enveloped viruses in suspension. Only results at 60 min contact time are presented.

Formulation set	Formulation (composition)	Virus	Virus titre reduction (log_{10})
Batch 1: formulation prepared in Cardiff	CA001 (40% (w/v) ethanol+0.1% (w/v) zinc sulphate+excipients)	MS2	4.18±0.12
	CA001G (0.1% (w/v) zinc sulphate+excipients)		1.07±0.03
	CA001EXCP (excipients)		0.22±0.07
	CA001 in presence of 0.06% (w/v) EDTA		0.39±0.02
Batch 2: formulation prepared by RB (December 2014)	RB-001 (40% (w/v) ethanol+0.1% (w/v) zinc sulphate+excipients)	MS2	1.26±0.07
	RB-001 in presence of 0.06% (w/v) EDTA		0.33±0.03
Batch 3: formulation prepared by RB (September 2015)	RB-002 (40% (w/v) ethanol+0.1% (w/v) zinc sulphate+excipients)	MS2	0.88±0.04
		F116	0.68±0.02
		AdV2	5.26±0.10
		Pv1	3.71*
	RB-002G (0.1% (w/v) zinc sulphate+excipients)	MS2	0.37±0.06
		F116	0.14±0.02
		AdV2	0.80±0.12
		Pv1	2.06*
	RB-002EXCP (excipients)	MS2	0.05±0.03
		F116	0.09±0.04
		AdV2	0.61±0.10
		Pv1	1.39*

* only performed once; AdV2: adenovirus type 2; Pv1: poliovirus type 1

Table 6.3: Summary of virucidal activities obtained for formulations containing both alcohol and zinc as active agents after different storage times. Formulations were stored at room temperature in dark environment.

Formulation set	Formulation (composition)	Storage time	Virus titre reduction (log_{10})
Batch 1: formulation prepared in Cardiff	CA001 (40% (w/v) ethanol+0.1% (w/v) zinc sulphate+excipients)	0.5 month	4.18±0.12
		13 months	0.59±0.08
Batch 2: formulation prepared by RB (September 2015)	RB-002 (40% (w/v) ethanol+0.1% (w/v) zinc sulphate+excipients)	1 month	1.07±0.05
		6 months	1.00±0.04
		12 months	0.89±0.06
	RB-002 in presence of 0.06% (w/v) EDTA	1 month	0.61±0.12
		6 months	0.55±0.04
		12 months	0.33±0.04

Besides, the activity of CA001 formulations decayed significantly with time (see Table 6.3 CA001 after 13 months storage in Batch 1) when formulation was stored at room temperature in dark environment. The pH of the formulation was measured every two weeks and it was constant over the storage period under analysis. RB-002 formulations also yielded lower virucidal efficacy against MS2 over 12 months of storage although observed activity reduction from 1 to 12 months was lower than the one observed for CA001. In addition, storage time of RB-002 seemed to have an impact on how formulation activity is affected by presence of chelating agent (EDTA, see Table 6.3). This may be an indication that presence of free zinc ions in formulation and its coordination may change over storage time (Maret, 2015). These data show storage time is also a factor to take into account for overall formulation activity against non-enveloped viruses (see Table 6.3).

Overall, the formulations containing both alcohol and zinc yielded significantly lower activities in the presence of a strong chelating agent towards zinc such as EDTA.

This points out the importance of zinc to produce the observed virucidal activity. The importance of zinc was also shown to be more relevant at longer contact times (30 min and 60 min) against bacteriophage MS2 (see Chapter 3). This could be associated to mass transfer resistance for zinc and alcohol to reach their respective target on bacteriophage. Mass transfer is associated to the diffusion capacity of those active ingredients in liquid phase to reach targets on virus surfaces and also to the diffusion of virions in a liquid suspension by convection for example (Yakimovich et al., 2012; Liu et al., 2015). In this case, it is possible that there is a series of mass transfer resistances between virus target sites and active ingredients, which could be caused by improper mixing for example**.** Furthermore, the divalent cations like zinc may play a role at increasing phage attachment rate to the bacterial host, which questions the mechanism of virus inactivation of the formulation system under study. Indeed, the differences observed in virucidal activity between bacteriophages and mammalian viruses could be related to a different dynamic of virus/host cell receptor interaction. The dual effect of zinc as a divalent cation essential for virus infectivity but also virucidal at certain concentrations has been reported in the literature (Date, 1979; Lazarczyk and Favre, 2008; Geist et al., 1987; Wei et al., 2012). For instance, it is possible that zinc ions have a much more pronounced dual role in the bacteriophage infectious cycle compared to the mammalian virus one. The addition of chelating agents to RB-002 against poliovirus suggests mammalian viruses could be blocked from entering the host (see section 3.3.6 in Chapter 3). That observation raises interesting questions about the differences of dynamic of viral attachment and binding of zinc ions to specific viral capsid receptors and effective usage of bacteriophages as valid surrogates when formulations containing cations are used.

Bacteriophage F116 and adenovirus were used to investigate the impact of zinc and alcohol on virus structure and to identify possible targets for virucidal action. It was found that the presence of both alcohol and zinc could be associated to a specific damage pattern on bacteriophage F116 where material inside phage head presented a distinct conformation (concentrated + expanded) with severe damage to tail. F116 DNA was observed to be a potential target for alcohol/zinc virucidal action as nucleic damage was observed through gel electrophoresis (see Chapter 4). On the other hand, transmission electron microscopy showed that adenovirus exposed to formulation containing both alcohol and zinc showed possible damages to the capsid, which may have led to a distinct uptake of the negative stain. The nano-indentation investigation

with adenovirus showed that all RB-002 formulations led to less stiff virus capsid (lower spring constant) although formulations containing zinc as active ingredient (RB-002 and RB-002G) yielded fragile viruses where their capsid was more susceptible to mechanical stress (lower breaking force). The capacity of viral capsid to withstand certain force and alter capsid conformation is crucial for different steps of the infectious cycle such as cell attachment, cell entry and release of viral genome (Greber, 2016). The higher mechanical susceptibility of the viral capsid may be a net result of capsomer displacement, protein conformation changes and pressure variation exerted by potential nucleic acid conformational changes (Bauer et al., 2015). Nevertheless, adenovirus DNA damage was not observed upon exposure to formulation through the protocol carried out in the present project.

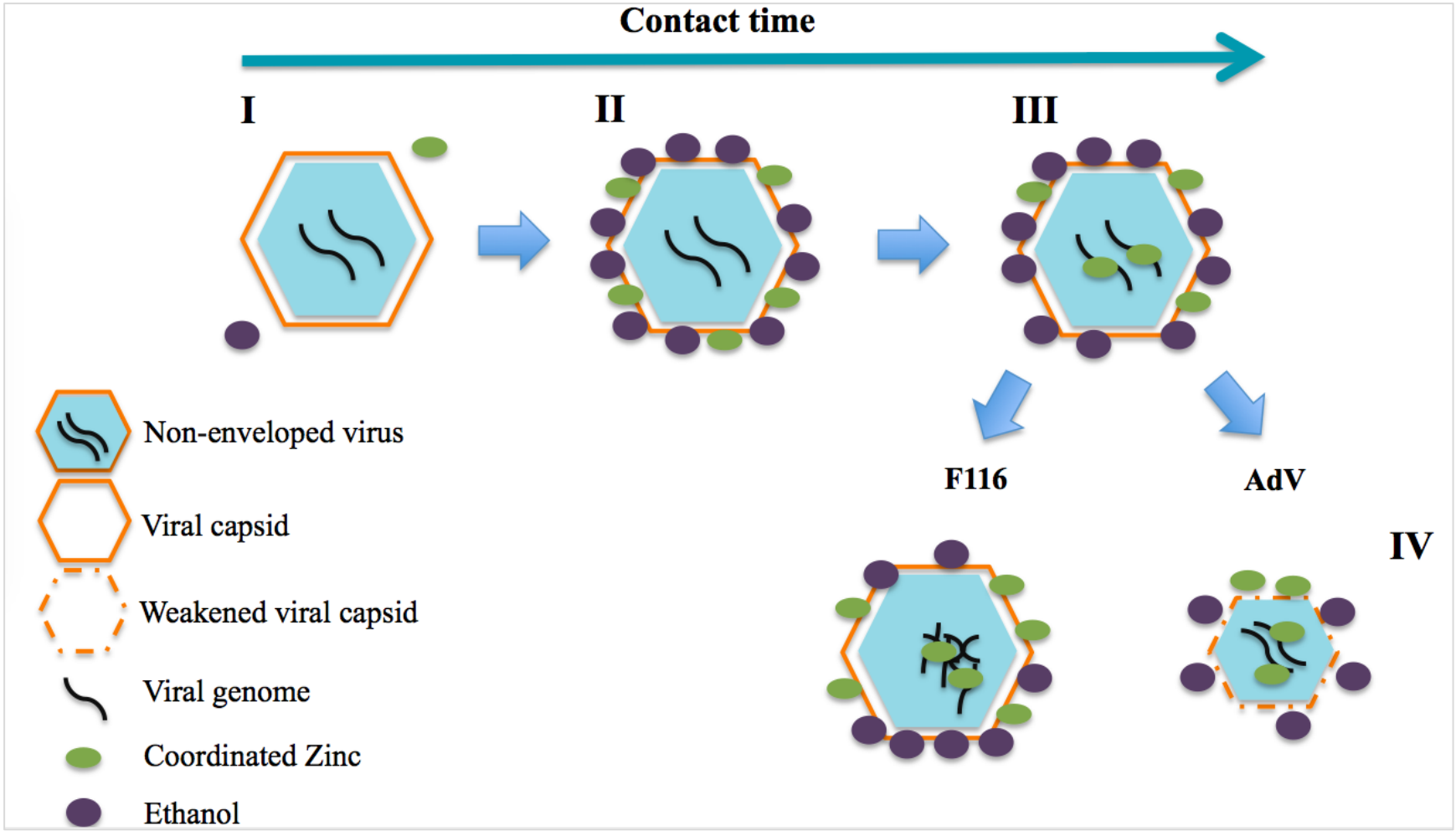
The virucidal activity differences observed for RB-002 formulation against different viruses could be due to the following factors: i) different targets for zinc action; ii) effect of zinc on phage attachment to bacterial host; iii) possible impairment of mammalian virus cell entry by an excess of zinc. Although bacteriophages susceptibility to RB-002 formulations was significantly lower than mammalian viruses, virucidal activity trends were similar between the tested mammalian viruses and bacteriophages. For example, the highest activities were consistently observed against mammalian viruses and bacteriophages when both alcohol and zinc were present as active ingredients, whereas the control formulation comprising zinc as sole active ingredient yielded lower inactivation of both bacteriophages and mammalian viruses. Therefore, bacteriophages MS2 and F116 could be used as preliminary indicators of virucidal activity associated to alcohol/zinc formulation system against mammalian viruses under pre-defined environmental conditions (pH, temperature, presence of absence of organic load and/or chelating agents). However, surrogacy capacity in terms of inactivation has not been proven for this particular formulation system and virucidal activity quantification despite biological attributes of MS2 and F116 being similar to Pv1 and AdV2, respectively. Sinclair et al. (2012) stresses out that underestimating or overestimating inactivation of an organism may impact negatively on the elaboration of a risk assessment and consequently jeopardize safety levels of a given disinfection protocol. In this project, inactivation of non-enveloped viruses by alcohol/zinc combination is underestimated by activity test in suspension against bacteriophage MS2 and F116, which potentially invalidate usage of those microorganisms as ultimate surrogates for mammalian viruses for the present formulation system.

6.2.2 Proposed mechanism of action by zinc/alcohol combination against non-enveloped viruses

Knowledge about a given biocide mechanism of action (MoA) is essential to optimise its use, understand its limitations as well as improve biocidal product design and detect potential resistance mechanisms by target organisms (Chapman, 2002). The discovery of potential targets for biocidal action against pathogen microorganisms is an essential part of the MoA elucidation. The proposed MoA for the combination of alcohol with zinc along contact time is presented in Figure 6.1.

Initially non-enveloped virus is in contact with formulation containing both alcohol and zinc, which will tend to promote internal capsid protein conformation changes and bind to potential zinc finger motifs on viral capsid protein as time progresses (Phase I and II Figure 6.1). The interaction zinc and alcohol with the viruses is likely to be time-dependent since virucidal efficacy increases significantly with contact time (see Chapter 3). At a later stage (Phase III Figure 6.1) the viral capsid is weakened and zinc ions could penetrate the virus more efficiently (EDAX data, Chapter 3) interacting with viral nucleic acid nucleic (Phase IV) yielding damage to F116 virions (DNA damage by electrophoresis, Chapter 4). On the other hand, the presence of ethanol and zinc led to weaker and reduced adenovirus capsid compromising its infectivity (Nano-indentation studies, Chapter 4). Unfortunately, it was not possible to identify successfully possible chemical species, such as zincate, involved at different stages of formulation virucidal action (Chapter 5). The proposed MoA for formulations comprising 40% (w/v) ethanol and 0.1% (w/v) zinc salt with excipients has its limitations as possible interaction and damage to mammalian virus nucleic acid still remains to be confirmed. Future experiments aiming to gather deeper look on the proposed MoA will be suggested by the end of the present chapter.

Figure 6.1: Proposed mechanism of action (MoA) of alcohol/zinc combination against non-enveloped viruses along contact time



6.3 Relevance of the project and impact on public health

The usage of zinc salts and oxides as antimicrobial agents is still relatively limited nowadays. The main usage of zinc is as a preservative in combination with other active ingredients in biocidal products used as pesticide in agriculture (Rajasekaran et al. 2016), as hand and skin antisepsis in household and healthcare products (Guthery et al., 2005) and as antifouling agent in paints for metal surfaces treatment (Ytreberg et al., 2010). The only example of biocidal product based on the combination of alcohol with zinc salt found to date is the usage of zinc pyrithione as an effective agent to increase antimicrobial activity persistence in skin antisepsis biocidal products. This combination allows higher activity for longer period of times in topical antiseptics containing alcohol as main active ingredient. Biocidal products based on that antimicrobial system is in compliance with FDA guidelines and used frequently as shampoos for control of dandruff in in shampoos for control of dandruff and surgical handrubs for example (FDA, 2007; Goncalves et al., 2011)**.** Usage of other zinc salts such as zinc sulphate (as used in the present study) in combination with alcohol has not been commercially reported yet.

Despite alcohol in concentrations between 59-90% (w/v) showing a fast acting and broad-spectrum of antimicrobial action (McDonell and Russel, 1999; Macinga et al., 2008; Alhmidi et al., 2017), persistence of action is relatively low as alcohol evaporate quickly and residual activity after short periods of time is compromised (Rutala and Weber, 2014; Alhmidi et al., 2017). Hence, there is a need to combine it with other active ingredients. Moreover, usage of 60-90% (w/v) alcohol in surface and hand disinfectants can be problematic mostly due to increased flammability and toxicity affecting user safety (Kramer et al., 2006). It is also known that volatile organic compounds (VOCs) generated by alcohol-based household products may have short and long-term adverse health effects on animal and human in indoor environments such as sensory irritation, allergies, asthma and leukemia (Dai et al. 2017). The European Union and several states within U.S. have put in place environmental directives imposing limitations on VOCs emission in industrial settings (EU Council, 2004; EPA, 2017). Therefore, it is recommended that alcohol concentrations are reduced in many of those products while maintaining or preferably enhancing overall antimicrobial activity. In

this context, the present study indicated that combination of zinc with relatively low alcohol concentration can be effective against resilient microorganisms like non-enveloped viruses while complying with environmental and safety guidelines.

Data from our study helped understand how the zinc with low alcohol amount (< 45% w/v) combination inactivates viruses and provided information on the limitations of the formulation like relatively long contact time for larger extent of virucidal action and stability over period of storage. In this context, this research is an important step towards the development of novel biocidal products, which are effective, less toxic, safer to handle and store as well as in compliance with environmental guidelines and market trends of VOCs reduction.

6.4 Conclusion

It is undeniable that biocides (active ingredients) in biocidal products play a key role in surface disinfection procedures. Those procedures are carried out in different areas ranging from household to healthcare and industry settings in order to prevent infections and product spoilage. Synergistic combinations of active ingredients aim to increase microbicidal efficiency while decreasing product development and production costs, reducing toxicity, enhance surface compatibility and increase compliance with environmental guidelines. The need for gathering information about the mechanism of microbicidal action associated to a biocide is essential for rational improvement, design, development and assessing the toxicological and environmental impact of a given product. In this context, the present project aimed to gather a deeper understanding of the mechanism of action associated to the combination of alcohol and zinc against non-enveloped viruses. Research on the mechanism of action of alcohol and metallic ions in combination provided insightful information about those active ingredients interaction with viruses and limitations.

The initial screening of virucidal activity allowed verifying the combination of both alcohol and zinc against bacteriophage MS2 was more effective than counterparts containing one of the active ingredients only and that zinc had a more pronounced impact at lower ethanol concentrations (ranging from 25% w/v to 40% w/v) and at longer contact times (see Chapter 2). Surprisingly, activity of formulation RB-002 (40% (w/v) ethanol + 0.1% (w/v) zinc salt; pH 10.5) against AdV2 and Pv1 in suspension

induced 5.26 and 3.71 $\log_{10}$ reduction in virus concentration at room temperature, respectively. Those virucidal activities were significantly higher than the ones obtained against bacteriophage MS2 and F116. Thus, some caution is required about the usage of MS2 and F116 as virus surrogates for that formulation system and experimental conditions. Formulation storage time was also shown to have an effect on virucidal efficacy as formulations prepared in Cardiff led to lower viral inactivation 13 months after initial preparation. That was also reflected on the impact chelating agents had on overall activity along time on formulations CA001 and RB-002 (see section 3.3.5, Chapter 3). Structural studies showed that presence of zinc and alcohol has an impact on adenovirus capsid mechanical properties, which could potentially affect the ability to attach and infect a host cell. It is hypothesized that the combination weakens the viral capsid enabling zing to penetrate the nucleocapsid and interacts with the viral nucleic acid. Electron microscopical investigations showed the F116 capsid was affected to some extent and F116 nucleic acid was shown to be a target for virucidal action against bacteriophage F116 where the presence of zinc could be detected by EDAX and nucleic acid damage was observed by agarose gel electrophoresis. With adenovirus, the formulation was shown to weaken the capsid but unfortunately, the protocol used to exemplify DNA damage in adenovirus did not show any nucleic acid damage following formulation exposure.

There was an attempt of formulation speciation by identification of possible chemical entities responsible for activity as well as zinc coordination. It was shown by NMR analysis that interaction of zinc salts with triethanolamine, one of the excipients of the formulation known to be a chelating agent, was not significant indicating excipients may not be affecting zinc activity. Other analytical chemistry techniques were performed; however, no chemical species could be identified. It was hypothesized that zincate would be the most predominant zinc salt form at pH 10.5 complexed with other formulation ingredients such as triethanolamine for instance (Long and Angino, 1977). Unfortunately, no complexation was observed and zincate could not be identified through LC-MS studies.

In summary, the combination of alcohol with zinc salt revealed to be promising on their own against non-enveloped viruses at room temperature and pH 10.5. It was showed that viral capsid and nucleic acid are potential targets for the observed virucidal action as hypothesized beforehand. Based on those observations, a mechanism of action for both active ingredients was proposed although some aspects of it are still unclear

such as the impact of the formulation on i) mammalian/virus receptors and ii) mammalian virus nucleic acid as well exact chemical entities responsible for virucidal action.

6.5 Current project limitations and prospects

The data obtained throughout this project is an asset towards the unravelling of mechanism of action of alcohol and zinc combination against non-enveloped viruses. Nevertheless, this project had some limitations, which should be acknowledged. The proposed mechanism of action is only valid at the experimental parameters chosen for virucidal efficacy screening and testing, i.e. pH 10.5, room temperature in absence of organic load) at a specific alcohol/zinc ratio. Moreover, the virucidal activity of the formulations was evaluated against viruses in suspension, which is intrinsically less challenging for the viruses when compared to tests on viruses adhered to inanimate surfaces (Klein, 2007). Hence, the observed viral inactivation may be an underestimation and testing of the biocide in different situations reflecting the end user experience should be carried out under compatible parameters (Brinch et al., 2016; Lambert et al., 2003). In this context, the present study can be seen as proof of principle justifying further work to understand how the combination of alcohol and zinc performs with non-enveloped viruses using more appropriate standard efficacy tests for different usage scenarios.

From this research, several questions arose and ideas for future experiments can be suggested. All these questions would also help understanding the possible differences between bacteriophages and mammalian viruses and their possible usage as surrogates or not

- What is the influence of different environmental parameters (pH, temperature, organic load on overall activity) on the virucidal activity of a given ratio of alcohol/zinc?
- How specific is the interaction of the formulation with the viral capsid? Is it mediated by zinc finger motifs? How does capsid conformation change along time? How does it affect virus attachment and cell entry?

- How specific is the interaction of zinc ions with viral nucleic acid? How severe is nucleic acid damage?
- What are the chemical species involved in virucidal activity and zinc coordination throughout contact time?

The influence of different key environmental parameters and different alcohol/zinc ratios would allow the kinetic modelling of inactivation kinetics under different experimental conditions and a better understanding of the mechanism of action against viruses (Wigginton et al., 2012). That could be a useful tool for the prediction of the virucidal activities and rapid development of a given biocidal product and rapid screening for the most suitable alcohol/metal ion ratio for further analysis. Additionally, the very same virucidal efficacy analysis should be performed against a wide range of non-enveloped viruses. It has been reported that related viruses may present different susceptibilities towards a given biocide (Baxter et al., 2007). This study could also highlight potential emergence of resistance mechanisms by viruses through selective pressure for example (Zhong et al., 2016).

The viral capsid mechanical properties (stiffness and fragility) were shown to be severely damaged by the combination of alcohol with zinc. Viruses may present zinc finger binding motifs on their capsid and/or proteins associated to viral nucleic acid conformation (Kirthi and Savithri, 2003; Chatuverdi and Shrivastava, 2005). Thus, it would be useful to investigate specific point of interaction of zinc with viral capsid proteins and the impact of their conformation and virus stability. It would also be an additional information about the dual effect of zinc as a divalent cation essential for virus infectivity but also virucidal at certain concentrations. The virus capsid conformational changes including pressure variation by nucleic acid along contact time could be analysed by small angle X-ray spectroscopy of highly purified viruses exposed to formulation (Bauer et al., 2015). Sodium dodecyl sulfate polyacrylamide gel electrophoresis of capsid proteins extracted after exposure to formulation could reveal potential protein alterations. Additionally, points of interaction of zinc ions with proteins could be revealed by in-gel screening of zinc ions through laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) (Becker et al., 2004).

The nucleic acid was demonstrated to be a target for virucidal action against bacteriophage F116 in this project although damage could not be observed for adenovirus. Extra-long range quantitative real-time PCR (LR-qPCR) could be

performed in order to quantify DNA damage as well as to estimate inactivation of virus exposed to formulation (Rodriguez et al., 2013). That experiment could provide rapid insightful information about extension of nucleic acid lesions on DNA and RNA viruses, whereas the gel analysis could exclusively provide a qualitative analysis for DNA viruses. The quantification of DNA damage would be an additional parameter to be taken into account into modelling the virus inactivation kinetics by alcohol and zinc.

In this project, it was shown that the role of zinc is essential for observed virucidal activity and that presence of different chelating agents (see Chapter 3) can affect significantly virus inactivation. Besides, it was observed that formulation chemistry may change with time and that virucidal activity will tend to decrease with long storage periods in dark environment. The chemical speciation of formulation and analysis of zinc coordination including the influence of external factors like pH and temperature would be important to predict virucidal activity decay and get insightful information about biocide limitations. It is recommended that analytical techniques like nuclear magnetic resonance (NMR) and direct injection tandem mass spectrometry are performed at different stages of formulation preparation.

Finally, it would be useful to evaluate activity of alcohol/zinc combination against a wide range of potential resilient pathogenic microorganisms like bacteria, spores and mycobacteria in order to attest for its usage as a suitable standalone disinfectant product in healthcare, household or industrial settings.

7. References

Aich, P.; Labiuk, S.; Tari, L.; Delbaere, L.; Roesler, W.; Falk, K.; Steer, R. and Lee, J. (1999). M-DNA: A Complex Between Divalent Metal Ions and DNA which Behaves as a Molecular Wire. Journal of Molecular Biology. Vol. 294, pp.: 477-485.

Aitken, C. and Jeffries, D. (2001). Nosocomial Spread of Viral Disease. Clinical Microbiology Reviews. Vol. 14, pp.: 528-546.

Alba, R.; Cots, D.; Ostapchuck, P.; Bosch, A.; Hearing, P. and Chillon, M. (2011). Altering the Ad5 Packaging Domain Affects the Maturation of the Ad Particles. PLoS One. Vol. 6, pp.: 1-10.

Alba, R.; Hearing, P.; Bosch, A. and Chillon, M. (2007). Differentual Amplification of Adenovirus Vectors by Flanking the Packaging Signal with attB/attP-PhiC31 Sequences: Implications for Helper-dependent Adenovirus Production. Virology. Vol. 367, pp.: 51-58.

Alhmidi, H.; Koganti, S.; Cadnum, J.; Rai, H.; Jencson, A. and Donskey, C. (2017). Evaluation of a Novel Alcohol-Based Surface Disinfectant for Disinfection of Hard and Soft Surfaces in Healthcare Facilities. Open Forum Infectious Diseases. Vol. 4, pp.: 1-3.

Allan, N., Omar, A., Harding, M. and Olson, M. (2011). A Rapid, High-throughput Method for Culturing, Characterizing and Biocide Efficacy Testing of both Planktonic Cells and Biofilms. In: Science against microbial pathogens: communicating current research and technological advances. A. Méndez-Vilas Ed., Madrid, Spain.

Allied Market Research (2017). Biocides Market by Type (Metallic Compounds, Halogen-Based Derivatives, Organic Acid, Sulfones, Phenol Based, and Others), and End Use Industry (Food and Beverages, Construction, Agriculture, Pharmaceuticals, Personal Care, Water Treatment, Paints & Coatings, and Others). Portland, USA

Amim, R., Oliveira, M., Janczak, J.; Rubinger, M.; Vieira, L.; Alves, L. and Zambolim, L. (2011). Syntheses, Characterization, Crystal Structure and Antifungal Activity of Four tetraphenylphosphonium bis(*N*-R-sulfonyldithiocarbimato)zincate(II) salts. Polyhedron. Vol. 30, pp.: 683-689.

Andersson, M.; Gustavsson, E. and Beck, O. (2008). Direct Injection LC-MS/MS Method for Identification and Quantification of Amphetamine, Metamphetamine, 3,4-Methylenedioxyamphetamine and 3,4-Methylenedioxymethamphetamine in Urine Drug Testing. Journal of Chromatography B. Vol. 861, pp.: 22-28.

Aoki, S. and Kimura, E. (2004). Zinc-Nucleic Acid Interaction. Chemical Reviews. Vol. 104, pp.: 769-787.

Aranha-Creado, H. and Brandwein, H. (1999). Application of Bacteriophages as Surrogates for Mammalian Viruses: A Case for Use in Filter Validation Based on Precedents and Current

Practices in Medical and Environmental Virology. PDA Journal of Pharmaceutical Science and Technology. Vol. 53, pp.: 75-82.

Arens, M. and Travis, S. (2000). Zinc Salts Inactivate Clinical Isolates of Herpex Simplex Virus in vitro. Journal of Clinical Microbiology. Vol. 38, pp.: 1758-1762.

ASTM International (2011a). E1052 Standard Test Method to Assess the Activity of Microbicides against Viruses in Suspension. Pennsylvannia, United States.

ASTM International (2011b). E1053 Standard Test Method to Assess Virucidal Activity of Chemicals Intended for Disinfection of Inanimate, Nonporous Environmental Surfaces. Pennsylvania, United States.

ASTM International (2013). E2011-13 Standard Test Method for Evaluation of Hygienic Handwash and Handrub Formulations for Virus-Eliminating Activity Using the Entire Hand.

Bae, J. and Schwab, K. (2008). Evaluation of Murine Norovirus, Feline Calicivirus, Poliovirus, and MS2 as Surrogates for Human Norovirus in a Model of Viral Persistence in Surface Water and Groundwater. Applied and Environmental Microbiology. Vol. 74, pp.: 477-484.

Bales, R. and Li, S. (1993). MS2 and Poliovirus transport in porous media: hydrophobic effects and chemical perturbations. Water Resources Research. Vol. 29, pp.: 957-963.

Baltimore, D. (1971). Expression of Animal Virus Genomes. Bacteriology Reviews. Vol. 35, pp.: 235-241.

Bauer, D.; Li, D.; Huffman, J.; Homa, F.; Wilson, K.; Leavitt, J.; Casjens, S.; Baines, J. and Evilevitch, A. (2015). Exploring the Balance between DNA Pressure and Capsid Stability in Herpesviruses and Phages. Journal of Virology. Vol. 89, pp.: 9288-9298.

Baugh, M. (2000). HIV: Reactive Oxygen Species, Enveloped Viruses and Hyperbaric oxygen. Medical Hypotheses. Vol. 55, pp.: 232-238.

Baxter, C.; Hofmann, R.; Templeton, M. (2007). Inactivation of adenovirus types 2, 5, and 41 in drinking water by UV light free chlorine, and monochloramine. Journal of Environmental Engineering. Vol. 133, pp.: 95-103.

Becker, J., Zoriy, M., Krause Buchholz, U.; Susanne Becker, J.; Pickhardt, C.; Przybylski, M.; Pompe, W. and Roedel, G. (2004). In-gel Screening of Phosphorus and Copper, Zinc, and Iron in Proteins of Yeast Mictochondria by LA-ICP-MS and Identification of Phosphorylated Protein Structures by MALDI-FT-ICR-MS after Separation with Two-dimensional Gel Electrophoresis. Journal of Analytical Atomic Spectrometry. Vol. 19, pp.: 1236-1243.

Beeton, M., Alves, D., Enright, M. and Jenkins, A. (2015). Assessing Phage Therapy against *Pseudomonas aeruginosa* using a *Galleria mellonella* Infection Model. International Journal of Antimicrobials Agents. Vol. 46, pp.: 196-200.

Bester, K. and Lamani, X. (2010). Determination of Biocides as well as Some biocide Metabolites from Façade run-off Waters by Solid Phase Extraction and High Performance Liquid Chromatographic Separation and Tandem Mass Spectrometry Detection. Journal of Chromatography A. Vol. 32, pp.: 5204-5214.

Blevins, S. & Bronze, M. (2010). Robert Koch and the "Golden Age" of Bacteriology. International Journal of Infectious Diseases. Vol. 14, pp.: 744-751.

Block, S. (2001). Chapter 1: Historical Review. In: Disinfection, Sterilization and Preservation 5th edition. Lippincott Williams and Wilkins, Philadelphia, USA.

Bloomfield, S. (2002). Significance of Biocide Usage and Antimicrobial Resistance in Domiciliary Environments. Journal of Applied Microbiology. Vol. 92, pp.: 144S-157S.

Bolton, S.; Kotwal, G.; Harrison, M.; Law, S.; Harrison, J. and Cannon, J. (2013). Sanitizer Efficacy against Murine Norovirus, a Surrogate for Human Norovirus, on Stainless Steel Surfaces when using Three Application Methods. Applied and Environmental Microbiology. Vol. 79, pp.: 1368-1377.

Borkow, G. and Gabbay, J. (2005). Copper as a Biocidal Tool. Current Medicinal Chemistry. Vol. 12, pp.: 2163-2175.

Borkow, G. and Monk, A. (2012). Fighting Nosocomial Infections with Biocidal Non-intrusive Hard and Soft surfaces. World Journal of Clinical Infectious Diseases. Vol. 2, pp.: 77-90.

Boyce, J. (2016). Modern Technologies for Improving Cleaning and Disinfection of Environmental Surfaces in Hospitals. Antimicrobial Resistance & Infection Control. Vol. 5, pp.: 1-10.

Brady, M., Evans, J. and Cuartas, J. (1990). Survival and Disinfection of Parainfluenza Viruses on Environmental Surfaces. American Journal of Infection Control. Vol. 18, pp.: 18-23.

Breathnach, A. (2013). Nosocomial Infections and Infection Control. Medicine. Vol. 41, pp.: 649-653.

Brinch, A.; Hansen, S.; Hartmann, N. and Baun, A. (2016). EU Regulation of Nanobiocides: Challenges in Implementing the Biocidal Product Regulation. Nanomaterials (Basel). Vol. 6, pp.: 33.

Brown, D., Westphal, M., Burlingham, B.; Winterhoff, U. and Doerfler, W. (1975). Structure and Composition of the Adenovirus Type 2 Core. Journal of Virology. Vol. 16, pp.: 366-387.

Bruns, R., Kaulen, J., Kretschik, M.; Kugler, M. and Uhr, H. (2004). R&D in Material protection: New biocides. In: Directory of Microbicides for the Protection of Materials. Kluwer Academic Publishers. Norwell, USA.

Byrne, M. and Kropinski, A. (2005). The Genome of the *Pseudomonas aeruginosa* Generalized Transducing Bacteriophage F116. Gene. Vol. 346, pp.: 187-194.

Campos, R.; Andrade, K.; Ferreira. P.; Bonjardim, C.; La Scola, B.; Kroon, E. and Abrahao, J. (2012). Virucidal Activity of Chemical Biocides against Mimivirus, a Putative Pneumonia Agent. Journal of Clinical Virology. Vol. 55, pp.: 323-328.

Carducci, A.; Verani, M.; Lombardi, R.; Casini, B. and Privitera, G. (2011). Environmental Survey to Assess Viral Contamination of Air and Surfaces in Hospital Settings. Journal of Hospital Infection. Vol. 77, pp.: 242-247.

Carrasco, C.; Luque, A., Hernando-Perez, M.; Miranda, R.; Carrascosa, J.; Serena, P.; Ridder, M.; Raman, A.; Gomez-Herrero, J.; Schaap, I.; Reguera, D. and de Pablo, P. (2011). Built-in Mechanical Stress in Viral Shells. Biophysics Journal. Vol. 100, pp.: 1100-1108.

Carubelli, R.; Schneider, J.; Pye, Q. and Floyd, R. (1995). Cytotoxic Effects of Autoxidative Glycation. Free Radical Biology & Medicine. Vol. 18, pp.: 265-269.

Caspar, D. and Klug, A. (1962). Physical Properties in the Construction of Regular Viruses. Cold Spring Harbor Symposia on Quantitative Biology. Vol. 27, pp.: 1-24.

CEN, prEN (2003). BS EN 13610:2002 Chemical Disinfectants. Quantitative Suspension Test for the Evaluation of Virucidal Activity against Bacteriophages of Chemical Disinfectants used in Food and Industrial areas. Test method and Requirements (phase 2, step 1).

CEN, prEN (2011). EN14476: 2011 Quantitative Suspension Test for the Evaluation of Virucidal Activity of Chemical Disinfectants and Antiseptics in Human Medicine (phase 2, step 1)

Cerf, O., Carpentier, B. and Sanders, P. (2010). Tests for Determining In-use Concentrations of Antibiotics and Disinfectants are Based on Entirely Different Concepts: "Resistance" has Different Meanings. International Journal of Food Microbiology. Vol. 136, pp.: 247-254.

Chambon, M., Bailly, J. and Peigue-Lafeuille, H. (1992). Activity of Glutaraldehyde at Low Concentrations against Capsid Proteins of Poliovirus Type 1 and Echovirus Type 25. Applied and Environmental Microbiology. Vol. 58, pp.: 3517-3521.

Chapman, J. (2003). Biocide Resistance Mechanisms. International Biodeterioration & Biodegradation. Vol. 51, pp.: 133-138.

Chatham, J. and Blackband, S. (2001). Nuclear Magnetic Resonance Spectroscopy and Imaging in Animal Research. ILAR Journal. Vol. 42, pp.: 189-208.

Chatuverdi, U. and Shrivastava, R. (2005). Interaction of Viral Proteins with Metal Ions: Role in Maintaining the Structure and Functions of Viruses. FEMS Immunology and Medical Microbiology. Vol. 43, pp.: 105-114.

Chatuverdi, U., Shrivastava, R. and Upreti, R. (2004). Viral Infections and Trace elements: A Complex Interaction. Current Science. Vol. 87, pp.: 1536-1554.

Chen, N.; Zheng, Y.; Yin, J.; Li, X. and Zheng, C. (2013). Inhibitory Effects of Silver Nanoparticles against Adenovirus Type 3 *in vitro*. Journal of Virological Methods. Vol. 193, pp.: 470-477.

Choi, Y. and Chang, J. (2013). Viral Vectors for Vaccine Applications. Clinical and Experimental Vaccine Research. Vol. 2, pp.: 97-105.

Colemann, J. (1992). Zinc Proteins: Enzymes, Storage Proteins, Transcription Factors, and Replication Proteins. Annual Reviews of Biochemistry. Vol. 61, pp.: 897-946.

Condell, O.; Iversen, C.; Cooney, S.; Power, K.; Walsh, C.; Burgess, C. and Fanning, C. (2012). Efficacy of Biocides Used in the Modern Food Industry To Control Salmonella enterica, and Links between Biocide Tolerance and Resistance to Clinically Relevant Antimicrobial Compounds. Applied and Environmental Microbiology. Vol. 78, pp.: 3087-3097.

Condezo, G.; Marabini, R.; Ayora, S.; Carazo, J.; Alba, R.; Chillon, M. and San Martin, C. (2015). Structure of Adenovirus Incomplete Particles Clarify Capsid Architecture and Show Maturation Changes of Packaging Protein L1 52/55k. Journal of Virology. Vol. 89, pp.: 9653-9664.

Crutchfield, C.; Lewis, E. and Zelickson, B. (1997). The Highly Effective Use of Topical Zinc Pyrithione in the Treatment of Psoriasis. Dermatology Online Journal. Vol. 3, pp.: 3-5.

Dai, H.; Jing, S.; Wang, H.; Ma, Y.; Li, L.; Song, W. and Kan, H. (2017). VOC Characteristics and Inhalation Health Risks in Newly Renovated Residences in Shangai, China. Science of the Total Environment. Vol. 577, pp.: 73-83.

Date, T. (1979). Kinetic Studies of the Interaction between MS2 Phage and F Pilus of *Escherichia coli.* European Journal of Biochemistry. Vol. 96, pp.: 167-175.

Davison, A., Benko, M. and Harrach, B. (2003). Genetic Content and Evolution of Adenoviruses. Journal of General Virology. Vol. 84, pp.: 2895-2908.

Dawson, D.; Paish, A.; Staffell, L.; Seymour, I. and Appleton, H. (2005). Survival of Viruses on Fresh Produce, using MS2 as Surrogate for Norovirus. Journal of Applied Microbiology. Vol. 98, pp.: 203-209.

DeForest, K. (1983) Principles of Viral Inactivity. In: Disinfection, Sterilisation and Preservation 3rd Edition. Lea & Febiger. Philadelphia, USA.

DIN (2013). EN 14476:2013-10 Chemical Disinfectants and Antiseptics – Quantitative Suspension Test for the Evaluation of Virucidal Activity in the Medical Area – Test Method and Requirements (Phase 2/Step 1). Berlin, Germany.

Do-Kyun, K.; Soo-Ji, K. and Dong-Hyun, K. (2017). Inactivation modelling of human enteric virus surrogates, MS2, Qbeta, and phiX175, in water using UVC-LEDs, a novel disinfecting system. Food Research International. Vol. 91, pp.: 115-123.

Doerfler, W. (1996). Chapter 67 Adenovirus. In: Medical Microbiology 4th Edition. Texas, United States.

Dunn, J. and Miller, M. (2014). Emerging Respiratory Viruses Other than Influenza. Clinics in Laboratory Medicine. Vol. 34, pp.: 409-430.

DVV (2012). Quantitative Pruefung der viruziden Wirksamkeit chemischer Desinfektions-mittel auf nicht-poroesen Oberflaechen. Hygiene and Medicine. Vol. 37, pp.: 78-85.

Ehrenfeld, E.; Domingo, E. and Roos, R. (2010). Chapter 7: Pathogenesis of Disease. In: The Picornaviruses. 1st Edition. ASM Press. Washigton, United States.

Elnifro, E.; Cooper, R.; Klapper, P. and Bailey, A. (2000). PCR and Restriction Endonuclease Analysis for Rapid Identification of Human Adenovirus Subgenera. Journal of Clinical Microbiology. Vol. 38, pp.: 2055-2061.

Environmental Protection Agency (EPA) (2017). Technical Overview of Volatile Organic Compounds (VOCs). https://www.epa.gov/indoor-air-quality-iaq/technical-overview-volatile-organic-compounds#references. Last update: April 2017. Date of access: Jan 2018.

EPA (2012). Product Performance Test Guidelines: Overview, Definitions, and General Considerations.

Eterpi, M.; McDonnell, G. and Thomas, V. (2009). Disinfection Efficacy against Parvoviruses Compared with Reference Viruses. Journal of Hospital Infections. Vol. 73, pp.: 64-70.

European Commission - Public Health (2009) http://ec.europa.eu/health/opinions/en/biocides-antibiotic-resistance/about-biocides-antibiotic-resistance.htm#7. Last access: August 2017.

European Commission (2013). General Report on REACH: Report from the Commission to the European Parliament Council, the European Economic and Social Committee and the Committee of the regions. Brussels, Belgium.

European Union Council (EU Council) (2004). Directive 2004/42/CE of the European Parliament and of the Council on the Limitation of Emission of Volatile Organic Compounds due to the Use of Organic Solvents in Certain Paints and Varnishes and Vehicle Refinishing Products and Amending Directive 1999/13/EC.

Farkas, K., Varsani, A. and Pang, L. (2014). Adsorption of Rotavirus, MS2 Bacteriophage and Surface-modified Silica Nanoparticles to Hydrophobic Matter. Food and Environmental Virology. Epub document ahead of print.

Feliciano, L.; Li, J.; Lee, J. and Pascall, M. (2012). Efficacies of Sodium Hypochlorite and Quaternary Ammonium Sanitizers for Reduction of Norovirus and Selected Bacteria during Ware-Washing Operations. Plos one. Vol. 7, pp.: 1-7.

Ferrario, D. and Rabbit, R. (2012). Analysis of the Proposed EU Regulation Concerning Biocide Products and its Opportunities for Alternative Approaches and a Toxicology for the 21st Century (t4 report). ALTEX. Vol. 29, pp.: 157-172.

Firquet, S.; Beaujard, S.; Lobert, P.; Sane, F.; Caloone, D.; Izard, D. and Hober, D. (2015). Survival of Enveloped and Non-Enveloped Viruses on Inanimate Surfaces. Microbes Environment. Vol. 30, pp.: 140-144.

Fitzgerald, K., Davies, A. and Russel, A. (1992). Sensitivity and Resistance of *Escherichia coli* and *Straphylococcus aureus* to Chlorhexidine. Letters in Applied Microbiology. Vol. 14, pp.: 33-36.

Flint, S. and Enquist, L. (2008). Chapter 2: Virological Methods. In: Principles of Virology 3rd Edition. ASM Press. United States.

Flora, S. and Pachauri, V. (2010). Chelation in Metal Intoxication. International Journal of Environmental Research and Public Health. Vol. 7, pp.: 2745-2788.

Fong, T. and Lipp, E. (2005). Enteric viruses of Humans and Animals in Aquatic Environments: Health Risks, Detection, and Potential Water Quality Asessement Tools. Microbiology and Molecular Biology Reviews. Vol. 69, pp.: 357-371.

Food and Drug Administration (FDA) (2007) Dandruff, seborrheic dermatitis, and psoriasis drug products for over-the-counter human use. Maryland, United States.

Fraise, A. (2013). Section 1 Part 1: Historical Introduction. In: Russel, Hugo & Ayliffe's Principles and Practice of Disinfection, Preservation and Sterilization 5th Edition. Wiley-Blackwell Publishing. Oxford, UK.

Furiga, A.; Pierre, G.; Glories, M.; Aimar, P.; Roques, C.; Causserand, C. and Berge, M. (2011). Effects of Ionic Strength on Bacteriophage MS2 Behavior and their Implications for the Assessment of Virus Retention by Ultrafiltration Membranes. Applied and Environmental Microbiology. Vol. 77, pp.: 229-236.

Gallandat, K. and Lantagne, D. (2017). Selection of a Biosafety Level 1 (BSL-1) Surrogate to Evaluate Surface Disinfection Efficacy in Ebola Outbreaks: Comparison of Four Bacteriophages. PLOS One. Vol. 12, pp.: 1-10.

Gaonkar, T.; Geraldo, I.; Shintre, M. and Modak, S. (2006). *In vivo* Efficacy of an Alcohol-Based Surgical Hand Disinfectant Containing a Synergistic Combination of Ethylhexylglycerin and Preservatives. Journal of Hospital Infection. Vol. 63, pp.: 412-417.

Geist, F.; Bateman, J.; Hayden, F. (1987). In vitro Activity of Zinc Salts against Human Rhinoviruses. Antimicrobial Agents and Chemotherapy. Vol. 31, pp.: 622-624.

Gelderblom, H. (1996). Chapter 41: Structure and Classification of Viruses. In: Medical Microbiology 4th Edition. Galveston (TX): University of Texas Medical Branch at Galveston. Texas, USA.

Gerba, C. (2015). Quaternary Ammonium Biocides: Efficacy in Application. Applied and Environmental Microbiology. Vol. 81, pp.: 464-469.

Gilson, R. and Durrant, M. (2009). Estimation of the pK_a Values of Water Ligands in Transition Metal Complexes Using Density Functional Theory with Polarized Continuum Model Solvent Corrections. Dalton Transactions. pp.: 10223-10230.

Goncalves, K.; Graziano, K. and Kawagoe, J. (2011). A Systematic Review of Surgical Hand Antisepsis Using an Alcohol Preparation Compared to Traditional Products. Revista da Escola de Enfermagem da USP. Vol. 46, pp.: 1483-1492.

Greber, U. (2016). Virus and Host Mechanics Support Membrane Penetration and Cell Entry. Journal of Virology. Vol. 90, pp.: 3802-3805.

Guidry, C.; Mansfield, S.; Sawyer, R. and Cook, C. (2014). Resistant Pathogens, Fungi, and Viruses. Surgical Clinics of North America. Vol. 94, pp.: 1195-1218.

Guo, J.; Wu, T.; Anderson, J.; Kane, B.; Johnson, D.; Gorelick, R.; Henderson, L. and Levin, J. (2000). Zinc Finger Structures in the Human Immunodeficiency Virus Type 1 Nucleocapsid Protein Facilitate Efficient Minus- and Plus-Strand Transfer. Journal of Virology. Vol. 74, pp.: 8980-8988.

Gustavsson, M.; Hellohf, A. and Backhaus, T. (2017). Evaluating the Environmental Hazard of Industrial Chemicals from Data Collected during the REACH Registration Process. Science of The Total Environment. Vol. 586, pp.: 658-665.

Guthery, E., Lawton, A. and Anderson, E. (2005). Zinc Pyrithione in Alcohol-Based Products for Skin Antisepsis: Persistence of Antimicrobial effects. Association for the Professionals in Infection Control and Epidemiology. Vol. 33, pp.: 15-22.

Gyurek, L. and Finch, G. (1998) Modeling Water Treatment Chemical Disinfection Kinetics. Journal of Environmental Engineering. Vol. 124, pp.: 783-793.

Hanlon, G.W. (2007). Bacteriophages: an appraisal of their role in the treatment of bacterial infections. International Journal of Antimicrobial Agents. Vol. 30, pp.: 118-128.

Hall, C. (1981). Nosocomial Viral Respiratory Infections: Perennial Weeds on Pediatric Wards. American Journal of Medicine. Vol. 70, pp.: 670-676.

Harrison, J.; Turner, R.; Joo, D.; Stan, M.; Chan, C.; Allan, N.; Vrionis, H.; Olson, M. and Ceri, H. (2008). Copper and Quaternary Ammonium Cations Exert Synergistic Bactericidal and Antibiofilm Activity against *Pseudomonas aeruginosa.* Antimicrobial Agents and Chemotherapy. Vol. 52, pp.: 2870-2881.

Hassan, M., Tuckman, H., Patrick, R.; Kountz, D. and Kohn, J. (2010). Cost of Hospital-Acquired Infection. Hospital Topics. Vol. 88, pp.: 82-89.

He, Y.; Mueller, S.; Chipman, P.; Bator, C.; Peng, X.; Bowman, V.; Mukhopadhyay, S.; Wimmer, S.; Kuhn, R. and Rossmann, M. (2003). Complexes of Poliovirus Serotypes with Their Common Cellular Receptor, CD155. Journal of Virology. Vol. 77, pp.: 4827-4835.

Hernando-Perez, M., Lambert, S., Nakatani-Webster, E.; Catalano, C. and de Pablo, P.J. (2014a). Cementing Proteins Provide Extra Mechanical Stabilization to Viral Cages. Nature Communications. Vol. 5, pp.: 1-8.

Hernando-Perez, M., Pascual, E., Aznar, M.; Ionel, A.; Caston, J.; Luque, A.; Carrascosa, J.; Reguera, D. and de Pablo, P.J. (2014b). The Interplay Between Mechanics and Stability of Viral Cages. Nanoscale. Vol. 6, pp.: 2702-2709.

Hijikata, N.; Tezuka, R.; Kazama, S.; Otaki, M.; Ushijima, K.; Ito, R.; Okabe, S.; Sano, D. and Funamizu, N. (2016). Bactericidal and Virucidal Mechanisms in the Alkaline Disinfection of Compost using Calcium Lime and Ash. Journal of Environmental Management. Vol. 181, pp.: 721-727.

Hogle, J. (2002). Poliovirus Cell Entry: Common Structural Themes in Viral Cell Entry Pathways. Annual Review of Microbiology. Vol. 56, pp.: 677-702.

Holah, J. (2013). Chapter 9: Cleaning and Disinfection Practices in Food Processing. In: Hygiene in Food Processing Principles and Practices 2nd Edition. Woodhead Publishing, Cambridge, UK.

Hollak, C.; vom Dahl, S.; Aerts, J.; Belmatoug, N.; Bembi, B.; Cohen, Y.; Collin-Histed, T.; Deegan, P.; van Dussen, L.; Giraldo, P.; Mengel, E.; Michelakakis, H.; Manuel, J.; Hrebicek, M.; Parini, R.; Reinke, J.; di Rocco, M.; Pocovi, M.; Sa Miranda, M.; Tylki-Szymanska, A.; Zimran, A. and Cox, T. (2010). Force Majeure: Therapeutic Measures in Response to Restricted Supply of Imiglucerase (Cerezyme) for Patients with Gaucher Disease. Blood Cells, Molecules, and Diseases. Vol. 44, pp.: 41-47

Hooker, J.; Kovacs, E. and Francis, M. (2003). Interior Surface Modification of Bacteriophage MS2. American Chemical Society. Vol. 126, pp.: 3718-3719.

Horie, M., Ogawa, H., Yoshida, Y.; Yamada, K.; Hara, A.; Ozawa, K.; Matsuda, S.; Mizota, C.; Tani, M.; Yamamoto, Y.; Yamada, M.; Nakamura, K. and Imai, K. (2008). Inactivation and Morphological Changes of Avian Influenza Virus by Copper Ions. Archives of Virology. Vol. 153, pp.: 1467-1472.

Hugbo, P. (1976). Additivity and Synergism *in vitro* as Displayed by Mixtures of Some Commonly Employed Antibacterial Preservatives. Canadian Journal of Pharmaceutical Sciences. Vol. 11, pp.: 17-20.

Hulisz, D. (2003). Efficacy of Zinc against Common Cold Viruses: An Overview. Journal of the American Pharmacists association. Vol. 44, pp.: 594-603.

Huttunen, R., Karppelin, M. and Syrjaenen, J. (2013). Review: Obesity and Nosocomial Infections. Journal of Hospital Infection. Vol. 85, pp.: 8-16.

Ichim, C. and Wells, R. (2011). Generation of High-Titer Viral Preparations by Concentration using Successive Rounds of Ultracentrifugation. Journal of Translational Medicine. Vol. 9, pp.: 137-144.

Ijaz, M. and Zhu, Y. (2014). Aqueous Alcoholic Microbicidal Compositions Comprising Zinc Ions. Patent WO 2014/006380.

Ijaz, M.; Rubino, J. and Zhu, Y. (2012). Aqueous Alcoholic Microbicidal Compositions Comprising Copper ions. Patent WO 2012/164254.

Ionidis, G.; Huebscher, J.; Jack, T.; Becker, B.; Bischoff, B.; Todt, D.; Hodasa, V.; Brill, F.; Steinmann, E. and Steinmann, J. (2016). Development and Virucidal Activity of a Novel Alcohol-Based Hand Disinfectant Supplemented with Urea and Citric Acid. BMC Infectious Diseases. Vol. 16, pp.: 1-10.

Ito, K.; Kajiura, T. and Abe, K. (2002). Effect of Ethanol on Antigenicity of Hepatitis B Virus Envelope Proteins. Japanese Journal of Infectious Diseases. Vol. 55, pp.: 117-121.

Ivanovska, I.; Wuite, G.; Joensson, B. and Evilevitch, A. (2007). Internal DNA Pressure Modifies Stability of WT Phage. PNAS. Vol. 104, pp.: 9603-9608.

Jeffrey, D. (1995). Chemicals Used as Disinfectants: Active Ingredients and Enhancing Additives. Revue Scientifique Et Technique. Vol. 14, pp.: 57-74.

Jones, M.; Bellamy, K.; Alcock, R. and Hudson, R. (1991). The Use of Bacteriophage MS2 as A Model System to Evaluate Virucidal Hand Disinfectants. Journal of Hospital Infection. Vol. 17, pp.: 279-285.

Jutkina, J.; Marathe, N.; Flach, C. and Larsson, D. (2018). Antibiotics and Common Antibacterial Biocides Stimulate Horizontal Transfer of Resistance at Low Concentrations. Science of The Total Environment. Vol. 616, pp.: 172-178.

Ka-Wai Hui, E. (2006). Reasons for the Increase in Emerging and Re-Emerging Viral Infectious Diseases. Microbes and Infection. Vol. 8, pp.: 905-916.

Kaehkoenen, E. and Nordstroem, K. (2008). Toward a Nontoxic Poison: Current Trends in (European Union) Biocides Regulation. Integrated Environmental Assessment and Management. Vol. 4, pp.: pp.: 471-477.

Kampf, G. (2016). Efficacy of Ethanol in Hand Hygiene against Adenoviruses. American Journal of Infection Control. Vol. 44, pp.: 1429.

Kampf, G.; Ostermeyer, C.; Werner, H. and Suchomei, M. (2013). Efficacy of Hand Rubs with A Low Alcohol Concentration Listed as Effective by A National Hospital Hygiene Society in Europe. Antimicrobial Resistance and Infection Control. Vol. 2, pp.: 1-7.

Karlstroem, A. and Levine, R. (1991). Copper Inhibits the Protease from Human Immunodeficiency Virus 1 by both Cysteine-dependent and Cysteine-independent Mechanisms. Proceedings of the National Academy of Sciences. Vol. 88, pp.: 5552-5556.

Kaushik, N.; Subramanu, C.; Anang, S. (2017). Zinc Salts block Hepatitis E Virus Replication by Inhibiting the Activity of Viral RNA-dependent RNA Polymerase. Journal of Virology. Vol. 92, pp.: 54-67.

Kay, M. (2011). State-of-the-art Gene-based Therapies: The Road Ahead. Nature Reviews Genetics. Vol. 12, pp.: 316-328.

Kennedy, M. and Parks, R. (2009). Adenovirus Virion Stability and the Viral Genome: Size Matters. The American Society of Gene and Cell Therapy. Vol. 17, pp.: 1664-1666.

Kirthi, N. and Savithri, H. (2003). A Conserved Zinc Finger Motif in the Coat Protein of *Tomato leaf curl Bangalore* Virus is Responsible for Binding to ssDNA. Archives of Virology. Vol. 148, pp.: 2369-2380.

Klein, D. (2007). Chapter 11: Microbiological Testing of Disinfectants and Decontaminants for Critical Surfaces. In: Disinfection and Decontamination: Principles, Applications and Related Issues. CRC Press. Boca Raton, United States.

Knipe, D.; Howley, P. and Griffin, D. (2006). Section II: Specific Virus Families. In: Fields Virology 5th Edition Vol.1. Lippincott Williams & Wilkins. Philadelphia, USA.

Kocwa-Haluch, R. (2001). Waterborne Enteroviruses as a Hazard for Human Health. Polish Journal of Environmental Studies. Vol. 10, pp.: 485-487.

Kojaoghlanian, T.; Flomenberg, P. and Horwitz, M. (2003). The Impact of Adenovirus Infection on the Immunocompromised Host. Medical Virology, pp.: 155-171.

Koonin, E. (1991). Genome Replication/Expression Strategies of Positive-Strand RNA Viruses: a Simple Version of a Combinatorial Classification and Prediction of New Strategies. Virus Genes. Vol. 5, pp.: 273/281.

Koonin, E.; Dolja, V. and Krupovic, M. (2015). Origins and Evolution of Viruses of Eukaryotes: The Ultimate Modularity. Virology, pp.: 2-25.

Korant, B. and Butterworth, B. (1976). Inhibition by Zinc of Rhinovirus Protein Cleavage: Interaction of Zinc with Capsid Polypeptides. Journal of Virology. Vol. 18, pp.: 298-306.

Kramer, A.; Galabov, A.; Sattar, S; Doehner, L.; Pivert, A.; Payan, C.; Wolff, M.; Yimaz, A. and Steinmann, J. (2006). Virucidal Activity of A New Hand Disinfectant with Reduced Ethanol Content: Comparison with Other Alcohol-Based Formulations. Journal of Hospital Infection. Vol. 62, pp.: 98-106.

Kuemel, G.; Schrader, S.; Zentgraf, H.; Daus, H. and Brendel, M. (1990). The Mechanism of The Antiherpetic Activity of Zinc Sulphate. Journal of General Virology. Vol. 71, pp.: 2989-2997.

Kutter, E.; Raya, R. and Carlson, K. (2005). Chapter 7: Molecular Mechanisms of Phage Infection. In: Bacteriophages: Biology and Applications. CRC Press. Florida, USA.

Kuzmanovic, D.; Elashvili, I.; Wick, C.; O'Connell, C. and Krueger, S. (2006). Quantification of RNA in Bacteriophage MS2-like Viruses Solution by Small-Angle X-Ray Scattering. Radiation Physics and Chemistry. Vol. 75, pp.: 359-368.

Labiuk, S. (2003). Cobalt, Nickel and Zinc-DNA Interactions: A Structural Study. Thesis submitted at University of Saskathewan. Sasketoon, Canada.

Labiuk, S.; Delbaere, L.; Lee, J. (2001). Gamma and Ultraviolet Radiation Cause DNA Crosslinking in The Presence of Metal Ions at High pH. Photochemistry and Photobiology. Vol. 73, pp.: 579-584.

Laksham, K.; Kalidoss, V.; Sivanantham, P.; Sambath, P.; Arunachalam, M. and Chinnakali, P. (2016). Household Biocide Use and Personal Safety Practices among Rural Population in South India: A Community-Based Study. Medycyna Pracy. Vol. 67, pp.: 599-604.

Lambert, R.; Johnston, M.; Hanlon, G. and Denyer, S. (2003). Theory of Antimicrobial Combinations: Biocide Mixtures – Synergy or Addition? Journal of Applied Microbiology. Vol. 94, pp.: 747-759.

Lara, H.; Ayala-Nunez, N.; Ixtepan-Turrent, L. and Rodriguez-Padilla, C. (2010). Mode of Antiviral Action of Silver Nanoparticles against HIV-1. Journal of Nanobiotechnology. Vol. 8, pp.: 1-10.

Lazarczyk, M. and Favre, M. (2008). Role of Zn^{2+}Ions in Host-Virus Interactions. Journal of Virology. Vol. 82, pp.: 11486-11494.

Lee, J.; Latimer, L. and Reid, R. (1993). A Cooperative Conformational Change in Duplex DNA Induced by Zn^{2+} and Other Divalent Metal Ions. Biochemistry and Cell Biology. Vol. 71, pp.: 162-168.

Li, G.; Brockman, J.; Lin, S.; Abnet, C.; Schell, L. and Robertson, J. (2012). Measurement of the Trace Elements Cu, Zn, Fe, and Mg and the Ultratrace Elements Cd, Co, Mn, and Pb in Limited Quantity Human Plasma and Serum Samples by Inductively Couples Plasma-Mass Spectrometry. American Journal of Analytical Chemistry. Vol. 3, pp.: 646-650.

Li, J. and Dennehy, J. (2011). Differential Bacteriophage Mortality on Exposure to Copper. Applied and Environmental Microbiology. Vol. 77, pp.: 6878-6883.

Liu, P.; Yuen, Y.; Hsiao, H.; Jaykus, L. and Moe, C. (2010). Effectiveness of Liquid Soap and Hand Sanitizer against Norwalk Virus on Contaminated Hands. Applied and Environmental Microbiology. Vol. 76, pp.: 394-399.

Liu, X.; Guo, Z.; Roache, N.; Mocka, C.A.; Allen, M. and Mason, M. (2015). Henry's Law Constant and Overall Mass Transfer Coefficient for Formaldehyde Emission from Small Water Pools under Simulated Indoor Environmental Conditions. Environmental Science & Technology. Vol. 49. pp.: 1603-1610.

Llauro, A.; Coppari, E.; Imperatori, F.; Bizzarri, A.R.; Caston, J.R.; Santi, L.; Cannistraro, S. and de Pablo, P.J. (2015). Calcium Ions Modulate the Mechanics of Tomato Bushy Stunt Virus. Biophysical Journal. Vol. 109, pp.: 390-397.

Long, D.; Angino, E. (1977). Chemical Speciation of Cd, Cu, Pb, and Zn in Mixed Freshwater, Seawater, and Brine Solutions. Geochimica et Cosmochimica Acta. Vol. 41, pp.: 1183-1191.

Louten, J. (2016). Chapter 14: Poliovirus. In: Essential Human Virology. 1st Edition. Academic Press (Elsevier). San Diego, United States.

Lu, R.; Li, Q.; Yin, Z.; Xagoraraki, I.; Tarabara, V. and Nguyen, T. (2016). Effect of Virus Influent Concentration on Its Removal by Microfiltration: The Case of Human Adenovirus 2. Journal of Membrane Science. Vol. 497, pp.: 120-127.

Luria, S. (1947). Reactivation of Irradiated Bacteriophage by Transfer of Self-Reproducing Units. Proceedings of the National Academy of Sciences. Vol. 33, pp.: 253.

Macinga, D.; Sattar, S.; Jaykus, L. and Arbogast, J. (2008). Improved Inactivation of Nonenveloped Enteric Viruses and Their Surrogates by a NovelAlcohol-Based Hand Sanitizer. Applied and Environmental Microbiology. Vol. 74, pp.: 5047-5052.

Magri, M.; Fidjeland, J., Joensson, H.; Albihn, A. and Vinneras, B. (2015). Inactivation of Adenovirus, Reovirus and Bacteriophages in Fecal Sludge by pH and Ammonia. Science of The Total Environment. Vol. 520, pp.: 213-221.

Magulski, T.; Paulmann, D.; Bischoff, B.; Becker, B.; Steinmann, E.; Steinmann, J.; Goronchy-Bermes, P. and Steinmann, J. (2009). Inactivation of Murine Norovirus by Chemical Biocides on Stainless Steel. BMC Infectious. Vol. 9, pp.: 1-7.

Maillard, J.-Y. (1994). Chapter 6: Effects of Biocides on Phage Nucleic Acid and Transduction. Bacteriophages and Biocides. Cardiff University. United Kingdom.

Maillard, J.-Y.; Hann, A.; Beggs, T.; Day, M.; Hudson, R. and Russel, A.D. (1995). Electronmicroscopic Investigation of The Effects of Biocides on *Pseudomonas aeruginosa* PA0 Bacteriophage F116. Journal of Medical Microbiology. Vol. 42, pp.: 415-420.

Maillard, J.-Y. (2001). Virus Susceptibility to Biocides: An Understanding. Reviews in Medical Microbiology. Vol. 12, pp.: 63-74.

Maillard, J.-Y. (2002). Bacterial Target Sites for Biocide Action. Journal of Applied Microbiology Symposium Supplement. Vol. 92, pp.: 16S-27S.

Maillard, J.-Y. (2005). Antimicrobial Biocides in The Healthcare Environment: Efficacy, Usage, Policies, and Perceived Problems. Therapeutics and Clinical Risk Management. Vol. 1 (4), pp.: 307-320.

Maillard, J.-Y.; Beggs, T.; Day, M.; Hudson, R. and Russel, A.D. (1993). Effect of Biocides on *Pseudomonas aeruginosa* Phage F116. Letters in Applied Microbiology. Vol. 17, pp.: 167-170.

Maillard, J.-Y.; Beggs, T.; Day, M.; Hudson, R. and Russel, A.D. (1994). Effect of Biocides on MS2 and K Coliphages. Applied and Environmental Microbiology. Vol. 60, pp.: 2205-2206.

Maillard, J.-Y.; Hann, A.; Beggs, T.; Day, M.; Hudson, R. and Russel, A.D. (1995). Electronmicroscopic Investigation of The Effects of Biocides on *Pseudomonas aeruginosa* PA0 Bacteriophage F116. Journal of Medical Microbiology. Vol. 42, pp.: 415-420.

Maillard, J.-Y.; Hann, A.; Beggs, T.; Day, M.; Hudson, R. and Russel, A.D. (1995b). Energy Dispersive Analysis of X-rays Study of The Distribution of Chlorhexidine Diacetate and Cetylpyridinium Chloride on the *Pseudomonas aeruginosa* Bacteriophage F116. Letters in Applied Microbiology. Vol. 20, pp.: 357-360.

Maillard, J.-Y., Sattar, S., and Pinto, F. (2013). Section 1 Chapter 9: Virucidal activity of microbicides. In: Russel, Hugo & Ayliffe's Principles and Practice of Disinfection, Preservation and Sterilization 5th Edition. Wiley-Blackwell Publishing. Oxford, UK.

Maillard, J.-Y., Beggs, T.; Day, M.; Hudson, R. and Russel, A.D.. (1996). Damage to *Pseudomonas aeruginosa* PAO1 Bacteriophage F116 DNA by Biocides. The Journal of Applied Bacteriology. Vol. 80. Pp.: 540-544.

Maillard, J.-Y., Hann, A. and Perrin, R. (1998). Resistance of *Pseudomonas aeruginosa* PAO1 Phage F116 to Sodium Hypochlorite. Journal of Applied Microbiology. Vol. 85, pp.: 799-806.

Makhluf, H. and Shresta, S. (2018). Development of Zika Virus Vaccines. Vaccines (Basel). Vol. 6, pp.: 1-9.

Marchetti, M.; Wuite, G. and Roos, W. (2016). Atomic Force Microscopy Observation and Characterization of Single Virions and Virus-Like Particles by Nano-Indentation. Current Opinion in Virology. Vol. 18, pp.: 82-88.

Maret, W. (2015). Analyzing Free Zinc (II) Ion Concentrations in Cell Biology with Fluorescent Chelating Molecules. Metallomics. Vol. 7, pp.: 202-211.

Maris, P. (1995). Modes of Action of Disinfectants. Revue Scientifique et Technique. Vol. 14, pp.: 47-55.

Martin, R. and Mariam, Y. (1979). Interaction Between Metal Ions and Nucleic Bases, Nucleosides and Nucleotides in Solution. In: Metal Ions in Biological Systems Vol. 28. Marcel Dekker. New York, US.

McDonell, G. and Russel, A.D. (1999). Antiseptics and Disinfectants: Activity, Action, and Resistance. Clinical Microbiology Reviews. Vol. 12, pp.: 147-179.

McDonnell, G. and Burke, P. (2011). Review: Disinfection: is it time to reconsider Spaulding?. Journal of Hospital Infection. Vol. 78, pp.: 163-170.

McDonnell, G. and Russel, A.D. (1999). Antiseptics and disinfectants: activity, action and resistance. Clinical Microbiology Reviews. Vol. 12, pp.: 147-179.

McDonnell, G. and Russel, A.D. (1999). Antiseptics and disinfectants: activity, action and resistance. Clinical Microbiology Reviews. Vol. 12, pp.: 147-179.

Michael, S.; Kilfoil, V.; Schmidt, M.; Amann, B. and Berg, J.M. (1992). Metal Binding and Folding Properties of a Minimalist Cys2His2 Zinc Finger Peptide. PNAS. Vol. 89, pp.: 4796-4800.

Miles, A. and Misra, S. (1938). The Estimation of The Bactericidal Power of the Blood. Journal of Hygiene. Vol. 38, pp.: 732-749.

Minor, P. (1996). Poliovirus Biology. Cell. Vol. 4, pp.: 775-778.

Morens, D. and Fauci, A. (2013). Emerging Infectious Diseases: Threats to Human Health and Global Stability. PLOS Pathogens. Vol. 9, pp.: 1-3.

Morina, F.; Jovanovic, L.; Mojovic, M.; Vidovic, M.; Pankovic, D. and Veljovic Jovanovic, S. (2010). Zinc-Induced Oxidative Stress in Verbascum Thapsus is Caused by An Accumulation of Reactive Oxygen Species and Quinhydrone in The Cell Wall. Physiologia Plantarum. Vol. 140, pp.: 209-224.

Myers, F. (2008). Biocidal Agents: Modes of Action and Correlation with Antibiotic Resistance. The Biomedical Scientist. Pp.: 227-231.

Nath, N., Fang, C and Dodd, R. (1982). Inactivation of DNA-Polymerase Associated with Hepatitis B Virus. Journal of Medical Virology. Vol. 10, pp.: 131-140.

Nathanson. N. and Kew, O. (2010). From Emergence to Eradication: The Epidemiology of Poliomyelitis Deconstructed. American Journal of Epidemiology. Vol. 172, pp.: 1213-1229.

Nguyen, T.; Easter, N.; Gutierrez, L.; Huyett, L.; Defnet, E.; Mylon, S.; Ferri, J. and Ai Viet, N. (2011). The RNA Core Weakly Influences the Interactions of Bacteriophage MS2 at Key Environmental Interfaces. Soft Matter. Vol. 7, pp.: 10449-10456.

Noda, N.; Watanabe, M.; Yamada, F. and Fujimoto, S. (1981). Virucidal Efficiency of Alcohols against Viruses in Liquid Phase. The Journal of the Japanese Association for Infectious Diseases. Vol. 55, pp.: 355-366.

Nwachuku, N. and Gerba, C. (2006). Health Risks of Enteric Viral Infections in Children. Review in Environmental Contamination and Toxicology. Vol. 186, pp.: 1-56.

Nyborg, J. and Peersen, O. (2004). That Zincing Feeling: The effects of EDTA on the behavior of zinc-binding transcriptional regulators. Biochemistry Journal. Vol. 281, pp.: e3-34.

O'Gorman, J. and Humphreys, H. (2012). Application of Copper to Prevent and Control Infection. Where are we now?. Journal of Hospital Infection. Vol. 81, pp.: 217-223.

Oestman, M.; Lindberg, R.; Fick, J.; Bjoern, E. and Tysklind, M. (2017). Screening of Biocides, Metals and Antibiotics in Swedish Sewage Sludge and Wastewater. Water Research. Vol. 115, pp.: 318-328.

Ortega Morente, E.; Fernandez Fuentes, M.A.; Grande Burgos, M.J.; Abriouel, H.; Perez Pulido, R. and Galvez, A. (2013). Biocide Tolerance in Bacteria. International Journal of Food Microbiology. Vol. 162, pp.: 13-25.

Ortega-Esteban, A.; Perez-Berna, A.; Menendez-Conejero, R.; Flint, S.; San Martin, C. and de Pablo, P.J. (2013). Monitoring Dynamics of Human Adenovirus Disassembly Induced by Mechanical Fatigue. Scientific Reports, Vol. 3, pp.: 1-7.

Park, G.; Barclay, L.; Macinga, D.; Charbonneau, D.; Pettigrew, C.A. and Vinje, J. (2010). Comparative Efficacy of Seven Hand Sanitizers against Murine Norovirus, Feline Calicivirus and GII.4 Norovirus. Journal of Food Protection. Vol. 73, pp.: 2232-2238.

Patel, S. (2015). Plant Essential Oils and Allied Volatile Fractions as Multifunctional Additives in Meat and Fish-Based Food Products. Food Additives & Contaminants: PartA: Chemistry, Analysis, Control, Exposure & Risk Assessment. Vol. 32, pp.: 1049-1064.

Paulus, W. (2004). Introduction to Microbicides. In: Directory of Microbicides for the Protection of Materials. Kluwer Academic Publishers. Norwell, USA.

Pecson, B.; Martin, L. and Kohn, T. (2009). Quantitative PCR for Determining the Infectivity of Bacteriophage MS2 upon Inactivation by Heat, UV-B Radiation, and Singlet Oxygen: Advantages and Limitations of An Enzymatic Treatment to Reduce False-Positive Results. Applied and Environmental Microbiology. Vol. 75, pp.: 5544-5554.

Pfaender, S.; Brinkmann, J.; Todt, D.; Riebesehl, N.; Steinmann, J.; Steinmann, J.; Pietschmann, T. and Steinmann, E. (2014). Mechanisms of Viral Inactivation methods against Hepatitis C Virus. Applied Environmental Microbiology. Vol. 81, pp.: 1616-1621.

Pinto, F. (2010). Thesis: Mechanisms of Action of PHMB-Based Biocides against Naked Virus. Cardiff University. Cardiff, UK.

Pinto, F., Maillard, J.-Y. and Denyer, S.P. (2010). Effect of Surfactants, Temperature, and Sonication on The Virucidal Activity of Polyhexamethylene Biguanide against The Bacteriophage MS2. Association for Professionals in Infection Control and Epidemiology. Vol. 38, pp.: 393-398.

Plowman, R.; Graves, N.; Griffin, M.; Roberts, J.A.; Swan, A.V.; Cookson, B. and Taylor, L. (2001). The Rate and Cost of Hospital Acquired Infections Occurring in Patients Admitted to

Selected Specialties of a District General Hospital in England and The National Burden Imposed. Journal of Hospital Infection. Vol. 47, pp.: 198-209.

Price, B. (2001). Chapter 4: Asepsis. In: Surgical Technology for the Surgical Technologist 2nd edition. Taylor & Francis. New York, USA.

Putnam, C.; Hammel, M.; Hura, G. and Tainer, J.A. (2007). X-ray Solution Scattering (SAXS) Combined with Crystallography and Computation: Defining Accurate Macromolecular Structures, Conformations and Assemblies in Solution. Quaterly Reviews of Biophysics. Vol. 40, pp.: 191-285.

Rabenau, H.; Kampf, G.; Cinatl, J. and Doerr, H.W. (2005). Efficacy of Various Disinfectants against SARS Coronavirus. Journal of Hospital Infection. Vol. 61, pp.: 107-111.

Rabenau, H.; Steinmann, Rapp, I.; Schwebke, I. and Eggers, M. (2014). Evaluation of A Virucidal Quantitative Carrier Test for Surface Disinfectants. PLOS One. Vol. 9, pp.: 1-10.

Raczek, N. (2004). Food and Beverage Preservation. In: Directory of Microbicides for the Protection of Materials. Kluwer Academic Publishers. Norwell, USA.

Rajasekaran, P.; Kannan, H.; Das, S.; Young, M. and Santra, S. (2016). Comparative Analysis of Copper and Zinc Based Agrichemical Biocide Products: Materials Characteristics, Phytotoxicity and *in vitro* Antimicrobial efficacy. Environmental Science. Vol. 3, pp.: 439-455.

Regenmortel, M.; Fauquet, C.; Bishop, D.; Carstens, E.B.; Estes, M.K.; Lemon, S.M.; Maniloff, J.; Mayo, M.A.; McGeoch, D.J.; Pringle, C.R. and Wickner, R.B. (2000). Virus Taxonomy: Classification and Nomenclature of Viruses. Seventh report of the International Committee on Taxonomy of Viruses. Academic Press. San Diego, United States.

Rexroad, J.; Evans, R. and Middaugh, C. (2006). Effect of pH and Ionic Strength on The Physical Stability of Adenovirus Type 5. Journal of Pharmaceutical Sciences. Vol. 95, pp.: 237-247.

Rodriguez, R.; Bounty, S. and Linden, K. (2013). Long-Range Quantitative PCR for Determining Inactivation of Adenovirus 2 by Ultraviolet Light. Journal of Applied Microbiology. Vol. 114, pp.: 1854-1865.

Russel, A.D. (2002). Introduction of biocides into clinical practice and the impact on antibiotic-resistant bacteria. Journal of Applied Microbiology Symposium Supplement. Vol. 92, pp.: 121S-135S.

Russel, A.D. (2003). Review: Biocide use and antibiotic resistance: the relevance of laboratory findings to clinical and environmental situations. Infectious Diseases. Vol. 3, pp.: 794-803.

Russel, A.D. (2003a). Similarities and differences in the responses of microorganisms to biocides. Journal of Antimicrobial Chemotherapy. Vol. 52, pp.: 750-763.

Rutala, W. and Weber, D. (2014). Selection of the Ideal Disinfectant. Infection Control and Epidemiology. Vol. 35, pp.: 855-865.

Rutala, W.; Peacock, J.; Gergen, M.; Sobsey, M.D. and Weber, D.J. (2006). Efficacy of Hospital Germicides against Adenovirus 8, A Common Cause of Epidemic Keratoconjunctivits in Health Care Facilities. Antimicrobial Agents and Chemotherapy. Vol. 50, pp.: 1419-1424.

Rutala, W.; Weber, D. and Healthcare Infection Control Practices Advisory Committee (HICPAC) (2017). Guideline for Disinfection and Sterilization in Healthcare Facilities. Center for Disease Control (CDC), United States.

Sagripanti, J. and Lightfoote, M. (1996). Cupric and Ferric Ions Inactivate HIV. AIDS Research and Human Retroviruses. Vol. 12, pp.: 333-336.

Sagripanti, J., Routson, L., Bonifacino, A.C. and Lytle, C.D. (1997). Mechanism of Copper-Mediated Inactivation of Herpes Simplex Virus. Antimicrobial Agents and Chemotherapy. Vol. 41, pp.: 812-817.

Sakudo, A., Onodera, T. and Tanaka, Y. (2010). Chapter 2.4: Inactivation of Viruses. In: Sterilization and Disinfection by Plasma 1st Edition. Nova Science Publishers. Tokyo, Japan.

San Martin, C. (2012). Review: Latest Insights on Adenovirus Structure and Assembly. Viruses. Vol. 4, pp.: 847-877.

Sanders, E. (2012). Aseptic Laboratory Techniques: Plating Methods. Journal of Visualized Experiments. Vol. 63, pp.: 3064.

Sattar, S.A.; Jacobsen, H.; Rahman, H.; Cusack, T. and Rubino, J.R. (1994). Interruption of Rotavirus Spread through Chemical Disinfection. Infection Control and Hospital Epidemiology. Vol. 15, pp.: 751-756.

Sauerbrei, A.; Eichhorn, U.; Scheibenzuber, M. and Wutzler, P. (2007). Hexon Denaturation of Human Adenoviruses by Different Groups of Biocides. Journal of Hospital Infection. Vol. 65, pp.: 264-270.

Sauerbrei, A.; Sehr, K.; Eichhorn, U.; Reimer, K. and Wutzler, P. (2004). Inactivation of Human Adenovirus Genome by Different Groups of Disinfectants. Journal of Hospital Infection. Vol. 57, pp.: 67-72.

Sauerbrei, A.; Sehr, K.; Brandstaed, A.; Heim, A.; Reimer, K. and Wutzler, P. (2004a). Sensitivity of Human Adenoviruses to Different Groups of Chemical Biocides. Journal of Hospital Infections. Vol. 57, pp.: 59-66.

SCENIHR – Scientific Committee on Emerging and Newly Identified Health Risks (2009). Assessment of the Antibiotic Resistance Effects of Biocides. European Commission. Directorate-General for Health & Consumers. pp.: 14-20.

Shaban, N.; Ali, A. and Masoud, M. (2003). Effect of Cadmium and Zinc Ethanolamine Complexes on Rat Brain Monoamine Oxidase-B Activity *in vitro*. Journal of Inorganic Biochemistry. Vol. 95, pp.: 141-148.

Sharp, D. and Leong, J. (1980). Inactivation of Poliovirus I (Brunhilde) Single Particles by Chlorine in Water. Applied and Environmental Microbiology. Vol. 40, pp.: 381-385.

Shirasaki, N.; Matsushita, T.; Matsui, Y.; Urasaki, T. and Ohno, K. (2009). Comparison of Behaviors of Two Surrogates for Pathogenic Waterborne Viruses, Bacteriophages Qß and MS2, during Aluminium Coagulation Process. Water Research. Vol. 43, pp.: 605-612.

Sinclair, R.; Rose, J.; Hashsham, S.; Gerba, C. and Haas, C.N. (2012). Criteria for Selection of Surrogates Used To Study the Fate and Control of Pathogens in the Environment. Applied and Environmental Microbiology. Vol. 78, pp.: 1969-1977.

Skinner, A. and Laurence, J. (2008). High-field Solution NMR Spectroscopy as A Tool for Assessing Protein Interactions with Small Molecule Ligands. Journal of Pharmaceutical Sciences. Vol. 97, pp.: 4670-4695.

Solomon, E.; Fino, V.; Wei, J. and Kniel, K.E. (2009). Comparative Susceptibilities of Hepatitis A Virus, Feline Calicivirus, Bacteriophage MS2 and Bacteriophage X-174 to Inactivation by Quaternary Ammonium and Oxidative Disinfectants. International Journal of Antimicrobial Agents. Vol. 33, pp.: 288-289.

Somani, S. and Ingole, N. (2010). Formulation of Kinetic Model to Predict Disinfection of Water by Using Natural Herbs. International Journal of Environmental Sciences. Vol. 2, pp.: 1344-1354.

Soule, H.; Duc, D.; Mallaret, M.; Chanzy, B.; Charvier, A.; Gratacap-Cavallier, B.; Morand, P. and Seigneurin, J.-M. (1998). Résistance des Vírus dans L'Environnement Hospitalar: Le Point sur L'Activité Virucide des Désinfectants Utilisés à L'état Liquid. Annales de Biologie Clinique. Vol.56, pp.: 693'703.

Souza, J.; Castro, C. and Bloch, C. (2000). Zinc Binding to Lambda Phage DNA Studied by Voltammetric Techniques. Journal of the Brazilian Chemical Society. Vol. 11, pp.:398-404.

Spaulding, E. (1957). Chemical Disinfection and Antisepsis in The Hospital. Journal of Hospital Research. Vol. 9, pp.: 5-31.

Springthorpe, V.; Grenier, J.; Lloyd-Evans, N. and Sattar, S.A. (1986). Chemical Disinfection of Human Rotaviruses: Efficacy of Commercially-Available Products in Suspension Tests. Journal of Hygiene. Vol. 97, pp.: 139-161.

Steinmann, J. (2001). Some Principles of Virucidal Testing. Journal of Hospital Infection. Vol. 48, pp.: S15-S17.

Steinmann, J.; Becker, B.; Bischoff, B.; Magulski, T.; Steinmann, J. and Steinmann, E. (2013). Virucidal Activity of Formulation I of The World Health Organization's Alcohol-Based

Handrubs: Impact of Changes in Key Ingredient Levels and Test Parameters. Antimicrobial Resistance and Infection Control. Vol. 2, pp.: 2-34.

Stockley, P.; Stonehouse, N. and Valegard, K. (1994). Molecular Mechanism of RNA Phage Morphogenesis. International Journal of Biochemistry. Vol. 26, pp.: 1249-1260.

Su, X. and D'Souza, D. (2012). Inactivation of Human Norovirus Surrogates by Benzalkonium Chloride, Potassium Peroxymonosulfate, Tannic Acid, and Gallic Acid. Foodborne Pathogens and Disease. Vol. 9, pp.: 829-834.

Suara, R. and Crowe, J. (2004). Effect of Zinc Salts on Respiratory Syncytial Virus Replication. Antimicrobial Agents and Chemotherapy. Vol. 48, pp.: 783-790.

Suchomel, M.; Gnant, G.; Weinlich, G. and Rotter, M. (2009). Surgical Hand Disinfection Using Alcohol: The Effects of Alcohol Type, Mode and Duration of Application. Journal of Hospital Infection. Vol. 71, pp.: 228-233.

Sulakvelidze, A., Alavidze, Z. and Glenn Morris, J. (2001). Bacteriophage Therapy. Antimicrobial Agents and Chemotherapy. Vol. 45, pp.: 649-659.

Susianto, G.; Farid, M.; Dhany, N. and Addy, H.S. (2014). Host Range for Bacteriophages that Infect Bacterial Blight Pathogen on Soybean. Procedia Environmental Sciences. Vol. 20, pp.: 760-766.

Sutherland, B.; Bennett P. and Sutherland J. (2006). Chapter: DNA Damage Quantitation by Alkaline Gel Electrophoresis. In: DNA Repair Protocols. Methods in Molecular Biology. 2nd Edition. Humana Press. New York, United States.

Tablan, O.; Anderson, L.; Arden, N.; Breinman, R.F.; Butler, J.C. and McNeill, M.M. (1994). Guideline for Prevention of Nosocomial Pneumonia. The Hospital Infection Control Practices Advisory Committee, Centers for Disease Control and Prevention. American Journal of Infection Control. Vol. 22, pp.: 247-292.

Tandon, R.; AuCoin, D. and Mocarski, E. (2009). Human Cytomegalovirus Exploits ESCRT Machinery in the Process of Virion Maturation. Journal of Virology. Vol. 83, pp.: 10797-10807.

Te Velthuis, A.; van den Worm, S.; Sims, A.; Baric, R.S.; Snijder, E.J. and van Hemert, M.J. (2010). Zn^{2+}Inhibits Coronavirus and Arterivirus RNA Polymerase Activity *in vitro* and Zinc Ionophores Block the Replication of These Viruses in Cell Culture. PLOS Pathogens. Vol. 6, pp.: 1-10.

Thurman, R.; Gerba, C. and Bitton, G. (1989). The Molecular Mechanisms of Copper and Silver Ion Disinfection of Bacteria and Viruses. Critical Reviews in Environmental Control. Vol. 18, pp.:295-315.

Tirel, E.; Bellamy, Z.; Adams, H.; Lebrun, V.; Duarte, F. and Williams, N.H. (2014). Catalytic Zinc Complexes for Phosphate Diester Hydrolysis. Angewandte Chemie. Vol. 126, pp.: 8385-8389.

Toropova, K.; Basnak, G.; Twarock, R.; Stockley, P. and Ranson, N.A. (2008). The Three-dimensional Structure of Genomic RNA in Bacteriophage MS2: Implications for Assembly. Journal of Molecular Biology. Vol. 375, pp.: 824-836.

Toropova, K., Stockley, P. and Ranson, N.A. (2011). Visualising A Viral RNA Genome Poised for Release from Its Receptor Complex. Journal of Molecular Biology. Vol. 408, pp.: 408-419.

Trilisky, E. and Lenhoff, A. (2007). Sorption Processes in Ion-Exchange Chromatography of Viruses. Journal of Chromatography A. Vol. 1142, pp.: 2-12.

Tucker, R. (1961). The Role of Magnesium Ions in the Growth of Salmonella Phage Anti-R. Journal of General Microbiology. Vol. 26, pp.: 313-323.

Tumpey, T. and Belser, J. (2009). Resurrected Pandemic Influenza Viruses. Annual Reviews of Microbiology. Vol. 63, pp.: 79-98.

Ueda, K.; Morita, J.; Yamashita, K. and Komano, T. (1980). Inactivation of Bacteriophage phi X174 by Mitomycin C in The Presence of Sodium Hydrosulfite and Cupric Ions. Chemico-Biological Interactions. Vol. 29, pp.: 145-158.

Urbani, A.; Bazzo, R.; Nardi, M.; Cicero, D.O.; De Francesco, R.; Steinkuehler, C. and Barbato G. (1998). The Metal Binding Site of The Hepatitis C Virus NS3 Protease: A Spectroscopic Investigation. The Journal of Biological Chemistry. Vol. 273, pp.: 18760-18769.

Urbanski, N. and Beresewicz, A. (2000). Generation of .OH Initiaded by Interaction of Fe^{2+} and Cu^{+} with Dioxygen; Comparison with The Fenton Chemistry. Acta Biochimica Polonica. Vol. 47, pp.: 951-962.

Van Engelenburg, F.; Terpstra, F.; Schuitemaker, H. and Moorer, W.R. (2002). The Virucidal Spectrum of A High Concentration Alcohol Mixture. Journal of Hospital Infection. Vol. 51, pp.: 121-125.

Von Barbro, I.; Toft, E. and Clemedson, C. (2003). Sensitising Properties of Active Substances in Biocidal Products Used in the Nordic Countries. Nordic Council of Ministers. Expertradet, Sweden.

Wales, A. and Davies, R. (2015). Co-Selection of Resistance to Antibiotics, Biocides and Heavy Metals and Its Relevance to Foodborne Pathogens. Antibiotics (Basel). Vol. 4, pp.: 567-604.

Wanaratana, S.; Tantilertcharoen, R.; Sasipreeyajan, J. and Pakpinyo, S. (2010). The Inactivation of Avian Influenza Virus Subtype H5N1 Isolated from Chickens in Thailand by Chemical and Physical Treatments. Veterinary Microbiology. Vol. 140, pp.: 43-48.

Warnes, S. and Keevil, C. (2011). Mechanism of Copper Surface Toxicity in Vancomycin-Resistant Enterococci Following Wet or Dry Surface Contact. Applied and Environmental Microbiology. Vol. 77, pp.: 6049-6059.

Wei, Z.; Burwinkel, M.; Palissa, C.; Ephraim, E. and Schmidt, M.F. (2012). Antiviral Activity of Zinc Salts against Transmissible Gastroenteritis Virus *in vitro*. Veterenary Microbiology. Vol. 160, pp.: 468-472.

Wessa, P. (2013). Free Statistics Software. Office for Research, development and education, URL http://www.wessa.net/

Wetz, K. and Kucinski, T. (1991). Influence of Different Ionic and pH Environments on Structural Alterations of Poliovirus and Their Possible Relation to Virus Uncoating. Journal of General Virology. Vol. 72, pp.: 2541-2544.

Whitehead K. and McCue, K.A. (2010) Virucidal Efficacy of Disinfectant Actives Against Feline Calicivirus, A Surrogate for Norovirus, in A Short Contact Time. American Journal of Infection Control 38: 26–30.

WHO – World Health Organization (2014). Global Alert and Response Ebola virus Disease Update – West Africa. http://www.who.int/csr/don/2014_08_19_ebola/en/. Last access: August 2014.

Wieck, S.; Olsson, O. and Kuemmerer, K. (2017). Consumer's Perception of Biocidal Products in Households. International Journal of Hygiene and Environmental Health. Vol. not specified.

Wigginton, K.; Pecson, B.; Sigstam, T.; Bosshardt, F. and Kohn, T. (2012). Virus Inactivation Mechanisms: Impact of Disinfectants on Virus Function and Structural Integrity. Environmental Science & Technology. Vol. 46, pp.: 12069-12078.

Wilton, T.; Dunn, G.; Eastwood, D.; Minor, P.D. and Martin, J. (2014). Effect of Formaldehyde Inactivation on Poliovirus. Journal of Virology. Vol. 88, pp.: 11955-11964.

Winand, R. (2010). Chapter 10: Electrodeposition of Zinc and Zinc Alloys. In: Modern Electroplating 5th Edition. John Wiley and Sons. New York, USA.

Withey, S.; Carmell, E.; Avery, L. and Stephenson, T. (2005). Bacteriophages – Potential for Application in Wastewater Treatment Processes. Science of the Total Environment. Vol. 339, pp.: 1-18.

Wong, K., Morgan, A. and Paranchych, W. (1974). Controlled Cleavage of Phage R17 RNA within The Virion by Treatment with Ascorbate and Copper. Canadian Journal of Biochemistry. Vol. 52, pp.: 950-958.

World Health Organization (WHO) (2010). Guide to Local Production: WHO-recommended Handrub Formulations. Geneva, Switzerland.

World Health Organization (WHO) (2014). Infection Prevention and Control of Epidemic- and Pandemic-prone Acute Respiratory Infections in Health Care. WHO Guideline I. Geneva, Switzerland.

Wright, S. and Bieluch, V. (1993). Selected Nosocomial Viral Infections. Heart & Lung. Vol. 22, pp.: 183-187.

Wu, X.; He, J.; Ding, H.; Zhu, Z.; Chen, J.; Xu, S. and Zha, D. (2015). Modulation of Zinc-Induced Oxidative Damage in *Solanum melongena* by 6-Benzylaminopurine Involves Ascorbate-Glutathione Cycle Metabolism. Environmental and Experimental Botany. Vol. 116, pp.: 1-11.

Yahya, M.; Straub, T. and Gerba, C. (1992). Inactivation of Coliphage MS2 and Poliovirus by Copper, Silver and Chlorine. Canadian Journal of Microbiology. Vol. 38, pp.: 430-435.

Yakimovich, A.; Gumpert, H.; Burckhardt, C.; Luetschg, V.A.; Jurgeit, A.; Sbalzarini, I.F. and Greber, U. (2012). Cell-Free Transmission of Human Adenovirus by Passive Mass Transfer in Cell Culture Simulated in a Computer Model. Journal of Virology. Vol. 86, pp.: 10123-10137.

Yamaguchi, Y.; Kumakura, A.; Sugasawa, S.; Harino, H.; Yamada, Y. and Shibata, K. (2007). Direct Analysis of Zinc Pyrithione Using LC-MS. International Journal of Environmental Analytical Chemistry. Vol. 86, pp.: 83-89.

Yamamoto, N.; Haller, W. and Hiatt, C. (1964). Mechanism of Inactivation of Bacteriophages by Metals. Biochimica et Biophysica Acta. Vol. 91, pp.: 257.

Yeap, J.; Kaur, S.; Lou, F.; DiCaprio, E.; Morgan, M.; Linton, R. and Li, J. (2015). Inactivation Kinetics and Mechanism of a Human Norovirus Surrogate on Stainless Steel Coupons via Chlorine Dioxide Gas. Vol. 82, pp.: 116-123.

Yoon, J.; Jekle, A.; Najafi, R.; Ruado, F.; Zuck, M.; Khosrovi, B.; Memarzadeh, B.; Debabov, D.; Wang, L. and Anderson, M. (2011). Virucidal Mechanism of Action of NVC-422, A Novel Antimicrobial Drug for The Treatment of Adenoviral Conjunctivitis. Antiviral Research. Vol. 92, pp.: 470-478.

You, J.; Zhang, Y.; Hu, Z. (2011). Bacteria and Bacteriophage Inactivation by Silver and Zinc Oxide Nanoparticles. Colloids and Surfaces B: Biointerfaces. Vol. 85, pp.: 161-16.

Young, D. and Sharp, D. (1985). Virion conformational forms and the complex inactivation kinetics of echovirus by chlorine in water. Applied and Environmental Microbiology. Vol. 49, pp.: 359-364.

Ytreberg, E.; Karlsson, J. and Eklund, B. (2010). Comparison of Toxicity and Release Rates of Cu and Zn from Anti-Fouling Paints Leached in Natural and Artificial Brackish Seawater. The Science of Total Environment. Vol. 408, pp.: 2459-2466.

Yuan, C.; Wei, Y. and Yang, P. (2006). DNA-Binding and Cleavage Studies of Zinc (II) Mixed-Polypyridyl Complex. Chinese Journal of Chemistry. Vol. 24, pp.: 1006-1012.

Yuasa, N.; Naganuma, A.; Sato, K.; Ikeda, M.; Kato, N.; Takagi, H. and Mori, M. (2006). Zinc is A Negative Regulator of Hepatitis C Virus RNA Replication. Liver International. Vol. 26, pp.: 1111-1118.

Zambon, M. (2014). Influenza and Other Emerging Respiratory Viruses. Medicine. Vol.42, pp.: 45-51.

Zar, J. (1999). Chapter 6: The Normal Distribution. In: Biostatistical Analysis 4th Edition. Prentice-Hall. New Jersey, United States.

Zhang, X.; Zhu, Y.; Gao, H. and Zhao, C. (2014). Solvolysis Mechanisms of RNA Phosphodiester Analogues Promoted by Mononuclear Zinc(II) Complexes: Mechanisic Determination upon Solvent Medium and Ligand Effects. Inorganic Chemistry. Vol. 53, pp.: 11903-11912.

Zhong, Q.; Carratala, A.; Nazarov, S.; Guerreiro-Ferreira, R.C.; Piccinini, L.; Bachmann, V.; Leiman, P.G. and Kohn, T. (2016). Genetic, Structural, and Phenotypic Properties of MS2 Coliphage with Resistance to CLO2 Disenfection. Environmental Science & Technology. Vol. 50, pp.: 13520-13528.

Zinchenko, A.; Sergeyev, V.; Yamabe, K.; Murata, S. and Yoshikawa, K. (2004). DNA Compaction by Divalent Cations: Structural Specificity Revealed by The Potentiality of A Designed Quaternary Compounds Diammonium salts. Chembiochem. Vol. 5, pp.: 360-386.