Mechanisms of action of polyhexamethylene biguanide-based biocides against non-enveloped virus

Federica Pinto

Thesis submitted for the Degree of Doctor of Philosophy

April 2010

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

TABLE OF CONTENTS

List of Figures

List of Tables

ABBREVATIONS

A260: Absorbance at 260 nm
A280: Absorbance at 280 nm
Ad5: Adenovirus type 5
BSA: Bovine serume albumine
CFU: Colony forming unit
cm: centimetre
CsCl: Cesium Chloride
Da: Dalton
DAB: 3,3' Diaminobenzidine
DI: Deionised
DMEM: Dulbecco's modified eagle medium
EIDs: Emerging infectious diseases
EM: Electron microscopy
g: Gravity
g-mole: molar mass
KDa: Kilo Dalton
KHz: Kilo hertz
Log_{10}: Base 10 logarithm
M: Molar
MATH: Microbial adhesion to hydrocarbon
Min: Minute
MOI: Multiplicity of infection
MPN: Most probable number
mRNA: messanger ribonucleic acid
MW: Molecular weight
ml: millilitre
NaCl: Sodium Chloride
OD: Optical density
PBS: Phosphate buffered saline
PEG: Polyethylene glycol
PFU: Plaque forming unit
pH: Potentiometric hydrogen ion concentration
PHMB: Polyhexamethylene biguanides
PPM: Part per million
Q10: Temperature coefficient per 10°C rise
RF: Reduction factor
RNA: Ribonucleic acid
RPM: Round per minute
SARS: Severe acute respiratory syndrome
sd: Standard deviation
SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SM Buffer: Sulphate magnesium buffer
Son: Sonication
T: Temperature in Celsius
TEM: Transmission electron microscopy
TSA: Tryptone soya agar
TSB: Tryptone soya broth

AKNOWLEDGEMENT

I would like to thank my supervisor Jean Yves Maillard for giving me the opportunity to start working in microbiology. During these 4 years, he supported me and he taught me how think scientifically. I also enjoyed the amusing France vs Italy friendly arguments.

I am very grateful to my supervisor Prof. Stephen Denyer, for his availability and for his important hints and advices that resulted vital during my periods of "lost". I consider him "The wise man of Microbiology".

I would like to acknowledge Paula McGeechan for her support and Arch Chemicals which financed my PhD project.

A big thank to Gareth Williams, for his valuable scientific help and his jokes which accompanied me during these years and mostly during the tedious suspension tests.

Thanks also to all the people of the Lab 1.49 and 1.11, the one who left and then one who came for their chats, jokes, and smiles.

I also want to thank my partner, friend and scientific consultant Stefano. He has been an essential presence in my life during the last ten years, with his patience, his passion in life and his ability to make me constantly laugh.

I am grateful to my parents, Anna and Peppe, and my sister Carolina who supported me emotionally all these years and understood my not easy choice to live abroad, far from them. They are the pillars of my life.

Finally thanks to all my friends in Cardiff who helped me to enjoy the time in this rainy town.

SUMMARY

Human viral pathogens place a serious threat on the healthcare system. Adequate and efficient disinfection procedures minimise the incidence of surface contamination and viral disease transmission. Biocide type, environmental conditions and viral characteristics (e.g. presence of envelope) influence the disinfection effectiveness. Thus, understanding the mechanisms of virucidal action is essential for improving disinfection efficacy. PHMB has a wide range of antimicrobial activities, but its action against viruses has been mostly tested on enveloped viruses, which appeared to be inactivated. This project aimed at understanding the mechanisms of action of two PHMB-based biocides, VANTOCIL^{TM}TG and COSMOCIL^{TM}CQ, against non-enveloped viruses including the bacteriophages MS2 and F116 used as model viruses, and the human Adenovirus type5. Biocides were tested in a suspension test at different conditions. Hydrophobicity tests, SDS-PAGE, DNA analysis, dynamic light scattering and transmission electron microscopy were performed.

Suspension tests at 20°C showed that PHMBs at 800 ppm reduced MS2 and Ad5 by 90%. Higher reduction was achieved against F116 (99%). Times of exposure did not increase the activity, whereas temperature had a great effect. At 30°C 99.99% of MS2 was inactivated after 10 min; the efficacy against Ad5 was also enhanced, but to minor extent. Despite a modest activity, PHMB interacted with the viral capsid producing damage which was very specific against Ad5. Viral aggregation played a key role in limiting virucidal efficacy of PHMB, especially concerning MS2. It is likely that the observed capsid damage caused the inactivation. However, the hypothesis of domain formation by PHMB and thus the inhibition of the virus-host interaction cannot be ruled out. The mechanism of binding PHMB molecules to the viruses was highly cooperative and occurred through first electrostatic and then hydrophobic forces.

Concluding, it is the first time that PHMB-based biocides were shown to interact with viral capsid resulting in virucidal activity against non-enveloped viruses. However, such activity is reduced by formation of viral aggregates. Results supported also the use of MS2 as a surrogate of human RNA non-enveloped viruses.

1. GENERAL INTRODUCTION

1.1. Historical background

1.1.1. Virology

Reconstructing the history of virus establishment within the human population is a big challenge. The beginning of animal domestication, the demography increase, and higher rates of commerce caused the spread of an increasing number of viral particles among populations and the development of new forms of virus.

Evidence of viral diseases can be found in ancient records: rabies was already mentioned in the Mesopotamian laws, and drawings illustrating the consequences of poliovirus infection were found on the Egyptian hieroglyphs (Flint *et al.*, 2004c).

Measures to control diseases were taken even before the discovery of microorganisms. For instance the variolation, consisting of inoculating healthy individuals with materials from a smallpox postule, was a quite common procedure in China and India by the 11^{th} century. In England in the 1770's the physician Edward Jenner understood the principal of viral immunization, even though the viral agent itself was still unknown. He inoculated an individual with the extracts from cowpox lesions, inducing only mild symptoms of the disease but protecting him from the more dangerous effects of smallpox. From these experiments the word vaccination (*vacca* = cow in Latin) was derived.

The famous experiment of Louis Pasteur, where he demonstrated that a boiled medium remained free of microorganisms as long as it was prevented contact with airborne microbes, proclaimed the end of the spontaneous generation hypothesis (Flint *et al.*, 2004c). Then, the next big step towards the science of microbiology occurred when the German physician Robert Koch associated for the first time pathologies with microorganisms. He developed a set of criteria for the identification of the agent responsible (pathogen) for specific diseases.

Koch's postulates are as follows:

- The microorganism must be found in abundance regularly in organisms suffering from the disease.
- The microorganism must be isolated from a diseased organism and grown in pure culture.
- The cultured microorganism should cause disease when introduced into a healthy organism.
- The microorganism must be re-isolated from the inoculated, diseased experimental host and identified as being identical to the original specific causative agent.

These criteria are still applied today.

Only in 1892 did the Russian botanist Dmitri Iwanowsk discover for the first time what we call viruses at the St. Petersburg Academy of Science. While studying mosaic tobacco disease in plants, he found that the agent causing the pathology was small enough to pass through ceramic filters used to trap all bacteria. He called them filterable agents.

In the 1898, Martinus Beijerinck independently discovered the tobacco mosaic virus identifying it as a small distinctive agent able to pass through the filter. During the same year two former students and assistants of Koch, Friedrich Loeffler and Paul Frosch, observed that the cause of foot-and-mouth disease, spread among the livestock, was also filterable. In addition, they understood that these filterable agents replicated only in their host organisms and not in solution as with bacteria.

These events are considered to be the beginning of virology.

Bacterial viruses were first characterised independently on two occasions at the beginning of the 1900's. In 1915 a British pathologist described the transformation of *Micrococcus* by a transmissible agent. Then, two years later, in 1917, a Canadian scientist, Félix Hubert d'Hérelle, observed the lysis of *Shigella* culture in broth and understood the viral nature of the event. He coined the term bacteriophage (Ackermann, 2003).

The first human virus described was the agent responsible for yellow fever in 1901. First the Cuban Carlos Juan Finlay in 1880 proposed that a

bloodsucking insect was responsible for transmission of the disease. A commission was established in 1899 to further investigate the aetiology of yellow fever, and one of the members, Dr. Jesse Lazear, demonstrated by sacrificing his own life that mosquitoes were responsible for the transmission.

The discovery of other human viruses progressed slowly, due to the danger and difficulties associated with the experiments, but in 1930 virology changed completely with the introduction of the electron microscope, which allowed the visualization of these agents.

In the last 50 years great progress has been made in understanding viral structures and the molecular basis of their replication cycle.

1.1.2. Disinfection practices

Although the concept of bacterial diseases began to be understood only in the nineteenth century, back in time disinfection and preservation procedures were already practiced (Fraise, 2004).

Humans carried out disinfection procedures hundreds of years ago, mostly related to religious practices (Maillard, 2001). Heat disinfection procedures, such as passing metal objects utilized in the kitchen through the fire before use, are well documented in the Bible. At the time of the Persian Empire, 450 BC, it is recorded the use of copper and silver vessels to keep the water potable, and the application of various substances, such as wine, vinegar and honey on wounds.

In the fourteenth century, to fight episodic scourges of plague, it was recommended to burn juniper branches and sulphur in rooms where ill people were resting.

Chemical disinfectants were used scientifically around 150 years ago, but empiric practices can be found in ancient times. In the 1763 ethanol was already used in hospital for dressing wounds; formulations of mercury, lead, arsenic, copper, iron and sulphur were introduced by a Swiss physician, Paracelsus, in 1493 as disinfectants.

In 1798 bleaching powder was made and then later used as a deodorant and disinfectant by Alcock later in 1827 (Fraise, 2004).

Some chemical disinfectants, which are still in use today, were discovered a long time ago. For instance, hydrogen peroxide was investigated in 1893 by Traugott, quaternary ammonium compounds were introduced in 1916 by Jacobs, and phenols were discovered in 1832 by Reichenbach.

After Kronig, Paul and a Japanese physical chemist Ikeda introduced the science of disinfection dynamics in 1916, numerous studies on the subject and on the hygiene concept lead to a better understanding of the world of disinfection. Since then several new biocides have been introduced and a significant amount of research on their activity against microorganisms has been performed.

The last 50 years has seen enormous advances in the prevention of infections, but many aspects regarding mechanisms of action, and possible synergistic effects remain unclear.

1.2. Viruses and the healthcare environment

Nosocomial infections are infections that develop as a result of a period in hospital or are produced by microorganisms and viruses acquired during hospitalisation. They may be endogenous, arising from an infectious agent present within a patient's body, or exogenous, transmitted from another source within the hospital (Aitken & Jeffries, 2001). Annually, 4-10% of hospitalized patients acquire infections, and it is estimated that it costs one billion pounds per year to the UK's National Health Service, and between 17 and 25 billion dollars for the USA (Schabrun & Chipchase, 2006). Moreover, nosocomial infections directly cause 5000 deaths and indirectly 15,000 deaths in the UK every year. A similar situation exists in the USA, where 17,500-70,000 patients die by an hospital acquired infection every year (Plowman *et al.*, 1999).

Schabrun & Chipchase (2006) reviewed published and unpublished studies from 1972 to 2004. The research indicated that nosocomial infections were due mainly to contaminated hospital equipment, such as cloths, uniforms,

bedrails, and catheters. Thirty-three percent of 33 diagnostic ultrasound probes were found contaminated, as well as 66% of swabs taken from equipment in constant use. The high level of contamination was of particular concern, especially for the large number of pathogenic, opportunistic and multi-resistant organisms detected on the sampled equipments.

Paediatric units (Hall *et al.*, 1980; Butz *et al.*, 1993) and wards with elderly patients (Aitken & Jeffries, 2001) are particularly exposed to seasonal outbreak of viral infections. In some situations when the origin and the transmission route of an outbreak are uncertain, the closure of the unit is required, increasing further the costs for the affected medical department. In the Outbreak Database (Hygiene and Environmental Medicine Charité – University Medicine Berlin, 2008), 2204 outbreaks have been registered from 1967 and in 1994 the closure of the unit was necessary. The most common pathogens which were responsible for the unit closure were influenza virus, Severe Acute Respiratory Syndrome coronavirus (SARS), *Streptococcus pneumoniae*, noravirus and rotavirus (Hansen *et al.*, 2007).

Valenti *et al.* (1980) found that viruses are the cause of 78% of nosocomial respiratory pathology, 71% of gastrointestinal illness, 5% of disseminated and blood infections and 2% of skin/soft tissue disease. When adenoviral outbreaks occurred in health care facilities, the infection rate was estimated to be over 25% (Rutala *et al.*, 2006).

Two to ten percent of hospital-acquired infections, often caused by contaminated drugs or fluids, developed in outbreaks. In a systematic review by Vonberg *et al.* (2007), the characteristics and the most frequent occasions in which contaminated substances occur are described.

Viruses are the most common cause of infectious disease acquired in indoor environments (Barker *et al.*, 2001). They cause mostly gastrointestinal and respiratory infections. From 1995 to 1996 rotavirus, astrovirus, Norwalk-like viruses and caliciviruses were responsible for 48% of all reported outbreaks of intestinal diseases (Barker *et al.*, 2001). Every year adults suffer two to five colds and infants get four to eight colds (Barker *et al.*, 2001), mostly caused by influenza, rhinoviruses and coronavirus. Person to person or person to surface contact are the main route of transmission of

viral infections. The viral load of an infected person can be incredibly high, faeces for instance shed up to 10^{12} enteric viruses per ml. Respiratory viruses spread even faster than the enteric ones via the airborne route (from a sneeze, viral particles travel 3 m at 20 m/s) and have a shorter incubation period (Boone & Gerba, 2007).

There are three main measures to control viral infections: i) reducing the number of viral sources (use of masks, gloves); ii) interrupting transmission (immunization); iii) reducing the number of disease-producing organisms (sterilization and disinfection) (Grossgebauer, 1970). Since viral infections are not always easy to treat and vaccines are not available yet for many viruses, prevention of their spread through disinfection and improved hygiene standards are important achievements especially in a community setting. The assessment of the hazard of viral contamination can be difficult, since the infectious dose can vary significantly with the pathogenicity of the organism and the immune status of the host (Barker *et al.*, 2001).

Various studies showed that viruses can persist on inanimate and animate surfaces for a prolonged time (Sattar *et al.*, 1987; Mbithi *et al.*, 1992; Tiwari *et al.*, 2006). Carpets may harbour viable virus for at least 12 days, even after routine vacuum cleaning (Cheesbrough *et al.*, 1997). Viruses from the gastrointestinal tract, such as polio- and rotavirus, can persist for approximately 2 months on surfaces (Kramer *et al.*, 2006b). SARS coronavirus, which appeared between the late 2002 to the first half of 2003 causing over 8,436 cases and 812 deaths, can persist for 3 days in faeces and 17 days in urine both at 20°C (Wang *et al.*, 2005).

There are several examples of large epidemics caused by viruses, such as the fatal Lassa fever (Arenaviridae) and the Marburg-Ebola agent (Filoviridae), the origin of which is still not clear, although rodents and primates are the suspected reservoirs. During the 1980s HIV virus erupted on two continents, North America and Africa, infecting thousands of people. Influenza A and B viral infections are among the most contagious diseases of humans. There have been 31 pandemics recorded since their discovery (e.g. Hong-Kong flu 1968-69, 0.75-1 million of deaths).

Emerging infectious diseases (EIDs) represent an important problem for public health. An EID is a new infection that appears for the first time or a

known one which increases in prevalence. In the past two decades, several countries have been facing big pandemics and outbreaks, such as the immunodeficiency viruses, the severe acute respiratory syndrome (SARS)-associated coronavirus and Infuenza A (H1N1). The latter posed a big threat to the whole world at the beginning of 2009; it has been recorded in forty countries, with a total of 17,410 cases and 115 deaths (WHO, 2009a).

Causes of emerging diseases fall into three categories: viral factors, human factors and ecological factors (Ka-Wai Hui, 2006). Viral factors involve the rapid evolution and environmental adaptability of the viral agents which cause EIDs. They are mostly RNA virus, which have a very high point mutation and recombination rate, and gene re-assortment. Human factors seem to have the most influence on disease emergence: population size increases, favouring an easy and quick spread of pathogens; travelling, migration and globalization of commerce enhance the spread of pathogens in different parts of the world (Maillard, 2001; Russell, 2004); social changes, such as urbanization, sexual practices, drug abuse and new medical care and technology (blood transfusion, transplant, contaminated health care facilities) also have big relevance (Kuiken *et al.*, 2003). Climatic changes and ecosystem disturbance are the main ecological factors involved. Some outbreaks, indeed, take place after extreme natural disasters, such as floods and droughts; an example is the emergence of hantavirus pulmonary syndrome (HPS) in 1993 in USA. After massive rainfall, the high production of fruit led to a population explosion of the deer mouse. These animals contaminated floors and blankets with their dropping (Ka-Wai Hui, 2006), spreading the virus among the human population.

1.2.1. Respiratory infections

Respiratory infections are caused by rhinoviruses, coronoviruses, influenza viruses A and B, and respiratory syncytial virus. They are a major health concern; it was estimated that children suffer about four to eight colds and adults have at least two a year (Barker *et al.*, 2001). They are also the main factor that caused the dramatic pandemics in the past, such as the Spanish

influenza in 1918, the Asian influenza in 1957 (Cox & Fukuda, 1998) and lately, in 2009 the big outbreaks of a new strain of Influenza A H1N1 (WHO, 2009b). Respiratory viruses have such success because of their short incubation time, between 1 to 8 days, and considerable transmissibility. Transmission occurs via the respiratory route, through small droplets originated by sneezing and coughing that can spread across large distances, and big droplets which are transmitted only by person-to-person or person-to-surface contact. In addition, influenza viruses have a quick antigenic drift, which means a rapid and unpredictable change of antigens on the viral surface (hemagglutinin and neuraminidase) that escape neutralisation by the antibodies already present in the human body (Cox & Fukuda, 1998).

These viruses are able to survive on surfaces for long time. For instance influenza A virus survives for more than 48 h on stainless steel and plastic surfaces; transfer of viable viral particles from stainless steel to hands was possible for 24 h, whereas from paper tissue to hand for only 15 min (Weber & Stilianakis, 2008). Furthermore Sattar and colleagues found that rhinovirus was able to survive on surfaces for several hours (Sattar *et al.*, 1983).

The prevention of respiratory virus can be accomplished by an early diagnosis of the disease in hospitals and healthcare places, and also by good disinfection and hygiene practices such as an efficient hand-washing or the use of gloves by the personnel (Aitken & Jeffries, 2001; Hota, 2004).

1.2.2. Gastrointestinal infections

Gastrointestinal infections are mainly caused by rotaviruses, noroviruses, enteroviruses and hepatitis A virus. They are transmitted through the faecal-oral route, but other routes can be considered such as contaminated surfaces and aerosol from episodes of vomiting. These infections are extremely relevant in developing countries, where it has been estimated that the deaths of 800,000 children under 5 years of age associated with gastrointestinal infections (Barker *et al.*, 2001). The Asian Rotavirus Surveillance Network

collected data from 2001 to 2003 and found that 43% of diarrhoea admissions were positive for rotavirus (Nelson *et al.*, 2008b).

Hepatitis A (HAV) is the most common cause of viral hepatitis. It is characterised by high morbidity and can be transmitted through contaminated food, such as filter-feeding shellfish and water (Barker *et al.*, 2001). Infected persons are highly contagious two weeks before the appearance of symptoms. The incidence of HAV is decreasing in many countries in the last decade because of immunization programmes (Keystone & Hershey, 2008), but it is still a big threat in developing areas due to poor sanitation and unhygienic conditions (Shapiro & Margolis, 1993).

Enteroviruses comprise picornaviruses, coxsackievirus A and B, poliovirus, and enteroviruses 68 to 71. Even though they often cause asymptomatic infections, enteroviruses, such as polio and coxsackie, can develop serious pathologies such as the paralysis of spinal or bulbar poliomelytis (Aitken & Jeffries, 2001).

Resistance to drying is common amongst gastrointestinal viruses. Hepatitis A was found to be still infective after several hours on hands and after several days on surfaces (Sattar *et al.*, 2000).

Isolation of infected persons and application of good hygienic practices are essential in preventing the spread of such viruses. Moreover, vaccination has helped to decrease the occurrence of such infections, especially those that are polio and HAV related.

1.2.3. Blood-borne diseases

The group of blood-borne viruses comprises hepatitis B (HBV), hepatitis C (HCV) and HIV, characterised by being able to induce persistent viraemia. The blood-borne viruses have been associated with nosocomial infections, where person-to-person, and patient-to-personnel transmissions occurred (Ross *et al.*, 2009), with alarming situations in many low-income countries where HIV transmission can be frequent if not endemic (Ganczak & Barss, 2008).

Transmission occurred mostly by transferring blood or other fluids through contaminated surfaces, medical devices, tissue and contaminated sharps. In a survey on sharp injuries at the Aga Khan University Hospital, Pakistan, during 2002-2007, 1,382 injuries were reported with the highest number associated with junior doctors (28.5%) followed by nurses (20.4%) (Zafar *et al.*, 2009). Vaccines exist only for hepatitis B (HBV).

Hepatis C is considered one of the major health problems worldwide; in fact it is estimated there are 170 million infected people. The virus, contrary to HBV, develops as a chronic infection in 50%-80% of cases (Helge *et al.*, 2009), and the high transcription error and the high turnover of viral particles enables the virus to escape the immune system. Currently the infection is treated with a combination of pegylated interferon-α and ribavirin.

Since the first epidemic occurred in the 1980s HIV, which is the agent causing AIDS, has infected over 42 million people. It is a human retrovirus and its disease is characterised by a deep immunodeficiency due to a quantitative and qualitative deficiency in the subset CD4+T lymphocytes (Parisa *et al.*, 2009). At present there are several vaccines under clinical trial or under study.

The control of blood-borne diseases can be mostly accomplished by education of the public and healthcare staff, in addition to good hygienic practices. In the UK different guidelines have been issued by the Department of Health concerning clearance of blood-borne viruses (UK Department of Health, 2007).

1.3. Viruses

Viruses can be defined by their properties which are summarized as follows:

- A virus is an infectious, obligate, intracellular parasite;
- The viral genome is either DNA or RNA;
- Within an appropriate host, the viral genome is replicated and leads to the synthesis of the other viral components by the cellular systems;

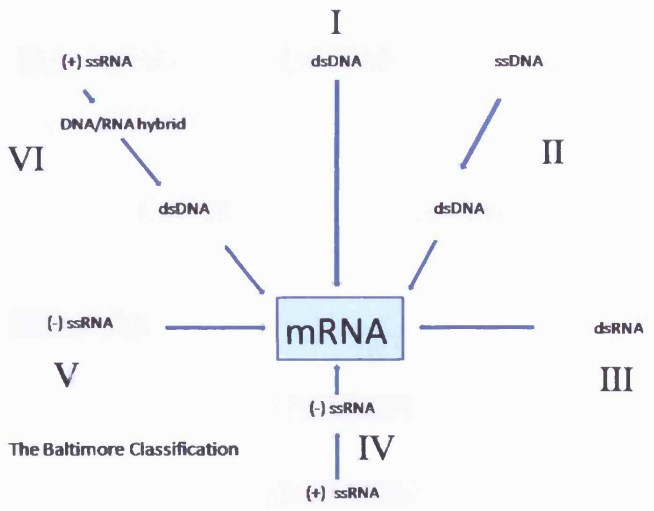
- Within the host cell, the infectious progeny particles (virions) are built *de novo* from the newly synthesized components;
- The infectious progeny virion is the vehicle for the transmission of the disease.

Viruses exist in various shapes, sizes and structural complexity.

There are two most commonly used classification systems. One, created in 1962 by Robert Lwoff and Paula Tournier, is based on the Linnaean hierarchical system (family, genera, species), which classified viruses principally on their shared properties. Four main characteristics are used in their classification: i) nature of nucleic acids; ii) symmetry of the capsid; iii) presence or absence of an envelope; iv) dimension of the capsid and the virion. Following the Lwoff system, the International Committee on Taxonomy of Viruses (ICTV) reported approximately 40,000 viruses isolated from plant, bacteria and animals, grouped in 3 orders, 56 families, 233 genera, and 1550 species with some other not yet assigned (International Union of Microbiologist Societies, 2002).

The second system is the Baltimore Classification System (Figure 1-1), edited by David Baltimore, and is based on the genetic system of each virus and describes the obligatory relationship between the viral genome and its mRNA (Flint *et al.*, 2004c).

Figure 1-1 The Baltimore classification system. Viruses are classified according to the nature and polarity of their genome. The translation process (production of mRNA) is the unique pathway taken into consideration.



Viruses are distinct among organisms, because of their very small size (20 nm-300 nm) and because they do not have any metabolic activity (Dulbecco, 1980). Viruses can infect bacteria, animal cells, and plants. Certain groups of virus possess a host in more than one Kingdom. For instance, rhabdovirus has hosts both in the Plantae and Animalia, partitiviruses in Plantae and Fungi. Moreover, several families infect across different Phyla (insects and vertebrates), such as flavivirus (Dengue virus, West Nile virus, yellow fever), and togavirus (Sindbis virus).

The main function of virions is to be effective in the transmission of the nucleic acid from one host to another; therefore it is essential to protect the genome from potentially lethal environmental factors, such as chemical compounds, enzymes, irradiation, or extreme pH and temperature.

Thanks to the electron and cryo-electron microscope, X-ray crystallography, and nuclear magnetic resonance (NMR) microscopy, the viral structures have been well defined up to the level of small proteins, even though complex viruses are relatively less studied.

A virus is composed of a protective coat (the capsid), the nucleic acid and in some viruses a lipidic envelope. In addition, some viruses also contain enzymes or other proteins necessary to their infection cycle.

The capsid is composed only of proteins, and is responsible for 60-90% of the total weight of the virus. The capsid proteins are coded by viral genes. The coat proteins display helical or icosahedral symmetry. The helical symmetry is common in the animal enveloped viruses and certain plant viruses and bacteriophage. They have a rod-like shape. The helical symmetry is considered an open structure, because any volume can be enclosed simply varying the length of the helix (Flint *et al.*, 2004d).

The icosahedral symmetry has 20 equilateral triangular faces with 5:3:2 axes of rotational symmetry. In contrast with the helical symmetry, it is considered a closed structure of fixed internal volume. In non-enveloped viruses the capsid itself is involved in the attachment to the host cell (Davies *et al.*, 2004).

Some non-enveloped viruses have a complex structure, with the presence of various proteins protruding from the capsid involved in specific functions. For instance, adenovirus has an icosahedral structure but with long projections at the 12 vertices called fibre attached to the 12 penton base. Each fibre terminates with a knob that is essential for binding the cell surface adenoviral receptor (Rux & Burnett, 2004).

The viral genome is located at the core of the capsid and it can be either DNA or RNA, and this may be single- or double-stranded. The proportion of the nucleic acid varies from about 1%, as in the influenza virus, to 50% in some bacteriophages. Viruses have just one copy of the genome (haploid), with the exception of retrovirus.

There are three mechanisms of packaging and condensing the nucleic acid in the capsid. The viral genome is in direct contact with the shell, which has proteins that interact with the nucleic acid. Using the same proteins to construct the shell and package the genome allows for small genetic material, which is advantageous for small viruses, such as picornaviruses and poliovirus (Flint *et al.*, 2004d). Another mechanism of packaging involves specialised proteins, such as the IVa2 protein in adenovirus (Zhang *et al.*, 2001). Finally, some viruses, such as papillomaviruses and simian

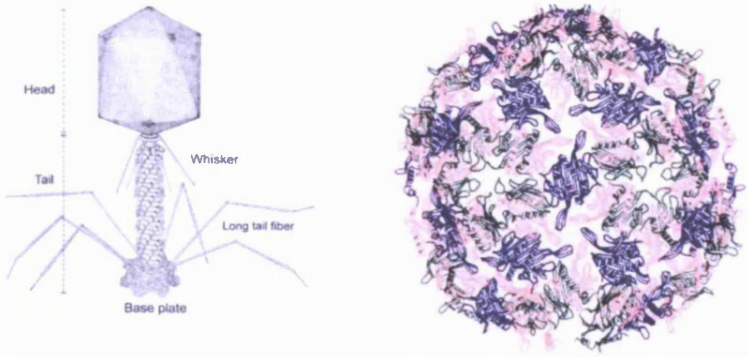
virus 40, contain cellular core histones, organizing the genome in a chromatin-like structure.

In the enveloped viruses, the envelope is composed of a lipid bilayer containing proteins with special functions. Depending on the virus, the envelope can have different origins, from the cytoplasmic membrane (e.g. influenza virus) or the nuclear membrane (e.g. herpes simplex virus). There are two kinds of proteins in the envelope, the glycoproteins and the matrix proteins. The first one is exposed on the outer part of the bilayers and has an important role in the infectivity of the virus. Matrix proteins are on the inner part of the envelope, in order to establish a bond between the envelope and the capsid.

1.3.1. Bacteriophages

Bacteriophages are viruses infecting bacterial cells. They can possess a tail, such as T phages or F116, and they can be of different sizes and structures. Tails consist of a central tube, through which the DNA passes during the infection process, surrounded by a sheath. At the tip of the tail several fibres can be present, which are involved in the attachment to the wall of the bacteria (Figure 1-2A). Other bacterial viruses are constituted just by a head, which has usually an icosahedral symmetry, as is the case for MS2 (Figure 1-2B) or P22.

Figure 1-2 Different structures of bacteriophages. A. Tailed bacteriophage T4 (Eiserling & Black, 1994). B. Icosahedral bacteriophage MS2.

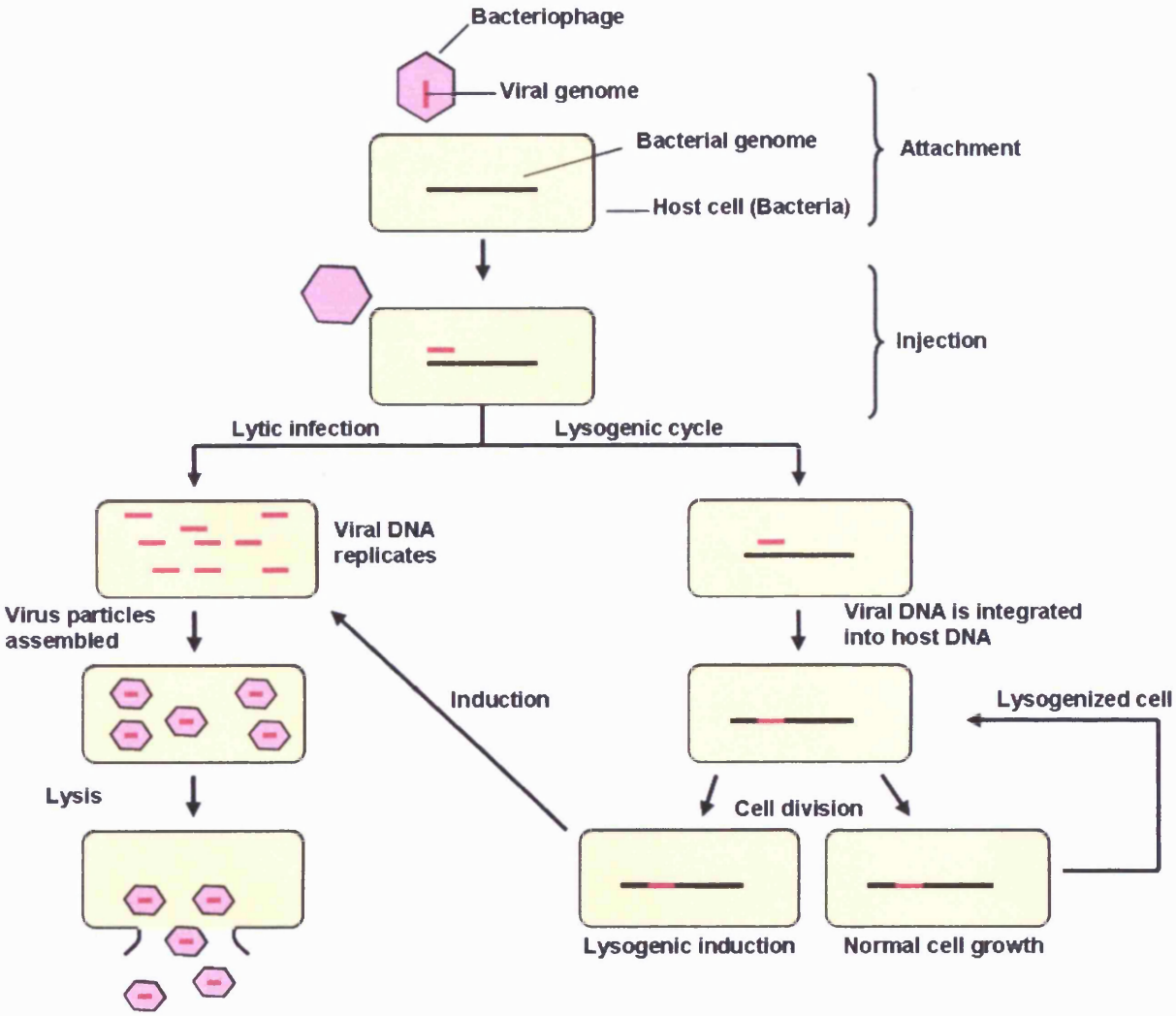


A **B**

Phages exist in three different stages: extracellular virions, replicative phage and prophage. All bacteriophages can be found as extracellular virions and, during their multiplication in the host cell, in a replicative stage. The stage of prophage is achieved when the bacteriophage is latent in the host cell and the viral genome is integrated into the bacterial chromosome. Those viruses able to enter this stage are called temperate. The prophage has the same genetic information as other viruses, but during the latent period (lysogeny) the expression of viral genes is blocked by a specific repressor codified by the virus itself (Kissmann *et al.*, 2008).

There are two kinds of infection cycles, the virulent and the lysogenic the latter being performed just by temperate phage (Figure 1-3).

Figure 1-3.Infection cycles of bacteriophages.



The infection cycle of virulent bacteriophages consists of attachment, adsorption, transcription and replication inside the host, maturation, and then release of the virions. The cycle lasts as little as 30 min. The phage-host attachment is highly specific. Once attached to the host, several changes happen on the cell membrane, leading to an increase of permeability in order to allow entry of the phage. These alterations cause metabolic changes and a rearrangement of all macromolecular synthesis. In fact, the cellular DNA replication is replaced by the DNA-RNA viral synthesis, and viral proteins initiate to be transcribed. Then, all components are assembled to form mature infectious phages, which are released outside the host cell after the rupture of the cell wall.

Temperate virions, in contrast, do not immediately generate mature bacteriophages, but their DNA persists indefinitely in the host cell. During the lysogenic state, the viral nucleic acid can integrate into the bacterial genome. A process linked to the lysogeny is transduction, where the bacterial DNA is transferred from one cell to another through a bacteriophage. The transduction can be either generalized, when any portion of the bacterial genome can be inserted in new viral particles, or specialized, when a specific group of bacterial genes is integrated into the genome of the virus, generally substituting some viral genes (Kissmann *et al.*, 2008). The transduction process has several applications in biology, such as providing an important mechanism for gene transfer, or supplying a model for viral oncogenesis (Dulbecco, 1980).

Phages can have receptor sites on different parts of the host cell. These so called somatic phages recognise sites located on the cell wall and are present at all times; they include members of the family Myoviridae, Siphoviridae, Podoviridae and Microviridae. Other phages have receptors located on specific sites on the host, such as the male-specific phages (Inoviridae and Leviviridae), that use sites on the pilus of host bacteria which are produced only under optimal conditions and in the logarithmic growth phase (Grabow, 2001).

Considering the increase in bacterial resistance to antibiotics, phage therapy is likely to have an important role in medical and veterinary infectious

diseases. In Eastern Europe phages are already commonly used as part of general care, as a treatment for dysentery, respiratory tract inflammation, otitis, meningitis, septicaemia and in application to wounds and burns (Duckworth & Gulig, 2002).

Recently, bacteriophages have been proposed for food industry as antimicrobials, and as a tool to detect pathogens. Phage have been used i) against salmonellosis (Berchieri *et al.*, 1991) and colibacillosis (Murray *et al.*, 2008) in poultry, ii) to reduce *E. coli* O157:H7 infection of sheep and cattle (Garcia-De-Lomas *et al.*, 2008), and iii) to prevent *Staphylococcus aureus* growth in raw milk (Kok *et al.*, 2000) and dairy products (Garcia *et al.*, 2007).

Phages have an important role in molecular biology: nowadays a multitude of studies on isolation, sequence analysis, mutagenesis, expression, and transduction of DNA in different hosts depend on them. Furthermore, the development of phage-display technology, the process of inserting new genetic material to be expressed on the phage surface, has been applied to recombinant antibodies, diagnostic procedures, and targeted therapies (Arap, 2005).

1.3.1.1. F116

F116 is a pilus-specific *Pseudomonas* phages and member of the Siphoviridae. It was first isolated from a strain of *P. aeruginosa* obtained from a Melbourne hospital by B.W. Holloway (Holloway *et al.*, 1960). Its head is icosahedral (70 nm in diameter) and it possesses a 70-nm-long noncontractile rod-shaped tail with helical symmetry. The genome is linear double-stranded DNA of 61.7 kb. F116 attaches to the host pili, whose retraction brings the phage in contact with the bacteria where the DNA is released into the cell (Byrne & Kropinski, 2005). Phage F116 is a temperate phage.

F116 has been used to determine the effect of disinfectants on several occasions (Maillard, 2001) due to the high resistance to some biocides

(Maillard, 2001) and because its large size facilitates analysis of structural damage (Maillard *et al.*, 1995).

1.3.1.2. MS2

Bacteriophages containing single-stranded RNA genome show an incredible adaptation to their parasitic life, a sophisticated way to control and coordinate the synthesis of all phage elements, and a specific way of assemblage. The RNA phages parasitizing *E. coli* have been used several times as a model to understand the mechanisms of infection. They are classified serologically into five groups, but group I, including MS2, f2, R17, R23, and group III, with Qβ, are the most relevant. RNA coliphages are about 24-26 nm in diameter, with a molecular mass that ranges from 3.6 x 10^6 to 4.2 x 10^6 Dalton. The RNA is around 30% of the total weight, and consists of a chain of 3300-4500 nucleotides (Hindley, 1973). One interesting property is the capacity of the RNA to exist as a compact folded structure due to the high degree of secondary structure, involving 63%-82% of the bases. The secondary structures consist mainly of hairpin-like structures, formed by a helical, hydrogen-bonded stem and a terminal loop of not less than 3 nucleotides (Weissman *et al.*, 1973). The ability to compact itself lets the RNA form quasispherical structures easily, even in solution, around which the capsid could assemble (Hohn, 1976).

One of the main proteins coded is the β subunit of the enzyme complex replicase. It is a very important feature of the RNA phages, which ensures their specificity and the efficacy to replicate just the phage RNA among all of the various RNA species in the cell (Blumenthal & Carmichael, 1979).

Coat proteins, in addition to their structural function, are also involved in translational repression. In the late phase of infection, when the demand for coat proteins is maximal, they bind specifically to a RNA segment that includes the start of the subunit replicase cistron, inhibiting its translation (Hindley, 1973).

F-RNA phages are often used as a surrogate/model for mammalian viruses (mostly enteric viruses) due to their physical structure, composition, and

morphology. Furthermore, they have been shown to have similar or higher resistance to unfavourable conditions and to disinfection processes (Grabow, 2001). Therefore, F-RNA phage have been used to assess the virological quality of raw and treated water (Havelaar *et al.*, 1993), and in laboratory experiments to test the viricidal properties of biocides (Dawson *et al.*, 2005; Koivunen & Heinonen-Tanski, 2005).

1.3.2. Mammalian viruses

Mammalian viruses infect eukaryotic cells, implying several differences to bacteriophages in their cycle and their strategies of infection. In a eukaryotic cell metabolic processes occur in separate compartments, mRNAs are processed before transcription and each eukaryotic RNA molecule codifies just one polypeptide. Instead, in bacteria mRNAs are polycistronic, so translation will yield several polypeptides (Kissmann *et al.*, 2008). Furthermore, mammalian cells do not have a peptidoglycan wall, so the entire virus very often enters the cell through endocytosis.

Depending on the type of virus, the host cell can be affected in different ways. Acute infection leads to the death of the cell and to the release of virions (cytopathic viruses, e.g. poliovirus, rotavirus); persistent infections yield virions without causing immediate cell death (non-cytopathic viruses). The latter are differentiated between chronic infections which are eventually cleared (e.g. measles virus), and latent infections that last the whole life of the host. Latent viral infections are characterised by the absence of expression of productive cycle viral genes, a reduction in the immune detection of the infected cell, and by the persistence of the viral genome intact as a chromosome in a non-dividing cell (e.g. neurone; herpes simplex virus, varicella-zooster virus), or as an autonomous self-replicating chromosome (e.g., Epstein-Barr virus), or integrated into the host chromosome (e.g., adeno-associated virus) (Flint *et al.*, 2004a).

Mammalian viruses can be differentiated by the type of genome (single- and double-stranded DNA or RNA), by the presence or not of the lipid envelope, or, for some families, by their replication strategies (Table 1-1).

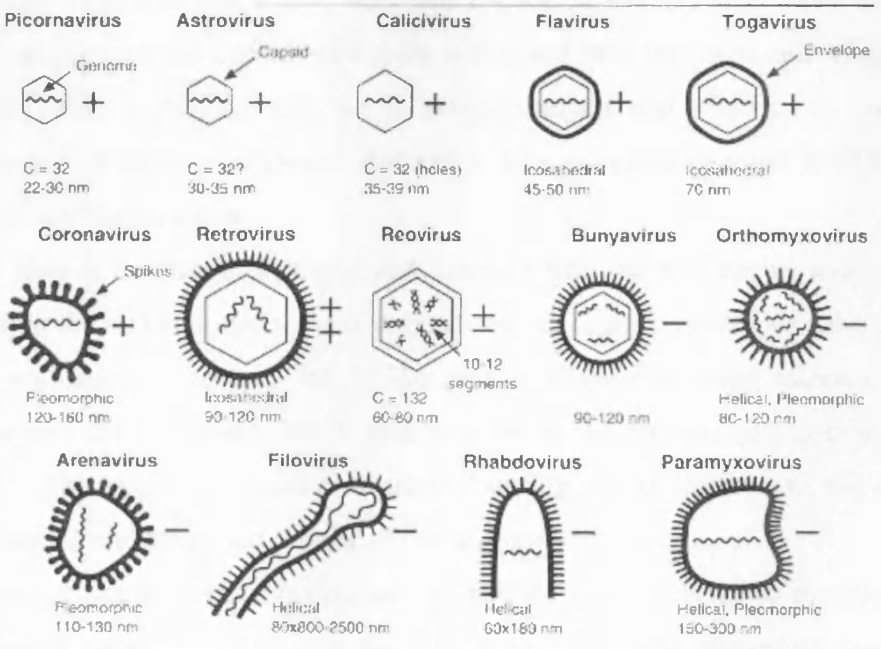
Table 1-1. Most important mammalian virus families and their main characteristics.

Genome	Genome strandedness	Infectivity of RNA	Symmetry of capsid	Non-lipidic (NLE) or lipidic (LE) envelope	Family	Examples
RNA	Single-stranded	+	Icosahedral	NLE	Picornaviridae	Enterovirus, poliovirus, coxsackievirus, rhinovirus
		-	Helical	LE	Rhabdoviridae	Rhabdovirus (Rabies), VSV (vesicular stomatitis virus), flanders virus
		-	Helical	LE	Orthomixoviridae	Influenza virus A, influenza virus B, influenza virus C
		+	Icosahedral	LE	Retroviridae	Virus inducing tumour (HIV, Leukosis viruses, Sarcoma viruses, etc.)
		+	Icosahedral	NLE	Caliciviridae	Norwalk virus, Sapporo virus, feline calcivirus
	Double-stranded		Icosahedral	NLE	Reoviridae	Reoviruses (respiratory enteric orphan virus)
DNA	Single-stranded		Icosahedral	NLE	Parvoviridae	Adeno-associated virus (defective viruses depending upon the multiplication of adenovirus)
	Double-stranded		Icosahedral	NLE	Adenoviridae	Adenovirus (infections in tonsils, adenoids, and lymphoid tissues)
			Icosahedral	NLE	Papovaviridae	Polyoma virus, SV40
			Icosahedral	LE	Herpesviridae	Herpes simplex, herpes varicella-zoster, equine abortion virus
			Complex	Lipid in the outer coat, not a real envelope	Poxviridae	Smallpox, vaccinia virus, molluscum contagiosum virus

RNA viruses are wide-spread parasites, responsible for many diseases (Figure 1-4). In the past 25 years several RNA viruses, such as HIV, hepatitis C virus, and severe acute respiratory syndrome coronavirus (SARS-CoV), have emerged. They have been either new to medical science or increased to such an extent that they raise concern for public health (Moya *et al.*, 2004).

Four characteristics are considered most relevant to understand their importance. First, their large population size: the number of viral particles in a given organism might be as high as 10^{12}. Second, natural selection is more efficient in a large population, such as the one produced during an explosive replication of RNA viruses in the host. A single infection, for instance, can produce an average of 100,000 viral copies in 10 h. Thirdly, RNA viruses posses the highest rate of mutation among all the organisms, due to the absence of a proofreading activity in their polymerase enzyme. Finally, the small size of their genome, ranging from 3 kb to 30 kb, when added to the high mutation rate leads to the development of new beneficial mutations in a short time (Moya *et al.*, 2004).

Figure 1-4. Shapes and dimensions of different families of RNA virus.



From Baron *et al.* (1996).

1.3.2.1. Adenovirus

Adenoviruses have the ability to infect a wide range of species and mostly attack the respiratory and gastrointestinal tissue. General symptoms are conjunctivitis, pharyngitis, pneumonia and diarrhoea. There are 51 human serotypes, subdivided into six subgroups (A to F) based on their ability to agglutinate erythrocytes of human, rat and monkey and on their oncogenicity in rodents (Russell, 2009). The Adenoviridae family comprises four genera: Aviadenovirus, Mastadenovirus, Atadenovirus, and Siadenovirus.

Thanks to X-ray crystallography and to cryo-electron microscopy, the structure of the adenovirus is clear. They are formed by a capsid, fibres, a core, and associated proteins. The capsid is icosahedral, non-enveloped and measures 80-110 nm with a molecular mass of 150-180 x 10^6 Da.

The capsid is formed of 252 capsomers. The main component is the homotrimeric hexon situated on the 20 faces with 240 copies; at the 12 fivefold apices there are penton bases and extended fibres (Russell, 2009). Each hexon consists of a hexagonal base with a central cavity, and its size can vary with the serotype. The penton capsomere is a covalent complex of two proteins: the homopentameric penton base and the homotrimeric fibre protein. The fibre has a tail, shaft and a knob. There are also other small polypeptides on the capsid with some associated with the virus core (Figure 1-5) (Table 1-2). The genome is not segmented and contains a single molecule of linear double-stranded DNA. The complete genome is 35800-36200 nucleotides long.

The fibre is the first component that interacts with the host tissue, and after binding the receptor, the virus is internalised via clathrin-coated vesicles and into endosomes (Russell, 2009). The rupture of the endosome releases the virus into the cytoplasm which then attaches to the nucleus and inserts the DNA. The immune response is induced during the attachment to the cell membrane and when the rupture of the endosome occurs.

Adenoviruses have been intensively studied for over 50 years as models of virus-cell interactions and also as gene vectors. Recently adenovirus type 5 has been used in gene therapy and genetic vaccination (Rexroad *et al.*, 2003; Maheshwari *et al.*, 2004). There are more than 160 human clinical trials

involving adenoviral vectors (Rexroad *et al.*, 2006). Adenoviruses have also been used as a model for testing virucidal efficacy of disinfectants (Sauerbrei *et al.*, 2004a), and a recent study recommended the use of serotypes 5 and 44 because of their high resistance to biocides (Sauerbrei *et al.*, 2004a).

Figure 1-5. A. Adenovirus diagram. B. Adenovirus micrograph. The arrows point to the detached fibres. Both images provided by ICTVdB (International Union of Microbiologist Societies, 2002).

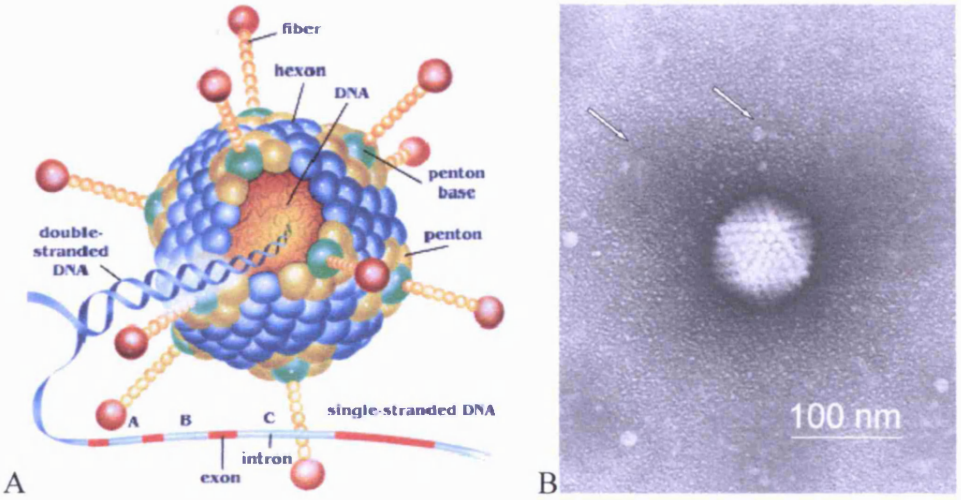


Table 1-2. Adenovirus structural proteins (Rux & Burnett, 2004).

TABLE 1. ADENOVIRUS STRUCTURAL PROTEINS

Polypeptide	*Molecular mass of monomer (Da)*[a]	*Number of residues in monomer*[a]	*Biochemical copy number of monomer*[a]	*Copy number of protein in current model*
II (hexon)	109,077	967	720 ± 7	240 Trimers
III (penton base)	63,296	571	56 ± 1	12 Pentamers
IIIa				
External portion	41,689	376[b]		
Internal portion	21,846	194[b]		
Total	63,535[c]	570[c]	68 ± 2[c]	60 Monomers
IV (fiber)				
N terminus + 2/8 of shaft	13,912	133[b]		
6/8 of shaft + C terminus	48,048	449[b]		
Total	61,960	582	35 ± 1	12 Trimers
V (core)	41,631	368	157 ± 1	—[d]
Terminal[e] (core)	~55,000	~500	2	—
VI				
Ordered portion	9,773	91[b]		
Disordered protein	12,345	115[b]		
Total	22,118[c]	206[c]	342 ± 4[c]	60 Hexamers
VII (core)	19,412	174	833 ± 19	—
VIII	15,390[c]	140[c]	127 ± 3[c]	—
IX	14,339	139	247 ± 2	80 Trimers
μ[f] (core)	~4,000	~36	~104	—

1.4. Biocides and mechanisms of viricidal action

Disinfection refers to the elimination or inactivation of contaminating microorganisms (Terpstra *et al.*, 2007). It can be carried out through physical removal, thermal kill, UV or gamma irradiation, or through the use of chemicals. Chemicals used on inanimate surfaces are called disinfectants, the ones used on skin and tissues are called antiseptics. Virus disinfection has the intention to interrupt a chain of infection. Amongst the viruses, those which are resistant outside the host body and are transmitted by air, dust and direct contact are most relevant (Grossgebauer, 1970).

The use of biocides, as a chemical substance capable of killing different forms of living organisms, can control the transmission of viruses in many environments, such as the home, public places, day-care centres, schools, hospitals and other healthcare institutions.

There are specific criteria relevant to developing and then selecting an appropriate biocide. Firstly, the compound has to be safe for the manufacturing workers, healthcare personnel and the patients. There have been cases of toxicity, such as glutaraldehyde-based biocides which caused dermatitis in 11% of the personnel in a dental surgery (Ravis *et al.*, 2003); sodium hypochlorite and chlorine gas have been responsible for occupational illness (Sattar, 2006). The environmental impact is also a factor to be considered when biocides are used. Biocides have been found in ground and surface water (Adolfsson-Erici *et al.*, 2002), affecting the quality of food and water and also possibly damaging the fauna by disrupting their hormone function (Murr & Goldman, 2005).

Antimicrobials used in routine disinfection in healthcare settings need to have a broad spectrum of activity, inactivating bacteria, fungi and viruses and to be effective after a reasonable contact time.

Improper use of biocides can have important consequences. There is a controversy on the efficacy of disinfection procedures. Some German scientists in two reviews (Dettenkofer *et al.*, 2004; Hota, 2004) stated that there is a lack of evidence which links the decrease of nosocomial infections to increasing cleaning and disinfection procedures in hospitals and health

care centres. This argument underlines the huge increase in using biocides and antiseptics, particularly in many household products, which is in contrast with the principle that disinfectants must be used when necessary and with a full appreciation of the factors influencing their activity. The uncritical use of biocides, especially at low concentrations, might cause the development of resistance by pathogenic organisms (Dettenkofer & Block, 2005). Lately, evidence has been found in laboratory-based studies of the ability of microorganisms to become resistant to antibiotics after that have developed resistance to biocides (Levy, 2002; Sattar, 2006).

The type of vehicles\surfaces on or in which the virus is present and the kind of virus itself has to be taken into consideration when the viricidal action is studied (Maillard, 2001).

Several factors, such as temperature, pH, organic load, concentration, contact time and virus structure influence the capacity of a biocide to kill or inactivate organisms (Thurman & Gerba, 1988b; Russell & McDonnell, 2000; Maillard, 2001).

pH can change the ionic composition of the biocide, affecting its binding to the virus target. Brion *et al.* (1999) found that iodine inactivates MS2 more at pH 7.0 than 8.0, even though some works demonstrated that inactivation of virus with iodine is more effective at alkaline pH (Alvarez & Obrien, 1982; Sobsey *et al.*, 1991). Furthermore, viruses have surface isoelectric points (pI) which are pH values at which virus proteins carry no net electrical charge. Consequently, at a pH below the pI, the viral particle has an overall net positive charge, above the pI, it carries a net negative charge. This can alter the sensitivity of target sites in or on the virus (Thurman & Gerba, 1988b), changing their spatial arrangement or inducing clumping or aggregation, perhaps by increasing virion hydrophobicity (Sharp & Leong, 1980; Vrijsen *et al.*, 1983).

The presence or absence of organic load is a significant influencing factor on the efficacy of the biocide, competing and interfering with the attachment of the biocide to virus targets (Wallbank *et al.*, 1978; Weber *et al.*, 1999; Ferrier *et al.*, 2004).

The mechanisms of inactivation by a biocide are various. Viruses present multiple target sites to the disinfectant. Generally, the viral structures

subjected to viricides are classified into four features: the envelope; the capsid; the glycoprotein receptors, and the viral genome.

The viral envelope, being highly lipophilic and negatively charged, is quite susceptible to a wide range of membrane-active agents (particularly cationic biocides). Hence, the enveloped viruses appear to be more sensitive to biocide than the non-enveloped viral particles.

The capsid is the most important part in protecting the virus from the outer environment. The efficacy of the biocide might depend on the size of the capsid itself; a larger capsid provides more target sites than a smaller one. This could explain why larger virus are generally more sensitive than smaller ones (Maillard, 2001). The main components of the capsid can be a target for disinfectants reacting with $-NH_2$ groups (e.g. glutaraldehyde, ethylene oxide), or with –SH groups (e.g. iodine, hydrogen peroxide).

Loss of viral infectivity due to biocides can be caused by the alteration of glycoproteins, which are, in fact, responsible for specificity, and for releasing viral genome into the host cytoplasm.

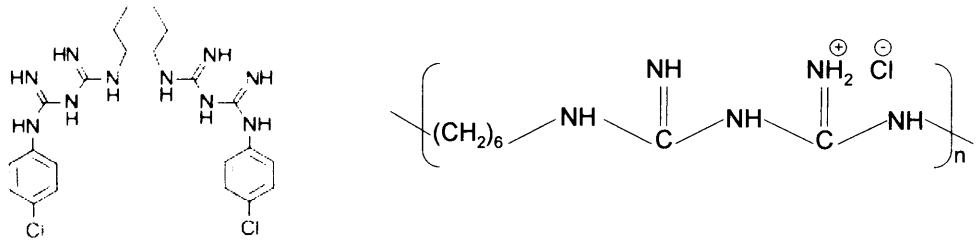
The nucleic acid is the infectious part of the virus and it is well protected. Its complete destruction will cause complete viral inactivation. The viral genome can differ in size, in content (DNA or RNA) and appearance (circular, linear, or segmented). In addition, it can be bound to the capsid proteins with different degrees of strength. It has been shown, for instance, that the helicoidal capsid is linked more closely to the genome than the icosahedral structure. Chlorine compounds (Charrel *et al.*, 2001), paracetic acid (Maillard, 2001; Jursch *et al.*, 2002), ozone and glutaraldehyde (Charrel *et al.*, 2001) have been found to alter the viral acid nucleic.

1.4.1. Biguanides

Biguanides are a wide range of compounds showing antimicrobial activity. Chlorhexidine is the most important and common biguanide used especially in veterinary antisepsis, in contact – lens solutions and in the dental field. It is one of a family N^1, N^5 – substituted biguanides (Figure 1-6) available in a number of pharmaceutical formulations. Chlorhexidine has a wide spectrum

of antibacterial activity. It is bacteriostatic at lower concentrations, inhibiting membrane enzymes and promoting leakage of cellular constituents. At higher concentrations bactericidal activity is demonstrated associated with coagulation of cytoplasmic constituents (Maillard, 2001). Its efficiency is reduced in presence of serum and organic matter, and due to its cationic nature, its activity decreases in the presence of soaps and other anionic compounds. Being a membrane – active agent, an interaction inducing alteration of the membrane is possible. Viricidal activity has been showed against such enveloped viruses as herpes simplex virus and HIV (Montefiori *et al.*, 1990; Wood & Payne, 1998; Baqui *et al.*, 2001), but not against non–enveloped viruses (Springthorpe *et al.*, 1986). A study with phage F116 showed that chlorhexidine caused structural damage to the F116 bacteriophage (Maillard *et al.*, 1995) and that it did not affect proteins (Maillard, 2001) or nucleic acid (Maillard, 2001).

Figure 1-6. Examples of biguanide disinfectants. A. Chlorhexidine. B. Polyhexamethylene biguanide



A B

Polymeric biguanides, for example polyhexamethylene biguanide (PHMB), have been used as antiseptics in medicine and as disinfectants in the food industry, and current applications include impregnation of fabrics to prevent microbial growth, water treatment, disinfection of various solid surfaces, prevention of *Salmonella* infection, and lately as a component in wound dressings (Mueller & Krebsbach, 2008).

Their composition is a mixture of polymeric biguanides of structure $[-(CH_2)_6.NH.C(=NH).NH.C.NH-]_n$, where n can vary from 2 to 40, resulting in a molecular mass range of 400-8000 (Allen *et al.*, 2006) (e.g.

VANTOCIL™ TG n=5.5). The antimicrobial activity is directly correlated with the number of polymers. Specifically, in bacteria, PHMB's induce phospholipid phase separation, causing loss of membrane function, with precipitation of intracellular constituents (Allen *et al.*, 2006). VANTOCIL™ TG, which is a commercial bactericide for use in industrial, institutional and domestic applications, causes domain formation of the acidic phospholipids of the cytoplasmic membrane, bringing permeability changes and altering enzyme function (Chawner & Gilbert, 1989b). This mechanism affects most bacteria, but it is less active against fungi. There is very little information regarding its viricidal activity.

It is likely that PHMBs can interact with viral cell membrane components. It was found active *in vitro* against Herpes simplex virus at a concentration of 0.02%, but was ineffective when tested in vivo on infected rabbit (Valluri *et al.*, 1997). Krebs *et al.* (2005) found PHMB active *in vitro* against both cell-free and cell-associated HIV-1, and able to interfere with viral binding and entry.

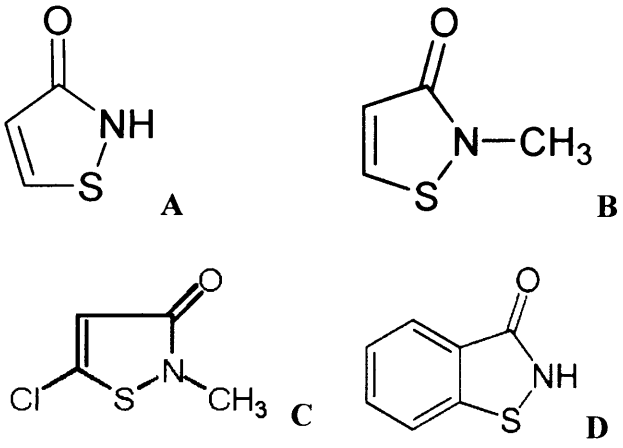
More detailed studies using HIV demonstrated that PEHMB (polyethylene hexamethylene biguanide) inhibited virus infection (binding and entry) by interacting with the HIV-1 co-receptor CXCR4 (Thakkar *et al.*, 2009). Other substituted biguanides, such as 1-isopropylbiguanide hydrochloride and 1,1-dimethylbiguanide hydrochloride, possess antiviral properties, in particular against Lee influenza virus grown in chick embryo (Pilcher *et al.*, 1961). Furthermore, alkyl substituted biguanides were shown to be effective against vaccinia and influenza viruses and their antiviral activity was directly correlated to the length of the alkyl chain (Fara *et al.*, 1974).

1.4.2. Isothiazolinones

The isothiazolinones are heterocyclic compounds, and three derivatives are used in significant amounts: methylisothiazolinone (MIT, MI), chloromethylisothiazolinone (CMIT, CMI, MCI), and benzisothiazolinone (BIT) (Mbithi *et al.*, 1992) (Figure 1-7).

They are antimicrobials used to control bacteria, fungi, and algae in cooling water systems, fuel storage tanks, pulp and paper mill water systems, oil extraction systems, wood preservation and antifouling agents (Nicoletti *et al.*, 1993; Vicini *et al.*, 2002; Critchley & Bentham, 2009). They are frequently used as preservatives in personal care products such as shampoos and other hair care products, as well as certain paint formulations. From 2002 to 2005, there was a notable increase use of isothiazolinones as a preservative, especially in the product category paints/lacquers (Flyvholm, 2005).

Figure 1-7. Isothiazolinone compounds. A. General structure. B. Methylisothiazolinone. C. Chloromethylsothiazolinone. D. Benzisothiazolinone.



1.5. Disinfection

1.5.1. Testing for viricidal activity

Official tests for approval of viricides are limited. In Europe there are just three organizations who provided protocols: in the United Kingdom DEFRA (Central Veterinary Laboratory, 1970), the Association Française de Normalisation in France (AFNOR, 1989), and the Deutsche Vereinigung zür Bekampfung der Viruskrankheiten in Germany (Anonymous, 1999). New

protocols have been developed, such as prEN14476 for testing viricidal activity in a suspension test (Anonymous, 2002), on fingerprints (Sattar & Ansari, 2002), and a protocol which uses contaminated hands (ASTM, 1999). However the main problem in developing a standard protocol to assess the virucidal activity of a biocide is the variability of resistance and survival of viruses, which makes the comparison between tests difficult.

The activity of chemicals against viruses is assessed by two principal methods: quantitative suspension and carrier tests.

The suspension test consists of mixing a known concentration of virus, with or without organic load, with a larger volume of the biocide at its use dilution. The mixture is kept for a defined contact time, at fixed pH and at a specific temperature, then after neutralising the biocide activity, viable viruses are titrated. Suspension tests are easy to perform but this test does not challenge the product strongly enough (Abad *et al.*, 1997). Therefore, they are only suitable for screening the activity of formulations under development and they are particularly recommended for viruses which are inactivated by drying.

Carrier tests simulate more realistic circumstances of disinfection practice, using animate or inanimate surfaces contaminated by viruses. The viruses are suspended in an appropriate soil load and a known volume of it is dried on a surface. The dried inoculum is exposed to the biocide for a specific contact time and temperature. Then the sample is eluted and titrated to determine the reduction of virus viability. The most important problem in this method is that some viruses do not survive after the drying process, hence appropriate control tests have to be performed (Sattar & Springthorpe, 2001).

Some factors must be considered in the design and performance of suspension tests and especially carrier tests, such as the type and the strain of virus(es) to be used, and the nature of the carrier, which can be inanimate or animate. Among the inanimate surfaces cylinders and discs of stainless steel, glass, or plastic have been used. For antiseptics, hands and fingerprints of human volunteers can been used. The neutralisation of the biocide is another important factor to take into account, because of its possible toxicity on the tested virus and on the host cells. Elimination of cytotoxicity and

control of the environmental parameters of the experiment (pH, temperature, organic load, contact time) are all important features in elaborating a protocol.

Some viruses can be difficult to propagate *in vitro* and may be hazardous to handle in the laboratory. To avoid these problems surrogate viruses, which are considered as a substitute of the interested organism, have been used (Ferrier *et al.*, 2004; Steinmann, 2004; Jimenez & Chiang, 2006; Kramer *et al.*, 2006a).

There are some considerations in selecting a surrogate virus. They must be easy to handle and stable on storage and as well on drying; they should have a high pool titre and they should possess a level of resistance to biocides that is representative of the class of virus under consideration. Moreover, disadvantages of using surrogates are the underestimation or overestimation of a products' activity or the resistance of naturally growing pathogens.

Bacteriophages have often been chosen as a surrogate for mammalian viruses, because of their ease to grow and enumerate. Coliphages MS2, K and ΦX174, being structurally similar to the enteric viruses and to poliovirus, have been used in viricidal tests as substitutes (Jones *et al.*, 1991; Davies *et al.*, 1993; Abad *et al.*, 1994; von Rheinbaben *et al.*, 2000; Maillard, 2001). They have also been used as indicators for enterovirus in waste water and polluted water (Kim *et al.*, 1980; Harakeh, 1984; Havelaar *et al.*, 1993; Brion & Silverstein, 1999).

1.5.2. Environmental factors, viral resistance and adaptation

Disinfectants can prevent contamination and transmission of infections. There are three different routes of transmission: *horizontal*, through person-to-person droplet infection and person-to-person intimate contact; *vertical* occurs during birth by transplacental infection or by vaginal passage; *zig-zag* is a very common way of transmission; it can be animal to insect to animal, or animal to animal (animate), or can be animal to fomite to animal/human (inanimate). The last route is the only one that can be meaningfully controlled by disinfection procedures.

Disinfection can be ineffective because of: environmental factors; type, structure and composition of the microorganisms; the ability of the microorganisms to degrade the compound (not valid with viruses) (Figure 1-8).

Figure 1-8. Classes of variables which influence biocide effectiveness.

Chemical disinfectant	Composition Concentration Temperature coefficient (Q_{10}) Concentration exponent
Environmental factors	pH Temperature Contact time Organic load (kind and quantity)
Virus	Concentration Degree of purity Virus carrier (skin, fabric, tile) Type of virus
Assays	Type of test (Suspension test, carrier test)

Among the environmental factors, organic load is the most common (Weber *et al.*, 1999). The organic matter can be represented by serum, blood, faeces, food residues, and earth. The interference can be attributed to the reaction that occurs between the organic materials and the biocides and to the possible protective layer that it is formed around the microorganisms (Russell, 2004). A study of the efficacy of biocides against Adenovirus 8 (Rutala *et al.*, 2006) showed that by adding organic load during the carrier tests, the viricidal activity was reduced from 4 to 1 Log_{10}. This loss of activity leaded to reclassification of some biocides, such as Chlorox [1:50] at 5 min, as no longer effective. Poschetto *et al.* (2007) found that different biocides were strongly influenced by organic load during their activity against Norovirus and Feline calicivirus. The concentration of an organic acid needed to be eight times higher to achieve the same viral reduction in clean and dirty conditions; similar results for the peroxide biocide were achieved which had to be ten times more concentrated.

pH can influence the virucidal activity by modifying the compound (protonation, dissociation), and by changing the surface properties of viral particles. For instance the efficacy of a formulation of silver and hydrogen peroxide against MS2 was reduced at higher pH (Pedahzur *et al.*, 2000).

The development of viral forms of resistance which alter the kinetics of the time/biocide efficacy reaction have been described although not extensively. Viruses show a variety of intrinsic and acquired mechanisms of resistance. The acquired mechanisms are distinct owing to single or multiple genetic mutations or to transmissible elements. The intrinsic ones are due to the structure of the viral particles themselves, such as the presence of an envelope, the virus surface properties or features arising due to the interaction between the biocide and the viral particles (McDonnel, 2007). Clumping is a phenomenon that develops after contact with disinfectants due mostly to a change of the viral surface properties. Viruses sometimes generate clumps in the cell, in the form of crystals. Then, after being released from the host, virions can remain clumped in water due to shifts in the pH or salt concentration, or associated with cellular debris (Floyd & Sharp, 1977). Clumps of 16 identically sized virions are required to form a protective coat around one virus of the same size, enough to protect it from contact with disinfectants. Factors that influence aggregate formation are i) the virus species and serotype, particularly the viral surface charge, ii) viral concentration, and iii) chemical properties of the surrounding medium, such as pH, ionic strength, and the nature of the ions present (Gassilloud & Gantzer, 2005). Studies showed that aggregation is correlated to the isoelectric point (pI) of viruses, in particular virions tend to be dispersed at their pI, even though it is not possible to find a general pattern because each virus responds differently in different buffers and pHs (Floyd & Sharp, 1979).

During disinfection procedures, aggregation can greatly influence biocide efficacy and can cause the underestimation of viable viral particles. The formation of clumps can, in fact, protect viruses which are trapped inside, avoiding the contact with biocide. As a consequence, some resistant subpopulations remain present in the medium or on the surface, as demonstrated with reovirus during bromine disinfection in water (Sharp *et*

al., 1975) and coxsackie virus after chlorine treatment (Jensen *et al.*, 1980). In addition, particulate matter can enhance further the formation of a layer around viruses and clumps, protecting viral particles from the environment (Thurman & Gerba, 1988b).

Another characteristic of viruses is their capacity for adaptation to the environment. The susceptibility of viruses to disinfectants can change after prolonged exposure, by modification of physical structures or by altering interactions with the host cells (Maillard, 2001).

Multiplicity reactivation is a further process of resistance, in which damaged and inactive viruses combine their resources, producing infectious virions (Thurman & Gerba, 1988b). This phenomena was discovered by Luria (1947) as the explanation for an increase in concentration of f2 after a previous reduction in viability due to disinfection. The process seems to happen when inactivated virions clump together and hybridize their gene pool.

In order to have truly functional disinfection and to avoid the viruses ability to acquire resistance to biocides, the capsid proteins have to be destroyed impeding the host-virus linkage and the released genome must be degraded and then dispersed such that the likelihood of multiplicity reactivation is diminished (Thurman & Gerba, 1988b).

1.6. Aims

The overall aim of the PhD project is to understand the mechanisms of action of PHMB-based biocides against viruses. Isothizolinone was used as a reference biocide.

Specifically this will be achieved through various steps:

- Investigating the interactions of PHMB-based and isothiazolinone-based disinfectants with bacteriophages (MS2 and F116), used as a virus model;
- Investigating the effects of PHMB-based disinfectants on phage capsid proteins;
- Electron microscopy investigations will be considered to analyze structural damages;
- Investigating the development of forms of resistance;
- Understanding the mode of action of PHMB against a mammalian virus, Adenovirus.

Since the viricidal activity of PHMB has not been well studied compared to the amount of investigations performed on other disinfectants, and since products based on biguanides are commonly used as bactericides, this project aims to produce preliminary data which will be useful for the enhancement of this category of biocides. Clearly, these results will have important implications for use of this type of biocide in healthcare.

2. GENERAL MATERIALS AND METHODS

2.1.Chemicals

2.1.1. Biocides

The biocides, COSMOCIL™ CQ, VANTOCIL™ TG, and PROXEL™ were provided by Arch Chemicals (Manchester, UK).

VANTOCIL™ TG contains 20% w/v (200000 ppm) polyhexamethylene biguanide hydrochloride (PHMB). The sample provided, batch bx1211, has a molecular weight of 2678. COSMOCIL™ CQ is a PHMB-based disinfectant, containing 20% w/v (200000 ppm) of the active compound with molecular weight of 3016. Concentrations used during the project were 2 ppm, 20 ppm, 200 ppm and 800 ppm of the active ingredient.

PROXEL™ ultra 10 is an isothiazolinone preservative biocide, composed of 1,2-benzisothiazol-3(2H)-one [9% - 12% w/v] and potassium hydroxide [2% - 5% w/v]. The concentration required for its use has been 400 ppm (0.04% w/v). PROXEL™ was used as a comparative biocide.

Biocides were prepared with sterile deionised water (Inorganic at 25°C 1 to > 15 MΩ-cm, TOC≤30 ppb) in pre-sterilized glass bottles and were stored for a maximum of 2 months in a dark environment at approx. 20°C.

2.1.2. Neutraliser

The neutralization step is an important passage during the suspension test in order to stop accurately the biocide activity at a time of exposure and to eliminate any toxicity if cell culture, embryonated eggs or animals are used to count the surviving viruses (Maillard, 2001). The neutralizer reacts with the active region of the compound which would bind and consequently inactivate the microorganism.

The neutraliser used for quenching all the studied biocides, both the PHMB-based and the isothiazolinone, was composed of Tween 80 [6% w/v], Lecithin [0.45% w/v], and L-Histidine [0.1% w/v]. Neutralisers were sterilized by autoclaving at 121°C for 15 min (British Pharmacopoeia, 2008) and store at 4°C.

2.2.Bacterial host culture

2.2.1. Propagation, enumeration and host suspension

Escherichia coli (NCIMB 9481) and *Pseudomonas aeruginosa* (NCIMB 10548) are the host bacteria for the MS2 and F116 phage respectively. They were grown in 10 ml TSB (Oxoid, Cambridge, UK) for 24 h at 37°C. Then the broth was centrifuged for 15 min at 3276 x*g* (Mistral 1000 centrifuge, MSE, London, UK) and the bacterial pellet was resuspended in 5 ml of phage buffer (SM buffer) (0.79% w/v (0.05M) Tris/HCl pH 7.4, 0.2 % w/v $MgSO_4$, 0.02% w/v gelatine).

Bacterial enumeration was performed through the drop counting method (Miles & Misra, 1938). The suspension was serially diluted by adding 100 µl of bacterial suspension in 900 µl of phage buffer and three 10 µl drops of the chosen dilution were plated on TSA plates. Three replicates were prepared for each suspension. After 24 h incubation at 37°C, the numbers of colonies for each drop were counted. Viable count and measurement of the optical density at 540 nm (Helios α UV-Vis spectrophotometer, Thermo Scientific, Waltham, USA) were performed for every new culture.

Furthermore, 20 cryogenic vials (Corning, NY, USA) of stock cultures were prepared by mixing 800 µl of bacteria suspension with 200 µl of glycerol (Fisher Scientific, Leicestershire, UK) and frozen at -80°C.

2.3. Phage

F116 (NCIMB 10882) is a temperate, pilus-specific, generalized transducing phage belonging to the Podoviridae family, infecting *P. aeruginosa*. It is non-enveloped with a head and a short tail with 6 fibers. The nucleocapsid is icosahedral, 60 nm in diameter, and composed by 72 capsomers per nucleocapsid. The genome is a linear double-stranded DNA, with a GC content of 63.2%. Its molecular weight (MW) is about 48 x 10^6 Da (International Committee on Taxonomy of Viruses, 2002).

MS2 (NCIMB 10108) belongs to Leviviridae family, and the host is *E. coli.* This non-enveloped coliphage consists of an icosahedral capsid without a tail. The capsid has a diameter of 24-26 nm and its molecular weight is 3.5 x 10^6 Da. The genome is not segmented and contains a single molecule of linear positive-sense, single-stranded RNA (International Committee on Taxonomy of Viruses, 2002). F116 was propagated at 30°C, whereas MS2 at 37°C.

2.3.1. Phage propagation and suspension

In order to have good bacteriophages infectivity (Mandel & Higa, 1970), agar and broth were supplemented with calcium chloride ($CaCl_2$). Taking into account results from Maillard (2001), different calcium chloride concentrations were tested, and the best result was found using $5x10^{-4}$ M $CaCl_2$ for MS2 and $1x10^{-3}$M $CaCl_2$ for F116. In addition, stocks of phage suspension (10^8 PFU/ml) were prepared to assure available viruses as required. For each species, 800 µl of phage suspension was mixed with 200 µl of glycerol in cryogenic vials and stored at -80°C.

The propagation of both phages was performed using broth culture and plate culture. The broth culture consisted of inoculating 10 ml of broth (Oxoid, Cambridge, UK) supplemented with $CaCl_2$ with the host bacteria ($5x10^7$ CFU/ml) and the bacteriophage (10^7 PFU/ml). The numerical ratio between the phage and the host was chosen according to the multiplicity of infection (MOI) results [see section 2.3.3]. After 24 h of incubation at 37°C in a shaking incubator at 70 RPM, the mixture was centrifuged for 15 min at 3276 x*g* (Mistral 1000 centrifuge, MSE, London, UK). The supernatant containing the phage particles was filtered through a 0.22 µm membrane filter (Millipore, Cork, Ireland) and stored at -4°C.

In the plate culture technique, phages were collected from infected plates where more than 30 plaques were observed. Five ml of SM buffer was poured into the plate, and the 65% agar layer [see section 2.3.2], where phages have been reproducing, was removed by a sterile plastic L-shape spreader and poured in a centrifuge tube. After being centrifuged for 15 min

at 11000 x*g* at 4°C (Rotor JA25.50, Avanti J-25, Beckman Coulter, Fullerton, USA), the supernatant was filtered through a 0.22 μm membrane filter (Millipore, Cork, Ireland) to avoid the passage of bacterial cells and large debris. The filtered phage suspension was stored at -4°C. A stock was mixed with 20% w/v glycerol and stored at -80°C.

2.3.2. Phage enumeration (soft overlay agar technique)

The plaque counting method was used to calculate phage concentration (plaque forming units per ml, PFU/ml). One hundred μl of the host cells (approx. 10^8 CFU/ml) and the diluted phage solution were mixed with a gelling agent (65% nutrient agar, Oxoid, Cambridge, UK) and poured in a plate on top of an already set nutrient agar. The use of 65% agar restricted the spread of bacteriophages only to the adjacent cells. It was assumed that each clear plaque was formed by the infection resulting from one virus. The phage-specific amount of $CaCl_2$ was added to 5 ml of 65% molten agar. The mixture, after being autoclaved, was kept in the water bath at 45°C, ready to be used for experiments.

After 24 h of incubation at the phage-specific temperature, plaques were counted and the PFU/ml was calculated.

2.3.3. Multiplicity of infection

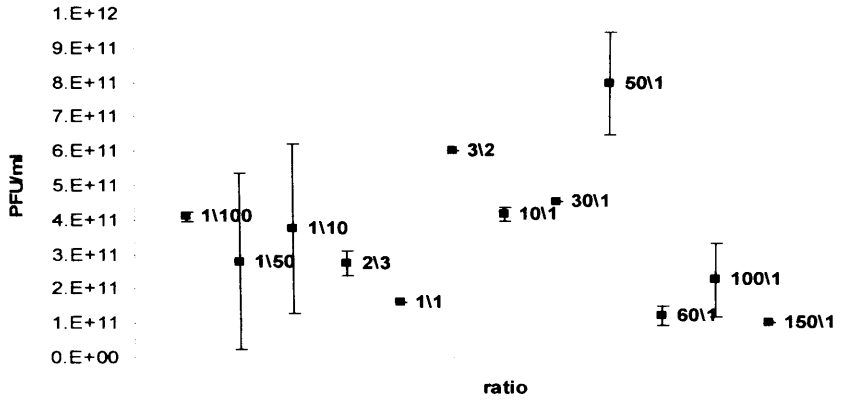
The multiplicity of infection (MOI) test was performed to calculate the infectivity capacity of phages. The result gave the optimum ratio between the host cell and the virus, which was used to get the highest yield of viral particles during the propagation in broth.

Phage suspension and host culture were mixed at different ratios in 10 ml of nutrient broth supplemented with $CaCl_2$ [see section 2.3.1] (Phage/Bacteria ratio: 100/1; 80/1; 50/1; 10/1; 3/2; 1/1; 2/3; 1/10; 1/50; 1/80; 1/100). An aliquot was taken from the inoculated bottle to measure the pH (Hanna Instruments Srl, Padova, Italy). The mixture was then incubated at 37°C.

After 24 h incubation, the broth was centrifuged at 3276 x*g* for 15 min (Mistral 1000, MSE, London, UK) and the supernatant was then filtered through a 0.22 µm membrane filter (Millipore, Cork, Ireland). The phage suspension was then serially diluted and enumerated. The ratio that gave the highest concentration of bacteriophage was determined.

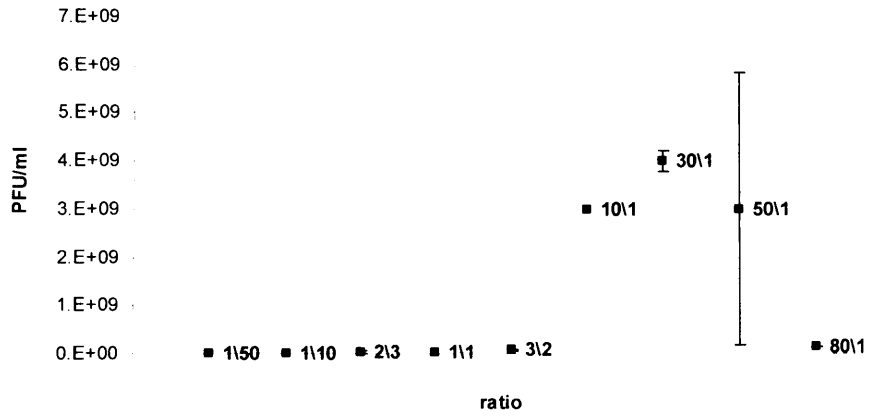
Optimum ratios were 50/1 for MS2 with *E. coli* and 30/1 for F116 with *P. aeruginosa* (Figure 2-1, Figure 2-2).

Figure 2-1. Multiplicity of infection graph of MS2 hosted by *E. coli*.



The ratio is bacteria/phage. Bars represent standard deviation.

Figure 2-2. Multiplicity of infection graph of F116 hosted by *P. aeruginosa*.



The ratio is bacteria/phage. Bars represent standard deviation.

2.4. Concentrated phage stock

Five ml of SM buffer was poured onto plates showing confluent lysis and was allowed to stand for 15 min. The top agar layer was then scraped and poured into a centrifuge tube. Then the mixture was centrifuged at 11000 x*g* for 15 min at 4°C and the supernatant was filtered through a 0.22 μm filter (Millipore, Cork, Ireland). After centrifuging at 19000 x*g* for 2 h at 4°C, the pellet was re-suspended into 1/10 of the initial volume. Starting from 10^9 PFU/ml, the final concentration obtained was approximately 10^{11} PFU/ml.

2.5. Purification of viral particles

2.5.1. Concentration of phage

The modified protocol was based on a method described by Sambrock & Russel (2001). The phage suspension prepared from confluent lysis plates was mixed with 10% w/v PEG 8000 and NaCl to achieve a final concentration of 1 M. The mixture was stirred and kept at 4°C for at least 5 h. It was then centrifuged at 11000 x*g* for 30 min at 4°C. The precipitate was resuspended in 1:20 of the initial volume of SM buffer and an equal volume of chloroform was added to extract PEG and cell debris. It was then vortexed for 30 sec and centrifuged at 3000 x*g* for 15 min at 4°C (J4-14 rotor, Avanti J20 XP centrifuge, Beckman Coulter, Fullerton, USA) to separate the aqueous from the organic phase. The aqueous phase was recovered and used during the isopycnic separation.

2.5.2. Isopycnic separation

CsCl (0.5 g/ml) was added to the concentrated viral suspension. Different densities of CsCl were prepared using SM buffer as diluents for MS2.

Table 2-1 showed the properties of the CsCl solutions used. According to the MS2 buoyant density in CsCl (1.38 g ml^{-1}) (Kuzmanovic *et al.*, 2003), three densities (ρ = 1.36 g ml^{-1}, ρ = 1.45 g ml^{-1}, ρ = 1.70 g ml^{-1}) were chosen

in order to detect the band containing the coliphage. A 13.5 ml polyallomer centrifuge tube (Beckman Coulter, Fullerton, USA) was filled with 2.6 ml of each CsCl density. A line was drawn outside the tube at the interface between ρ = 1.36 g ml^{-1} and ρ = 1.45 g ml^{-1}. The virus suspension (5.2 ml) was layered on top of the gradient and then centrifuged at 200000 x*g* for 8 h at 20°C (Vti 65 rotor, Optima LE-80K Ultracentrifuge, Beckman Coulter, Fullerton, USA). The band corresponding to the phage (in the ρ = 1.36 g ml^{-1} zone) was harvested by gently inserting a sterile needle to remove it. The infectivity was checked.

The purified phage was dialysed (Spectra/Por Float-A-Lyzer, MWCO 3500 Da) overnight against SM buffer and then filtered through a 0.22 µm filter (Millipore, Cork, Ireland). Aliquots of 1 ml were stored at -80°C.

Table 2-1. Densities and respective concentrations of CsCl solutions prepared in 10 ml of SM buffer.

Density ρ (g/ml)	M (g-mole/L)	CsCl (g)	SM buffer (ml)
1.36	2.92	4.92	8.74
1.45	3.60	6.00	8.50
1.70	5.65	9.50	7.50

From Sambrock & Russel (2001).

2.6. Statistical analysis

All experiments were repeated three times, except the suspension test which was performed five times. All data were transformed into Log_{10} to assure that data were normally distributed.

Any differences among results were analyzed by parametric tests, one-way ANOVA test and t-test using Statistica 7.0 software (StatSoft Inc., Tulsa, USA). If the analysis of variance resulted in significance, post-hoc Tukey test was performed. When the data were not normally distributed, even after being transformed, the Mann-Whitney U Test was used.

2.7. Validation

The drop count technique and the phage enumeration technique have been validated by performing the experiments 10 times. The resulting data were subjected to a One Way ANOVA in Statistica 7.0 software package (StatSoft Inc, Tulsa, USA). Validation was deemed successful when $P>0.05$ and the data was normally distributed according to the Anderson-Darling test (Bacterial drop counting method ANOVA, $F_{1,20}=0.66$, $P>0.05$; phage enumeration technique ANOVA, $F_{1,18}=0.11$, $P>0.05$).

3. EFFECT OF PHMBs ON THE VIABILITY OF F116 AND MS2 PHAGES

3.1. Introduction

The use of biocides is crucial for the control of microbial contamination and infection in the healthcare environment (Maillard, 2001). Although the number and the use of products containing an antimicrobial are increasing, there is generally a lack of understanding of their mechanisms of action against viruses. Several biocides (e.g. quaternary ammonium compounds, biguanides, phenolics) have been described to have a virucidal activity against enveloped viruses, but only low efficacy against certain non-enveloped ones (Wood & Payne, 1998; Krebs *et al.*, 2005; Sauerbrei *et al.*, 2006). The reason for such diversity of activity has been based on the interaction of these chemical agents with the viral envelope and an absence of activity against the protein capsid (Maillard, 2001).

Testing the virucidal activity of biocides is undertaken mainly with two tests: the suspension test and the carrier test. Suspension test is less challenging to the microorganisms, but is essential as a first screening of the efficacy of a new or previously un-tested biocide. It is important that the environmental conditions (pH and temperature) are kept constant for the whole treatment and that a defined contact time in ensured by the use of a neutralizer able to quench the biocide activity. In Europe the suspension tests most commonly used are the German DVV (Anonymous, 1999), the French AFNOR (AFNOR, 1989) and the European EN 13610 (CEN, 2002a). They all require a 4 Log_{10} reduction of viral concentration, but are based on different parameters, such as the biocide concentration and the volumes ratio used. Moreover, the German guideline recommends as a standard test virus strains of polio 1 (wild type), adenovirus type 2, vaccinia virus and papovavirus SV40, whereas the French test mentions polio 1 (vaccine type), adenovirus type 5 and vaccinia virus (Steinmann, 2001). In the USA, the ASTM guideline (ASTM, 1989b) requires a lower reduction (3 Log_{10}) and suggests more standard virus strains: influenza virus, rhinovirus, herpes simplex, polio and vaccinia virus (Bellamy, 1995).

The carrier test is a simulation of "real life", where a known concentration of a viral suspension is dried on surfaces (different types) and then treated with the biocide. The challenge for the microorganisms is higher than in a suspension test, but the main obstacle in performing this test is the risk of inactivation of the virus during the drying step (Maillard, 2001). In Europe there are several types of surface suggested (Sattar *et al.*, 1987; Sattar *et al.*, 2003; Ferrier *et al.*, 2004), but there is no standard procedure. However in the USA, the ASTM developed a carrier test standard procedure (ASTM, 1989a).

The main obstacle in developing a standard protocol, which allows a comparison of results, is the choice of a representative virus. The heterogeneity of viral response to biocides and therefore the difficulties to find a standard response pattern are well-known. The only attempt was made by Klein and Deforest 40 years ago (Klein & Deforest, 1965). They explained the nature of viral inactivation by the chemical properties of the virus surface. They divided viruses into three groups (A, B and C) based on their lipidic nature that consequently influences their sensitivity to chemical agents. Table 3-1 summarises the sensitivity to biocides based on the Klein-Deforest classification. The lipophilic property is based on the adsorption to lipids, such as cholesterol, palmitic acid, and hexadecylamine, on particle destruction by diethylether, and as well by inactivation using lipophilic germicides, such as the quaternary ammonium compounds (QACs) (Prince & Prince, 2001).

Table 3-1 Sensitivity to biocides based on Klein-Deforest classification.

Klein-Deforest category	**Solubility**	**Lipidic membrane**	**Sensitivity**
A	Lipophilic	+	High (Herpes, HIV, Myxoviruses)
B	Hydrophilic	-	Low (Poliovirus, Rhino virus, Coxsackie virus)
C	Intermediate	-	Moderate (Adenovirus, SV-40, Rotavirus)

MS2 was often used as a surrogate virus. In a study of Koivunen *et al.* (2005), MS2 was found to be resistant to peracetic acid; a dose of 0.07%-0.17% w/v was needed to produce 1-1.5 Log_{10} reduction. They also found that combined treatments, such as peracetic acid and UV or hydrogen peroxide and UV, did not achieve synergistic effects.

Other investigations conducted *in vitro* using MS2 as a surrogate, showed 0.5 Log_{10} reduction after treatment with 70% ethanol over 60 sec (Woolwine & Gerberding, 1995), and approximately 0.35 Log_{10} reduction with 0.5% chlorhexidine gluconate/70% ethanol within 60 sec (Woolwine & Gerberding, 1995). The only high level reduction (2.29 Log_{10}) of MS2 was achieved in a carrier test (fingerprints) through the use of plain soap for an exposure time of 30 seconds (Davies *et al.*, 1993).

PHMBs are cationic agents which interact with the acidic bacterial membrane lipids causing phase separation and domain formation. Such mechanism provoke lethal leakage of cytoplasmic materials (Gilbert & Moore, 2005). A polymer size-dependent activity was shown by PHMB, where larger molecules produce larger domains and therefore more membrane disruption (Broxton *et al.*, 1984). Thereby, it is likely that PHMB-based biocides are more active against enveloped viruses, such as herpes simplex virus and HIV (Wood & Payne, 1998; Baqui *et al.*, 2001).

3.1.1. Inactivation kinetics

The disinfection reaction rate was analysed by Chick (1908) by exposing bacteria to a disinfectant at a constant concentration and enumerating the surviving organisms at successive times. He considered the reaction analogous to a bi-molecular chemical reaction, such as:

$$xN + nC \xrightarrow{k_1} p \text{ (inactive microorganisms)}$$

where N is number of organisms, C is the compound concentration and p is the product (number of inactive microorganisms).

Considering the concentration of the disinfectant constant and the reaction irreversible, it can be described by the rate law:

$$dN/dt = -kN$$

where dN/dt is the rate of change of the viable count of organisms with time t (survival ratio)

k_1 is the reaction rate constant

N_t is the number of organisms at time t

t is time

If N_0 is the number of organisms at time 0, the above equations can be integrated to a pseudo first-order relationship:

$$N_t/N_0 = e^{-kt} \qquad \text{or}$$

$$Ln(N_t/N_0) = -kt$$

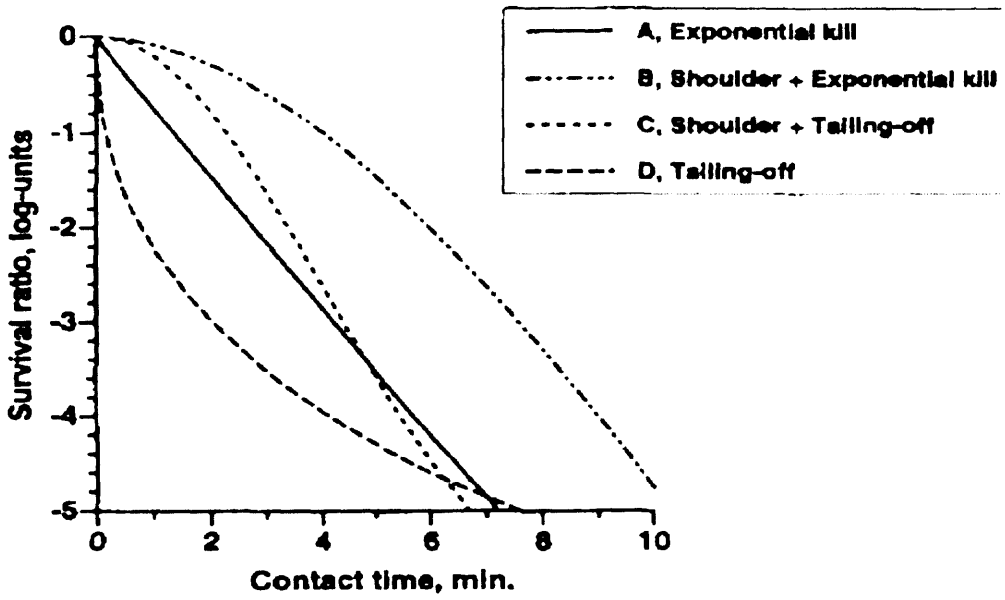
For this relationship, to be valid, it has to follow some assumptions (Hoff & Akin, 1986):

- Viruses are discrete units equally susceptible to the disinfectant;
- Viruses and disinfectants have to be uniformly distributed in the medium;
- The disinfectant is stable (concentration and chemical composition) through the duration of the experiment;
- The medium where the reaction occurs does not have any interfering substances.

However, these conditions are frequently not fulfilled in real life. Figure 3-1 is a semi-log plot of the inactivation rate against contact time. The ideal first-order inactivation kinetic (exponential kill, curve A) can be affected by several environmental conditions (type of organisms, medium, pH,

temperature, biocide) causing a deviation from the linear relationship (Churn *et al.*, 1984).

Figure 3-1. Inactivation curve of virus caused by disinfectant action (Gyurek & Finch, 1998).



Different behaviours could occur. The "shoulder" effect (curves B and C) can be caused by an improper mixing, by a delay due to diffusion of the disinfectant, by the necessity of multiple targets for the viral inactivation or/and the presence of aggregates. The "tailing off" curve (D) describes a rapid initial inactivation followed by a decrease in the killing rate yielding to a complete lack of activity. It can be attributed to an depletion of the disinfectant concentration, or to the presence of more resistant subpopulations caused by clumping or capsid conformational changes (Hoff, 2002).

Polyhexamethylene biguanide (PHMB) is a cationic antimicrobial agent used for over 40 years in the healthcare environment, food and water industries. Although its interaction with viruses has not been described, PHMB has been found active against HIV-1 (Krebs *et al.*, 2005). Other

biguanide derivatives were shown to be effective against polio, vaccinia and influenza viruses (Fara *et al.*, 1974).

3.2. Aims

The aims of this chapter are to assess the effect of VANTOCIL™ TG and COSMOCIL™ CQ on the viability of the phage F116 and MS2. The isothiazolinone-based biocide, PROXEL™ was used as a comparative biocide.

Specifically:

- Verify the toxicity of a tween-based neutraliser on the tested microorganisms;
- Assess the neutraliser ability to quench the antimicrobial activity of the PHMB-based and isothiazolinone-based biocides;
- Assess the virucidal activity of the two PHMB-based and the isothiazolinone-based biocides against MS2 and F116.

3.3. Materials and methods

3.3.1. Neutraliser tests

Prior to any virucidal activity tests, the neutraliser toxicity test on the organisms (hosts and phages) and the neutraliser efficiency test to measure the ability to quench the biocidal activity were performed. Measurement of pH of the phage-neutraliser mixture was taken after performing the serial dilution (pH meter, Hanna Instruments Srl, Padova, Italy).

3.3.1.1. Neutraliser toxicity test

One ml of the overnight host cell suspension (10^7 CFU/ml) [see chapter 2, section 2.2] was mixed with 9 ml of neutraliser (6% w/v tween 80, 0.45% w/v lecithin, and 0.1% w/v L-histidine). After 10 min contact, 1 ml of sample was taken and serially diluted (10-fold). Five drops of 10 µl of three different dilutions were plated on agar in duplicate. Plates were then incubated for 24h at 37°C for *Escherichia coli* and 30°C for *Pseudomonas aeruginosa*. The reduction factor (RF) was calculated as Log_{10} (CFU/ml initial inoculum) – Log_{10} (CFU/ml after exposure).

The procedure was similar with the phages. One ml of phage suspension [see chapter 2, section 2.3] and 9 ml of neutraliser were mixed together for 10 min. Then 1 ml of the sample was withdrawn and serially diluted (10-fold). Two dilution factors (in duplicate) were used to enumerate the phage concentration [see chapter 2, section 2.3]. The reduction factor (RF) was calculated as Log_{10} (PFU/ml initial inoculum) – Log_{10} (PFU/ml after exposure).

3.3.1.2. Neutraliser efficacy test

This experiment was performed to assess the efficiency of the neutraliser to halt the virucidal activity of the biocides. Different relatives proportion of disinfectants and neutraliser (1/1 and 2/1) were used in this investigation to reflect the different experiments. The reduction factor (RF) was calculated. The pH was also measured.

The biocide and neutraliser were mixed at ratios of 1/1 (5 ml + 5 ml) and 2/1 (8 ml + 4 ml). Then 1 ml of the overnight bacterial host cell (10^7 CFU/ml) was poured into the mixture and left for 10 min. One ml of the experimental sample was taken and serially diluted (10-fold). Five drops of 10 µl of three different dilution factors were plated in duplicate, and incubated at the bacteria-specific temperature for 24h.

Regarding the bacteriophage, 1 ml of phage suspension (10^7 PFU/ml) was added to the mixture of biocide and neutraliser at the relative proportions, 1/1 (5 ml + 5 ml) and 2/1 (8 ml + 4 ml). After 10 min, 1 ml of sample was serially diluted (10-fold). Two dilution factors were plated in duplicate to measure the PFU/ml [see chapter 2, section 2.3]. Controls consisted of testing the microorganism with the neutraliser mixed with sterile water, instead of biocide. The reduction factor (RF) was calculated as Log_{10} (CFU/ml or PFU/ml control) – Log_{10} (CFU/ml or PFU/ml after exposure).

3.3.2. Suspension test

The purpose of the suspension test was to determine the virucidal activity of the biocides against F116 and MS2. The pH was measured as described above.

Biocides were tested at 20°C. Four different contact times, 1, 5, 10 and 30 min, were considered according to a previous study on biocide interaction with bacteriophages (Maillard, 2001). Testing contact times over 30 min would not be representative of the use of the biocide in the disinfection procedure.

One ml of phage solution was added to 9 ml of biocide, and after the appropriate contact time, 1 ml was added to 9 ml of neutraliser. After 2 min contact, 1 ml of sample was taken and serially diluted (10-fold). Two dilution factors were plated in duplicate to measure the surviving phages. Controls consisted of testing the microorganisms against sterile water instead of PHMB.

The reduction factor (RF) was calculated as Log_{10} (PFU/ml control) – Log_{10} (PFU/ml after exposure). A decrease in concentration greater than 4 Log_{10}

was necessary to consider the biocide active against the virus, as defined in prEN: 13610 (CEN, 2002a).

3.3.3. Statistical analysis

Significant differences were assessed by ANOVA and t-test [see Chapter 2, section 2.6]. Pearson's correlation and determination factor were calculated between the survival ratio, $\ln(N_1/N_0)$, and contact time after each treatment (Statistica 7.0, StatSoft Inc, Tulsa, USA).

3.4. Results

3.4.1. Neutraliser tests

3.4.1.1. Neutraliser toxicity test

3.4.1.1.a. Host culture

Escherichia coli and *Pseudomonas aeruginosa* (Table 3-2) were not affected by the neutraliser showing no significant differences in CFU/ml between the control and after treatment (*E. coli*: t-test, $P>0.05$; *P. aeruginosa*: t-test, $P>0.05$).

Table 3-2. Neutraliser toxicity test against *E. coli* and *P. aeruginosa*.

	CFU/ml before treatment ± sd	**CFU/ml after treatment ± sd**	**RF**
E. coli	8.96 ± 0.07	9.20 ± 0.20	0*
P. aeruginosa	9.18 ± 0.28	9.17 ± 0.19	0.01 ± 0.24

* 0 means no reduction.

RF: reduction factor.

Data are expressed as Log_{10}. Mean and standard deviation of three experiments.

3.4.1.1.b. Phages

The neutraliser was determined as not toxic to either MS2 or F116 (MS2: t-test, $P>0.05$; F116: t-test, $P>0.05$) (Table. 3-3).

Table. 3-3 Neutraliser toxicity test against MS2 and F116.

	PFU/ml before treatment ± sd	**PFU/ml after treatment ± sd**	**RF**
MS2	11.40 ± 0.00	11.38 ± 0.29	0.01 ± 0.28
F116	8.02 ± 0.63	8.39 ± 0.75	0*

* 0 means no reduction.

RF: reduction factor.

Data are expressed as Log_{10}. Mean and standard deviation of three replicates.

3.4.1.2. Neutraliser efficacy test

3.4.1.2.a. Host culture

The neutraliser quenched efficiently the activity of VANTOCIL™ TG at both relatives proportion of biocide and neutraliser against *E. coli* and *P. aeruginosa* (*E. coli*: ratio 1/1 ANOVA, $F_{1,4}$=8.40, P>0.05; ratio 2/1 ANOVA, $F_{1,4}$=7.73, P>0.05. *P. aeruginosa*: ratio 1/1 ANOVA, $F_{1,4}$=2.61, P>0.05; ratio 2/1 ANOVA, $F_{1,4}$=0.59, P>0.05).

COSMOCIL™ CQ activity was halted when the ratio of neutraliser/biocide was 1/1 (*E. coli*: ratio 1/1 ANOVA, $F_{1,4}$=0.64, P>0.05. *P. aeruginosa*: ratio 1/1 ANOVA, $F_{1,4}$=2.65, P>0.05) and against *P. aeruginosa* at ratio 2/1 (ANOVA, $F_{1,4}$=0.37, P>0.05) (Table 3-4; Table 3-6). However at the ratio 2/1, the number of surviving *E. coli* after the treatment was significantly higher (*E. coli*: ratio 2/1 ANOVA, $F_{1,4}$=8.55, P≤0.05.). Since the increased number of bacterial cell was low, these results were considered as natural variation among counts.

The neutraliser was also efficacious in stopping any bactericidal activity of PROXEL™ (*E. coli*: ratio 1/1 ANOVA, $F_{1,4}$=0.39, P>0.05; ratio 2/1 ANOVA, $F_{1,4}$=0.61, P>0.05. *P. aeruginosa*: ratio 1/1 ANOVA, $F_{1,4}$=0.22, P>0.05; ratio 2/1 ANOVA, $F_{1,4}$=0.37, P>0.05) (Table 3-6).

Table 3-4. Neutraliser efficacy test on VANTOCIL™ TG against *E. coli* and *P. aeruginosa*.

VANTOCIL™ TG	Ratio	pH	CFU/ml control ± sd	CFU/ml after treatment ± sd	RF ± sd
E. coli	1/1	6.82 ± 0.005	9.08 ± 0.10	8.87 ± 0.05	0.21 ± 0.15
	2/1	6.81 ± 0.01	9.04 ± 0.05	9.15 ± 0.07	0*
P. aeruginosa	1/1	6.80 ± 0.005	9.63 ± 0.24	9.39 ± 0.16	0.24 ± 0.22
	2/1	6.83 ± 0.005	9.47 ± 0.10	9.54 ± 0.17	0*

* 0 means no reduction.

The relatives proportion of biocide and neutraliser.

RF: reduction factor.

Data are expressed as Log_{10}. Mean and standard deviation of three replicates.

Table 3-5. Neutraliser efficacy test on COSMOCIL™ CQ against *E. coli* and *P. aeruginosa*.

COSMOCIL™ CQ	Ratio	pH	CFU/ml control ± sd	CFU/ml after treatment ± sd	RF ± sd
E. coli	1/1	6.85 ± 0.04	9.08 ± 0.10	9.03 ± 0.03	0.04 ± 0.09
	2/1	6.90 ± 0.02	9.04 ± 0.05	9.13 ± 0.04	0*
P. aeruginosa	1/1	7.34 ± 0.06	9.63 ± 0.24	9.40 ± 0.15	0.24 ± 0.21
	2/1	7.41 ± 0.08	9.47 ± 0.10	9.52 ± 0.15	0*

* 0 means no reduction.

The relatives proportion of biocide and neutraliser.

RF: reduction factor.

Data are expressed as Log_{10}. Mean and standard deviation of three replicates.

Table 3-6. Neutraliser efficacy test on PROXELTM against *E. coli* and *P. aeruginosa*.

PROXEL™	Ratio	pH	CFU/ml control ± sd	CFU/ml after treatment ± sd	RF ± sd
E. coli	1/1	8.49 ± 0.06	9.78 ± 0.54	9.79 ± 0.40	0*
	2/1	8.80 ± 0.06	9.78 ± 0.54	9.52 ± 0.88	0.26 ± 0.33
P. aeruginosa	1/1	8.66 ± 0.19	10.07 ± 0.03	10.03 ± 0.05	0.04 ± 0.05
	2/1	8.78 ± 0.02	10.42 ± 0.23	10.42 ± 0.22	0.35 ± 0.26

*0 means no reduction

The relatives proportion of biocide and neutraliser.

RF: reduction factor.

Data are expressed as Log10. Mean and standard deviation of three replicates.

3.4.1.2.b. Phages

The virucidal activity of the two PHMB-based biocides was stopped effectively by the neutraliser (Table 3-7; Table 3-8) against both bacteriophages, MS2 (VANTOCIL™ TG: ratio 1/1 t-test, $P>0.05$; ratio 2/1 t-test, $P>0.05$. COSMOCIL™ CQ: ratio 1/1 t-test, $P>0.05$; ratio 2/1 t-test, $P>0.05$) and F116 (VANTOCIL™ TG: ratio 1/1 t-test, $P>0.05$; ratio 2/1 t-test, $P>0.05$. COSMOCIL™ CQ: ratio 1/1 t-test, $P>0.05$; ratio 2/1 t-test, $P>0.05$).

The neutraliser was also effective in halting any virucidal activity of PROXEL™ (Table 3-9). MS2 (ratio 1/1 t-test, $P>0.05$; ratio 2/1 t-test, $P>0.05$) and F116 (ratio t-test, $P>0.05$; ratio 2/1 t-test, $P>0.05$) did not show any significant reduction.

Table 3-7. Neutraliser efficacy test on VANTOCIL™ TG against MS2 and F116.

VANTOCIL™ TG	Ratio	pH	PFU/ml before treatment ± sd	PFU/ml after treatment ± sd	RF ± sd
MS2	1/1	7.12 ± 0.01	8.79 ± 1.50	8.46 ± 1.82	0.33 ± 0.33
	2/1	7.22 ± 0.04	8.79 ± 1.50	8.45 ± 1.77	0.34 ± 0.34
F116	1/1	7.39 ± 0.19	11.17 ± 0.14	10.71 ± 0.63	0.23 ± 0.26
	2/1	7.49 ± 0.16	11.17 ± 0.14	10.71 ± 0.81	0.29 ± 0.44

The relatives proportion of biocide and neutraliser.

RF: reduction factor.

Data are expressed as Log_{10}. Mean and standard deviation of three replicates.

Table 3-8. Neutraliser efficacy test on COSMOCIL™ CQ against MS2 and F116.

COSMOCIL™ CQ	Ratio	pH	PFU/ml before treatment ± sd	PFU/ml after treatment ± sd	RF ± sd
MS2	1/1	7.23 ± 0.07	7.90 ± 0.27	7.60 ± 0.35	0.31 ± 0.31
	2/1	7.25 ± 0.09	7.90 ± 0.27	7.72 ± 0.30	0.18 ± 0.04
F116	1/1	7.55 ± 0.00	9.37 ± 0.58	9.13 ± 0.77	0.24 ± 0.20
	2/1	7.63 ± 0.02	9.37 ± 0.58	8.92 ± 0.93	0.45 ± 0.43

The relatives proportion of biocide and neutraliser.

RF: reduction factor.

Data are expressed as Log_{10}. Mean and standard deviation of the three replicates.

Table 3-9. Neutraliser efficacy test on PROXEL™ against MS2 and F116.

PROXEL™	Ratio	pH	PFU/ml before treatment ± sd	PFU/ml after treatment ± sd	RF ± sd
MS2	1/1	8.63 ± 0.06	9.02 ± 0.30	8.78 ± 0.48	0.24 ± 0.21
	2/1	8.93 ± 0.06	9.02 ± 0.30	8.87 ± 0.45	0.15 ± 0.16
F116	1/1	8.59 ± 0.04	10.06 ± 0.81	9.87 ± 1.03	0.19 ± 0.28
	2/1	8.82 ± 0.13	10.06 ± 0.81	9.89 ± 1.04	0.17 ± 0.26

The relatives proportion of biocide and neutraliser.

RF: reduction factor.

Data are expressed as Log_{10}. Mean and standard deviation of three replicates.

3.4.2. Suspension test

3.4.2.1. PHMB-based biocides – VANTOCIL™ TG and COSMOCIL™ CQ

3.4.2.1.a. MS2

VANTOCIL™ TG, at 200 ppm, reduced the coliphage MS2 viability by approximately 90% (1 Log_{10} reduction) (Table 3-10). The range of Log_{10} reduction varied from 0.14 to 2.02 Log_{10}, and longer contact times did not increase the efficiency of the biocide (ANOVA, $F_{1,16} = 0.03$; $P>0.05$).

At 800 ppm, the biocide activity was still low (Table 3-11) and did not produce more than 2 Log_{10}, with high variability among replicates. Contact time did not enhance the VANTOCIL™ TG efficacy (ANOVA, $F_{1,16} = 0.71$; $P>0.05$). The reduction factors at the two concentrations did not differ significantly (t-test, $P>0.05$), even though the figures appeared higher at 200 ppm.

The coliphage resulted to be more resistant to COSMOCIL™ CQ. At 200 ppm its concentration was reduced by less that 90% after 1, 5 and 10 min contact time, whereas after 30 min the reduction was significantly higher than after the other treatment period (ANOVA, $F_{1,16}=11.78$, $P\leq0.05$), but still lower than 1 Log_{10} (Table 3-12). Increasing the concentration to 800 ppm, the biocide was able to decrease the phage viability of 1.07 ± 0.30 after 30 min, although the contact time was not influencing significantly the virucidal activity (ANOVA, $F_{1,16}=1.61$, $P>0.05$).

Table 3-10. Suspension test results. VANTOCIL™ TG at a concentration of 200 ppm against MS2.

VANTOCIL™ TG		MS2		
Contact time (minute)	**pH**	**PFU/ml before treatment ± sd**	**PFU/ml after treatment ± sd**	**Mean RF ± sd**
1	7.91 ± 0.03	8.77 ± 0.09	7.71 ± 0.63	1.06 ± 0.61
5	7.91 ± 0.03	8.77 ± 0.09	7.62 ± 0.54	1.15 ± 0.53
10	7.91 ± 0.03	8.77 ± 0.09	7.65 ± 0.55	1.12 ± 0.55
30	7.90 ± 0.05	8.77 ± 0.09	7.62 ± 0.60	1.15 ± 0.64

RF: reduction factor. Data are expressed as Log_{10}. Mean and standard deviation of five replicates.

Table 3-11. Suspension test results. VANTOCIL™ TG at a concentration of 800 ppm against MS2.

VANTOCIL™ TG		MS2		
Contact time (minute)	**pH**	**PFU/ml before treatment ± sd**	**PFU/ml after treatment ± sd**	**Mean RF ± sd**
1	7.61 ± 0.18	8.88 ± 0.91	8.44 ± 1.30	0.44 ± 0.67
5	7.64 ± 0.21	8.88 ± 0.91	8.27 ± 1.16	0.61 ± 0.59
10	7.64 ± 0.23	8.88 ± 0.91	8.11 ± 1.22	0.77 ± 0.85
30	7.64 ± 0.22	8.88 ± 0.91	7.86 ± 0.91	1.02 ± 0.43

RF: reduction factor. Data are expressed as Log_{10}. Mean and standard deviation of five replicates.

Table 3-12. Suspension test results. COSMOCIL™ CQ at a concentration of 200 ppm against MS2.

COSMOCIL™ CQ		MS2		
Contact time (minute)	**pH**	**PFU/ml before treatment ± sd**	**PFU/ml after treatment ± sd**	**Mean RF ± sd**
1	7.75 ± 0.13	9.73 ± 0.61	9.75 ± 0.51	0*
5	7.75 ± 0.09	9.73 ± 0.61	9.77 ± 0.56	0*
10	7.78 ± 0.09	9.73 ± 0.61	9.62 ± 0.53	0.11 ± 0.19
30	7.74 ± 0.11	9.73 ± 0.61	9.13 ± 0.83	0.60 ± 0.22

*0 means no reduction

RF: reduction factor. Data are expressed as Log_{10}. Mean and standard deviation of five replicates.

Table 3-13. Suspension test results. COSMOCIL™ CQ at a concentration of 800 ppm against MS2.

COSMOCIL™ CQ		MS2		
Contact time (minute)	**pH**	**PFU/ml before treatment ± sd**	**PFU/ml after treatment ± sd**	**Mean RF ± sd**
1	7.58 ± 0.16	8.17 ± 0.46	7.63 ± 0.69	0.54 ± 0.36
5	7.64 ± 0.16	8.17 ± 0.46	7.40 ± 0.45	0.77 ± 0.45
10	7.69 ± 0.15	8.17 ± 0.46	7.40 ± 0.52	0.77 ± 0.40
30	7.66 ± 0.10	8.17 ± 0.46	7.10 ± 0.49	1.07 ± 0.30

RF: reduction factor. Data are expressed as Log_{10}. Mean and standard deviation of five replicates.

3.4.2.1.b. F116

The number of viable F116 phage was not affected by VANTOCIL™ TG at 200 ppm (Table 3-14). The activity at different contact times did not differ (ANOVA; $F_{1,16} = 0.56$; $P>0.05$). In fact, longer contact time did not increase the efficacy of VANTOCIL™ TG.

Raising the concentration of the biocide to 800 ppm, the activity of VANTOCIL™ TG increased greatly, even though 4 Log_{10} reduction was not achieved (Table 3-15). The range of reduction factors varies from 1.53 to 3.04 Log_{10} PFU/ml. The time of exposure did not increase the virucidal activity (ANOVA; $F_{1,16} = 0.69$; $P>0.05$).

The other polyhexamethylene biguanide compound, COSMOCIL™ CQ, showed the same activity as VANTOCIL™ TG. At 200 ppm the Log_{10} reduction achieved varied from 0.45 Log_{10} at 1 min contact time to 0.85 Log_{10} for the highest time of exposure (Table 3-16).

At 800 ppm, F116 was more affected by the biocide which decreased its viability by 99 %. In fact, COSMOCIL™ CQ achieved a reduction factor of a minimum of 2.17 at 1 min to a maximum of 2.63 at 30 min (Table 3-17). At both concentration the contact time did not influence the reduction of

F116 (200 ppm: ANOVA; $F_{1,16} = 0.25$; $P>0.05$. 800 ppm: ANOVA; $F_{1,16} = 1.55$; $P>0.05$).

Table 3-14. Suspension test results. VANTOCIL™ TG at a concentration of 200 ppm against F116.

VANTOCIL™ TG		F116		
Contact time (min)	**pH**	**PFU/ml before treatment ± sd**	**PFU/ml after treatment ± sd**	**Mean RF ± sd**
1	8.02 ± 0.24	10.08 ± 0.66	10.03 ± 0.69	0.05 ± 0.22
5	8.01 ± 0.15	9.61 ± 0.98	9.67 ± 1.01	0*
10	8.01 ± 0.14	9.61 ± 0.98	9.75 ± 1.17	0*
30	8.00 ± 0.10	9.80 ± 0.75	9.95 ± 0.62	0*

*0 means no reduction

RF: reduction factor. Data are expressed as Log_{10}. Mean and standard deviation of five replicates.

Table 3-15. Suspension test results. VANTOCIL™ TG at a concentration of 800 ppm against F116.

VANTOCIL™ TG		F116		
Contact time (min)	**pH**	**PFU/ml before treatment ± sd**	**PFU/ml after treatment ± sd**	**Mean RF ± sd**
1	7.67 ± 0.03	10.42 ± 0.18	8.28 ± 0.39	2.17 ± 0.43
5	7.70 ± 0.02	10.42 ± 0.18	8.05 ± 0.28	2.40 ± 0.29
10	7.73 ± 0.04	10.42 ± 0.18	8.04 ± 0.39	2.40 ± 0.43
30	7.70 ± 0.06	10.42 ± 0.18	7.89 ± 0.47	2.56 ± 0.54

RF: reduction factor. Data are expressed as Log_{10}. Mean and standard deviation of five replicates.

Table 3-16. Suspension test results. COSMOCIL™ CQ at a concentration of 200 ppm against F116.

COSMOCIL™ CQ		F116		
Contact time (min)	**pH**	**PFU/ml before treatment ± sd**	**PFU/ml after treatment ± sd**	**Mean RF ± sd**
1	7.62 ± 0.17	9.81 ± 0.48	9.35 ± 0.70	0.44 ± 0.45

5	7.73 ± 0.16	9.81 ± 0.48	9.09 ± 0.94	0.72 ± 0.72
10	7.73 ± 0.13	9.81 ± 0.48	9.09 ± 1.04	0.72 ± 0.84
30	7.73 ± 0.15	9.81 ± 0.48	8.95 ± 1.08	0.86 ± 0.88

RF: reduction factor. Data are expressed as Log_{10}. Mean and standard deviation of five replicates.

Table 3-17. Suspension test results. COSMOCIL™ CQ at a concentration of 800 ppm against F116.

COSMOCIL™ CQ		**F116**		
Contact time (min)	**pH**	**PFU/ml before treatment ± sd**	**PFU/ml after treatment ± sd**	**Mean RF ± sd**
1	8.01 ± 0.07	10.18 ± 0.66	8.45 ± 0.77	1.73 ± 0.40
5	8.00 ± 0.08	10.18 ± 0.66	8.32 ± 0.83	1.86 ± 0.39
10	8.08 ± 0.03	10.18 ± 0.66	8.02 ± 0.85	2.16 ± 0.37
30	8.02 ± 0.03	10.18 ± 0.66	7.99 ± 0.99	2.19 ± 0.48

RF: reduction factor. Data are expressed as Log_{10}. Mean and standard deviation of five replicates.

3.4.2.2. PROXEL™

3.4.2.2.a. MS2

PROXEL™ was tested against MS2 at the highest in use concentration of 400 ppm. It did not affect the coliphage concentration, resulting in no reduction at the different contact times tested (Table 3-18). There was no significant difference in reduction over time (ANOVA, $F_{1,16} = 0.59$; $P>0.05$).

During some experiments, it was possible to see some increase in the number of virions compared to the control. These increases could have occurred as an expression of replicate variability or as a consequence of aggregates breaking up by the tween-based neutraliser. The differences were not statistically significant (t-test, $P>0.05$).

Table 3-18. Suspension test results. PROXEL™ at a concentration of 400 ppm against MS2.

PROXEL™		MS2		
Contact time (min)	**pH**	**PFU/ml before treatment ± sd**	**PFU/ml after treatment ± sd**	**Mean RF ± sd**
1	7.81 ± 0.08	9.98 ± 0.90	10.00 ± 0.88	0*
5	7.83 ± 0.07	9.98 ± 0.90	10.13 ± 0.66	0*
10	7.84 ± 0.08	9.98 ± 0.90	10.10 ± 1.08	0*
30	7.84 ± 0.03	9.98 ± 0.90	10.22 ± 0.92	0*

*0 means no reduction

RF: reduction factor. Data are expressed as Log_{10}. Mean and standard deviation of five replicates.

3.4.2.2.b. F116

The isothiazolinone PROXEL™ at 400 ppm was examined against the tailed phage F116. Results showed that the activity of the biocide was low

(Table 3-19). Increased contact time did not improve the efficacy of the compound against F116 (ANOVA, $F_{1,16} = 0.32$; $P>0.05$).

Table 3-19. Suspension test results. PROXEL™ at a concentration of 400 ppm against F116.

PROXEL™		F116		
Contact time (min)	**pH**	**PFU/ml before treatment ± sd**	**PFU/ml after treatment ± sd**	**Mean RF ± sd**
1	7.95 ± 0.45	10.36 ± 0.23	10.36 ± 0.22	0.00 ± 0.19
5	8.07 ± 0.33	10.36 ± 0.23	10.30 ± 0.17	0.06 ± 0.15
10	8.06 ± 0.33	10.36 ± 0.23	10.34 ± 0.28	0.02 ± 0.24
30	8.05 ± 0.36	10.36 ± 0.23	10.25 ± 0.22	0.11 ± 0.16

RF: reduction factor. Data are expressed as Log_{10}. Mean and standard deviation of five replicates.

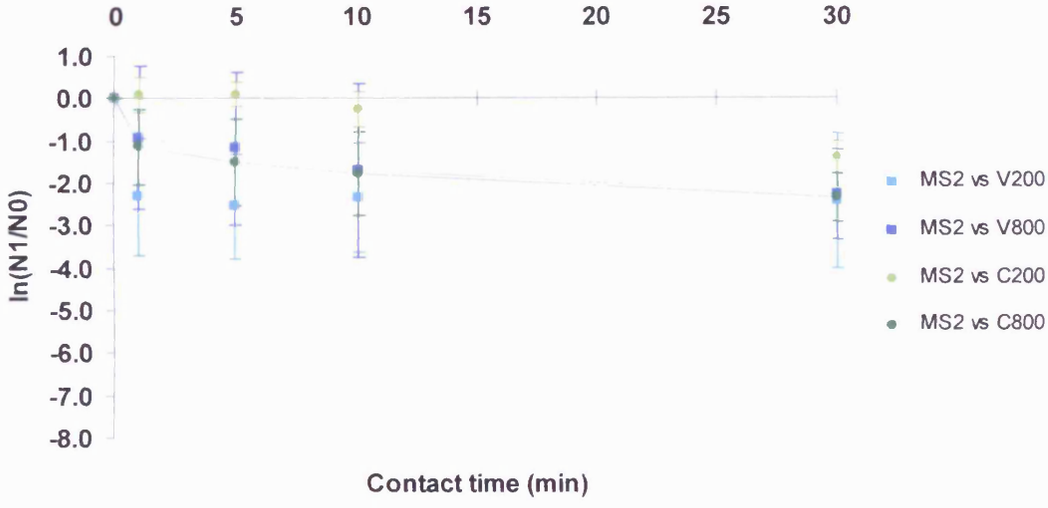
3.4.3. Inactivation kinetics

3.4.3.1. PHMB

3.4.3.1.a. MS2

The disinfection kinetic curve plots the survival ratio (N_1/N_0) against the contact time. The relationship between the inactivation rate and the time of exposure at the two different concentrations was not linear (Table 3-20) except the case of COSMOCIL™ CQ at 200 ppm. Treatment with VANTOCIL™ TG and COSMOCIL™ CQ against MS2 generally produced generally "tailing off" curves, indicating reducing activity as contact time increased (Figure 3-2).

Figure 3-2. Disinfection kinetics of MS2 treated with PHMB biocides at 200 and 800 ppm.



N_1/N_0 is the survival ratio, where N_1 is the PFU/ml after the treatment; and N_0 is PFU/ml before treatment.

V200: VANTOCIL™ TG at 200 ppm; V800: VANTOCIL™ TG at 800 ppm. C200: COSMOCIL™ CQ at 200 ppm; C800: COSMOCIL™ CQ at 800 ppm.

The error bars describe the standard deviation of 5 replicates.

Table 3-20. Linear regression analysis: Correlation coefficients for MS2 inactivation. Treatment with VANTOCIL™ TG and COSMOCIL™ CQ at 200 ppm and 800 ppm.

Phage	Treatment	r	R^2	*P*
MS2	VANTOCIL™ TG 200 ppm	-0.42	0.18	0.48
MS2	VANTOCIL™ TG 800 ppm	-0.86	0.74	0.06
MS2	COSMOCIL™ CQ at 200 ppm	-0.98	0.96	
MS2	COSMOCIL™ CQ at 800 ppm	-0.80	0.65	0.10

3.4.3.1.b. F116

The treatment against tailed phage F116 showed a similar pattern to MS2 (Figure 3-3.). The decrease in the number of phage over time did not follow first-order kinetics ($P>0.05$ all cases; Table 3-21); indeed after an initial decrease in F116 number, the inactivation curves reached a plateau where the biocide action ceased. VANTOCIL™ TG at 200 ppm showed some increase in PFU (N_1/N_0 increasing); this is likely to represent experimental variation.

Figure 3-3. Disinfection kinetics of F116 treated with PHMB biocides at 200 and 800 ppm.

N_1/N_0 is the survival ratio, where N_1 is the PFU/ml after the treatment; and N_0 is PFU/ml before treatment.

V200: VANTOCIL™ TG at 200ppm; V800: VANTOCIL™ TG at 800ppm. C200: COSMOCIL™ CQ at 200 ppm; C800: COSMOCIL™ CQ at 800 ppm.

The error bars describe the standard deviation of 5 replicates.

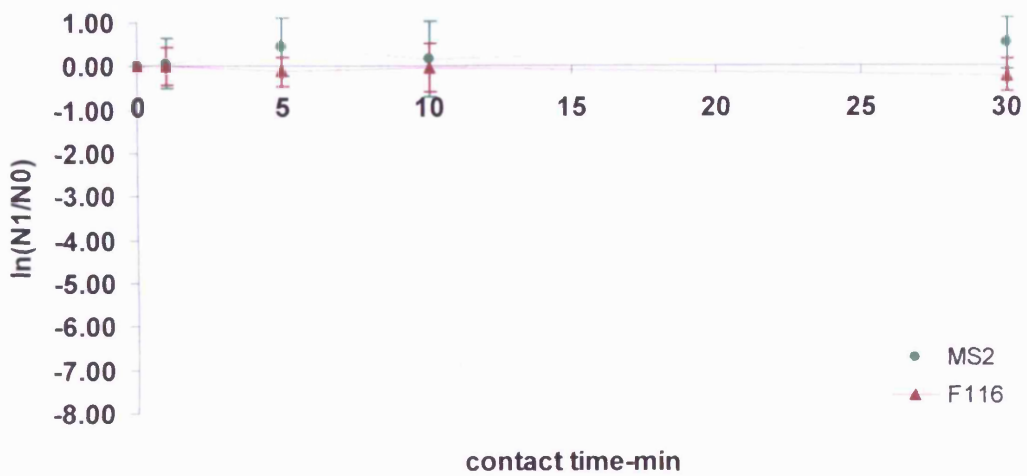
Table 3-21. Linear regression analysis: Correlation coefficients for F116 inactivation kinetic curves. Treatment with VANTOCIL™ TG and COSMOCIL™ CQ at 200ppm and 800ppm.

Phage	Treatment	r	R^2	*P*
F116	VANTOCIL™ TG 200 ppm	0.86	0.74	0.06
F116	VANTOCIL™ TG 800 ppm	-0.52	0.27	0.36
F116	COSMOCIL™ CQ at 200 ppm	-0.71	0.51	0.18
F116	COSMOCIL™ CQ at 800 ppm	-0.56	0.32	0.32

3.4.3.2. PROXEL™

PROXEL™ was found to be inactive against both bacteriophages even at long exposure time (Figure 3-4). No significant linear relation was found.

Figure 3-4 Disinfection kinetics for F116 and MS2 treated with PROXEL™ at 400 ppm.



N_1/N_0 is the survival ratio, where N_1 is the PFU/ml after the treatment; and N_0 is PFU/ml before treatment.

The error bars describe the standard deviation of 5 replicates.

3.5. Discussion

The F-specific coliphage MS2 is used as a model of enteric viral behaviour and as an indicator of faecal pollution in environmental waters (Bichai & Barbeau, 2006; Ogorzaly & Gantzer, 2006; Clevenger *et al.*, 2007), since it is found frequently in sewage and in animal faeces, and similarly to human enteric viruses (noroviruses, polioviruses), it is well adapted to the intestinal tract (Dawson *et al.*, 2005).

MS2 is also used as a model to test biocide efficacy, because of its structural similarity and a comparable resistance profile to enteric and non-enveloped respiratory viruses (Jones *et al.*, 1991; Davies *et al.*, 1993; Koivunen & Heinonen-Tanski, 2005; Sickbert-Bennett *et al.*, 2005; Park *et al.*, 2007). It has been used as a surrogate for clinically-important RNA non-enveloped viruses, such as picornaviridae and calciviridae, to evaluate the efficacy of hand hygiene agents (Sickbert-Bennett *et al.*, 2005).

Studies on the virucidal activity of PHMB-based biocides are very few. There are no reports on COSMOCIL^{TM} CQ. VANTOCIL^{TM} TG tests against some mammalian viruses (foot & mouth disease virus, rotavirus and Polio type3) showed reduction factors less than 4 Log_{10} (P. McGreechan, Arch Chemicals, personal communication), reflecting our suspension test results with MS2. The bis-biguanide Chlorhexidine showed the same efficacy as the PHMBs investigated here. It was able to inactivate 90% of the coliphage titre both in suspension tests (Maillard, 2001) and in carrier tests using glass slides and ceramic tiles (Woolwine & Gerberding, 1995).

The MS2 resistance observed here against PHMB-based biocides is in line with the literature reporting similar reduction in coliphage number (Davies *et al.*, 1993; Woolwine & Gerberding, 1995).

F116 instead was slightly more susceptible to the biocides (99% of reduction after 30 min) than MS2. In previous studies, chlorhexidine was found unable to inactivate F116 (Maillard, 2001), a result confirmed by the lack of damage of the phage proteins (Maillard, 2001) and nucleic acid

(Maillard, 2001). There are no other studies on virucidal activity against F116 by PHMB-based disinfectants or other compounds.
However, other biocides appeared to be more effective in inactivating F116. For instance, treatment with 0.001 % sodium hypochlorite for 30 sec and damaged severely the phage structure, inactivating F116 infectivity (Maillard, 2001).

As MS2 and F116 showed, the general trend demonstrated that the virucidal activity of the two PHMBs is low and that even while increasing the time of exposure the inactivation rate of both phages remained unchanged. The main disinfection pattern observed was the "tailing off" curve, which has been well characterised during water disinfection (Sharp *et al.*, 1975; Jensen *et al.*, 1980). It can be caused by depletion of the active compound in the solution, or by the presence of resistant subpopulations due to conformational aspects or aggregate formation. Since the concentration of both biocides was in excess, aggregation or/and conformational impediments might be the possible reasons for the limited sensitivity. This will be further investigated by electron microscopy and hydrophobicity tests.

In conclusion, the two PHMB-based biocides, VANTOCIL™ TG and COSMOCIL™ CQ, showed low virucidal activity at 20°C with both model viruses, MS2 and F116. The lack of interaction between the PHMB and the viral capsid may be the explanation for low activity. Another possible explanation can be extrapolated from the disinfection curve, which leads to the hypothesis of clump formation. Aggregation could hinder dramatically the virucidal activity of these biguanides limiting contact between viruses and biocides.

4. STRUCTURAL DAMAGE

4.1. Introduction

Biocides can cause structural damage to viruses. Targets will differ according to biocide chemistry. Initial interactions can be based on covalent binding, or on ionic and hydrophobic forces (Lambert, 2004). Since capsid and genome are the major components of non-enveloped viruses (Kissmann *et al.*, 2008), proteins and nucleic acids are then considered potential targets for a biocide.

The capsid is formed by proteins and it is in direct contact with the environment. It is likely that biocides such as hydrogen peroxide, glutaraldehyde and hypochlorite could induce damage, reacting with amino and sulphydryl groups (McDonnell & Russell, 1999). Specifically, aldehydes were found to react irreversibly with the amino groups by alkylation (Lambert, 2004). In fact, aldehyde-based products have been shown to posses virucidal activity against norovirus (Magulski *et al.*, 2009) and poliovirus (Bailly *et al.*, 1991) based on the interactions with the amino group of the lysine residues on the capsid (Chambon *et al.*, 1992; Chambon *et al.*, 2004).

Oxidizing agents (peracetic acid and hydrogen peroxide) belong to another biocide category. They have a wide spectrum of activity among viruses (Sauerbrei *et al.*, 2004a; Lombardi *et al.*, 2008; Martin *et al.*, 2008) and their action involves the oxidation of N-H and S-H groups of the capsid proteins (Lambert, 2004). Similar mechanisms of action were found during ozone disinfection, which caused inactivation of the bacteriophage f2 (Kim *et al.*, 1980), norovirus (Hudson *et al.*, 2007) and enteric virus (Roy *et al.*, 1981) through peroxidation of proteins (Murray *et al.*, 2008).

The genome is the infectious part of the virus and its contact with the biocide can occur only in the presence of a broken head or through capsid permeability (Maillard, 2001). Several biocides appear to damage viral genome (RNA and DNA) (Maillard, 2001; Li *et al.*, 2004), which is not, however, always correlated with complete viral inactivation. Chlorine dioxide, for instance, damaged poliovirus 1 genome, but genome analysis underestimated viral inactivation (Simonet & Gantzer, 2006). Sauerbrei *et*

al. (2004b) showed that, even though the genome of some adenovirus serotypes was destroyed by 2.5% of liposomal povidone-iodine and by 0.5% peracetic acid, the degree of genome degradation was not comparable to the loss of viral infectivity.

Among the diverse techniques employed to assess structural damage, SDS-page and nucleic acid agarose gel electrophoresis have been widely used (Churn *et al.*, 1984; Maillard, 2001; Sauerbrei *et al.*, 2004b; Sauerbrei *et al.*, 2007). In the last decade, in addition to the usual application in morphological studies (Chichon *et al.*, 2009; Liu *et al.*, 2009; Zhang *et al.*, 2009), electron microscopy has been used to understand the mechanisms of action of biocides against viruses by observing the magnitude and type of structural damage (Prince *et al.*, 1993; Maillard *et al.*, 1995; Wutzler & Sauerbrei, 2000).

Only a few studies have specifically involved the biguanide chlorhexidine. For example, SDS-PAGE electrophoresis suggested that no damage occurred to the protein capsid of F116 exposed to the biguanide (Maillard, 2001) and this result was in agreement with the lack of inactivation measured in a suspension test (Maillard, 2001). In addition, studying the effect of several biocides on the F116 genome, Maillard et al (2001) observed that while peracetic acids affected significantly the viral DNA, chlorhexidine did not.

Therefore, there is a need to further explore the interactions between PHMB-based biguanides and biological macromolecules. PROXEL™ was not used anymore in the next investigations since the lack of activity observed in the suspension tests (Chapter 3).

4.2. Aims

This chapter focuses on identifying the damage caused to phage structure following treatment with the two PHMB biocides, VANTOCIL™ TG and COSMOCIL™ CQ.

The aims are to:

- Examine the nature of damage by electron microscopy;

- Study the possible alterations to phage proteins through sodium dodecyl sulphate polyacrylamide gel electrophoresis;
- Investigate the interaction between viral genome (DNA) and PHMB.

4.3. Materials and methods

4.3.1. Transmission electron microscopy

Transmission electron microscopy was used in an attempt to visualize any structural damage caused by the biocides.

One ml of concentrated phage stock (F116 and MS2 at 10^{11} PFU ml^{-1}) [see Chapter 2] was treated with 1 ml of biocide at 200 ppm and 800 ppm. After 10 min exposure, 20 µl of sample was mixed with 30 µl of 2% methylamine tungstate for 10 min. A drop of the negatively stained phage was placed on a nickel- or copper-coated grid and left drying for 20 min. The excess liquid was removed with filter paper. The grid was examined in a Philips EM 400T electron microscope operating at 80 kV accelerating voltage at a magnification of x28000-44000. The control consisted of MS2 or F116 treated with distilled deionised water instead of PHMBs.

4.3.2. Transmission electron microscopy – purified MS2

Since purified MS2 was used during some assessments, comparisons of structural damage between purified and un-purified suspension were undertaken. Smaller volumes were used. The purified MS2 (0.1 ml) was mixed with 0.1 ml of VANTOCIL™ TG and COSMOCIL™ CQ to achieve a final concentration of 200 and 800 ppm. The control consisted of using distilled water instead of the biocide. The protocol followed that described in section 4.3.1.

4.3.3. Protein analysis

4.3.3.1. Lowry method – protein quantification

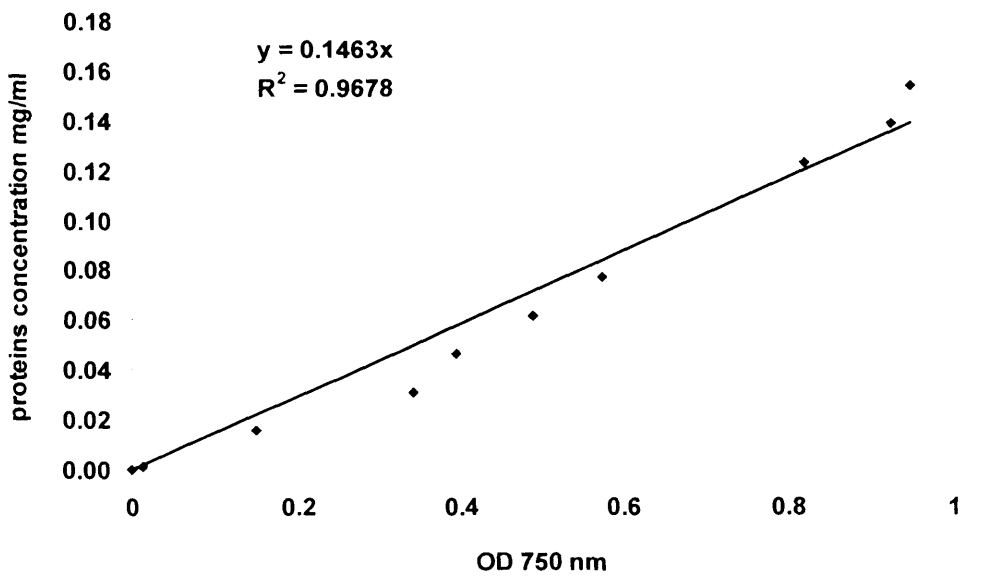
The Lowry procedure is a widely-used protein assay, first described by Lowry *et al.* (1951). Here, it was used to measure the quantity of phage proteins prior to protein electrophoresis.

The reagents used were: reagent A (2% w/v Na_2CO_3, 0.1 M NaOH); reagent B (0.5 w/v $CuSO_4 \cdot 5H_2O$; 1% w/v Na_2Tartrate$\cdot 2H_2O$); reagent C (50 ml of A

and 1 ml of B); and 2N Folin-Ciocalteu reagent (50% w/v 2N Folin-Ciocalteu reagent) (Sigma, St. Louis, MO, USA).

After treatment with biocides [see 4.3.3.2.a], 200 µl of the mixture was mixed with 1 ml of reagent C and allowed to stand at room temperature in the dark for 20 min. 0.1 ml of Folin-Ciocalteu reagent was added and left to react for 30 min at room temperature in the dark. The optical density (O.D.) was then measured at 750 nm against a blank, which consisted of water and reagent C and Folin mixed following the same protocol used for the protein samples. The amount of protein was calculated using a standard curve made from bovine serum albumin (BSA) (Acros Organics, Geel, Belgium) as follows: a stock solution of BSA of 1 mg/ml was prepared. After mixing the Lowry reagents with different dilutions of the BSA stock, the OD_{750} was measured. A curve plotting protein concentration against the OD_{750} (calibration curve) was constructed (Figure 4-1).

Figure 4-1 BSA calibration curve.



4.3.3.2. PhastSystem – SDS-PAGE

The PhastSystem (Amersham Pharmacia Biotech AB, Buckinghamshire, UK) is a quick and highly reproducible technique to perform SDS-PAGE

electrophoresis. It works with small pre-cast gels and it is able to detect small amounts of peptides and proteins (Scherberich *et al.*, 1989). Prospectively this system is appropriate to analyse protein yields from small particles such as bacteriophages and mammalian viruses.

4.3.3.2.a. Sample preparation – pilot experiment

Concentrated stock of F116 and MS2 [see Chapter 2] were used to perform the SDS-PAGE. A volume of the phage suspension (0.375 ml) was mixed with 0.125 ml of PHMB or PROXEL^{TM} to get a total volume of 0.5 ml. The PHMB concentrations tested were 200 and 800 ppm. Proxel at 400 ppm was used as a comparative biocide. The control was treated with deionised water instead of biocide.

After 5 min contact time, 0.2 ml was withdrawn to perform the Lowry titration and 0.3 ml was used for the following experiment. Cold acetone (-20°C) (1.2 ml) was added to the 0.3 ml of sample and then incubated for 5 hours at -20°C to precipitate the proteins. After centrifugation at 19000 x*g* for 15 min at 4°C, the pellet was air dried keeping the tubes open in the laminar flow cabinet for 10-15 min. The pellet was re-suspended in 20 µl of sample buffer (0.125M Tris-HCl, pH 6.8, 20% v/v glycerol, 4% w/v SDS, 10% v/v 2-mercaptoethanol, 0.005% w/v bromophenol blue) (Laemmli, 1970), and boiled for 5 min. In order to precipitate any insoluble materials, a quick centrifugation (10 sec) was performed before loading 10 µl of sample on the SDS-Polyacrylamide 20% homogeneous gel. Low range molecular weight marker (M.W. 6500-66000) (Sigma, St. Louis, MO, USA) was used, 5 µl of the marker was directly loaded onto the gel.

4.3.3.2.b. Sample preparation – refined experiment

In order to clarify the protein band profile on the gel [see Results], the purified MS2 suspension was used instead of the concentrated suspension [see Chapter 2]. PROXEL^{TM} was not tested further, because of the established lack of activity [Chapter 3] and damage [Section 4.4.3.2.b] to MS2. The protocol followed was according to section 4.3.3.2.a.

4.3.3.2.c. Controls

Controls consisted of the reacting mixture without the phage using the same protocol as for the phage suspension preparation [see Chapter 2]. Specifically, plates with 65% agar were inoculated only with the bacteria and incubated for 24 h. The upper layer was removed and centrifuged for 15 min at 11000 x*g* at 4°C (Rotor JA25.50, Avanti J-25, Beckman Coulter, Fullerton, USA). After filtering the supernatant through 0.22 μm membrane filter, samples was prepared for the SDS-PAGE electrophoresis [see 4.3.3.2.a]. Only *E. coli* was used and only the two PHMBs were tested as biocides.

4.3.3.2.d. Gel preparation

SDS-Polyacrylamide 20% homogeneous gel (GE Healthcare Bio-Sciences AB, Uppsale, Sweden) was used, following the Separation Technique File No. 110 (Anonymous, 1986a). Silver staining according to Development Technique File No. 210 was used (Anonymous, 1986b).

4.3.4. Genome analysis

One of the possible biocide targets is the genome of the virus. Two different treatments were performed to assess the effect of PHMB on phage DNA. The analysis was performed only with F116. Firstly entire phage particles were treated in a suspension test and then the phage DNA was extracted and the damage analysed, providing information on the ability of PHMB to penetrate the capsid.

Secondly, extracted phage DNA was directly treated with the biocides in order to assess the potential interaction between PHMB and phage nucleic acid.

4.3.4.1. Treatment of F116 with PHMBs

4.3.4.1.a. Phage treatment

Two ml of phage suspension (10^{10} PFU/ml of F116) was mixed with 2 ml of PHMB. Distilled water was used as a control. After 5 min of contact time the mixture was centrifuged at 19000 x*g* (J4-14 rotor, Beckman, Fullerton, USA) for 20 min at 15°C. The pellet was re-suspended with 1 ml of ultra pure water. The sample was centrifuged again for 20 min at 13000 x*g* (MiniSpin, Eppendorf, Hamburg, Germany), and the pellet re-suspended with 200 µl of PBS buffer.

The DNA was extracted using QIAamp DNA Mini Kit (QIAGEN, West Sussex, UK) following the manufacture's protocol for suspension samples.

The DNA concentration was estimated by measuring the absorbance at 260 nm (A_{260}), which is the peak of light adsorption for nucleic acids (Biophotometer, Eppendorf, Hamburg, Germany). Since DNA is associated with protein, the purity of the extracted DNA is measured from the ratio of A_{260} and A_{280}, where A_{280} is the absorbance at 280 nm where the protein peak light adsorption occurred. An A_{260}/A_{280} ratio between 1.7 and 2.0 represents a high-quality DNA sample.

4.3.4.1.b. Restriction endonuclease and DNA digestion

Restriction endonucleases are enzymes able to cleave DNA at specific sites on each of the two sugar-phosphate backbones of the double helix. Different endonucleases yield different sets of cuts, but one endonuclease will always cut a particular base sequence the same way, no matter what DNA molecule it is acting on (Alberts *et al.*, 2002).

The use of restriction endonucleases should generate nucleotide segments, increasing the possibility to detect alterations on the F116 genome caused by PHMB. The analysis was performed to investigate possible general damage to the genome; it was not focused on any specific sequence.

Two different endonucleases were used, Apa I (Sigma, St. Louis, MO, USA) and Hind III (Sigma, St. Louis, MO, USA). The tool NEBcutters V2.0 (Vincze *et al.*, 2003) was used to find the restriction enzymes' cut

positions on the F116 genome. The complete F116 genome sequence was taken from the NCBI GenBank database (NCBI, 2009; accession number NC_006552). Table 4-1 summarises the characteristics of the two enzymes.

Table 4-1. Summary of restriction endonuclease characteristics and cut positions on F116 DNA.

Restriction endonuclease	Recognition sequences	Cut position	Cutters
Apa I	5'... G G G C C▾C ... 3' 3'... C▴C C G G G ... 5'	7328/7324 46535/46531	2 cutters
Hind II	5'... A▾A G C T T ... 3' 3'... T T C G A▴A ... 5'	19901/19905 32323/32327 62757/62761	3 cutters

Restriction endonuclease cleavage is accomplished by incubating the enzyme with the DNA under appropriate reaction conditions. Hence, 20 µl of extracted DNA (from 0.6 to 1.12 µg/ml) was mixed with 2 µl of the ten-fold concentrated restriction buffer (Sigma, St. Louis, MO, USA). The restriction endonuclease was added at a concentration of 10 Unit/µg DNA. The mixture was then incubated for 1h at 37°C or 30°C with HindIII and Apa I respectively to allow the cleavage reaction to proceed. The reaction was halted by incubating the sample at 65°C for 10 min.

4.3.4.1.c. Electrophoresis

Gels for electrophoresis were prepared using agarose for routine use (Sigma, St. Louis, MO, USA). The 1% w/v agarose was prepared in Tris-borate-EDTA buffer with 0.033% v/v of ethidium bromide. The gel tray was left to set and refrigerated until needed.

Ten µl of DNA (from 0.6 to 1.12 µg/ml) extracted from each samples (which consisted of the phage treated with VANTOCIL™ TG and COSMOCIL™ CQ at 800 ppm and the control) were mixed with 5 µl of running buffer (Sigma, St. Louis, MO, USA). Fifteen µl of samples and 10

µl of DNA ladder (Sigma, St. Louis, MO, USA) were added to the wells of the 1% agarose gel and the electrophoresis was performed at 80 V for 1 h using a PowerPac™ Basic power supply (Bio-Rad Laboratories Ltd, Hempstead, UK). The gel was then viewed under UV light using Bio-Rad Gel Doc 1000 and Bio-Rad Molecular Analyst software (Bio-Rad Laboratories Ltd, Hempstead, UK).

4.3.4.2. Treatment of F116 DNA with PHMBs

Twenty µl of extracted DNA (from 0.4 to 0.9 µg/ml) was mixed directly with 20 µl of biocides to achieve a PHMB final concentration of 800 ppm. After 5 min the biocide activity was quenched adding 20 µl of neutraliser (see Chapter 2, section 2.1.2). 140 µl of PBS buffer was added to give a final volume of 200 µl as suggested by the manufacturer of the extraction kit. The nucleic acid was extracted again using the QIAamp DNA Mini Kit (QIAGEN West Sussex, UK) and prepared for agarose electrophoresis [see 4.3.4.1.c].

4.3.5. Statistical analysis

Electron microscopy data were collected during the observation of 30 different fields/micrograph. Results were analysed by non parametric tests, $\chi 2$ test using Statistica 7 software (StatSoft, Tulsa, USA).

SDS-page was repeated three times, whereas DNA analysis was performed twice.

4.4. Results

4.4.1. Transmission electron microscopy

Electron microscopy was used to qualitatively measure damage caused to the phage following biocide treatment; electron microscopy observations cannot be linked to inactivation data (Maillard *et al.*, 1995).

In order to categorise the phage damage, representative samples of 150-200 phages were randomly observed. Each observation was then placed in one of several different categories according to the structural damage. The categories for F116 were: intact phage (Figure 4-2A); head (Figure 4-3A); empty head (Figure 4-2D); tail (Figure 4-2B); collapsed head (Figure 4-2C); broken head (Figure 4-2B); broken head with tail (Figure 4-2B). MS2 observation was separated into just two groups: intact phage (Figure 4-4A) and broken capsid (Figure 4-5B). Only 10 min exposure time was considered in the investigation, since contact time did not previously appear to affect significantly the efficacy of the biocides [see Chapter 2].

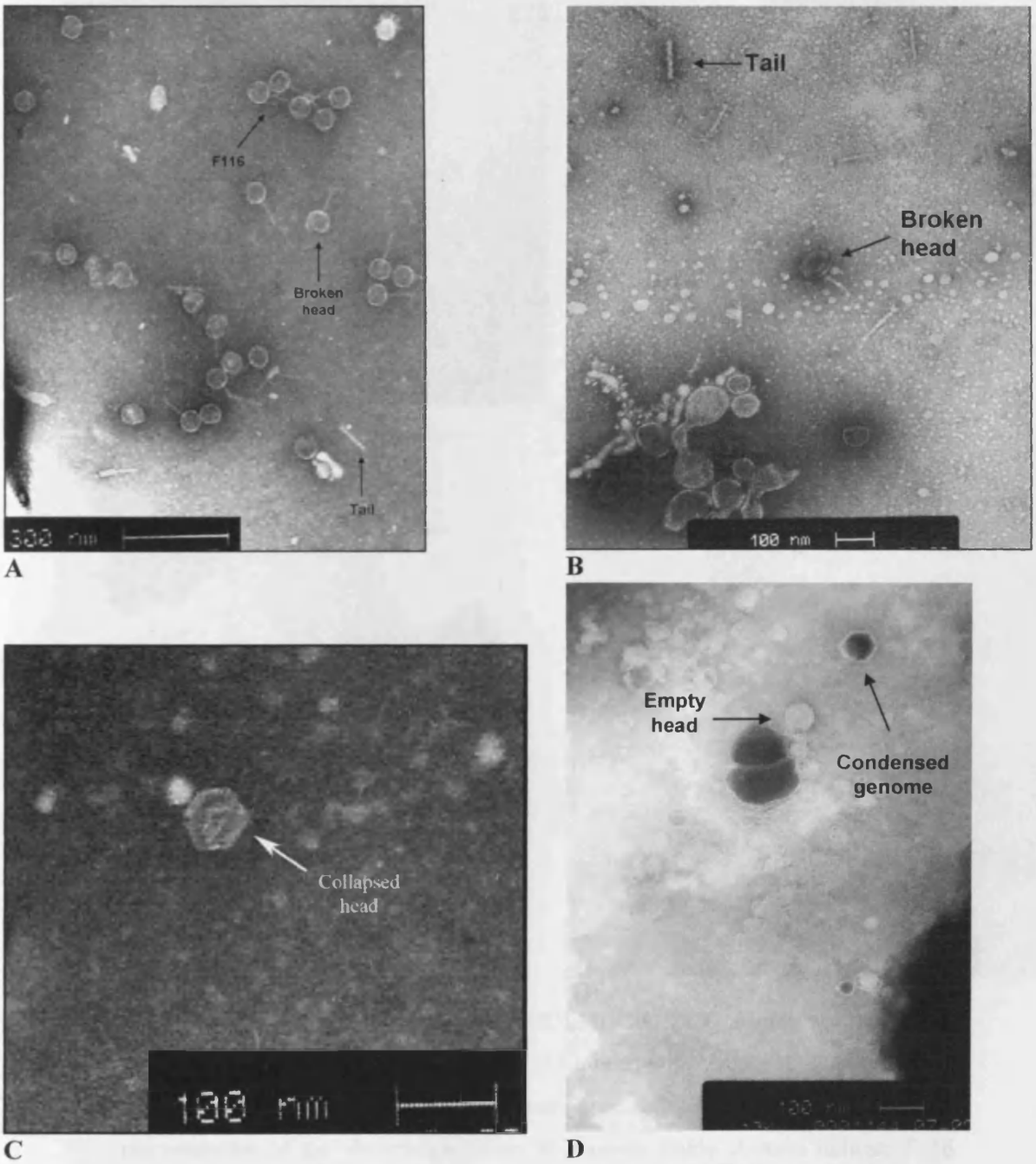
The untreated sample of F116 comprised of mainly intact phages, 55.1%, and 20% of full heads with no tail. The presence of heads without tails in the control might be caused by mechanical stresses during filtration of the phage suspension to eliminate any debris and bacterial cells. Significant damage to the capsid after PHMB treatment was visible in the micrographs (χ^2 test, $P \leq 0.01$) (Figure 4-2, Figure 4-3). The treated samples contained mostly empty heads, 37% and 49%, and a smaller amount of broken heads, 12% and 13%, for VANTOCIL^{TM} TG and COSMOCIL^{TM} CQ, respectively (Table 4-2).

Similar results to F116 were obtained with the coliphage. 99.2% of the control was the intact phage, whereas the amount of broken capsid increased remarkably (χ^2 test, $P \leq 0.01$) to 58.9% and 28% after treatment with VANTOCIL^{TM} TG and COSMOCIL^{TM} CQ, respectively (Table 4-3).

In addition to capsid damage, aggregates were visible in the treated phage micrographs (Figure 4-3, Figure 4-5). This occurred mostly after treatment at 800 ppm and it was more noticeable with MS2 (Figure 4-5); aggregates of

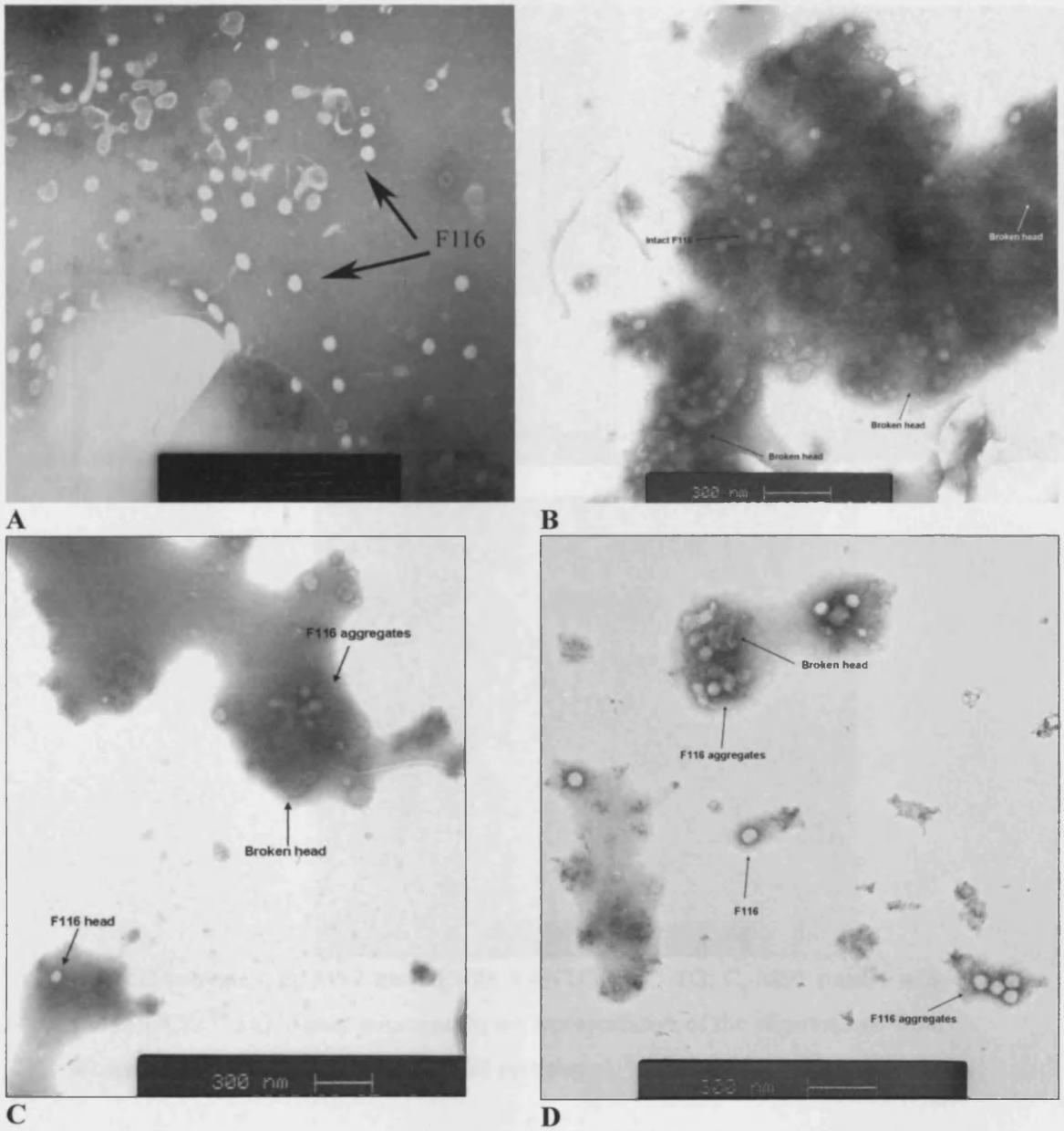
several viral particles, intact and damaged, were observed in 30 separate fields.

Figure 4-2. Micrographs of F116 treated with PHMBs at 200 ppm.



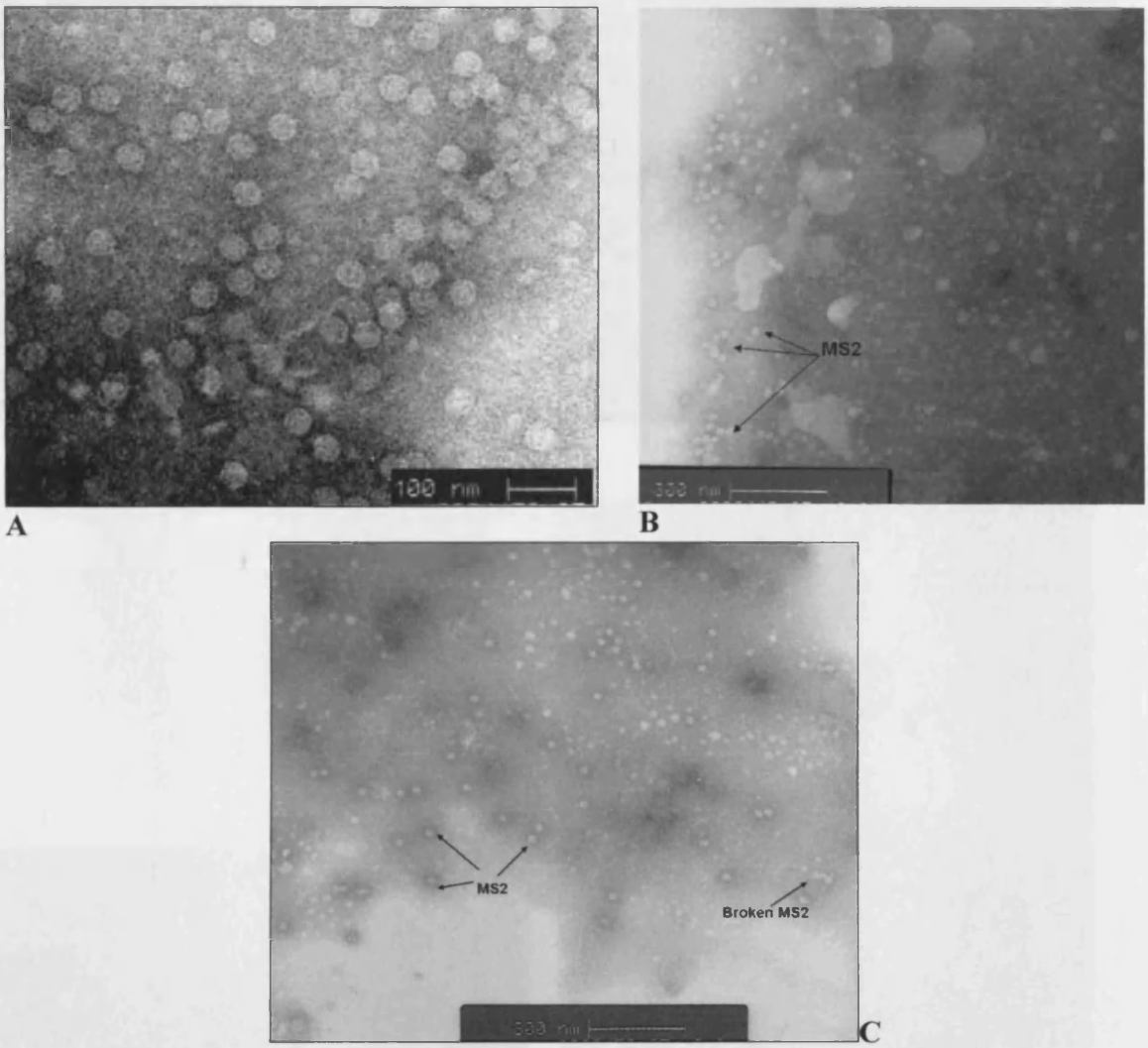
A, F116 untreated; B and C, F116 treated with VANTOCIL™ TG; D, F116 treated with COSMOCIL™ CQ. These micrographs are representative of the observations from 30 separate fields. Arrows indicate F116 particles.

Figure 4-3. Micrographs of F116 treated with PHMB at 800 ppm.



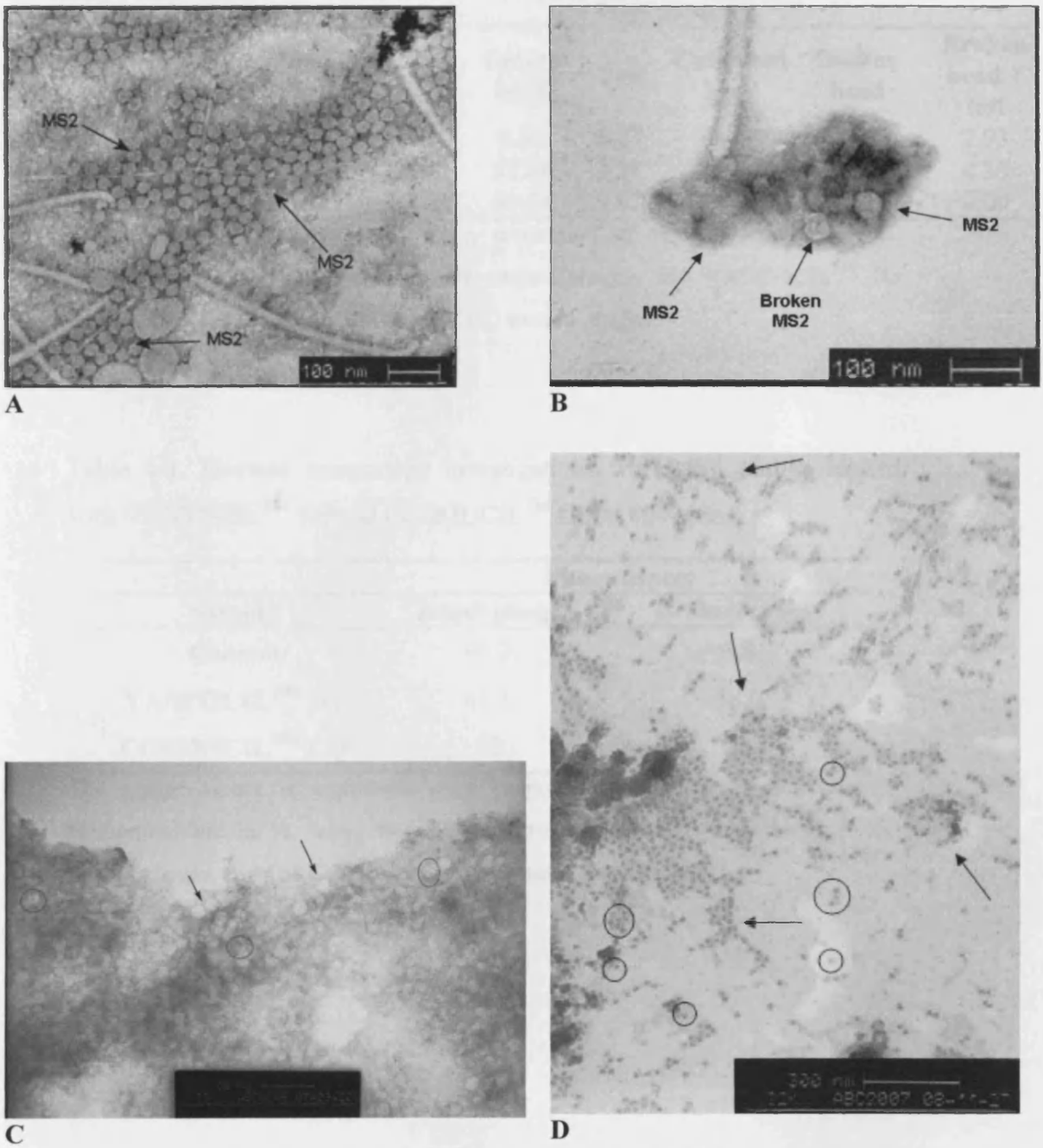
A, F116 untreated; B, F116 treated with COSMOCIL™ CQ, aggregates are present with intact and broken heads; C and D, F116 treated with VANTOCIL™ TG, small aggregates with intact and broken heads are visible. These micrographs are representative of the observations from 30 separate fields. Arrows indicate F116 particles.

Figure 4-4 Micrographs of MS2 treated with PHMB at 200 ppm.



A, MS2 untreated; B, MS2 treated with VANTOCIL™ TG; C, MS2 treated with COSMOCIL™ CQ. These micrographs are representative of the observations from 30 separate fields. Arrows indicate MS2 particles.

Figure 4-5 Micrographs of MS2 treated with PHMB at 800 ppm.



A, MS2 untreated; B and C, MS2 treated with VANTOCIL™ TG; D, MS2 treated with COSMOCIL™ CQ. Aggregates (arrows) and damaged phage (circles) are shown in pictures C and D. These micrographs are representative of the observations from 30 separate fields.

Table 4-2. Electron microscopy investigations with F116 phages treated with VANTOCIL™ TG and COSMOCIL™ CQ at 800 ppm.

Sample	Phage aspect						
	Intact phage	Head	Empty head	Tail	Collapsed head	Broken head	Broken head + tail
Control	55.12	19.02	6.83	9.27	2.44	4.39	2.93
VANTOCIL™ TG	12.42	24.84	37.89	3.11	4.97	12.42	4.35
COSMOCIL™ CQ	13.25	15.89	49.01	7.95	0.66	13.25	0.00

The phage aspect is expressed as a proportion of the population viewed. Proportions are in %, taken from 205 control phages, 161 VANTOCIL™ TG treated phages, and 151 COSMOCIL™ CQ treated phages.

Table 4-3. Electron microscopy investigations with MS2 phages treated with VANTOCIL™ TG and COSMOCIL™ CQ at 800 ppm.

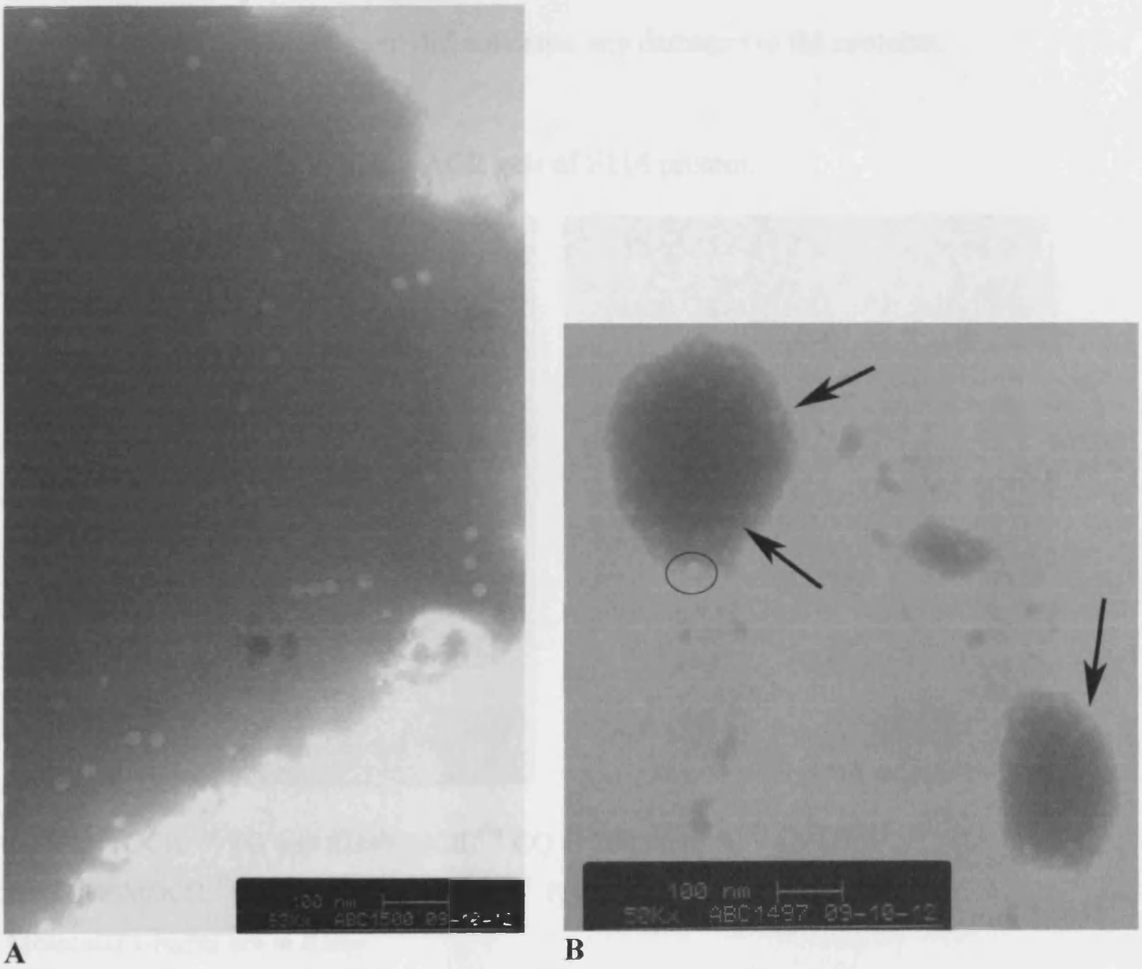
Sample	Phage aspect	
	Intact phage	Broken capsid
Control	99.2	0.8
VANTOCIL™ TG	41.1	58.9
COSMOCIL™ CQ	72	28

The phage aspect is expressed as a proportion of the population viewed. Proportions are in %, taken from 250 control phages, 124 VANTOCIL™ TG treated phages, and 168 COSMOCIL™ CQ treated phages.

4.4.2. Transmission electron microscopy – purified MS2

The untreated sample showed a preparation of the coliphage without any residual bacterial debris (Figure 4-6A). Unfortunately, when the samples were treated with PHMBs, it was impossible to observe clearly any phages in the micrographs. Furthermore, the micrographs were mostly black and it is likely that the cationic biocides reacted strongly with the negative stain. Only one observation of treated phage could be made (VANTOCIL™ TG at 200 ppm) and few damaged phages were visible (Figure 4-6B). The assessment was performed twice, with the same result.

Figure 4-6. Micrographs of purified MS2.



A B

A, un-treated phage; B, MS2 treated with VANTOCIL™ TG at 200 ppm. Arrows show MS2. Circles show broken capsid.

4.4.3. Protein analysis

4.4.3.1. F116

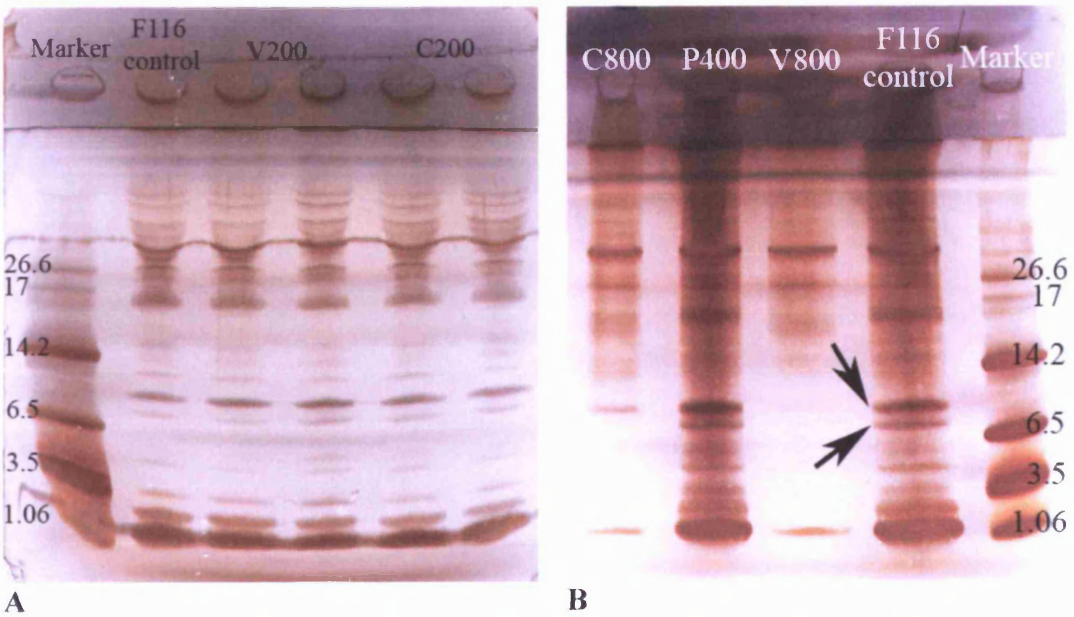
Contrary to MS2 [see section 4.4.3.2], it was not necessary to purify F116. The protein profile revealed approximately 15 bands consistent with other works (Byrne & Kropinski, 2005).

The quantity of protein loaded onto the gel was in the range of 0.4-0.8 mg/ml and the protein pattern was highly reproducible (n=3).

Protein bands from F116 treated with VANTOCIL™ TG and COSMOCIL™ CQ at 200 ppm did not differ from the control (Figure 4-7 A). On increasing the concentration of the PHMB to 800 ppm, the F116 protein profile changed as some protein bands disappeared, particularly P14 and P15 (Byrne & Kropinski, 2005) of respectively 12.5 KDa and 7.5 KDa molecular weight (Figure 4-7 B).

The isothiazolinone at 400 ppm did not cause any damages to the proteins.

Figure 4-7. Silver-stained SDS-PAGE gels of F116 protein.



A **B**

A, VANTOCIL™ TG and COSMOCIL™ CQ at 200 ppm; B, VANTOCIL™ TG and COSMOCIL™ CQ at 800 ppm and PROXEL™ at 400 ppm at 20°C. Molecular weights are in KDa.

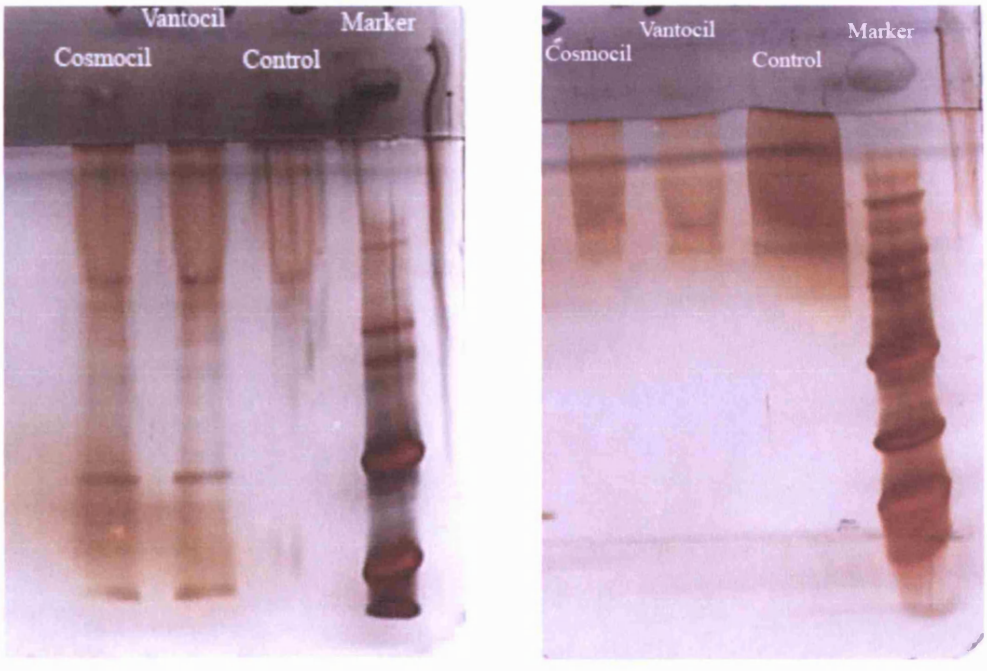
Arrows indicates proteins P14 (12.5KDa) and P15 (7.5 KDa) (Byrne & Kropinski, 2005).

4.4.3.2. MS2

4.4.3.2.a. Controls

Controls consisted of analyzing the mixture without phage inoculation, in order to assess the background bands produced by the presence of bacterial debris. Even though protein patterns were highly inconsistent between the experiments (n=3), bands derived from bacterial residuals were always present (Figure 4-8).

Figure 4-8. SDS-PAGE of mixture without phage. Treatment was performed with VANTOCIL™ TG and COSMOCIL™ CQ at 800ppm.



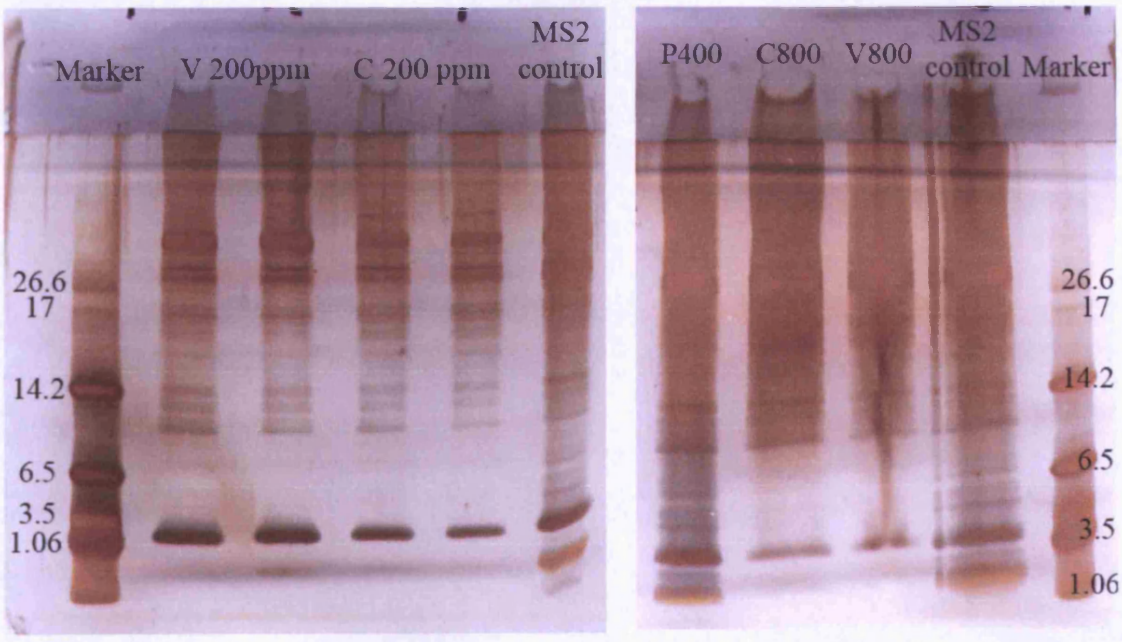
A B

4.4.3.2.b. Pilot experiment

The pilot experiment of SDS-PAGE with the coliphage MS2, revealed the necessity to purify the phage suspension (Figure 4-9). Gels with the un-purified coliphage suspension were overloaded with bands derived from bacterial debris, making difficult detection of any alterations in the two protein bands comprising MS2, the coat protein and A protein (Kuzmanovic

et al., 2006). Moreover, the reproducibility of the proteins profiles was not consistent.

Figure 4-9. Silver-stained SDS-PAGE gels of MS2 phages treated with VANTOCIL™ TG and COSMOCIL™ CQ at 200 and 800ppm and PROXEL™ at 400 ppm at 20°C.



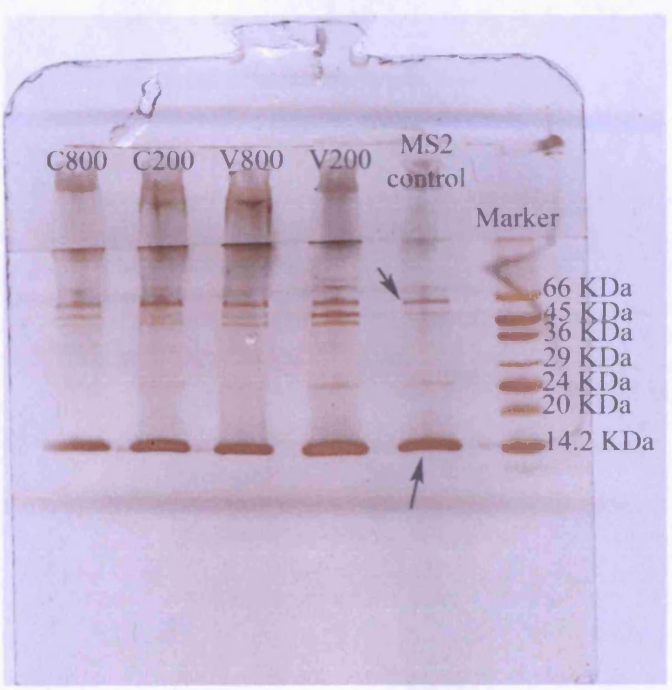
The phage suspension was not purified.

4.4.3.2.c. Refined experiment

Figure 4-10 shows the protein analysis performed on the purified MS2. The protein pattern arising from the untreated phage (control; Figure 4-10) comprised of 3 main protein bands and was highly reproducible between experiments (n=3). The quantity of protein loaded on the gel was between 1.2 and 1.5 mg/ml.

The bands presented in the untreated sample corresponded to the coat protein (13.7 KDa) and A protein (44 KDa). The band at approximately 24 KDa represented the coat protein homodimer (Kuzmanovic *et al.*, 2006). PHMB treatment at 20°C did not alter the phage protein profile.

Figure 4-10. Silver-stained SDS-PAGE gels of MS2 phages treated with VANTOCIL™ TG and COSMOCIL™ CQ at 200 ppm and 800 ppm at 20°C.



The arrows indicate the two proteins which formed MS2, coat protein (13.7 KDa) and A protein (44 KDa).

4.4.4. Genome analysis

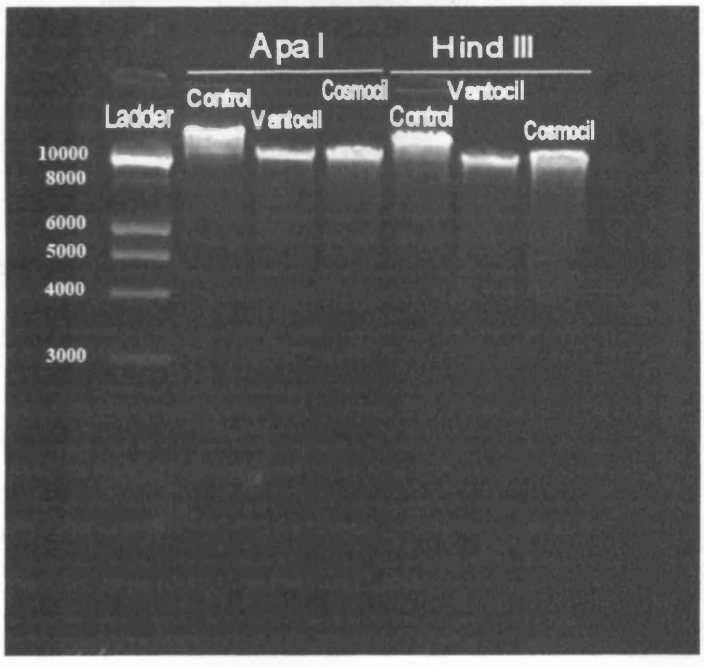
4.4.4.1. Treatment of F116 with PHMBs

The DNA concentration was in the range of 20-70 μg/ml, and a high purity was achieved after the extraction (A_{260}/A_{280} from 1.78 to 1.92). The experiment was performed twice, providing consistent results.

As Figure 4-11 shows, the restriction enzymes did not cut the nucleic acids as expected [see section 4.3.4.1.b]. Indeed the 3 or 4 segments expected following treatment with, respectively, ApaI and HindIII did not arise either for untreated or treated samples.

The treatment with the two PHMBs at 800 ppm altered the DNA profile, producing bands of lower molecular weight on the agarose gel compared with the untreated sample (Figure 4-11).

Figure 4-11. Agarose gel of DNA of F116 using two different endonucleases, ApaI and HindIII.



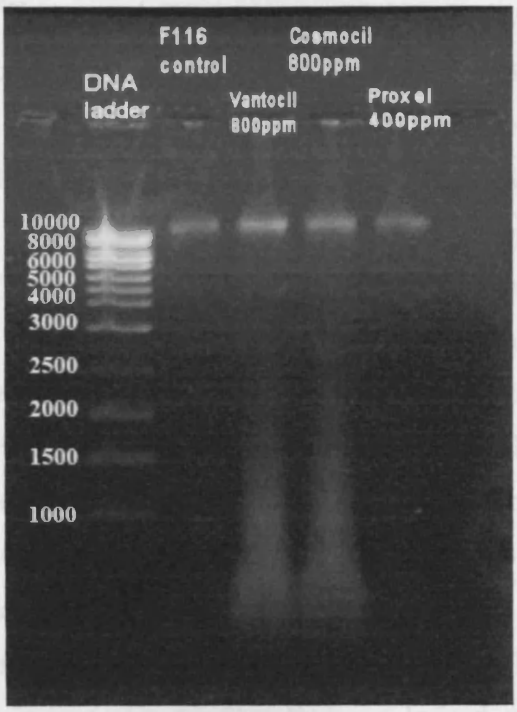
The PHMB treatment at 800 ppm was on the entire phage.

4.4.4.2. Treatment of F116 DNA with PHMBs

The quantity of DNA treated directly with PHMB or water was in the range of 0.12-0.24 µg in 20 µl, with a purity of A_{260}/A_{280}=1.71-1.82. The results were consistent between the experiments (n=2).

PHMB did not show any activity against the nucleic acid. It did not alter the DNA pattern (Figure 4-12).

Figure 4-12. Agarose gel of the DNA of F116.



The treatment was directly on the genome.

4.5. Discussion

This chapter investigated the interactions between the PHMBs and the protein and nucleic acid, macromolecules of the two phages.

The electron microscopy investigation showed a variety of structural damage to F116 (Figure 4-2 and Figure 4-3). The proportion of intact phages (head + tail only) decreased from 51% (control) to 12-13% (treated). The damage, mostly observed after the treatment with VANTOCIL^{TM} TG and COSMOCIL^{TM} CQ at 800 ppm, resulted principally in empty heads. These findings suggested that PHMB might have a precise target on the capsid that leads to the release of the genome. Whether this process was responsible for F116 inactivation is still to be verified. However, a 99% (2 Log_{10}) reduction in infectivity was observed in the suspension test after treatment with 800 ppm [see Chapter 3], which is higher than might be estimated from the structural damage observed under the electron microscope. Such discrepancy could be possibly explained by steric hindrance. In fact, the decreased infectivity could be also caused by an impediment of the phage to interact with the specific receptor on the host membrane due to the attachment of PHMB molecules to the F116 capsid. Moreover, visible structural changes might be a late manifestation of functional failure/damage which affects infectivity. These results are, however, partially in line with previous findings. Indeed, another biguanide-based biocide, chlorhexidine, was found to produce only small amount of damage to the F116 phage (Maillard *et al.*, 1995), but in this case it was consistent with the low reduction factor measured in a suspension test (Maillard, 2001).

The first observation during the DNA analysis was the absence of cleavage by the restriction enzymes (Figure 4-11). This might be explained by high F116 DNA methylation that impeded the action of the enzymes (Kissmann *et al.*, 2008). Secondly, both biocides interacted differently with the DNA according to whether or not the DNA was associated with the capsid. In fact, the migration of the DNA bands on the agarose gel extracted from the treated phage appeared altered, possibly because of a slight conformational

change. Conversely, when the nucleic acid was treated directly with the PHMBs, no conformational alteration was observed (Figure 4-12). Allen *et al.* (Allen *et al.*, 2006) analysed the interaction between PHMB and various nucleic acids (DNA, RNA, double and single strand). Interestingly, they found that the biocide bound cooperatively to the nucleic acids, causing precipitation of the complex PHMB/DNA(RNA) but without damaging the integrity of the macromolecule. These results appeared to conflict with the nucleic acid analysis, because if precipitation had occurred then higher molecular weight bands would be expected on the gel. In contrast, lower molecular weight bands were observed. It is likely that the association with phage proteins inhibited the complex formation and thus the precipitation.
The SDS-PAGE of the F116 phage gave approximately 15 bands (Figure 4-7). The protein profile of the phage treated with the biocides, when compared with the untreated, revealed an altered pattern only when VANTOCIL™ TG and COSMOCIL™ CQ were used at 800 ppm. Only two proteins, P14 and P15, resulted to be affected. P15 has not been associated to a function yet, whereas P14 was identified as one of the three virion structural proteins (Byrne & Kropinski, 2005). These findings might explain the structural damage observed, confirming the mechanism of action as deduced from the electron microscopy investigation. Overall, these observations on F116 suggest that PHMB molecules, after breaking the capsid, enter the viral particle and interact with the DNA. A similar mechanism of action was also found between chlorine and poliovirus (O'Brien & Newman, 1979). In fact, the poliovirus RNA appeared to be sensitive to chlorine degradation only when associated with the capsid, and only then the release occurred.

Regarding the coliphage MS2, the electron microscopy investigations revealed damage to the capsid after the treatment with VANTOCIL™ TG and COSMOCIL™ CQ (50% and 28 % of broken heads) (Figure 4-4 and Figure 4-5). The low amount of damage probably reflects the low virucidal activity seen in the suspension tests [Chapter 3]. Moreover, the SDS-PAGE gels showed a lack of damage to the capsid proteins (Figure 4-10). This investigation appears to contradict what found during the electron

microscopy investigation (presence of broken capsid). However, the reducing SDS-PAGE technique used 2-mercaptoethanol which reduces disulphide bonds, overcoming forms of tertiary protein folding and breaking up quaternary protein structure (Hames, 2002). Therefore, any alterations detected are referred only to the primary sequence and structure. Another result revealed by the electron microscope images was the increased presence of aggregates as the concentration of PHMB increased. Further investigation is needed to assess whether the MS2 clumping reflected a preparation artefact or a consequence of the contact with the biocides. The latter could further explain the low virucidal activity, since aggregation could have hindered any activity (Sharp *et al.*, 1975).

In conclusion, PHMB showed a different interaction with the two phages.
F116 appeared to be affected more by the biocides, which initially provoked capsid breakage allowing the PHMB inside the viral head. Secondly, an interaction with the DNA occurred, producing a possible a conformational alteration. Damage by PHMBs was also visible on MS2 capsid, but possibly modifying only the tertiary or quaternary structure of the capsid proteins and not affecting the primary structure, as observed with F116.
Overall, the PHMB appeared to interact with proteins and DNA. Further studies about the nature of the interactions need to be performed.

5. PHAGE RESISTANCE TO PHMBs

5.1. Introduction

The virucidal activity of biocides can vary depending on the type of virus and the environmental conditions (Maillard, 2001). The presence of organic load, contact time and temperature can alter greatly the interaction between the biocide and the virus (Russell, 2003) [see Chapter 1].

Viruses react differently with different biocides (Chambon *et al.*, 1994; Sauerbrei *et al.*, 2004a; Sauerbrei *et al.*, 2004b). The only attempts to classify viruses based on their susceptibility to antimicrobial compounds were first performed in 1965 (Klein & Deforest, 1965) then modified in 1983 (Klein & Deforest, 1983). They found that the presence of the lipidic envelope was responsible for higher susceptibility; therefore "lipophilic" viruses (e.g. Herpes simplex virus) were considered more sensitive, especially to biocides such as cationic surfactants (e.g. QACs), chlorhexidine, isopropanol and chloroform.

Little is known about penetration of disinfectants into viruses. However, in a study by Maillard *et al.* (1995) about the mechanisms of action of chlorhexidine diacetate and cetylpyridinium chloride against the bacteriophage F116, a positive relationship between the virucidal activity and the degree of binding to the viral surface was observed.

Viruses show various forms of resistance (McDonnel, 2007). Some are acquired mechanisms, such as genetic mutations and multiplicity reactivation. The latter was first described by Luria (1947) and consisted of the exchange of genetic material among viral particles, inactive and active, developing new infective particles. Other forms of resistance associated with intrinsic viral features are related to their surface properties (McDonnel, 2007). pH and salt concentration, for instance, change the charge and therefore the hydrophobicity of the virus (Thurman & Gerba, 1988b). These properties can influence the viral distribution in a hydrophilic environment, inducing clump formation. Aggregation was found responsible for the low virucidal activity of some biocides (Sharp *et al.*, 1975; Jensen *et al.*, 1980); more details on aggregation can be found in Chapter 1.

The adaptation to new environmental conditions, such as prolonged contact with a biocide, has to be considered a further way to acquire resistance. In this context, it was observed that F116 was becoming more resistant to sodium hypochlorite (Maillard, 2001) after several exposures. Another study on acquired resistance showed that poliovirus gradually developed resistance to chlorine inactivation after repeated cycles of sublethal exposure (Bates *et al.*, 1977).

5.1.1. Temperature coefficient

Most disinfectants increase their activity with an increase in temperature. Their dependence on temperature can be expressed by the temperature coefficient per degree rise (Θ) or per 10°C rise (Q_{10}).

The relationship between temperature and biocide activity follows an Arrhenius-type dependency (Lambert, 2003):

$$K_T = K_{20} \times \Theta^{(T-20)}$$

Where K_T is the disinfection rate constant at temperature T;

K_{20} is the disinfection rate constant at 20°C;

Θ is the temperature coefficient per degree.

If the concentration is constant during the reaction, Θ is calculated as:

$$\Theta^{(T_1-T_2)} = t_1/t_2$$

Where t_1 is the extinction time for a given population at temperature T_1

t_2 is the extinction time at temperature T_2.

Therefore the Q_{10} value can be calculated as (Smith, 2004):

$$Q_{10} = [\text{Time to kill at } T_1] / [\text{Time to kill at } (T_1+10)°C]$$

Q_{10} values for disinfectants vary from 1.5 for formaldehyde, between 3 and 5 for phenols, between 30 and 50 for aliphatic alcohols (Bean, 1967), to almost 300 for ethylene glycol monoethyl ether (Smith, 2004).

5.2. Aims

Previous investigations [see Chapter 3] suggested that aggregation was a possible cause of the low virucidal activity of PHMB-based biocides. MS2 was chosen as the model organism to test this hypothesis. This was studied by:

- Comparing the surface properties of MS2 after treatment;
- Assessing the effect of temperature, sonication and surfactants on the efficacy of VANTOCIL™ TG and COSMOCIL™ CQ;
- Measuring the degree of clumping by dynamic light scattering following treatment.

5.3. Materials and methods

5.3.1. Hydrophobicity tests

These tests measure the hydrophobic character of the bacteriophage surface and how the presence of the PHMB might influence that property.

5.3.1.1. Adsorption to lipids

The experiment is based on the original performed on animal viruses by Klein and Deforest (1983) and modified for bacteriophages by Maillard (2001). A 5% w/v cholesterol emulsion was prepared adding 0.01% v/v of Tween 80 to increase the lipid solubility. The suspension was kept at 4°C and used at room temperature during the experiments.

The biocide concentrations tested were 2, 20, 200 and 800 ppm. One ml of MS2 suspension (10^{10} PFU/ml) was mixed with 9 ml of PHMB biocide or deionised water for the control. After 5 min contact time, 1 ml was mixed with 4 ml of the cholesterol emulsion. The mixture was constantly agitated at 150 RPM for 30 min at room temperature and then centrifuged twice for 30 min at 5000 xg at 20°C in order to separate the lipidic phase from the aqueous phase. The resultant phage content in the supernatant was measured by plaque formation. The experiment was repeated three times in duplicate.

The percentage of MS2 partitioned was calculated as: (PFU/ml adsorbed to the cholesterol in the control/ PFU/ml adsorbed to the cholesterol suspension after treatment) x 100.

5.3.1.2. Microbial adhesion to hydrocarbon

The microbial adhesion to hydrocarbon (MATH) method uses hydrocarbons to measure the hydrophobicity (Denyer *et al.*, 1993) of microorganisms. Hexadecane was utilised in this experiment. The biocide concentrations tested were 2 ppm, 20 ppm, 200 ppm, and 800 ppm.

One ml of MS2 suspension (10^{10} PFU/ml) was mixed with 9 ml of PHMB biocide or deionised water for the control for 5 min. Then 1 ml of mixture was mixed with 0.2 ml of hexadecane and held at room temperature for 10 min. After 60 sec of vortexing, the two phases were allowed to separate at room temperature for further 10 min. The amount of phage in the aqueous phase was measured. The experiment was repeated three times in duplicate. The percentage of MS2 partitioned was calculated as: (PFU/ml adsorbed to the hexadecane in the control / PFU/ml adsorbed to the hexadecane after the biocide treatment) x 100.

5.3.2. Dynamic light scattering

Dynamic light scattering (N4plus, Beckman Coulter, Fullerton, USA) was used to measure the size of the aggregates of phage before and after PHMB treatment.

Purified virus suspension [see Chapter 2, section 2.5] was dialysed overnight against 0.25M KCl and then filtered through 0.22 μm filter. Four different concentrations were tested, 2, 20, 200 and 800ppm at three different temperatures; 10°C, 20°C and 40°C.

Particle size measurement occurred after 5 minutes of phage/biocide contact time, and ran for 200 sec and repeated in triplicate using a laser angle of 90°. The mean of the 3 measurements was used to categorise the size of aggregates. Different size categories were chosen to simplify the reading of results. The threshold of the smaller category size was chosen according to the theory of "the 16 particles aggregate". This theory established that an aggregate formed of 16 particles of identical diameter is needed to capture one particle inside the aggregate, giving complete protection from the medium around (Thurman & Gerba, 1988a). In the case of MS2 (diameter 24 nm) the threshold calculated was 60 nm, with the assumption that aggregates are globular.

5.3.3. Sonication treatment

In order to further investigate the existence of aggregation and its influence on the efficacy of PHMB, sonication was used with the intention of breaking up aggregates. Sonication consisted of treating phages with ultrasound waves at a specific frequency and amplitude, which are not lethal to the virus. The waves separate the aggregates into single particles (Burleson *et al.*, 1975).

Sonication treatment was added to the standard suspension test [see Chapter 3, section 3.3.3] and lasted for the duration of the contact time. It was performed in a Soniprep 150 sonicator (MSE, London, UK) at 23 KHz and at a wave amplitude of 10 μm. In addition, sonication was performed at two different temperatures; 10°C where the test vessel was kept in a water bath, and at room temperature. However in this case the temperature increased in the vessel to 30°C between 1 and 5 min and to 40°C between 10 and 30 min. Control tests were performed to check phage survival under such conditions without the presence of biocide. The pH and temperature were measured after the experiments.

5.3.4. Effect of temperature (Q_{10})

Suspension tests were performed at different temperatures, from 10°C to 50°C with increments of 10°C. The temperature was kept constant using a water bath, and the solutions were equilibrated at the precise temperature before starting the experiment. The suspension test was performed as described in Chapter 3 Section 3.3.3. pH and temperature were recorded. Controls were performed to check phage survival after exposure to the different temperatures. The experiments were repeated three times.

5.3.5. Formulations

Three formulations comprising VANTOCIL™ TG (800 ppm) and a range of surfactants at different concentrations were tested in a standard

suspension test [see Chapter 3, section 3.3.3]. These formulations, labelled F1, F2, F3 (Arch Chemicals, Manchester, UK) are described in Table 5-1.

Table 5-1 Characteristics of the three VANTOCIL™ TG-based formulations.

Formulation code	Composition
F1 (pH 4.20)	DI water 67.37% VANTOCIL™ TG 25% Armeen 2MCD 5%* 32% HCl 2.63%
F2 (pH 5.70)	DI water 70% VANTOCIL™ TG 25% Neodol 23-6.5 5%*
F3 (pH 5.70)	DI water 70% VANTOCIL™ TG 25% Synperonic 91/6 5%*

*Armeen 2MCD: Cocodimethylamine; Neodol 23-6.5: alcohol ethoxylate; Synperonic 91/6: Polyoxyethylene (6) C9-C11 alcohol

5.3.6. Suspension test – Purified MS2

Since the purified MS2 has been used in several assessments, a suspension test using this phage preparation was performed. The two PHMB biocides were tested only at 800 ppm at the highest time of exposure, 30 min. The protocol was as described in Chapter 2.

5.3.7. Protein analysis

SDS-PAGE was performed as described in Chapter 4 section 4.3.2. Treatment with PHMB was performed at 40°C.

5.3.7.1. Eosin Y staining

In addition to the normal SDS-page, attachment of the biocides to phage proteins was measured qualitatively with a colourimetric assay based on the property of PHMB to change the pK of certain indicators, such as Eosin Y (Gilbert & Moore, 2005). The protein separation was run in duplicate gels. One gel was silver stained, whereas the other was dyed with Eosin Y. The gel was first fixed in 7% v/v acetic acid for 10 min and then for another 20 min in 50% v/v methanol. A washing step was performed for 20 min. The gel was finally soaked in 10% w/v sodium acetate (100 ml), 0.024% w/v Eosin Y (250 ml) and water (150 ml) (Gilbert & Moore, 2005), until pink bands were visible (1 to 3 h). A last step of washing was performed to wash away all the dye not attached to the biocide.

Controls, which consisted of PHMB mixed with deionised water (final concentration 200 ppm and 800 ppm) and processed through the cold acetone precipitation, were performed to assess the presence of the biocide in solution after the SDS-PAGE protocol (negative control). No pink bands resulted, indicating that the free biocide in the medium (not attached to the virus) was washed out during the procedure.

5.3.8. Statistical analysis

The phage concentration measured in the controls was used to calculate the Log_{10} reduction after treatment. Each suspension test was repeated three times in duplicate. Statistical analysis was performed using Statistica 7.0 software (StatSoft Inc., Tulsa, USA). Any differences among results were analyzed by the parametric one-way ANOVA test. Where analysis showed significant differences a post-hoc Tukey test was used to underline which couple of variables produced the difference. Light scattering results were analyzed by the non parametric tests, $\chi 2$ test and Kruskal-Wallis ANOVA.

A Pearson correlation coefficient was calculated for the inactivation kinetic curves at each treatment (sonication and temperature).

The Q_{10} coefficient was calculated for each 10°C increment. A fitted line was drawn for each treatment temperature and the equation was used to

extrapolate the time of exposure required to achieve 4 Log_{10} reduction. The estimated contact time was then used to calculate the Q_{10} coefficient, as: time to kill at T_2 / time to kill at T_1.

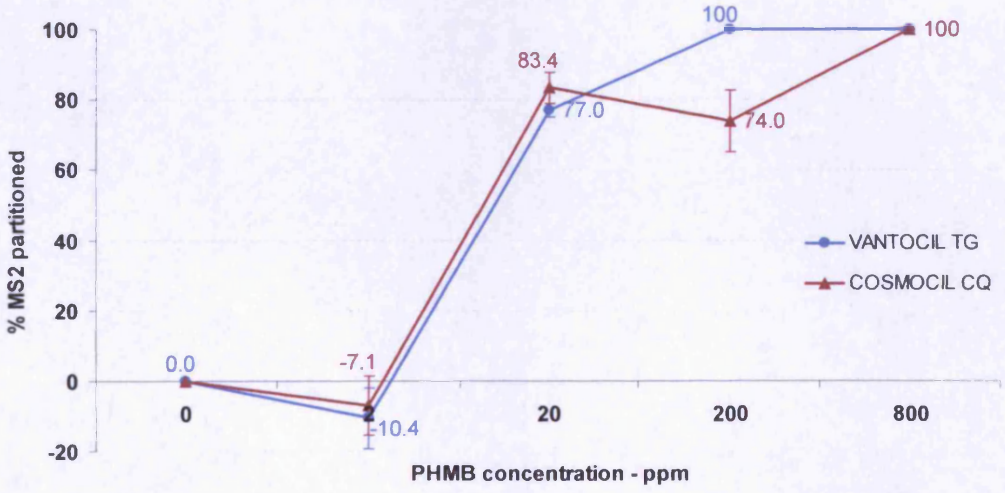
5.4. Results

5.4.1. Hydrophobicity tests

5.4.1.1. Cholesterol test

The treatment with PHMBs altered the hydrophobic properties of the coliphage. The MS2 partitioned in the lipidic (cholesterol) phase increasing significantly with concentration (Cholesterol test: VANTOCIL™ TG ANOVA, $F_{1,8} = 7.4$, $P \leq 0.05$; COSMOCIL™ CQ, ANOVA, $F_{1,8} = 162.1$, $P \leq 0.05$). At 800 ppm the phage was found entirely (100%) in the hydrophobic phase (Figure 5-2). The negative results observed with lower concentrations of PHMB, might be explained by the addition of Tween 80 in the cholesterol suspension. Tween 80 is a surfactant which might help disaggregation of clumps to produce a higher count.

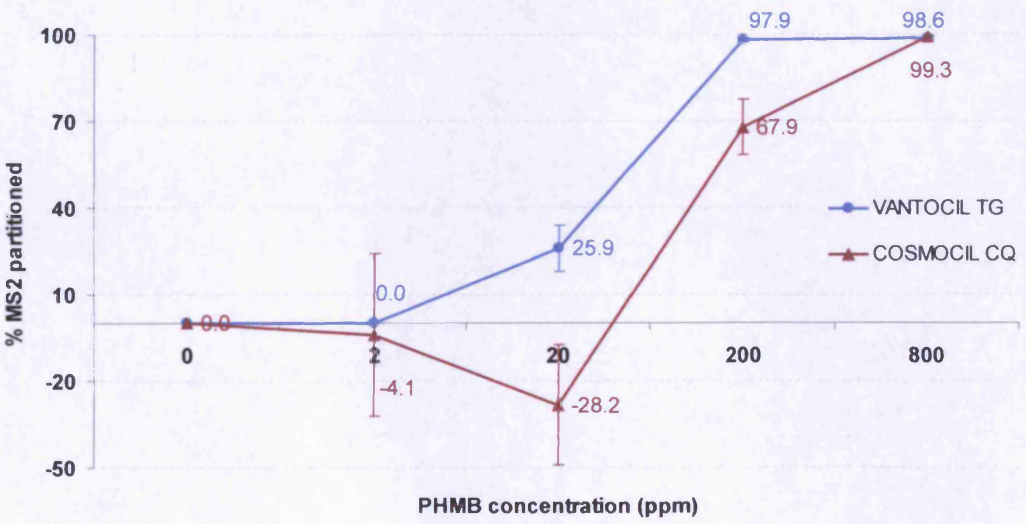
Figure 5-1. Cholesterol test of VANTOCIL™ TG and COSMOCIL™ CQ against MS2. MS2 partitioning in the lipidic phase (cholesterol). Bars are standard deviation.



5.4.1.2. MATH

The MATH confirmed the results achieved with the cholesterol test. The number of phages recovered in the aqueous phase in the control was taken into account when the effect of the biguanides on hydrophobicity was calculated. This number was considered as the number of phage prior to treatment. VANTOCIL™ TG and COSMOCIL™ CQ clearly modified the surface properties of the phage. The hydrophobicity increased significantly with increasing PHMB concentration: all of the phage particles were partitioned with the hydrocarbon (hexadecane) phase after treatment with both PHMBs at 800 ppm (MATH test: VANTOCIL™ TG, ANOVA, $F_{1,8}$ = 146.4, $P \leq 0.05$; COSMOCIL™ CQ, ANOVA, $F_{1,8}$ = 32.6, $P \leq 0.05$) (Figure 5-2).

Figure 5-2 MATH tests of VANTOCIL™ TG and COSMOCIL™ CQ against MS2. MS2 partitioning in the hydrocarbon phase. Bars are standard deviation.



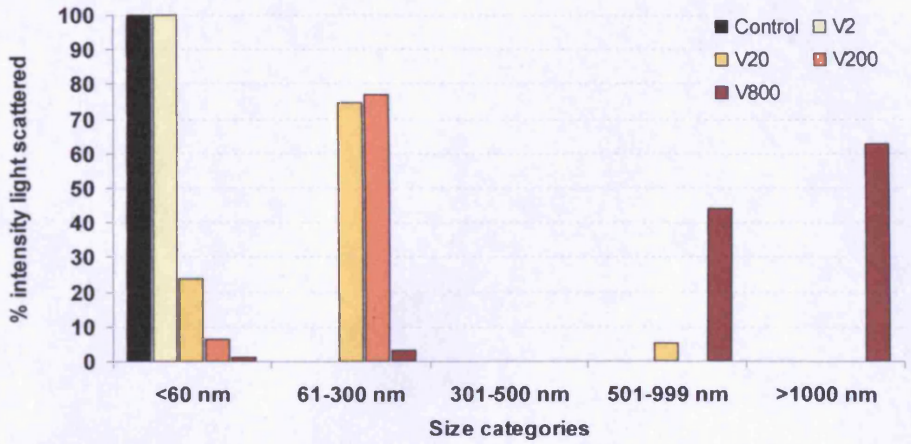
5.4.2. Dynamic light scattering

Dynamic light scattering is a technique which determines the size distribution of particles in suspension. When a beam of light passes through a suspension, particles scatter light which is detected by a device. The detector measures the time-dependent fluctuations of the scattered intensity. These fluctuations arise from the random thermal (Brownian) motion of the particles, and the distance between them is therefore constantly varying. The time dependent fluctuations are then analysed to calculate the particle size distribution (Berne & Pecora, 1976). Here, to simplify the readings, arbitrary size range categories were used [see section 5.3.2]. Results were expressed as % of intensity which reflects the mean value of the number of viral particles/aggregates within the size range taken on three separate readings.

Controls which represented the pre-treatment distribution of the phage in the medium (KCl), were mainly characterised by single phage particles (from Figure 5-3 to Figure 5-8).

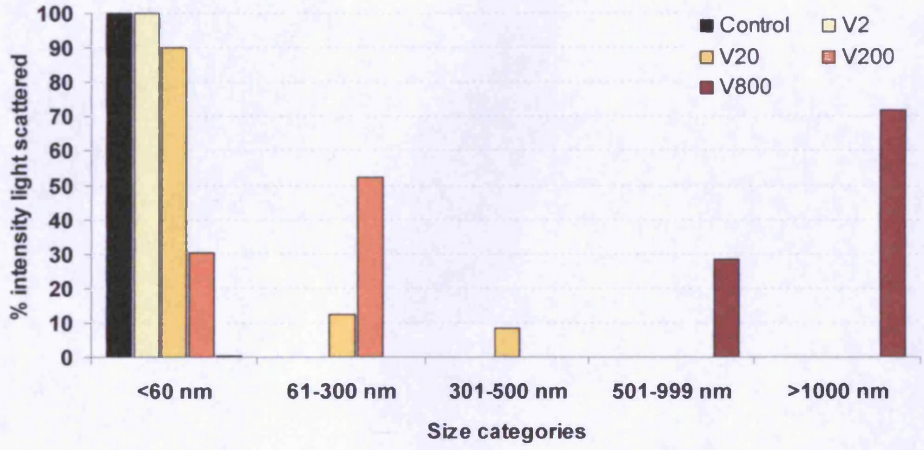
At 10°C and 20°C as the concentration of VANTOCIL^{TM} TG (Figure 5-3 and Figure 5-4) or COSMOCIL^{TM} CQ (Figure 5-6 and Figure 5-7) increased, the size of the particles/aggregates increased significantly (X^2 test, $P \leq 0.01$). At 800 ppm, all MS2 particles were in aggregates of a size into the micrometer range (Figure 5-3, Figure 5-4, Figure 5-6, and Figure 5-7). When the treatment was performed at 40°C, the aggregates were much smaller than at 10°C and 20°C, their diameter being of approximately 300 nm (Figure 5-5 and Figure 5-8)

Figure 5-3. Dynamic light scattering. MS2 treated with VANTOCIL™ TG at 10°C.



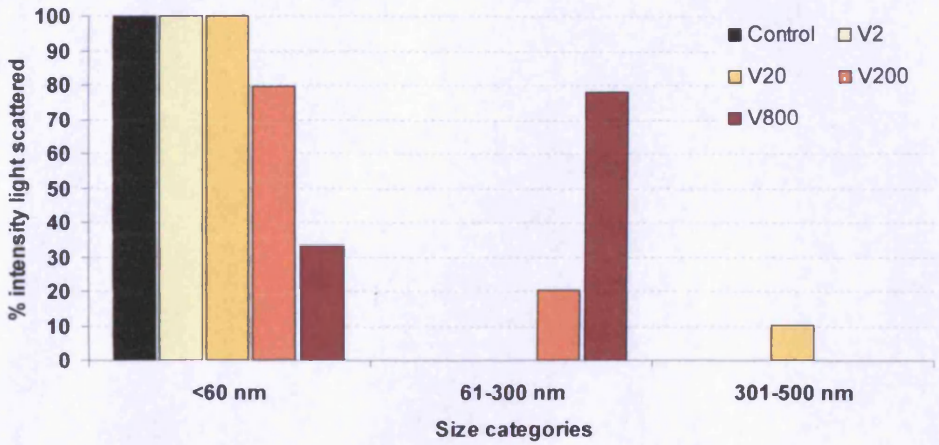
Distribution of five size groups of MS2 aggregates as a function of the percentage intensity of the light scattered. V2: 2 ppm, V20: 20 ppm, V200: 200 ppm, V800: 800 ppm.

Figure 5-4. Dynamic light scattering. MS2 treated with VANTOCIL™ TG at 20°C.



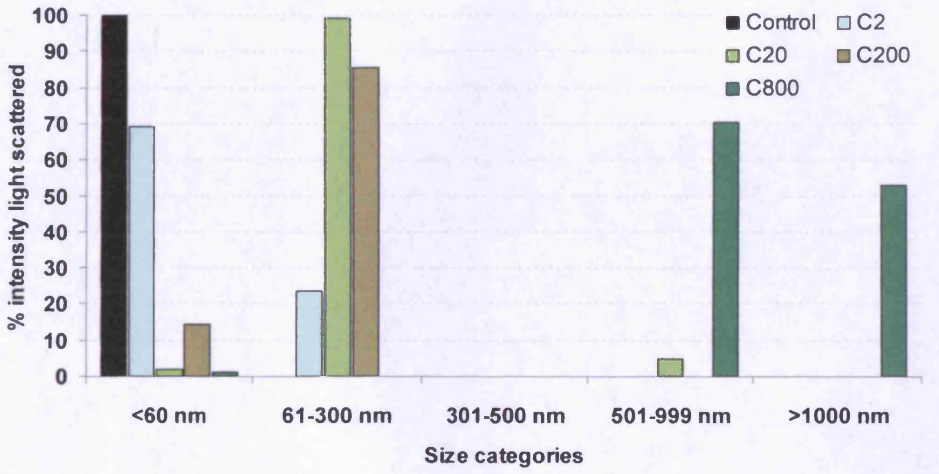
Distribution of five size groups of MS2 aggregates as a function of the percentage intensity of the light scattered. V2: 2 ppm, V20: 20 ppm, V200: 200 ppm, V800: 800 ppm.

Figure 5-5. Dynamic light scattering. MS2 treated with VANTOCIL™ TG at 40°C.



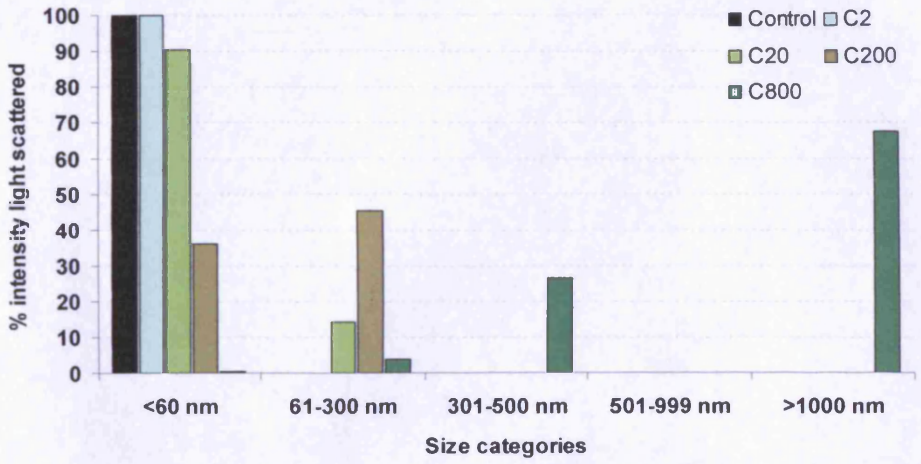
Distribution of five size groups of MS2 aggregates as a function of the percentage intensity of the light scattered. V2: 2 ppm, V20: 20 ppm, V200: 200 ppm, V800: 800 ppm.

Figure 5-6. Dynamic light scattering. MS2 treated with COSMOCIL™ CQ at 10°C.



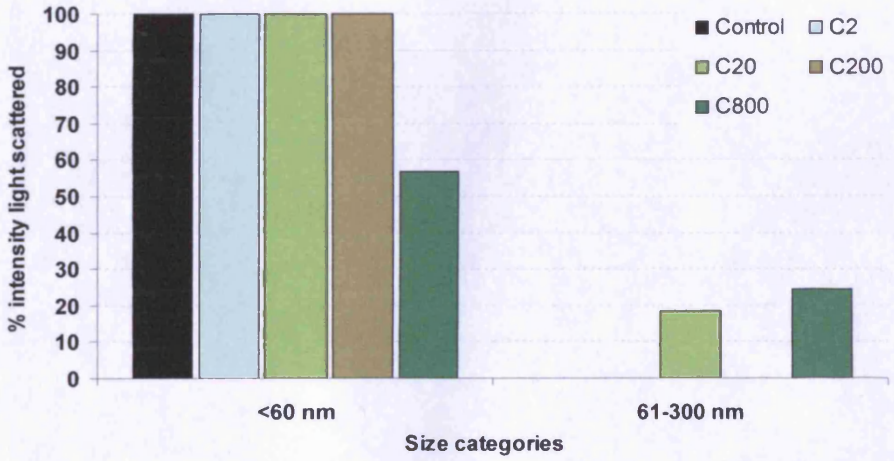
Distribution of five size groups of MS2 aggregates as a function of the percentage intensity of the light scattered. C2: 2 ppm, C20: 20 ppm, C200: 200 ppm, C800: 800 ppm.

Figure 5-7. Dynamic light scattering. MS2 treated with COSMOCIL™ CQ at 20°C.



Distribution of five size groups of MS2 aggregates as a function of the percentage intensity of the light scattered. C2: 2 ppm, C20: 20 ppm, C200: 200 ppm, C800: 800 ppm.

Figure 5-8. Dynamic light scattering MS2 treated with COSMOCIL™ CQ at 40°C.



Distribution of five size groups of MS2 aggregates as a function of the percentage intensity of the light scattered. C2: 2 ppm, C20: 20 ppm, C200: 200 ppm, C800: 800 ppm.

5.4.3. Suspension tests under different conditions

5.4.3.1. Sonication treatment

Sonication treatment did not kill the bacteriophage. However, a significant increase in number was observed (t-test, $P>0.05$) (Table 5-2). These results can be explained by the presence of small number of aggregates in the phage suspension prior to the treatment, which were then broken up by the sonication. These revised baselines were taken in account when reduction factors were calculated.

Table 5-2 Effect of sonication on phage survival without biocide.

	Phage numbers (Log_{10} PFU/ml)		
	Pre-sonication	**Post-sonication**	**RF**
10°C	9.38 ± 0.32	9.80 ± 0.57	0.42 ± 0.16
30°/40°C	9.51 ± 0.14	10.09 ± 0.10	0.58 ± 0.08

Son 30°/40°C= constant sonication, sample temperature ranged from 30° to 40°C. RF: Log_{10} reduction after 30 min exposure.

Sonication was performed at two different temperatures 10°C and 30°C/40°C [see Material and Methods] and affected the virucidal activity of the compounds. At 10°C, PHMB activity was still low (1-2 Log_{10}). However, after 30 min of exposure, VANTOCIL™ TG was more effective than the treatment without sonication, increasing significantly the reduction of MS2 (ANOVA, $F_{1,8}=35.8$, $P>0.05$) (Table 5-3). When the vessel was kept at room temperature, the sample temperature ranged from 30° to 40°C. The activity approximated 3 Log_{10} reduction after 10 min of exposure with both biocides, VANTOCIL™ TG and COSMOCIL™ CQ (Table 5-3), and contact time enhanced significantly the virucidal activity (VANTOCIL™ TG ANOVA $F_{1,8}=399.1$, $P\leq0.05$; COSMOCIL™ CQ ANOVA $F_{1,8}=219.7$, $P\leq0.05$).

Table 5-3 Suspension tests of VANTOCIL™ TG and COSMOCIL™ CQ at 800 ppm with sonication.

		RF			
	T (°C)	**Contact time (min)**			
		1	**5**	**10**	**30**
VANTOCIL™ TG	10	0.37 ± 0.24	0.36 ± 0.20	0.58 ± 0.05	1.89 ± 0.28
	30/40*	0.82 ± 0.06	1.43 ± 0.12	3.43 ± 0.21	5.20 ± 0.98
COSMOCIL™ CQ	10	0¥	0¥	0.93 ± 0.30	0.55 ± 0.13
	30/40	0.22 ± 0.15	1.22 ± 0.11	2.74 ± 0.49	5.42 ± 0.08

*temperature increased with duration of sonication: 30°C (1-5 min) and 40°C (10-30 min)

¥ No reduction observed.

RF: Log_{10} reduction factor. Values are the mean of three replicates.

5.4.3.2. Temperature effect

The effect of temperature on coliphage survival was tested. No significant viral viability reduction was found; however at some temperatures, specifically 30°C and 40°C, significant increases in MS2 number was observed (Table 5-4). This can be explained by the presence of some aggregates prior to the treatment which were eventually broken up by higher temperatures. In fact, at higher temperatures, the increased kinetic energy in the system can break electrostatic and hydrophobic bonds, responsible for the formation of the viral aggregates (Haynie, 2001).

Table 5-4. Effect of temperature on phage survival without biocide.

	Phage numbers (Log_{10}PFU/ml)		
	Pre-temperature treatment	**Post- temperature treatment**	**RF**
10°C	11.13 ± 0.02	11.02 ± 0.01	0.12 ± 0.02
20°C	10.80 ±0.01	10.60 ± 0.01	0.21 ± 0.01
30°C	9.86 ± 1.09	10.11 ± 1.15	0.24 ± 0.24*
40°C	10.04 ± 0.38	10.33 ± 0.52	0.29 ± 0.02*
50°C	10.98 ± 0.03	10.51 ± 0.01	0.47 ± 0.04

* indicate a significant increase in MS2 concentration.

RF: Log_{10} reduction after 30 min exposure.

Values are the mean of three replicates.

The virucidal activity of VANTOCIL™ TG and COSMOCIL™ CQ at 800 ppm was notably influenced by the temperature. Generally, an increase of activity was associated with a rise in temperature. At 20°C, 0.52 Log_{10} reduction was achieved after 5 min contact time by VANTOCIL™ TG, whereas at 30°C the reduction factor increased by 1 Log_{10} (Table 5-5). Higher reductions in number were observed at 40°C, with 4 Log_{10} after 10 min for both PHMB biocides (Table 5-5).

The efficacy of VANTOCIL™ TG virucidal activity increased significantly with time of exposure only at temperatures over 30°C (30°C ANOVA, $F_{1,8}$=65.9, $P \leq 0.05$; 40°C ANOVA, $F_{1,8}$=199.8, $P \leq 0.05$; 50°C ANOVA, $F_{1,8}$=122.7, $P \leq 0.05$).

The same result was found with COSMOCIL™ CQ, only at 30°C, 40°C and 50°C There was a significant relationship between reductions and contact time (30°C ANOVA, $F_{1,8}$=93.9, $P \leq 0.05$; 40°C ANOVA, $F_{1,8}$=24.4, $P \leq 0.05$; 50°C ANOVA, $F_{1,8}$=50.9, $P \leq 0.05$). The activity of the two PHMB-based biocides did not differ significantly (t-test, $P > 0.05$).

Table 5-5. Suspension tests of VANTOCIL™ TG and COSMOCIL™ CQ at 800 ppm against MS2 at different temperatures.

	T (°C)	RF			
		Contact time (min)			
		1	5	10	30
VANTOCIL™ TG	10	0.43 ± 0.54	0.45 ± 0.28	0.50 ± 0.08	0.56 ± 0.36
	20	0.43 ± 0.34	0.52 ± 0.05	0.88 ± 0.17	0.50 ± 0.02
	30*	0.64 ± 0.09	1.39 ± 0.09	1.46 ± 0.10	2.76 ± 0.34
	40*	1.49 ± 0.05	2.39 ± 0.30	4.10 ± 0.02	5.19 ± 0.30
	50*	2.49 ± 0.15	5.30 ± 0.42	5.79 ± 0.38	5.36 ± 0.28
COSMOCIL™ CQ	10	0.40 ± 0.54	0.42 ± 0.26	0.12 ± 0.12	0.50 ± 0.24
	20	0.54 ± 0.36	0.76 ± 0.45	0.77 ± 0.40	1.07 ± 0.30
	30*	0.61 ± 0.05	1.75 ± 0.31	2.39 ± 0.03	2.60 ± 0.03
	40*	2.26 ± 0.29	3.04 ± 0.63	3.56 ± 0.24	4.79 ± 0.09
	50*	2.46 ± 0.19	3.05 ± 0.37	3.92 ± 0.41	5.33 ± 0.16

* Significant relation between reduction factors and contact time.

RF: Log_{10} reduction factor. Values are the mean of three replicates.

Q_{10} is the factor which describes the rate by which a reaction increases for every 10°C rise of temperature. In disinfection practice, the rate represents the time to kill the same number of microorganisms at T_1 and T_1+10°C. Q_{10}

thus describes the biocide activity dependency on temperature. In these studies it was calculated with reference to the time needed to kill 4 Log_{10} as predicted from the fit between reduction factor and contact time [see Material and Methods, Statistical analysis].

Q_{10} values demonstrated the high influence of temperature on VANTOCIL™ TG activity mostly between 20°C and 30°C (Table 5-6).

COSMOCIL™ CQ activity was affected by temperature similarly, however a higher Q_{10} was found between 10°C and 20°C rise. The highest value occurred again when the temperature rose from 20°C to 30°C (Table 5-6).

Table 5-6. Q_{10} of VANTOCIL™ TG and COSMOCIL™ CQ.

	Q_{10}			
	10°C/ 20°C	**20°C/ 30°C**	**30°C/ 40°C**	**40°C/ 50°C**
VANTOCIL™ TG	1.1	3.9	2.0	1.2
COSMOCIL™ CQ	2.3	2.5	1.8	1.1

5.4.3.3. Formulations

Formulations are based on VANTOCIL™ TG combined with different surfactants [see Materials and Methods]. Their virucidal activity against MS2 was low, even after a long time of exposure (Table 5-7).

Table 5-7 Suspension test of three formulations.

VANTOCIL™ TG 800ppm		**RF**			
	T (°C)	**Contact time (min)**			
		1	**5**	**10**	**30**
F1	20	0.39 ± 0.14	0.99 ± 0.25	0.51 ± 0.11	0.27 ± 0.58
F2	20	0.10 ± 0.08	0.00 ± 0.15	0.87 ± 0.17	0.88 ± 0.06
F3	20	0[¥]	0[¥]	0.02 ± 0.29	0[¥]

[¥] No reduction observed.

RF: Log_{10} reduction factor. Values are the mean of three replicates.

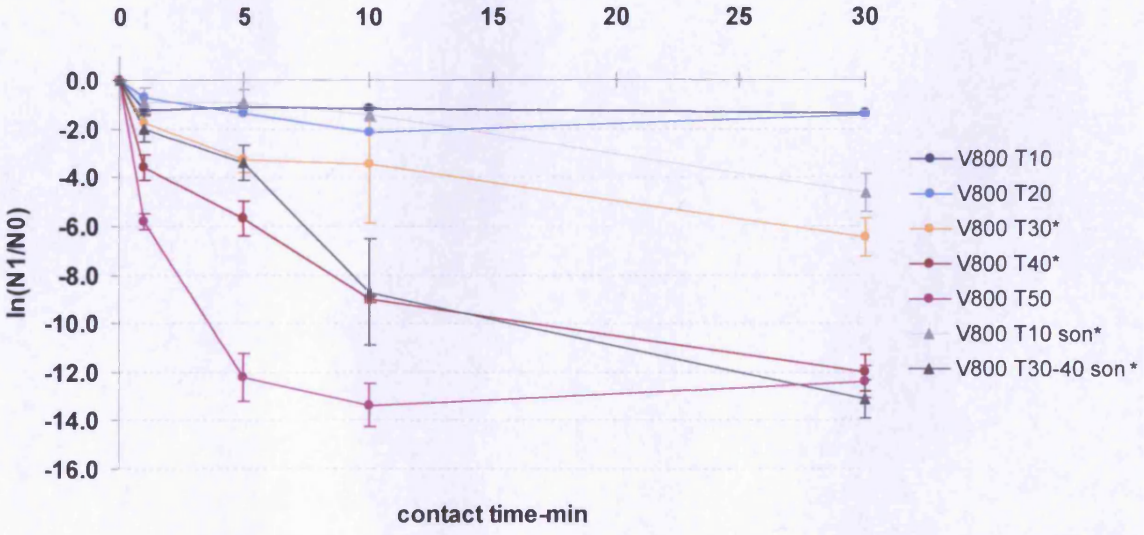
5.4.3.4. Inactivation kinetic

Inactivation kinetic curves plot the survival ratio (N_1/N_0), where N_1 is the viable count at time t and N_0 is the viable count at time 0, against contact time. Efficient phage inactivation would ideally show a significant linear relationship for the full time course (first-order kinetics).

VANTOCIL™ TG achieved significant linearity at 30°C (r=-0.93, $P \leq 0.05$), 40°C (r=-0.88, $P \leq 0.05$), and when sonication was used at 10°C: (r=-0.98, $P \leq 0.05$) and at 30/40°C (r=-0.95, $P \leq 0.05$) (Figure 5-9).

A linear relation was observed only during the treatment with sonication at room temperature when COSMOCIL™ CQ was used (r=-0.98, $P \leq 0.05$) (Figure 5-10).

Figure 5-9. The efficacy of VANTOCIL™ TG at 800 ppm against MS2 established by suspension test.

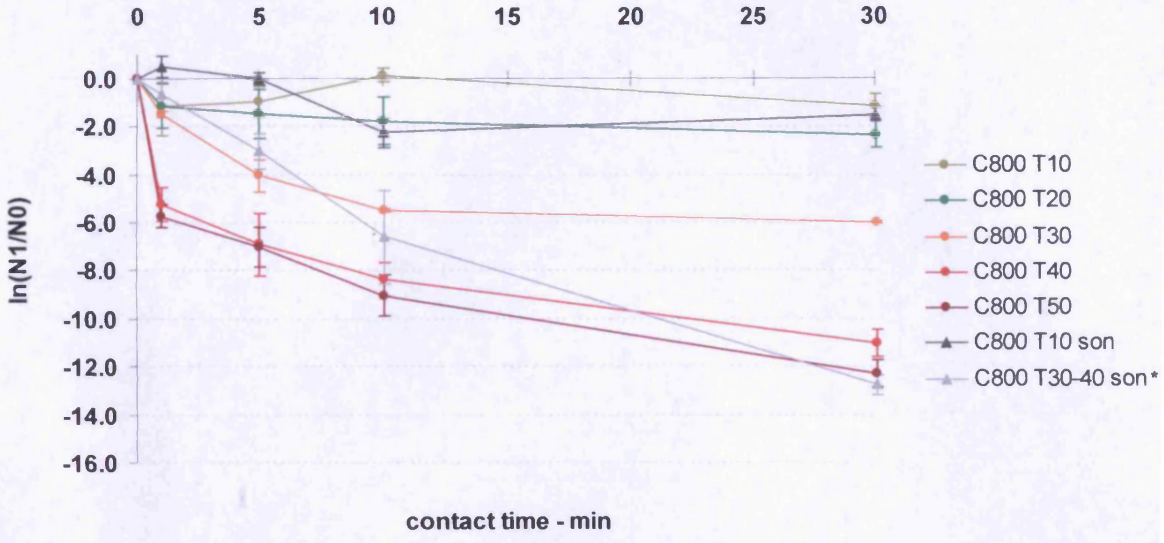


* significant Pearson coefficient.

Values are the mean of three replicates. Bars represent standard deviation.

Grey lines represent treatments with sonication while coloured lines represent treatment without sonication at different temperatures (from 10°C to 50°C).

Figure 5-10 The efficacy of COSMOCIL™ CQ at 800 ppm against MS2 established by suspension test.



* significant Pearson coefficient.

Values are the mean of three replicates. Bars represent standard deviation.

Grey lines represent treatments with sonication while coloured lines represent treatment without sonication at different temperatures (from 10°C to 50°C).

5.4.3.5. Suspension test – Purified MS2

A further investigation on the virucidal activity of VANTOCIL™ TG and COSMOCIL™ CQ was performed using the purified MS2 as phage suspension. This preparation has been used in other assessments (SDS-PAGE, dynamic light scattering) and an analysis was needed in order to assess if any changes in activity occurred when the phage was purified from bacterial debris.

Using the purified phage suspension only had a minor effect on VANTOCIL™ TG (ANOVA, $F_{1,4}$=25.98, $P \leq 0.05$) and no effect on COSMOCIL™ CQ (ANOVA, $F_{1,4}$=0.42, $P > 0.05$) (Table 5-8).

Table 5-8. Suspension tests results of purified MS2 treated with VANTOCIL™ TG and COSMOCIL™ CQ at 800 ppm.

	T(°C)	Purified MS2 RF	Un-purified MS2 RF
VANTOCIL™ TG	20	1.20 ± 0.24*	0.50 ± 0.02*
COSMOCIL™ CQ	20	0.95 ± 0.06	1.07 ± 0.30

* significantly different.

Time of exposure was 30 min.

RF: reduction factor expressed in Log_{10}.

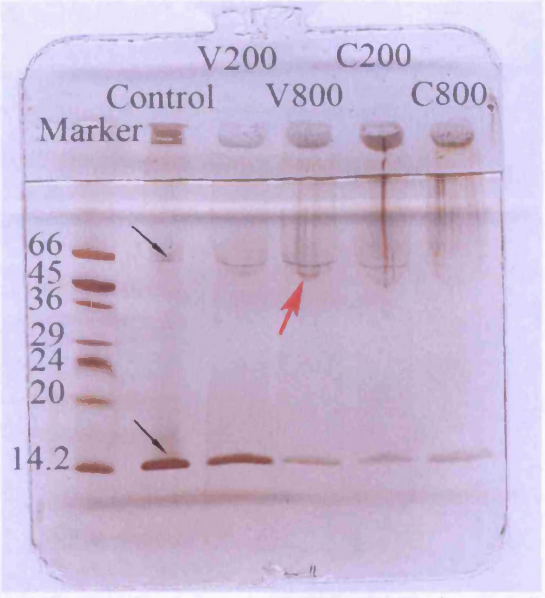
Results from suspension tests with the un-purified phage are taken from Chapter 2.

5.4.4. Protein analysis

The treatment with PHMBs at 40°C had an effect on the protein profile of MS2 (Figure 5-11); a new band of approximately 40 KDa was visible after the treatment at both PHMB concentrations (200 and 800 ppm). In addition, a loss of intensity of the coat protein band occurred.

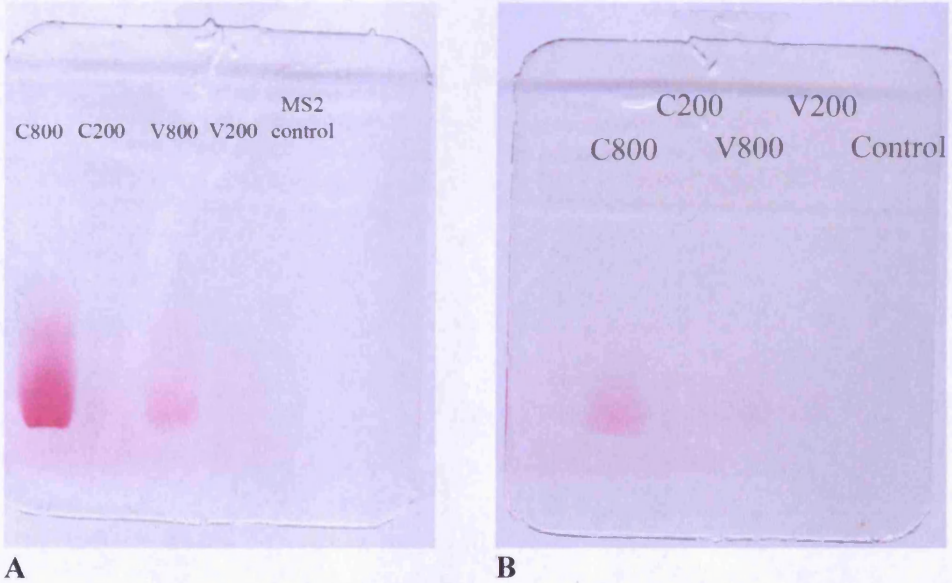
The gel stained with Eosin Y was performed at both 20°C and 40°C. Results showed the presence of PHMB on the phage capsid protein and the intensity increased with the concentration (Figure 5-12). At 20°C brighter pink bands were observed.

Figure 5-11. SDS-PAGE gels of MS2 phages treated with VANTOCIL™ TG and COSMOCIL™ CQ at 40°C.



The black arrows indicate the two proteins which formed MS2, coat protein (13.7KDa) and A protein (44KDa), the red arrow indicate the new band (40KDa). Proteins sizes are in KDa.

Figure 5-12. Eosin Y stained SDS-PAGE gels of MS2 phages treated with VANTOCIL™ TG and COSMOCIL™ CQ.



A **B**

The pink bands represent the band PHMB. A: treatment at 20°C. B: treatment at 40°C.

5.5. Discussion

This chapter examines the possible importance of aggregation in reducing the virucidal activity of the PHMB-based biocides, VANTOCIL™ TG and COSMOCIL™ CQ, against a non-enveloped virus. By testing the biocide in combination with temperature, sonication or surfactants, aggregation could be shown to be a relevant factor affecting the efficacy of the biocide. The role of aggregation in preventing virucidal activities is controversial. Some authors reported the role of aggregation as a viral resistance mechanism to chemical biocides, such as bromine against reovirus (Sharp *et al.*, 1975), and to physical agents, such as UV light against vaccinia virus (Sharp & Kim, 1966). Conversely, other authors concluded that aggregation did not influence the inactivation of poliovirus I by chlorine (Sharp & Leong, 1980). Some additional reasoning has been based on the use of disinfection kinetics, which has been described to be analogous to a chemical reaction (Chick, 1913). Thus it has been postulated that to achieve an efficient inactivation of viruses the reaction should follow a first order kinetic. Differences in viral structure, biocides and environmental conditions will cause a deviation from first order kinetics. Observing the kinetic curves, a "tailing off" effect was visible mostly after treatment at 10°C, 20°C and 50°C for VANTOCIL™ TG, and at all treatments except with sonication at room temperature for COSMOCIL™ CQ. The "tailing off" shape is usually explained by biocide depletion or by the presence of a more resistant viral subpopulation due to aggregation, conformational change of the viral capsid or genetic variation (Hoff & Akin, 1986).

In this study, one of the factors responsible for the departure from first-order kinetics in the case of VANTOCIL™ TG inactivation against MS2 at 20°C appeared to be aggregation. A significant linear relationship was found after treatments at 30°C and 40°C and when clump sizes were measured to be at a maximum of 155 ± 11.5 nm. Presumed clump sizes were much smaller at higher temperatures than the ones measured at 10°C and at 20°C, where clumps were more than 1000 nm in size. PHMB treatment at 50°C did not produce a first order inactivation kinetic. However, it can be noted that the

activity was fast and Log_{10} reduction observed at this temperature was at the limit of detection of the assay. Temperature also influenced also the activity of COSMOCIL™ CQ, leading to a reduction in the aggregate size as temperature rose. However, a significant linear relationship was not observed.

Q_{10} values for disinfectants, which describe the temperature effect, vary widely (4 for phenol, 45 for ethanol, 300 for ethylene glycol monoethyl ether) (Smith, 2004). VANTOCIL™ TG and COSMOCIL™ CQ exhibited temperature coefficients from 1.5 to 4, similar to those measured for PHMB bactericidal activity against *E. coli* (Broxton *et al.*, 1983). The highest value was achieved at the temperature step of 20°C/30°C for both biocides. VANTOCIL™ TG was the most affected by temperature, its activity increasing by a factor of 4 from 20°c to 30°C. Modest variation in Q_{10} over defined temperature ranges should be treated with caution given the wide range observed in different biocide classes. It appears likely that the temperature depending of MS2 interaction is similar for both PHMBs.

Sonication enhanced significantly the reduction of phage number only at 10°C over an exposure time of 30 min with VANTOCIL™ TG, and after 10 min with COSMOCIL™ CQ. Furthermore, significant first-order kinetics occurred when the biocides were tested in combination with sonication, which was used to enhance biocide efficacy presumably because of its capacity for breaking up clumps (Burleson *et al.*, 1975).

Formulations are often used to enhance the efficacy of disinfection. The concept is based on a potentiating action, where the activity of the compounds enhance each other (Jimenez & Chiang, 2006). Among surfactant properties, solubilisation ability is important, as well as their ability to break up microbial clumps. Here, the three surfactant-containing formulations did not increase the virucidal activity of VANTOCIL™ TG. No evaluation was undertaken to see if the added surfactants had any inhibitory effect on PHMB activity.

Temperature had a profound affect on the activity of PHMB against a non-enveloped virus. In addition to dispersing the viruses uniformly due to the thermal energy, temperature may also have altered the stability of viral capsid. Such properties have been extensively studied for drug design,

inactivation strategies for vaccine development, and for enhancement of antiviral activity (Newman *et al.*, 2003; Ausar *et al.*, 2006; Hilmer *et al.*, 2008; Nelson *et al.*, 2008a). For instance, temperature-induced changes occurred in the quaternary structure of Norwalk virus capsid, which were characterised by a loss of continuity in the icosahedral geometry (Ausar *et al.*, 2006). Canine parvovirus capsid responded to increased temperature by higher DNA exposure and by an increased permeability to negative stain (Nelson *et al.*, 2008a). In this study raised temperature did not affect phage survival. However, it is possible that an increase in capsid permeability enhanced the penetration of PHMB and thus increased and its virucidal activity in a temperature-dependent manner. Notably the Eosin Y stained gel showed a lower amount of PHMB on the capsid after the treatment at 40°C, meaning that a possible penetration of VANTOCIL™ TG and COSMOCIL™ CQ inside MS2 could have occurred.

In conclusion, aggregation appeared to account, at least in part, for the low virucidal activity of PHMB at 20°C. Raising the temperature of disinfection had a significant role in enhancing the efficacy of the PHMB biocides, helping virion dispersion, as shown by light scattering, and possibly increasing virus susceptibility due to protein conformational changes.

6. MAMMALIAN VIRUSES AND PHMBs

6.1. Introduction

Adenovirus was first isolated from adenoids of infected patients in 1953; since then more than 100 different species have been identified from reptiles, birds and mammals. Human adenoviruses are classified in 51 serotypes (Russell, 2009) and cause respiratory infection, conjunctivitis and severe gastro-enteric dysentery. Adenovirus type 2 and 5 (group C) are the most studied and present all over the world (Rux & Burnett, 2004).

Control of viral contamination is important to prevent the spread of infections, especially in the hospital environment (Eterpi *et al.*, 2009). Adenoviruses were found responsible for several nosocomial infections and outbreaks (Bruckova *et al.*, 1980; Alpert *et al.*, 1986; Hong *et al.*, 2001). Furthermore, in recent years they have been considered relevant viral pathogens of immunocompromised patients. Adenoviruses groups A, B and C have been isolated from bone marrow and haematopoietic stem cell transplant recipients, and the serotype 5 was found to be often associated with respiratory manifestations and hepatitis (Echavarria, 2008).

For over 50 years adenoviruses have been used as models of virus-cell interaction; currently their application is focused on gene therapy (cancer treatment, genetic disease, and vaccination) because of their ability to infect a variety of non-dividing cells (Cusack, 2005). In addition, their use in studies on the virucidal properties of biocides against human virus has been widely recognized (Gaustad *et al.*, 1974; Wichelhaus *et al.*, 2006; Raffo *et al.*, 2007; Garcia-De-Lomas *et al.*, 2008; Vieira *et al.*, 2008). Specifically, adenovirus type 5 is considered as a reference virus (Sauerbrei *et al.*, 2004b; Sauerbrei *et al.*, 2007; Eterpi *et al.*, 2009), since it has a higher resistance to biocides compared to other adenovirus strains of the groups C and D (Sauerbrei *et al.*, 2004a). In Europe it has also been recommended as a surrogate organism for testing disinfectants against human pathogenic viruses (German Association for the Control of Virus Diseases, 1990; European Committee for Standardization, 1996; Fachausschuss "Virusdesinfektion" der Deutschen Vereinigung zur Bekampfung der Viruskrankheiten und des Robert Koch-Instituts, 2003). A detailed

knowledge of its structure and infection cycle is available in the literature (Rexroad *et al.*, 2003; Maheshwari *et al.*, 2004; Rexroad *et al.*, 2006; Russell, 2009), helping the mechanistic interpretation of the results.

Investigations on the virucidal activity of PHMB-based biocides are very few. Recognising their reactivity with lipidic membranes, PHMBs are likely to interact with the viral cell membrane component. Indeed, it was found active against enveloped viruses, such as HIV (Krebs *et al.*, 2005; Thakkar *et al.*, 2009) and Herpes simplex virus (Valluri *et al.*, 1997). Some substituted biguanides have antiviral properties against influenza virus grown in chick embryo (Pilcher *et al.*, 1961). So far, however, their efficacy against non-enveloped viruses has not been tested.

This study was based on previous findings with the coliphage MS2 treated with PHMB-based biocides [Chapters 3 to 5]. Ad5 was chosen as a reference virus, since its high resistance and its relevance among the clinically important viruses.

6.2. Aims

The chapter aims to investigate the virucidal activity of VANTOCIL™ TG and COSMOCIL™ CQ against human Adenovirus type 5.

Specifically:

- To assess the virucidal activity of two PHMB-based biocides, VANTOCIL™ TG and COSMOCIL™ CQ against the human adenovirus type 5 (Ad5);
- To investigate the structural damage of Ad5 caused by the PHMBs;
- To investigate the interactions between PHMBs and the Ad5 viral particles.

6.3. Materials and methods

6.3.1. Cell culture

6.3.1.1. Establishing 293T from frozen stock

293T cell stocks were kindly supplied by Dr. Carol Rickards, Cytomegalovirus and Adenovirus Research group, Cardiff University. Cells were rapidly thawed by briefly immersing the vial in a 37°C water bath (2-3 min constant agitation). Upon thawing, the outside of the vial was immediately wiped with 70% ethanol, and after transferring the contents into a centrifuge tube (50 ml), 10 ml of complete growth medium (DMEM, 10% v/v heat inactivated foetal bovine serum, 50 units/ml penicillin, 0.05 mg/ml streptomycin and 0.1 mg/ml neomycin) was added gently (drop by drop, so as not to shock the cells). The mixture was centrifuged at 153 x*g* (Avanti J-14, Beckman Coulter, Fullerton, USA) for 10 min at 20°C. The supernatant was discarded and gently 20 ml of complete medium was added. The content was then transferred to the flask. The culture was incubated in a 5% CO_2 humidified incubator at 37°C.

Cell density was monitored daily. Cells were fed twice a week and usually split once a week when 50-60% confluent growth was reached.

6.3.1.2. 293T passaging

Cells were passaged when the monolayer reached 50% confluence. All media were pre-warmed to 37°C in a water bath. Growth medium was removed from the T75 flask (Corning Incorporated, NY, USA) and the cells were washed twice with 5 ml of PBS. Three ml of trypsin-EDTA (0.53 mM EDTA, 0.05% w/v trypsin) was added and after a short shake, it was withdrawn immediately. The flask was then incubated in a 5% CO_2 incubator at 37°C for 1-2 min. After checking under the microscope (Leica inverse microscope, objective 10X) if the cells were sloughed off, 10 ml of new complete medium was poured into the flask to stop the trypsinization. The cell suspension was then transferred in a new T75 flask following the

dilutions described in Table. 6-1. Cells were then placed in a 5% CO_2 incubator at 37°C and checked daily. The next passage occurred when cells reached 50% confluence.

Table. 6-1 Dilution factors and volumes used during cell 293T passage.

Dilution	Media in fresh T75 flask	Resuspension media	Cell suspension aliquot	Total volume in T75 flask
1:2	15	10	5	20
1:4	17	10	3	20
1:8	18	10	2	20
1:10	18.4	10	1.6	20

Volumes are in ml.

6.3.1.3. Frozen 293T cell preparation

After trypsinization [see section 6.3.1.2], cell suspensions were transferred in a centrifuge tube (50 ml). The cell were counted with an haemocytometer to DIN/ISO regulations double net ruling (Fisher Scientific, Leicestershire, UK) and then centrifuged at 153 x*g* for 10 min at 20°C. The pellet was re-suspended with the "freezing" medium (DMEM, 10% v/v heat inactivated foetal bovine serum, 50 units/ml penicillin, 0.05 mg/ml streptomycin and 0.1 mg/ml neomycin, 10% v/v dimethyl sulfoxide) in order to get 2-5 x 10^6 cells/ml. One ml was transferred into cryogenic vials (Corning, NY, USA) which were placed upright in a styrofoam box full of cotton wool. The box was transferred to a -80°C freezer for 24 h and then vials were placed in a liquid nitrogen container.

6.3.2. Adenovirus type 5

6.3.2.1. Propagation

Adenovirus type 5 (Ad5) stocks were kindly supplied by Dr. Carol Rickards, Cytomegalovirus and Adenovirus Research group, Cardiff University.

The growth medium was removed from the flask and 5 ml of pre-warmed serum-free medium was added. Cells were infected with 0.1 ml of the virus/cell suspension (multiplicity of infection 1-10 PFU/ml). The infected cells were incubated for 1h at 37°C to let the virus bind to the cells. Fifteen ml of complete growth medium was then added and incubation undertaken at 37°C under 5% CO_2 until cytopathic effects were visible (usually 2-3 days).

6.3.2.2. Viral extraction from infected cells

Infected cells were removed from the bottom of the flask with a scraper. The contents of the flask were withdrawn and transferred into a 50 ml centrifuge tube. A centrifugation step was performed at 345 x*g* for 5 minutes at 20°C (Avanti J-14, Beckman Coulter, Fullerton, USA). After discarding the supernatant, the pellet was washed with PBS. All pellets were pooled into one tube and centrifuged again at 345 x*g* for 5 min.

The pellet was re-suspended in 1 ml per flask of PBS and an equal amount of tetrachloroethylene was added. The mixture was then shaken vigorously in order to break up the cells to release the virus. A last centrifugation step at 345 x*g* for 5 min was performed to separate the 2 layers, the lower layer with the cell debris and the top one with the virus. The top layer was then removed and aliquots of 1 ml were stored at -80°C in PBS.

6.3.2.3. Titration

Immunostaining was used to titrate Ad5. The protocol is based on Bewig & Schmidt (2000).

24 well-plates (Corning Incorporated, NY, USA) were seeded with $5x10^5$ cells/well in 2 ml of complete growth medium and cells were allowed to adhere overnight. The complete medium was substituted by 0.5 ml of serum-free medium and 100 µl of the diluted viral suspension (approx. from 10^2 to 10^4 PFU/ml) was inoculated onto the well in duplicate. After 1 h of

incubation at 37°C under 5% CO_2, 1 ml of complete medium was added to the wells. The plates were incubated for 48 h at 37°C in 5% CO_2.

When cytopathic effects were visible (after 48 h) the medium was aspirated and the wells were air dried. Gently 1 ml of ice cold 50/50 acetone/methanol was added and incubated at -20°C for 10 min. The acetone/methanol was aspirated and a wash step in 0.5 ml of PBS with 1% w/v BSA was repeated 3 times.

The goat anti-adenovirus primary antibody (Millipore AB1056) was diluted 1 in 5000 in PBS + 1% BSA and 0.5 ml was added to each well. The 24 well-plates were incubated at 37°C in 5% CO_2 for 1h. The primary antibody was removed aspiring with the pipette and the cells were washed 3 times with 0.5 ml of PBS+1%BSA.

The secondary antibody (anti-goat hrp) (donkey anti goat/sheep IGG HRP, Millipore AB324P) was diluted 1 in 1000 in PBS + 1% BSA. 0.5 ml was inoculated into each well and then incubated at 37°C in 5% CO_2 for 1h. After removing with the pipette the secondary antibody, a further wash step in 0.5 ml of PBS + 1%BSA repeated 3 times was performed.

The metal enhanced DAB (3,3' diaminobenzidine) substrate (PIERCE 34065) was made up by diluting 10-fold the DAB/Metal concentrate by adding the peroxide buffer supplied with the kit. The 1X substrate solution was stable for several hours at 4°C.

500 µl of DAB substrate was added to each well and incubated for 10 min at room temperature (20°C). The DAB substrate was then removed and 0.5 ml of PBS was pipetted into the wells.

Using the inverse microscope (Leica, objective 10X and 20X) the number of infected cells (coloured purple/brown) were counted in 5 fields of view using an objective and a dilution that gave 5-50 positive cells (infected cells).

The PFU/ml was calculated as: [(infected cells per field) x (fields per well)] / [volume virus (ml) x dilution factor]. The number of fields per well was determined from Table 6-2. Objective 20x was usually used when 24-well plates were used.

Table 6-2. Number of fields per well.

Objective lenses	Fields per well		
	12-well plate	24-well plates	96-well plates
4x	19	10	1.6
5x	30	16	2.6
10x	150	79	12.6
20x	594	313	50

6.3.3. Concentration and purification of adenovirus

6.3.3.1.a. Concentration of adenovirus type 5

The modified protocol previously described by Sambrock & Russel (2001) was applied to mammalian viruses. The viral solution prepared from a minimum of 8 flasks T162 (162 cm^2) was mixed with 10% w/v of PEG 8000 and NaCl (final concentration of 1M). The mixture was stirred and held at 4°C overnight. It was then centrifuged at 11000 x*g* for 30 min at 4°C (JA25-50 rotor, Avanti J-25 centrifuge, Beckman Coulter, Fullerton, USA). The precipitate was dissolved 1:10 in the initial volume of PBS and an equal volume of chloroform was added to extract PEG and cell debris. A vortex step of 30 seconds was performed and then the mixture was centrifuged at 3000 x*g* for 15 min at 4°C to separate the aqueous from the organic phase. The aqueous phase was recovered and used for isopycnic separation.

6.3.3.2. Isopycnic separation

0.5 g of CsCl per ml of viral suspension was added. The different densities of CsCl were prepared using PBS as diluent.

Table 2-1 showed the characteristics of the CsCl solutions used. According to Ad5 buoyant density in CsCl, between 1.32 and 1.34 g ml^{-1} (International Committee on Taxonomy of Viruses, 2002), a 13.5 ml polyallomer centrifuge tube (Beckman Coulter, Fullerton, USA) was filled with 2.6 ml of the following densities of CsCl, ρ = 1.32 g ml^{-1}, ρ = 1.45 g ml^{-1}, ρ = 1.70 g ml^{-1}. A line was drawn outside the tube at the interface between ρ = 1.32 g ml^{-1} and ρ = 1.45 g ml^{-1}.

Table 6-3. Densities and respective concentrations of CsCl solutions prepared in 10ml of SM buffer used during the experiments.

Density ρ (g/ml)	M (g-mole/L)	CsCl (g)	PBS (ml)
1.32	2.49	4.20	8.93
1.45	3.60	6.00	8.50
1.70	5.65	9.50	7.50

From Sambrock & Russel (2001).

The virus suspension (5.2 ml) was layered on top of the gradient and then centrifuged at 200000 x*g* for 8 h at 20°C (Vti 65 rotor, Optima LE-80K Ultracentrifuge, Beckman Coulter, Fullerton, USA). The band corresponding to the virus (in the $\rho = 1.32$ g ml^{-1} zone) was harvested by gently inserting a sterile needle to remove it. The infectivity was checked as described in section 6.3.2.3.

The purified virus was dialysed overnight (Spectra/Por Float-A-Lyzer, MWCO 3500 Da) against PBS and then filtered through 0.22 μm filter (Millipore, Cork, Ireland). Purified adenovirus was stored in aliquots of 1 ml at -80°C.

6.3.4. Preliminary cytotoxicity tests

The toxicity of PHMB molecules was tested against 293T cells. Various methods were tested to neutralize any cytopathic effects: i) use of neutraliser A (6% Tween 80, 0.45% Lecithin and 0.1% L-Histidine); ii) use of neutraliser B (2% Tween 80, 0.45% Lecithin and 0.1% L-Histidine); iii) use of neutraliser C (6% Tween 80, 0.45% Lecithin and 0.1% L-Histidine in DMEM); iv) use of AMICON ULTRA 0.5 10 KDa (Millipore, Billerica, USA).

The test with the various neutralisers consisted of mixing 1 ml of growth medium with 9 ml of VANTOCIL™ TG and COSMOCIL™ CQ at 200 and 800 ppm for 5 min. Sterile water was used as the control. One ml of the mixture was mixed with 9 ml of neutraliser and after 2 min, a 10-fold dilution step was performed. Four dilutions were plated (1, 10, 10^{-2}, 10^{-3}) on

cells seeded a day before on a 24-well plate. Specifically, the growth medium of the cells was replaced with 0.5 ml of serum-free medium. A sample (diluted neutralised biocide) volume of 0.1 ml was inoculated into medium and after 1h incubation at 37°C under 5% CO_2, 1 ml of complete medium was added. Each dilution was plated in triplicate. Cytopathic effects were checked after 24 h, 48 h, and 72 h.

The experiment performed with the device AMICON ULTRA 0.5 10 KDa was carried out with and without the use of the neutraliser (6% Tween 80, 0.45% Lecithin and 0.1% L-Histidine). One ml of growth medium was mixed with 9 ml of PHMB biocides at 800 ppm or water as control. After 5 min, 1 ml of the mixture was mixed with 9 ml of neutraliser, when the neutralization step was included in the experiment. 0.5 ml was then loaded into the AMICON ULTRA 0.5 10 KDa in duplicate and a centrifugation step of 5 and 10 min at 14000 x*g* at 20°C was performed. The filter device was removed and placed upside down in a clean centrifuge tube and spun for 2 min at 1000 x*g* to recover the ultrafiltrate. The growth medium was removed from the wells with $5x10^5$ cells seeded for 24 h the previous day and replaced with 0.5 ml of serum-free medium. Cells were inoculated with 0.1 ml of the ultrafiltrate (undiluted and 10-folder diluted) in duplicate for 1 h at 37°C in 5% CO_2. Then 1 ml of complete medium was added and cytopathic effects were checked after 24 h, 48 h, and 72 h.

6.3.5. Sephadex™ column system

In order to overcome the cytotoxicity of PHMB on 293T cells, a gel filtration methods using Sephadex™ were also used (Geller *et al.*, 2009). Gel filtration separates molecules according to their size through a gel packed in a column.

6.3.5.1. Preparation of Sephadex™ media

Sephadex™ G-25 comes in powder. It was re-suspended in 20 ml g^{-1} of PBS for at least 3 hours. The swelled gel was autoclaved. The volume

achieved after swelling was 6 ml per g of Sephadex™. After decanting the supernatant, the excess of buffer was removed to obtain a suspension with a volume ratio of Sephadex™ G-25/PBS of 1/1. It was stored at 4°C.

6.3.5.2. Preparation of Sephadex™ columns

Columns were prepared with a 1 ml syringe packed with sterile (UV) cotton wool attached to a drilled 1.5 ml centrifuge tube. The syringe was filled with 1 ml of sterile Sephadex™. The whole system was placed in a 50 ml centrifuge tube and centrifuged at 4500 x*g* for 1 min. The centrifugation step was necessary to pack the column in the syringe and to eliminate the excess of PBS. After replacing the 1.5 ml centrifuge tube with the released buffer, the Sephadex™ column was ready to use.

6.3.5.3. Control experiments

Three different controls were performed:
- elimination of cytotoxicity against 293T cells;
- non-retention of Ad5 in the Sephadex™ column after filtration;
- neutralization of the potential virucidal activity of PHMB.

Cytotoxicity tests were performed by mimicking the suspension test experiment without the use of Ad5. 293T cells were seeded in a 24-well plate. After incubation at 37°C in 5% CO_2 overnight, the medium was replaced with 0.5 ml of serum-free medium and 0.1 ml of the undiluted and 10-fold diluted biocide solution filtered through the column [see section 6.3.6]. Cells were incubated for 1h and then 1 ml of complete medium was added. The presence of cytopathic effect was monitored at 24 h, 48 h and 72 h.

The retention of virus in the column was assessed mixing 0.5 ml of the viral suspension with sterile water for the defined contact time, and then filtered through the Sephadex™ column and titrated with the most probable number (MPN) assay explained in section 6.3.6. The reduction factor was calculated as Log_{10}(MPN/ml before filtration) – Log_{10}(MPN/ml after filtration).

The efficiency of neutralization of possible PHMB virucidal activity by the Sephadex™ column system was tested. 0.5 ml of the biocide solution at 800 ppm or sterile water (control) was filtered through the column before mixing 0.45 ml of these solutions with 50 µl of the viral suspension (10^4 PFU/ml). After 10 min contact time, viral titer was obtained as described in 6.3.2.3. The reduction factor was calculated as Log_{10}(PFU/ml control) – Log_{10}(PFU/ml after treatment). The reduction factor should not excess 0.5 Log_{10} as recommended by EN 14476+A1 (CEN, 2002b) to be considered effective.

6.3.6. Suspension test

The suspension test was performed at 20°C and 40°C, and repeated three times.

24 well-plates were seeded with $5x10^5$ cells/well in 2 ml of complete growth medium and allowed to adhere overnight. 0.5 ml of Ad5 suspension (10^7-10^8 PFU/ml) was mixed with 4.5 ml of VANTOCIL™ TG and COSMOCIL™ CQ at 200 and 800 ppm. After the appropriate contact time (1, 5, 10 and 30 min), 0.5 ml of the mixture was placed in the Sephadex™ column and centrifuged at 400 x*g* for 1 min to separate the virus from the biocides. The mixture was further diluted 2 and 8 times after the treatment respectively at 200 and 800 ppm, in order to eliminate the cytotoxicity of PHMB on the 293T cells. The viral titer in the filtrate was then evaluated with the most probable number (MPN) assay. Specifically, the cell medium was replaced by 0.5 ml of serum-free medium, and 0.1 ml of the filtrate was inoculated. After an incubation period of 1h at 37°C under 5% CO_2, 1 ml of complete medium was added. Three 10-fold dilutions in 4 replicates were plated. Positive and negative wells for cytopathic effects were checked after 48h and 72h. The number of positive wells for each dilution factor was used to determine the most probable number of organisms (causing the positive results) per unit volume. The software utilised for the MPN calculation was MPN Calculator VB6 version (Curiale, 2004). Reduction factors were calculated as MPN/ml control – MPN/ml after treatment.

6.3.7. Hydrophobicity test

6.3.7.1. MATH

The same protocol used with MS2 [see Chapter 5] was applied for Ad5.

Hexadecane was utilised in this experiment. The biocide concentrations tested were 2 ppm, 20 ppm, 200 ppm, and 800 ppm.

0.5 ml of Ad5 suspension (10^8 PFU/ml) was mixed with 4.5 ml of PHMB biocides or deionised water (control) for 5 min. Then 1 ml of the mixture was mixed with 0.2 ml of hexadecane and hold at room temperature for 10 min. After 60 sec of vortexing, the two phases were allowed to separate at room temperature for 10 min. The amount of Ad5 in the aqueous phase was titrated with the immunostaining technique [see section 6.3.2.3]. The experiment was repeated three times in duplicate.

The percentage of Ad5 partitioned was calculated as: [(PFU/ml adsorbed to the hexadecane in the control / PFU/ml adsorbed to the hexadecane after the biocide treatment) x 100].

6.3.7.2. Cholesterol test

The experiment followed the same procedure of that performed with the phage MS2 [see Chapter 5].

A 5% w/v cholesterol emulsion was prepared adding 0.01% v/v of Tween 80 to increase the lipid solubility. The suspension was kept at 4°C and used at room temperature during the experiments.

The biocide concentrations tested were 2 ppm, 20 ppm, 200 ppm, and 800 ppm. A volume of adenovirus suspension (0.5 ml, 10^8 PFU/ml) was mixed with 4.5 ml of PHMB biocide or deionised water (control). After 5 min of contact time, 1 ml was mixed with 4 ml of the cholesterol emulsion. The mixture was constantly agitated at 150 RPM for 30 min at room temperature and then centrifuged twice for 30 min at 5000 x*g* at 20°C (Mistral 1000, MSE, London, UK), in order to separate the lipidic phase from the aqueous phase. The resultant quantity of virus in the supernatant was titrated through

the immunostaining technique [see section6.3.2.3]. The experiment was repeated three times in duplicate.

The percentage of Ad5 partitioned was calculated as: [(PFU/ml adsorbed to the cholesterol in the control/ PFU/ml adsorbed to the cholesterol suspension after the biocide treatment) x 100].

6.3.8. Dynamic light scattering

Dynamic light scattering (N4plus, Beckman Coulter, Fullerton, USA) was used to measure the size of the aggregates of the virus before and after the treatment with the PHMB-based biocides [see Chapter 5]. The purified virus suspension [see section 6.3.3] was dialysed overnight against PBS and then filtered through 0.22 μm filter. Four different concentrations of PHMBs were tested, 2, 20, 200 and 800 ppm at 10°C, 20°C and 40°C.

Dynamic light scattering measurement were run for 200 sec, repeated 3 times using a laser angle of 90°. The mean of the 3 measurements was used to categorise the size of aggregates. In order to simplify the reading, arbitrary size categories have been chosen. The threshold of the smaller category size was selected according to the theory of "the 16 particle aggregate". This theory established that an aggregate formed of 16 particles of identical diameter is needed to protect one particle, giving complete protection from the medium around (Thurman & Gerba, 1988a). In the case of Ad5 (diameter 100 nm) the threshold calculated was 250 nm, with the assumption that aggregates are globular.

6.3.9. Protein analysis

The PhastSystem (Amersham Pharmacia Biotech AB, Buckinghamshire, UK) was used. The protocol used for MS2 [see Chapter 4, section 4.3.2] was applied to Ad5. Titration of proteins was performed by the Lowry method [see Chapter 4, section 4.3.2.1]

6.3.9.1. Sample preparation

The purified Ad5 in PBS [see section 6.3.3] was used to perform SDS-PAGE. A volume of the viral suspension (0.375 ml) (10^7 PFU/ml) was mixed with 0.125 ml of the PHMB biocide to get a total volume of 0.5 ml. The concentrations of biocide tested were 200 and 800 ppm. The control was treated with deionised water instead of biocide.

After 5 min contact time, 0.2 ml was withdrawn to perform the Lowry titration and 0.3 ml was used for the ongoing experiment. Four volumes of cold acetone (-20°C) (1.2 ml) were added to the 0.3 ml of sample and then incubated overnight at -20°C to precipitate the proteins and to eliminate any acetone soluble interferences. After a centrifugation at 19000 x*g* for 15 min at 4°C, the supernatant was discharged and the sample was air dried. The pellet was re-suspended in 20 µl of 2x sample buffer, and boiled for 5 min. In order to precipitate any insoluble materials, a quick centrifugation (10 sec) was performed before loading 10 µl of sample on the gel. Low range (MW 6500-66000) and a wide range (MW 6500-205000) molecular weight markers were used (Sigma, St. Louis, MO, USA). Five µl was directly loaded on the gel.

6.3.9.2. Gel preparation

SDS-Polyacrylamide 20% homogeneous gel (GE Healthcare Bio-Sciences AB, Uppsale, Sweden) was used, following the separation technique file No. 110 (Anonymous, 1986a). Silver staining development technique file No. 210 was used (Anonymous, 1986b).

6.3.9.3. Eosin Y staining

Attachment of PHMB to viral proteins was measured qualitatively with the colourimetric assay Eosin Y (Gilbert & Moore, 2005) which was used for MS2 [see Chapter 5, section 5.3.6.1]. The same sample which was used for the silver stained SDS-PAGE gel was processed through the Eosin Y procedure.

6.3.10. Electron microscopy

A transmission electron microscope was used to investigate structural damage on the virus capsid caused by the PHMBs. Twenty µl of concentrated virus stock (10^{10} PFU ml^{-1}) was treated with 20 µl of biocide to achieve a final concentration of 200 and 800 ppm. The treatment was performed at 20°C. After 10 min exposure, the sample was mixed with 40 µl of 2% methylamine tungstate for 15 min. Ten µl of the negatively stained virus mix was placed on a nickel- or copper-coated grid and left drying for 20 min. The excess liquid was removed with filter paper. The grid was examined in a Philips EM 400T electron microscope operating at 80 kV accelerating voltage at magnification of X28000-44000. Control consisted of Ad5 treated with deionised water instead of PHMBs.

6.3.11. Statistical analysis

Suspension tests, hydrophobicity tests and the SDS-PAGE were repeated three times, whereas dynamic light scattering was performed twice. The electron microscopy was carried out on one sample, and 15 different fields were considered.

Statistical analysis was performed using Statistica 7.0 software (StatSoft Inc, Tulsa, USA). Statistical differences were tested through the analysis of variance (one-way ANOVA), and Tukey test was used as a post-hoc test.

Light scattering results were analyzed by non parametric tests, $\chi 2$ test.

Pearson correlation coefficient was calculated for the inactivation kinetic curves.

6.4. Results

6.4.1. Preliminary cytotoxicity tests

The use of three different neutralisers did not remove the toxicity of the PHMB compounds. In addition, the neutralisers were found to be toxic to the cells as well. Cytopathic effects were visible after 24h incubation (Table 6-4).

The Amicon Ultra 0.5 10 KDa centrifugal filter devices provide fast ultrafiltration with a cut-off of 10 KDa, enough to release PHMB molecules (2.5 to 3.1 KDa) but retain the virus (150-180 x 10^3 KDa). When the PHMB solution was inoculated on the cells after being filtrated through the device, the presence of cytotoxicity was observed after 24 h incubation. The same results were achieved with and without the use of the neutraliser (Table 6-4).

Table 6-4. Preliminary cytotoxicity tests results.

	% cytopathic effects			
	24 h			
	Neutraliser A	**Neutraliser B**	**Neutraliser C**	**Amicon Ultra 0.5**
VANTOCIL™ TG	100	100	100	100
COSMOCIL™ CQ	100	100	100	100

Values are % of wells presenting cytopathic effects. Results relative to the undiluted filtrate.

6.4.2. Sephadex™ column system

A further method was considered in order to eliminate the cytotoxicity of molecules tested. The method was based on Sephadex™ column suggested by Geller *et al* (2009). Sephadex™ is usually used in gel filtration. It is a bead-formed gel prepared by cross-linking dextran with epichlorohydrin. There are several types of Sephadex™ and the degree of cross-linking influences the extent to which macromolecules pass through. Sephadex™

G-25 has a fractionation range for globular proteins of 1000–5000 molecular weight (GE Healthcare, 2009).

6.4.2.1. Controls

Three controls were undertaken prior to performing the actual suspension test: elimination of the cytotoxicity of PHMB molecules, the non-retention of viral particles in the Sephadex™ column and the neutralization efficacy of the Sephadex™ column.

PHMB molecules appear to be highly cytotoxic to the 293T cell line used to grow the Ad5 [see section 6.4.1]. Therefore the elimination of the cytotoxicity was essential in order to assess any virucidal activity of two PHMB-based biocides. The assessment was performed using VANTOCIL™ TG and COSMOCIL™ CQ at 100, 200 and 800 ppm. Only with the lowest concentration, 100 ppm, did the Sephadex™ column eliminated cytotoxicity, since no effects on the cells were visible after 72 h of incubation (Table 6-5). Results were consistent along the experiments (n=3).

Table 6-5. Cytotoxicity test results after filtration through Sephadex™ columns.

	% cytopathic effects					
	VANTOCIL™ TG			COSMOCIL™ CQ		
	100 ppm	200 ppm	800 ppm	100 ppm	200 ppm	800 ppm
24h	0	100	100	0	25	100
48h	0	100	100	0	50	100
72h	0	100	100	0	100	100

Values indicate the % of wells presenting cytopathic effects. Results relative to the undiluted filtrate.

The retention of Ad5 in the column was measured. The initial viral titre was approximately 10^8 PFU/ml. Reduction factors were calculated as the

difference between the viral titre before and after the filtration through the Sephadex™ column. Reductions of 0.37 ± 0.17, 0.15 ± 0.30, 0.05 ± 0.35, 0.00 ± 0.28 for time of exposure of 1, 5, 10 and 30 min were measured (Table 6-6). The contact time did not affect the retention of the virus in the column (ANOVA, $F_{1,8}$=0.72, P>0.05). Concluding, reductions of viral titre after filtering though the Sephadex™ column were not significant, even if aggregates were present. In fact, Sephadex™ column is based on gel filtration chromatography, therefore only molecules smaller than the pore size of the bead-formed gel (1000–5000 Da) are trapped inside the column. So viral aggregates (>100 KDa) leave the column even faster than individual particles.

Table 6-6. Non-retention test of Ad5 by the Sephadex™ column.

Contact time (min)	Pre - MPN/ml	Post - MPN/ml	RF
1	7.23 ± 0.15	6.85 ± 0.17	0.37 ± 0.17
5	7.23 ± 0.15	7.08 ± 0.29	0.15 ± 0.30
10	7.23 ± 0.15	7.18 ± 0.35	0.05 ± 0.35
30	7.23 ± 0.15	7.23 ± 0.29	0.00 ± 0.28

RF: reduction factors expressed in Log_{10}

Values are the mean of three replicates.

The last control performed was on the efficacy of the Sephadex™ column system to neutralise the activity of PHMB. A 0.5 Log_{10} difference between the viral titre before and after the treatment was considered the maximum value to accept the validity of the system (EN 14476+A1, CEN, 2002b).
The reduction factors of VANTOCIL™ TG and COSMOCIL™ CQ were 0.13 ± 0.14 and 0.12 ± 0.13, respectively. Controls consisted of mixing the Ad5 and the filtrate from previous filtrations containing only distilled water, instead of the biocides. The PFU/ml of control did not differ significantly from the PFU/ml of the mixture of Ad5+filtrate from previous filtrations containing the PHMBs (t-test P>0.05). The reduction factors were less than 0.5 Log_{10} (Table 6-7), therefore the neutralization was considered valid.

Table 6-7. Ad5 neutralisation efficacy test.

	PFU/ml control	PFU/ml after	RF
VANTOCIL™ TG	7.81 ± 0.17	7.62 ± 0.12	0.09 ± 0.12
COSMOCIL™ CQ	7.81 ± 0.17	7.69 ± 0.12	0.10 ± 0.12

The biocides concentration tested was 800 ppm.

RF is reduction factor. Values are expressed in Log_{10} and are the mean of three replicates.

6.4.3. Virucidal activity – suspension test

VANTOCIL™ TG and COSMOCIL™ CQ were tested at 200 and 800 ppm. The cytotoxicity tests showed that at 100 ppm PHMB toxicity against cells was not present. Such a concentration was not considered in the suspension tests for two reasons: 200 and 800 ppm are the manufacturer's recommended concentrations and compare directly the results of the human virus with the phage MS2. The treatment was also performed at 40°C as well as 20°C, since the higher virucidal activity observed with MS2 at raised temperatures [see Chapter 5]. In order to test the biocides at 200 and 800 ppm without having cytotoxic effects, samples were diluted before inoculating 293T cells to get a final PHMB concentration of 100 ppm. Furthermore, as the Sephadex™ column system did not retain any viral particles [see 6.4.2.1], the reductions observed were considered result only of the activity of the two biocides.

VANTOCIL™ TG tested at 200 ppm at 20°C reduced the Ad5 titre by factors ranging from 0.41 to 1.06 Log_{10} (Table 6-8). The contact time significantly influenced the activity (ANOVA, $F_{1,8}=4.74$, $P\leq0.05$), specifically the efficacy at 5 min differed from 30 min (Tukey test, $P\leq0.05$). At 800 ppm, reductions of 1 Log_{10} (90%) were achieved after 5, 10 and 30 min contact. These reductions in viral concentration were significantly higher than following 1 min exposure (ANOVA, $F_{1,8}=16.84$, $P\leq0.05$) where no reduction was found (Table 6-8). Comparing 200 and 800 ppm results, the highest concentration significantly enhanced the virucidal activity only at the 10 min time exposure (t-test, $P\leq0.05$), whereas a significantly lower

reduction was found after 1 min (t-test, $P \leq 0.05$). Times of exposure of five and thirty min did not differ in activity (t-test, $P > 0.05$).

COSMOCIL^{TM} CQ was tested only at 30 min contact time. Reductions of 0.03 ± 0.27 Log_{10} and 1.33 ± 0.17 Log_{10} were found respectively at 200 and 800 ppm (Table 6-8). Comparing with VANTOCIL^{TM} TG, a significant difference only occurred at 200 ppm (t-test, $P \leq 0.05$). No further investigation on COSMOCIL^{TM} CQ activity was considered, as it showed lack of activity at 200 ppm and an efficacy similar to VANTOCIL^{TM} TG at 800 ppm.

VANTOCIL^{TM} TG was also tested at 40°C. Reductions of up to 1.74 Log_{10} were observed (Table 6-8), and contact time did not affect the virucidal activity (ANOVA, $F_{1,8}=2.69$, $P > 0.05$). A comparison of the activity at 20°C showed that the higher temperature significantly enhanced the ability to kill Ad5 after 1 and 30 min of exposure (t-test, $P \leq 0.05$) (Table 6-8).

Table 6-8. Suspension test. Activity of VANTOCIL™ TG and COSMOCIL™ CQ against Ad5.

			RF			
	T °C	**Concentration (ppm)**	**Contact time (min)**			
			1	**5**	**10**	**30**
VANTOCIL™ TG	20	200*	0.41 ± 0.14	0.32 ± 0.47	0.67 ± 0.12	1.06 ± 0.16
		800¥	0.09 ± 0.14	1.06 ± 0.20	1.24 ± 0.16	0.77 ± 0.32
	40	800	0.75 ± 0.33	1.74 ± 0.56	1.09 ± 0.60	1.42 ± 0.22
COSMOCIL™ CQ	20	200	NT	NT	NT	0.03 ± 0.27
		800	NT	NT	NT	1.33 ± 0.17

* Significant difference between 5 and 30 min contact time.

¥ Significant difference between 1 and all other tested contact times.

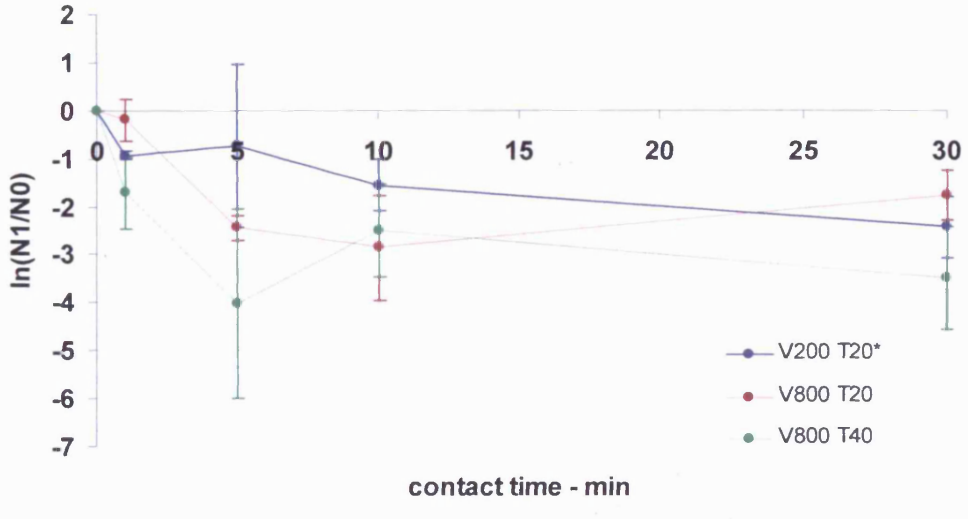
RF: reduction factor. Values are express as Log_{10} and are the mean of three replicates.

NT: not tested.

Figure 6-1 shows the inactivation kinetics of VANTOCIL™ TG tested at 200 and 800 ppm and at two different temperatures, 20°C and 40°C.

Only the treatment at 20°C with a concentration of 200 ppm achieved significant linearity (r=-0.91, $P \leq 0.05$). No linear relationship was detected during the other two treatments. Furthermore, a high variability was observed at 40°C (Figure 6-1).

Figure 6-1. Inactivation kinetics for VANTOCIL™ TG at 200 and 800 ppm against Ad5 tested at 20°C and 40°C by suspension test.



* significant Pearson coefficient.

V200 T20: 200 ppm at 20°C. V800 T20: 800 ppm at 20°C. V800 T40: 800 ppm at 40°C.

Values are the mean of three replicates. Bars represent standard deviation.

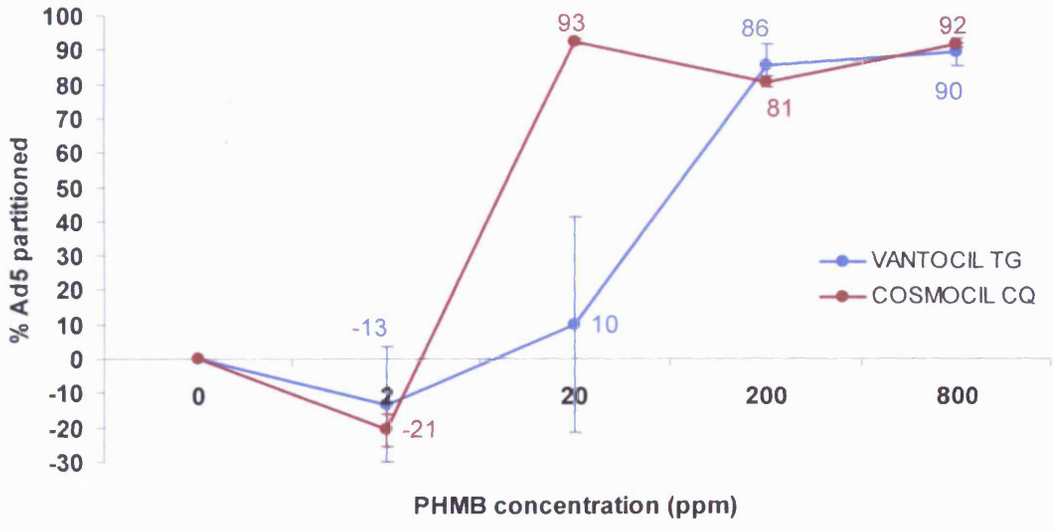
6.4.4. Hydrophobicity test

6.4.4.1. MATH

Ad5 surface properties were altered by the presence of the two biocides. The virus become more hydrophobic as the concentration of the PHMB increased (Figure 6-2). Regarding VANTOCIL™ TG, at 200 and 800 ppm

90% of the virus was found in the hydrocarbon phase, a significantly higher proportion compared to 2 and 20 ppm (ANOVA, $F_{1,8}$=24.9, $P \leq 0.05$). COSMOCIL™ CQ, instead, significantly changed the hydrophobicity of Ad5 after the treatment at 20 ppm (ANOVA, $F_{1,8}$=32.9, $P \leq 0.05$).

Figure 6-2 MATH test of Ad5 treated with VANTOCIL™ TG and COSMOCIL™ CQ.

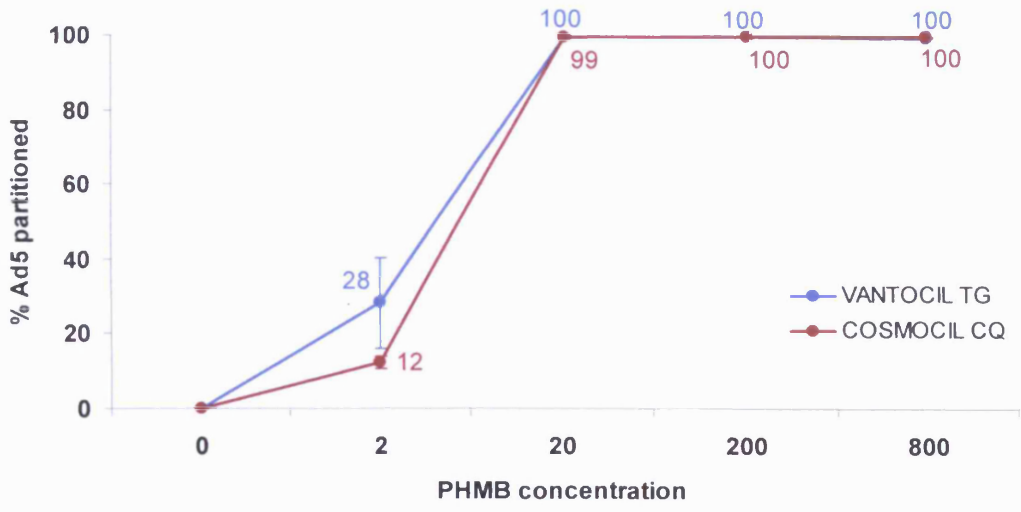


Percentage of Ad5 partitioned in the hydrocarbon phase.
Values are the mean of three replicates. Bars are standard deviation.

6.4.4.2. Cholesterol test

The cholesterol test confirmed what was already found with the MATH test. The hydrophobicity of the Ad5 surface increased significantly with the increasing PHMB concentration of VANTOCIL™ TG (ANOVA, $F_{1,8}$=103.8, $P \leq 0.05$) and of COSMOCIL™ CQ (ANOVA, $F_{1,8}$=9427.7, $P \leq 0.05$). The percentage of virus partitioned in the lipid phase was around 100% following the treatment at 20 ppm (Figure 6-3).

Figure 6-3. Cholesterol test of Ad5 treated with VANTOCIL™ TG and COSMOCIL™ CQ.



Percentage of Ad5 partitioned in the lipidic phase (cholesterol).
Values are the mean of three replicates. Bars are standard deviation.

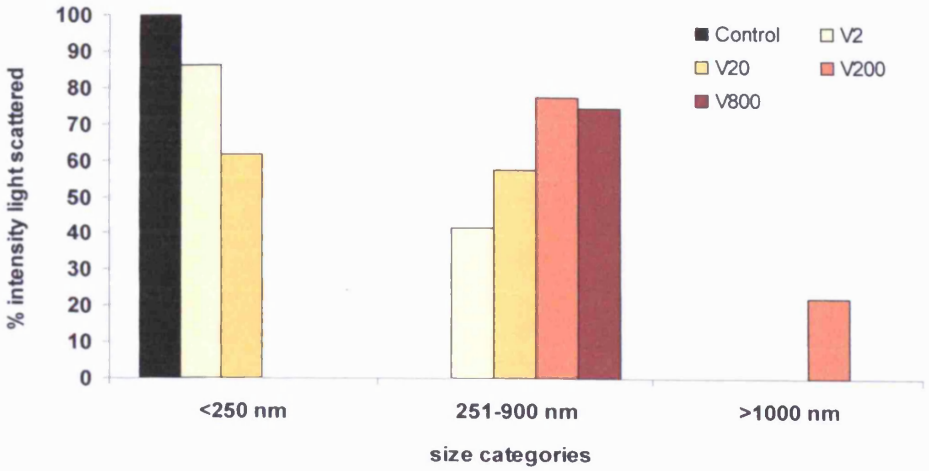
6.4.5. Dynamic light scattering

Results of the dynamic light scattering showed an overall increase in the number of aggregates as the concentration of the PHMB increased. Controls, which described the distribution of adenovirus before the biocide treatment, consisted mostly of single particles (from Figure 5-3 to Figure 6-9).

At the three different temperatures (10°C, 20°C, 40°C), the increasing VANTOCIL™ TG concentration increased significantly the number of clumps, comparing with the control (χ^2 test, $P \leq 0.01$). Interestingly, at 200 ppm the aggregates size measured over a micrometer at all temperatures, decreasing at 800 ppm to 430 ± 94 nm at 10°C and 779 ± 25 nm at 20°C. At 40°C, instead the clump size remained above one micrometer.

COSMOCIL™ CQ concentration also enhanced the size of aggregates at 10°C, 20°C and 40°C (χ^2 test, $P \leq 0.01$). Measurements up to a micrometer occurred at 2 and 20 ppm at 10°C, at 800 ppm at 20°C and at 20 ppm at 40°C (Figure 6-7, Figure 6-8, Figure 6-9).

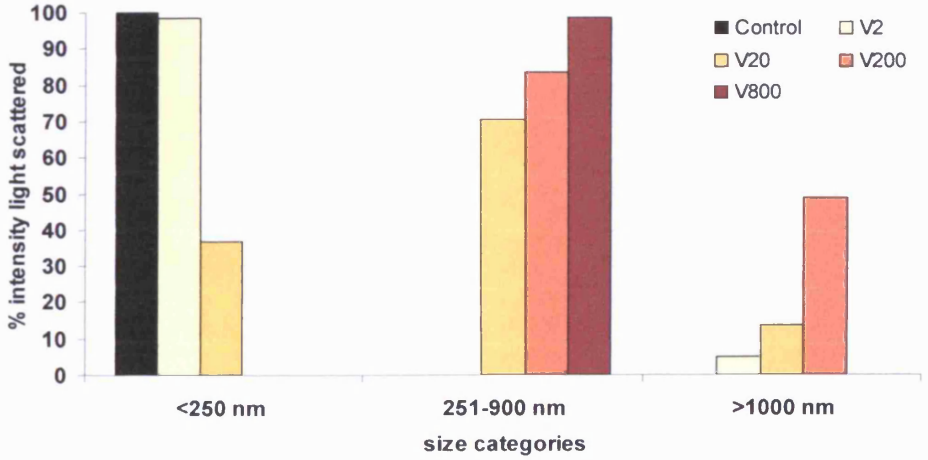
Figure 6-4 Dynamic light scattering. Ad5 treated with VANTOCIL™ TG at 10°C.



Distribution of three size groups of Ad5 aggregates as a function of the percentage intensity of the light scattered. The magnitude of each histogram is proportional to the percentage of the total scattered intensity caused by the particles of the corresponding group size.

V2: 2 ppm, V20: 20 ppm, V200: 200 ppm, V800: 800 ppm.

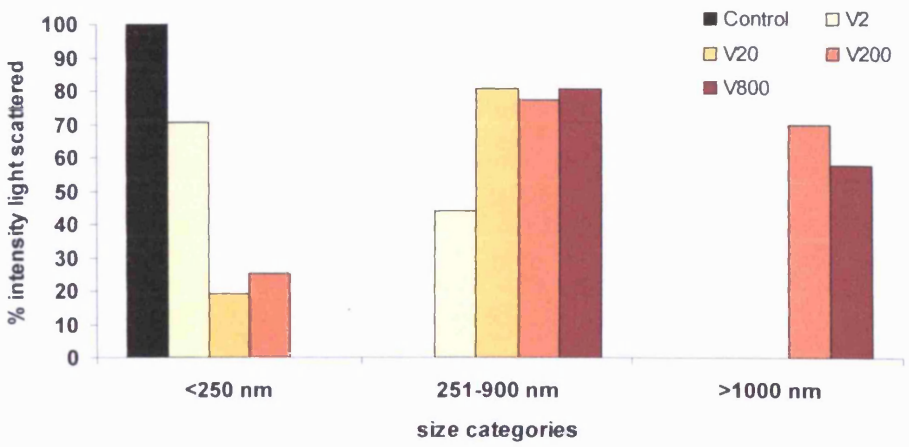
Figure 6-5. Dynamic light scattering. Ad5 treated with VANTOCIL™ TG at 20°C.



Distribution of three size groups of Ad5 aggregates as a function of the percentage intensity of the light scattered. The magnitude of each histogram is proportional to the percentage of the total scattered intensity caused by the particles of the corresponding group size.

V2: 2 ppm, V20: 20 ppm, V200: 200 ppm, V800: 800 ppm.

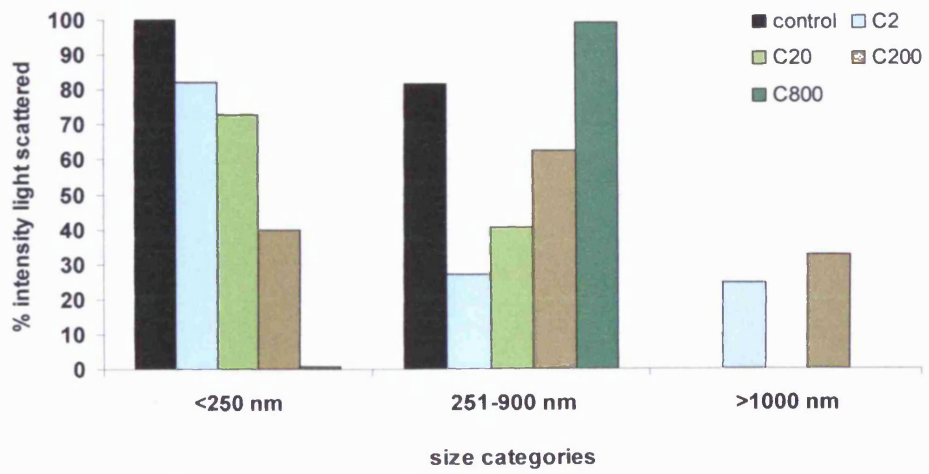
Figure 6-6 Dynamic light scattering. Ad5 treated with VANTOCIL™ TG at 40°C.



Distribution of three size groups of Ad5 aggregates as a function of the percentage intensity of the light scattered. The magnitude of each histogram is proportional to the percentage of the total scattered intensity caused by the particles of the corresponding group size.

V2: 2 ppm, V20: 20 ppm, V200: 200 ppm, V800: 800 ppm.

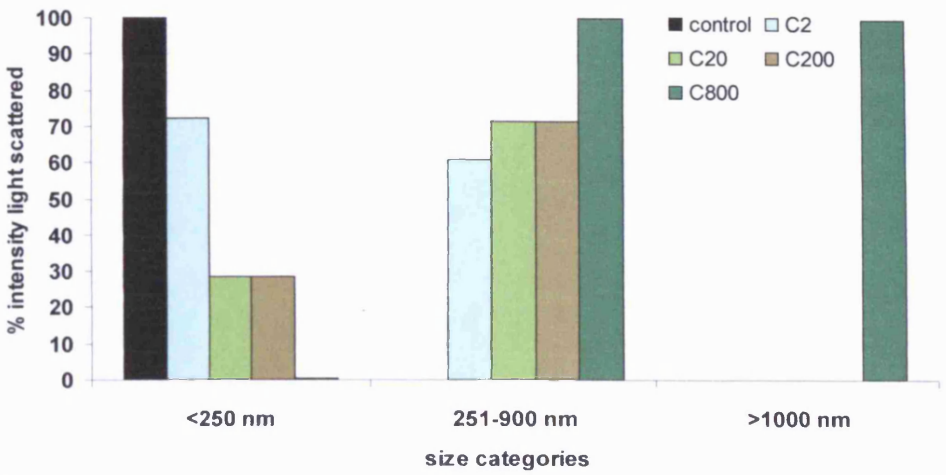
Figure 6-7. Dynamic light scattering. Ad5 treated with COSMOCIL™ CQ at 10°C.



Distribution of three size groups of Ad5 aggregates as a function of the percentage intensity of the light scattered. The magnitude of each histogram is proportional to the percentage of the total scattered intensity caused by the particles of the corresponding group size.

C2: 2 ppm, C20: 20 ppm, C200: 200 ppm, C800: 800 ppm

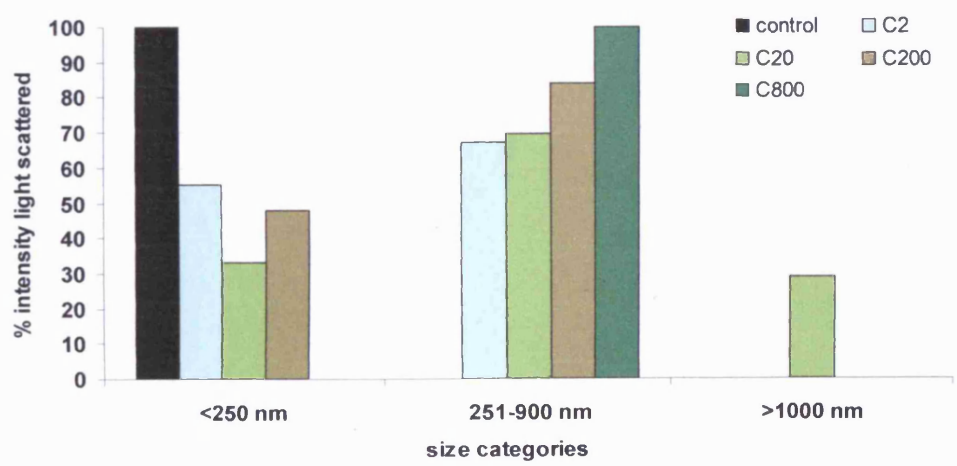
Figure 6-8 Dynamic light scattering. Ad5 treated with COSMOCIL™ CQ at 20°C.



Distribution of three size groups of Ad5 aggregates as a function of the percentage intensity of the light scattered. The magnitude of each histogram is proportional to the percentage of the total scattered intensity caused by the particles of the corresponding group size.

C2: 2 ppm, C20: 20 ppm, C200: 200 ppm, C800: 800 ppm

Figure 6-9 Dynamic light scattering. Ad5 treated with COSMOCIL™ CQ at 40°C.



Distribution of three size groups of Ad5 aggregates as a function of the percentage intensity of the light scattered. The magnitude of each histogram is proportional to the percentage of the total scattered intensity caused by the particles of the corresponding group size.

C2: 2 ppm, C20: 20 ppm, C200: 200 ppm, C800: 800 ppm

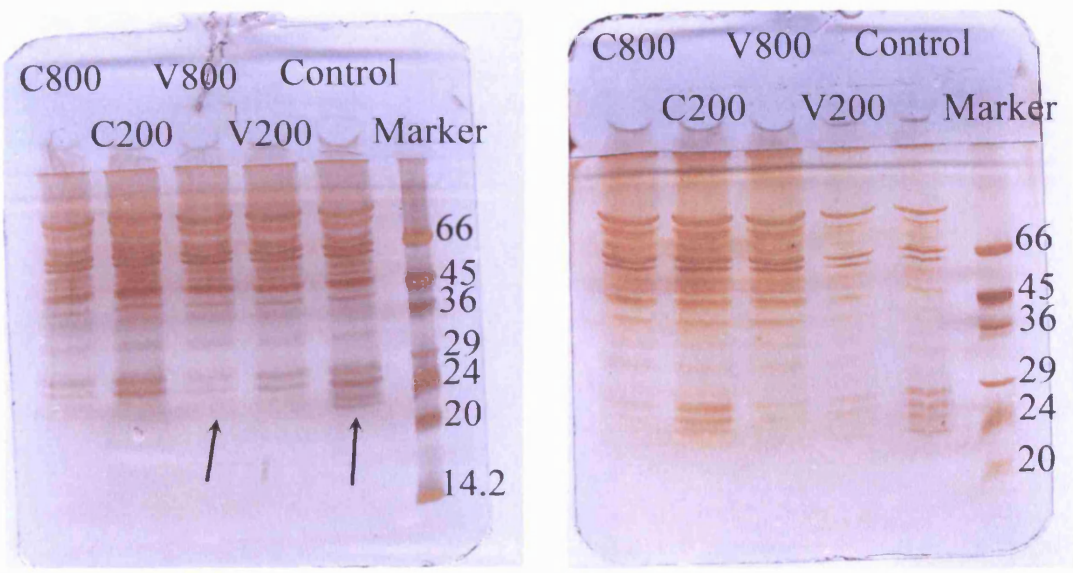
6.4.6. Protein analysis

The SDS-PAGE analysis was performed after treatment at 20°C and 40°C. The Ad5 protein pattern was consistent during the replicates (n=3) at both temperatures. The amount of proteins in the samples was in the range of 0.07 to 0.2 mg/ml.

Fourteen bands were present in the control. After contact with VANTOCIL^{TM} TG at 200 ppm, the two lowest molecular weight bands disappeared. According to the Ad5 proteins profile, the band of 22 KDa corresponds to the minor coat protein VI (Rux & Burnett, 2004), whereas the one at 19KDa correspond to the core protein VII (Chatterjee *et al.*, 1985). This profile was enhanced by treatment at a higher concentration, 800 ppm. No differences were observed in the protein pattern between 20°C and 40°C (Figure 6-10).

The gel stained with the Eosin Y showed the PHMB attached to the viral capsid. The amount of PHMB on the capsid is higher when treated at 20°C (brighter pink bands). The intensity of the stain increased with the concentration of the active compound (Figure 6-11).

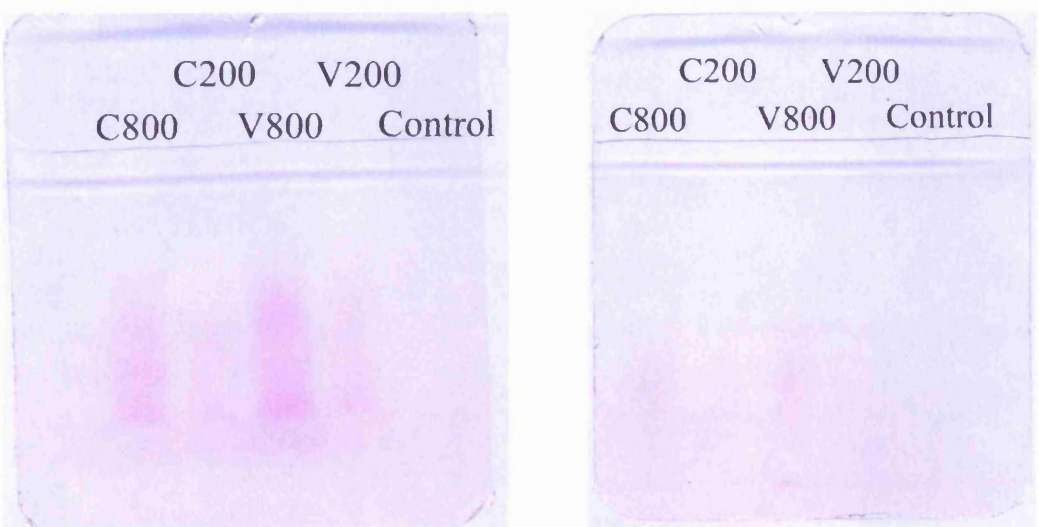
Figure 6-10 SDS-PAGE of Ad5 treated with VANTOCIL™ TG and COSMOCIL™ CQ at 200 and 800 ppm. A. treatment at 20°C. B. treatment at 40°C.



A B

Protein molecular weights are in KDa. Arrows indicate the two proteins, the coat protein VI and the core protein VII, affected by the treatment.

Figure 6-11. Eosin Y stained SDS-PAGE gels of Ad5 treated with VANTOCIL™ TG and COSMOCIL™ CQ at 200 and 800 ppm. A. treatment at 20°C. B. treatment at 40°C.



A B

The pink bands represent the PHMB bands.

6.4.7. Transmission electron microscopy

The electron microscopy investigation revealed a variety of damage to the Ad5 capsid (Figure 6-12 and Figure 6-13).

Representative samples of 120-150 viruses were observed in 15 separate fields for each treatment. Each observed virus was placed into two categories, intact and broken. The un-treated sample was composed mainly of intact Ad5 (94%), whereas the PHMB treated samples showed significantly higher amount of damaged particles (χ^2 test, $P \leq 0.01$) (Table 6-9). 36% and 56% of broken capsid were observed after contact with VANTOCIL™ TG at respectively 200 and 800 ppm. Similar results were obtained for COSMOCIL™ CQ, 28% and 61% of damaged viruses after treatement with 200 and 800 ppm of the biocide.

Various types of damages were observed in the micrographs. After PHMB treatment at 200 ppm broken capsid was the main damage visible (Figure 6-12 C-D). Increasing the concentration of the active compound to 800 ppm, led to a collapsed head (

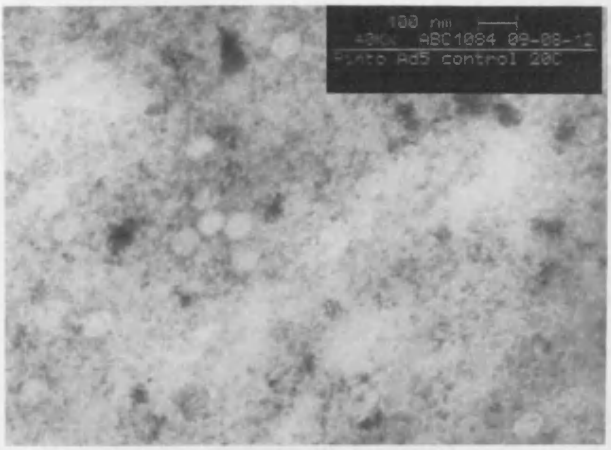
Figure 6-13 A-B) and precise breakage of the capsid (arrows in Figure 6-13 A). Furthermore, genetic material outside the viral particle was observed (circles in Figure 6-13 B-C).

Table 6-9. Electron microscopy investigations with Ad5 treated with VANTOCIL™ TG and COSMOCIL™ CQ at 200 and 800 ppm.

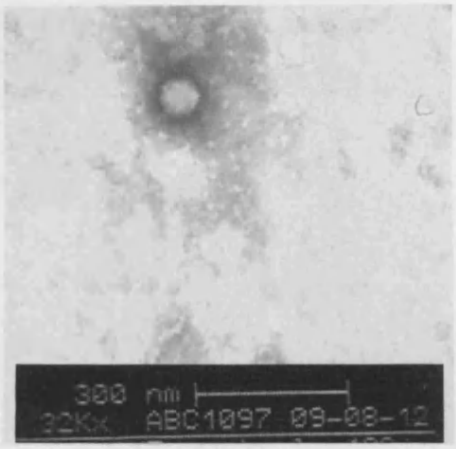
	Intact	Broken
Control	94	6
V200	64	36
V800	44	56
C200	72	28
C800	39	61

Proportion are in percentage taken from 128 control viruses, 148 VANTOCIL™ TG 200 ppm treated viruses, 148 VANTOCIL™ TG 800 ppm treated viruses, 137 COSMOCIL™ CQ 200 ppm treated viruses, 110 COSMOCIL™ CQ 800 ppm treated viruses.

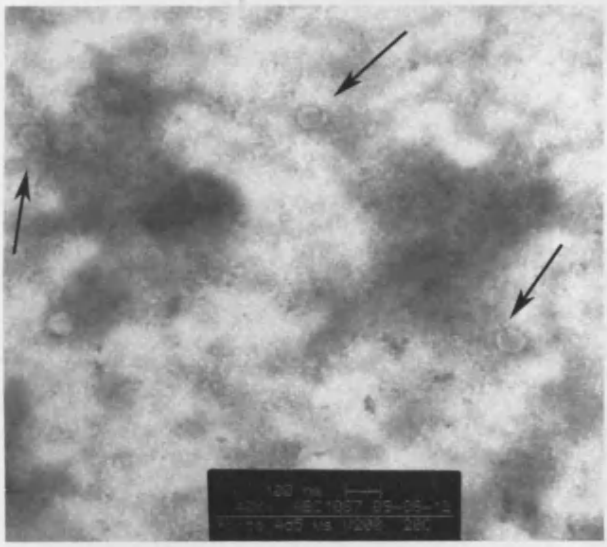
Figure 6-12. Micrographs of Ad5 treated with PHMBs at 200 ppm.



A



B

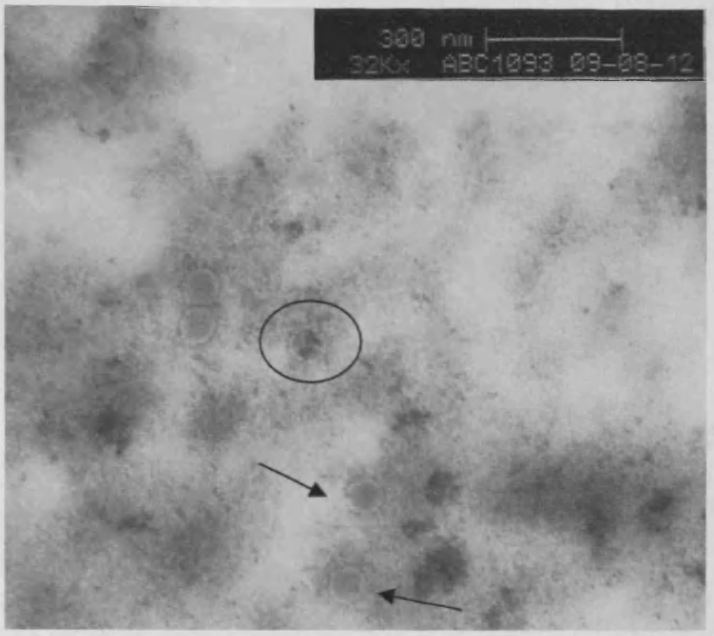


C

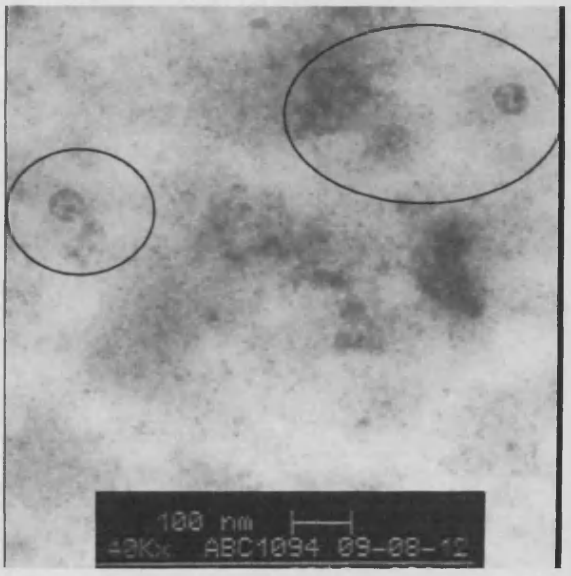
D

A-B) Ad5 untreated. C) Ad5 treated with VANTOCIL™ TG. D) Ad5 treated with COSMOCIL™ CQ. Arrows in figure C indicate damage capsid. These micrographs are representative of observations from 15 separate fields.

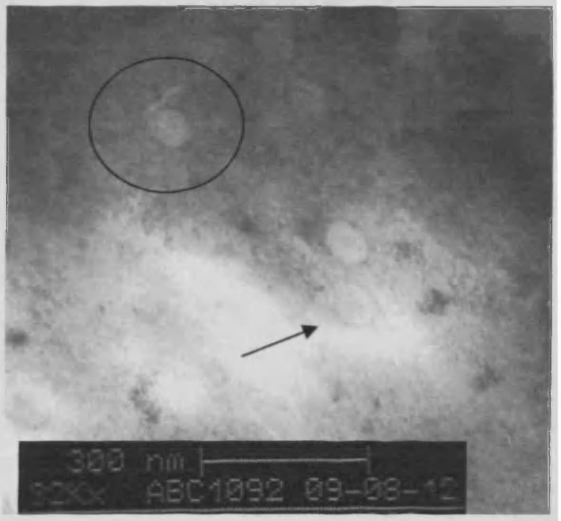
Figure 6-13. Micrographs of Ad5 treated with PHMB at 800 ppm.



A



B



C

A-B) Ad5 treated with VANTOCIL™ TG. C) Ad5 treated with COSMOCIL™ CQ. Arrows in figure A and C indicate precise damage of capsid, circles indicate collapsed capsid (A-B) and the released genetic materials (B-C) These micrographs are representative of observation from 15 separate fields.

6.5. Discussion

This chapter investigated the virucidal activity of PHMB-based biocides against an human non-enveloped virus and their possible interactions.

One of the major problems when testing the virucidal activity of a compound is to overcome the biocide-induced cytotoxicity on the cell line (Sattar *et al.*, 2003). Various attempts have been made, such as the use of dilution (Wood & Payne, 1998) which required an initial high viral titre to be able to detect 4 Log_{10} reduction. A washing step for the cells after the attachment of the virus to the host (Gordon *et al.*, 1993), the use of neutralisers (Woolwine & Gerberding, 1995; Rutala *et al.*, 2006) and the use of foetal bovine serum or bovine serum albumine (Gordon *et al.*, 1993) have been other possibilities. However, not all such methods assured the elimination of cytotoxicity and the biocide neutralization. PHMB was found to be cytotoxic against the 293T cell line. A dilution step and washing step were not applicable, since cytotoxicity was observed even after 1h and a high viral titre was required. After several failed attempts at neutralization, a Sephadex™ column system (Geller *et al.*, 2009) resulted the efficient elimination of cytotoxicity and neutralising the PHMB activity in 1 min.

The ability of biocides to kill adenoviruses varied widely, according to the serotype and the biocides used. Previous studies with enteric adenoviruses (type 40 and 41) showed that free chlorine was effective at killing the two serotypes (Thurston-Enriquez *et al.*, 2003a; Baxter *et al.*, 2007), but type 40 and 41 showed more resistance than other serotypes to UV treatment (Thurston-Enriquez *et al.*, 2003b; Baxter *et al.*, 2007). Adenovirus type 8, which is associated with epidemic keratoconjunctivitis, was inactivated (3 Log_{10} reduction) by 0.55% ortho-phtalaldehyde, 2.4% glutaraldehyde and 1900 ppm chlorine after 1 and 5 min; whereas the biguanide chlorhexidine did not cause any reduction ($\leq$ 1 Log_{10}) (Rutala *et al.*, 2006). Concerning Ad5, 2-butanone peroxide was able to reduce the titre by >4 Log_{10} after 60 min contact time. Other biocides, such ethanol, sodium hypochlorite, glutaraldehyde and peracetic acid based compounds resulted active against the serotype 5 even in the presence of blood and bovine serum albumin

(Eterpi *et al.*, 2009). However, chlorhexidine gluconate, which is a biguanide, appeared enable to remove the adenovirus from contaminated eyelid specula (Hutchinson *et al.*, 2000). Such lack of activity is partially in line with the findings from the two PHMB-based biocides tested here, VANTOCIL™ TG and COSMOCIL™ CQ. Low reductions ($\leq$1.5 Log_{10}) occurred after up to 30 min contact time at 20°C. The concentration of the active compound did not influence the efficacy, whereas by increasing the temperature to 40°C, a slight but significant enhancement of the virucidal activity was observed. Such result might be explained by morphological alterations of the viral capsid at 40°C which might help the action of the PHMB. In fact, Rexroad *et al.* (2003) found that a capsid degradation and loss of penton capsomers occurred in adenovirus type 5 subjected to a temperature higher than 45°C.

The disinfection kinetic analysis generally showed a lack of linear relationship between the inactivation rate and contact time. A "tailing off" effect was visible instead, which is usually explained by biocide depletion or by the presence of more a resistant viral subpopulation due to aggregation, conformational change of the viral capsid or genetic variation (Hoff & Akin, 1986). Here, the formation of clumps might be the cause and was highlighted by the light scattering analysis, which showed aggregates up to a micrometer in size after the treatment with PHMB. Furthermore, Ad5 surface hydrophobicity increased after contact with VANTOCIL™ TG and COSMOCIL™ CQ. Since the pI values of the adenovirus major capsid proteins are between 5 and 5.89 (Favier *et al.*, 2004), the virus charge during the treatment (pH 7.5) is negative, thus attracting the positively charged PHMB molecules. Such electrostatic interaction will reduce the charge on the Ad5 capsid, so that viral particles may aggregate in a hydrophilic medium. This hypothesis is supported by previous work where aggregation was found responsible for preventing virucidal activities (Sharp & Kim, 1966; Sharp *et al.*, 1975; Siyikanchana *et al.*, 2008).

Structural damage was visible during the electron microscopy investigation, especially the treatment with PHMB at 800 ppm which induced precise points of disruption on the capsid, often with the release of the genome. Such damage was also confirmed by the SDS-PAGE gel, where the two

proteins, the minor coat protein VI (22 KDa) and the core protein VII (19 KDa), were altered. The coat protein is possibly associated with the protein IIIa and the penton base and mediates the disruption of the endosomal membrane to allow the transport of the virus towards the nucleus (Russell, 2009). Since the proteins IIIa and penton base appeared to be the weakest part of the adenovirus capsid (Rexroad *et al.*, 2003), the damage caused by the PHMB to the protein VI could explain the broken capsid. The additional damage to the core protein VII could support such an hypothesis, since its function is in viral assembly interacting with the viral DNA (Zhang & Arcos, 2005). Furthermore, the SDS-PAGE gel stained with the Eosin Y showed the presence of PHMB on the virus at 20°C, confirming the attachment of the biocide to the adenovirus, possibly through electrostatic forces as explained by the change in viral surface hydrophobicity. At 40°C the intensity of the Eosin Y stained bands is decreased, indicating a lower amount of PHMB associated to the capsid. Higher capsid damage and therefore a higher penetration of the biocide could explain such result, confirming also the enhancement of the virucidal activity observed at 40°C.

Comparing Ad5 with the bacteriophage MS2, some similarities were observed. The reduction factors caused by PHMB at 20°C against the human virus were in agreement with the values obtained against the coliphage at the same temperature. Differences were visible as the temperature was increased. So, while the virucidal activity against Ad5 increased moderately, the MS2 titre was reduced up to 4-5 Log_{10}. According to the results, the degree of aggregation of the two viruses appeared to be different. The temperature seemed to affect only the aggregate size of MS2, although the surface properties of both viruses were similar (both viruses negatively charged at pH 7); they both increased their hydrophobicity and PHMB molecules were found attached to both viral capsids. Further investigations are needed to understand the causes of such a difference in sensitivity. It is possible that the strength of hydrophobic forces in the clumps might be different for the two viruses, with lower energy necessary to break up the MS2 aggregates. In addition, Ad5 has a more complex structure than the coliphage, with various proteins composing the capsid. Such complexity

could increase the forces involved (hydrophobic, electrostatic and Van der Waals) in the formation of aggregates and in the viral particle itself. In a study of the evaluation of the effect of temperature on the time-dependent MS2 inactivation, thermodynamic parameters were evaluated: an activation energy of 50.5 kJ/mol was required to develop an MS2 transition state that resulted in loss of infectivity (Anders & Chrysikopoulos, 2006). Conversely, an activation energy of 88 kJ/mol was required for breaking the capsid of adenovirus (Svensson & Persson, 1984). Another significantly difference between Ad5 and MS2 is in their genome. The RNA of MS2 contains several coat protein-binding sites, supporting the hypothesis that the genome is closely bound to the phage capsid (Koning *et al.*, 2003). Therefore, any breakage to the capsid caused by the PHMB could damage the MS2 nucleic acid, resulting in loss of infectivity. Additionally, Ad5 possesses a double-stranded DNA genome, whereas the phage has a single-stranded RNA. The human virus with damaged genome is still able to infect host cells (Seth, 1999), and because the genome is a double-stranded DNA, it can be processed by the DNA repair machine of the host cell (Eischeid *et al.*, 2009). This might explain further the low reductions achieved by the PHMB compared to the MS2.

In conclusion, PHMB was found to interact with the human adenovirus, altering the surface properties and damaging the capsid protein. Reductions lower than 2 Log_{10} were achieved, possibly due to aggregation and to high infectivity of the genome. Some of the characteristics of interaction were shared with the phage MS2.

7. FINAL DISCUSSION

7.1. Viruses and public health

There are thousands of different viruses which infect animal, plant and bacteria. They are obligate parasites, presenting various compositions, shapes, sizes and complexity.

Human viral pathogens place a serious strain on health and healthcare systems. Infections outbreaks are often a result of viral infections (Butz *et al.*, 1993; Aitken & Jeffries, 2001; Bont, 2009; Osbourn *et al.*, 2009) with 2322 cases registered from 1967 in the Outbreak Database (Hygiene and Environmental Medicine Charité – University Medicine Berlin, 2008). The most clinically important viruses are summarised in Table 7-1.

Table 7-1. Summary of the clinically most important virus families.

Family	Important species	Genome	Envelopment
Herpesviridae	Epstein-barr virus, herpes simplex type 1, herpes simplex type 2, varicella-zoster virus	dsDNA	Enveloped
Hepadnoviridae	Hepatitis B	dsDNA and ssDNA	Enveloped
Flaviviridae	Hepatitis C	+ssRNA	Enveloped
Picornaviridae	Poliovirus, Hepatitis A, coxsackievirus	+ssRNA	Non-enveloped
Retroviridae	HIV	+ssRNA	Enveloped
Adenoviridae	Adenovirus 5, 2, 8, 40, 41	dsDNA	Non-enveloped
Orthomyxoviridae	Influenza virus	-ssRNA	Enveloped
Caliciviridae	Noroviruses, caliciviruses, astroviruses, and small round-structured viruses	+ssRNA	Non-enveloped
Reoviridae	Reoviruses and rotaviruses	dsRNA	Non-enveloped

ds, double-stranded; +ss, positive single-stranded; -ss, negative single stranded.

Several viral characteristics are responsible for such high occurrence. First, viruses possess high infectivity, for instance one PFU (referring to viruses that form plaques) is required to cause the infection of 1% of an healthy adult population (Schiff *et al.*, 1984). Secondly, viruses can survive at different pHs and temperatures (Duizer *et al.*, 2004; Koopmans & Duizer, 2004), and for long periods of time on surfaces and fomites (Tiwari *et al.*, 2006; Weber & Stilianakis, 2008). Finally, transmission can occur through many routes including: person-to-person droplets; person-to-person intimate contact; animal to insect to human; or animal to human and, during birth by transplacental infection.

Nowadays, the main strategy to control viral diseases is vaccination and the use of antiviral drugs (Flint *et al.*, 2004b). Such methods are quite effective; however, vaccines and drugs are not always available for human viral pathogens and judging the hazard of viral contamination can be difficult, since the infectious dose can vary significantly with the pathogenicity of the organism and the immune status of the host (Barker *et al.*, 2001). Therefore good disinfection practices used for prevention are essential. Several types of biocide are available on the market, and their efficacy in killing viruses differs widely. The type of virus, the biocide used and the environmental conditions are features that influence the result of the disinfection process. Viral susceptibility varies amongst species, but the presence of an envelope, for instance, was found to be a relevant characteristic of less resistance to the action of biocides. Antimicrobial used in routine disinfection in healthcare settings needs to have a broad spectrum of activity, inactivating bacteria, fungi and viruses and to be effective after a reasonable contact time. Therefore, understanding the mechanisms of action of a specific biocide is essential for improving disinfection quality.

PHMB is a compound with a wide range of antimicrobial activity. It is active against Gram–positive and Gram–negative bacteria (Chawner & Gilbert, 1989a; Allen *et al.*, 2006). Moreover, PHMB is commonly used in contact lens fluids and for treatment of *Acanthamoeba keratitis* by damaging the plasma membrane (Khunkitti *et al.*, 1997; Hsin-Chiung *et al.*, 2009). The activity of PHMB against viruses has not been extensively

studied. The few available studies focused on enveloped viruses (Valluri *et al.*, 1997; Krebs *et al.*, 2005; Thakkar *et al.*, 2009), which appeared to be inactivated by the compound because of their lipidic membrane being the main target of the biocide (Broxton *et al.*, 1984; Chawner & Gilbert, 1989b).

This project aimed at understanding the mechanisms of action of two PHMB-based biocides against non-enveloped viruses. The investigation was initially performed with two bacteriophages, MS2 and F116 used as model viruses. It was then restricted to the coliphage MS2 principally because of its physical structure and its similar resistance profile to the enteric viruses under unfavourable conditions and disinfection processes (Grabow, 2001). Subsequently, the human viral pathogen Adenovirus type 5 was considered in the investigation.

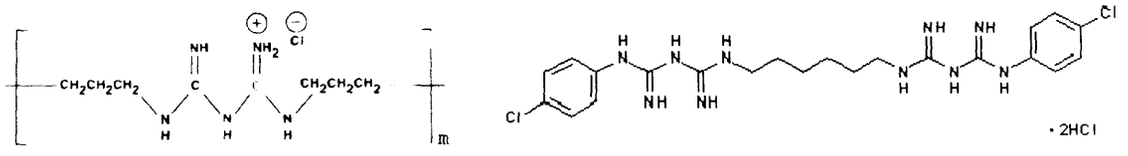
7.2. Virucidal activity of PHMB

Chlorhexidine is a biguanide compound commonly used as an antimicrobial (Figure 7-1), completely inhibiting herpes simplex virus and HIV after 30 sec at 1200 and 2000 ppm (Baqui *et al.*, 2001). HIV was also inactivated by a chlorhexidine–based formulation at 400 ppm after 15 sec of exposure (Montefiori *et al.*, 1990). However, no activity was observed against non–enveloped viruses (Springthorpe *et al.*, 1986). Against bacteriophage, 1% chlorhexidine was able to reduce 90% of the MS2 titre after 20 min contact time in suspension tests (Maillard, 2001), whereas 5000 ppm inactivated the coliphage by 1 Log_{10} in carrier tests (Woolwine & Gerberding, 1995). No activity was observed against phage F116 (Maillard, 2001).

PHMB is a polymeric biguanide (Figure 7-1) and it has been previously tested against enveloped viruses. It was found active *in vitro* against both cell-free and cell-associated HIV-1 (Krebs *et al.*, 2005). Different results were observed with different strains of the virus. Specifically, it completely inactivated cell-free HIV IIIB at 3200 ppm and cell-free HIV BaL at 600 ppm after 10 min contact. PHMB at 100 ppm also appeared effective *in vitro* against herpes simplex virus (Valluri *et al.*, 1997).

Such reductions are higher than determined here against two bacteriophages and a non-enveloped mammalian virus (Table 7-2). In fact reductions of 1 Log_{10} were achieved with both PHMB-based biocides at 800 ppm against MS2 and Adenovirus type 5 after 30 min at 20°C. In contrast, the biocides were more effective with the bacteriophage F116, reducing its titre of 2.5 Log_{10}.

Figure 7-1. Biguanide structures. A) PHMB. B) Chlorhexidine.



A B

The main disinfection kinetic pattern observed for the phages and Ad5 was the "tailing off" curve, which has been well characterised for water disinfection (Jensen *et al.*, 1980). Two main mechanisms could explain such a pattern: a depletion of the active compound in the solution, or the presence of resistant subpopulations arising from conformational character or aggregate formation. Since the quantity of both biocides was in excess, aggregation or/and conformational impediments might be the most plausible reasons. Different mechanisms for MS2 and Ad5 were explored by further detailed investigations. The efficacy against MS2 was greatly enhanced by increasing temperature (>4 Log_{10}) (Table 7-2), which also brought about a reduction in the size of aggregates. Therefore, aggregation was considered to have an influential effect on virucidal activity against MS2 at 20°C. In addition, temperature enhanced the efficacy of the PHMB biocides, VANTOCIL™ TG and COSMOCIL™ CQ, possibly increasing virus susceptibility due to conformational changes. Conversely, even though aggregation was observed with Ad5 after the treatment, temperature did not affect the activity and the size of clumps unlike with MS2 (Table 7-2). Clear structural damage was observed where the primary structure of a coat protein and a core protein were altered. Two characteristics could explain

the higher resistance of Ad5 compared to the coliphage: the nature of its genome and the higher complexity of the human virus.

Table 7-2. Summary of suspension test results for VANTOCIL™ TG and COSMOCIL™ CQ against F116, MS2 and Ad5.

	T (°C)	Concentration (ppm)	RF			
			VANTOCIL™ TG		COSMOCIL™ CQ	
			Contact time (min)			
			1	30	1	30
F116	**20**	**200**	0.05 ± 0.22	0	0.45 ± 0.45	0.85 ± 0.88
		800	2.17 ± 0.43	2.56 ± 0.54	1.73 ± 0.40	2.19 ± 0.48
MS2	**20**	**200**	0.44 ± 0.67	1.02 ± 0.43	0	0.60 ± 0.22
		800	0.43 ± 0.34	0.50 ± 0.02	0.54 ± 0.36	1.07 ± 0.30
	40	**800**	1.49 ± 0.05	5.19 ± 0.30	2.26 ± 0.29	4.79 ± 0.09
Ad5	**20**	**200**	0.41 ± 0.14	1.06 ± 0.16	NT	0.03 ± 0.27
		800	0.09 ± 0.14	0.77 ± 0.32	NT	1.33 ± 0.17
	40	**800**	0.75 ± 0.33	1.42 ± 0.22	NT	NT

RF: reduction factor. Values are in Log_{10} ± standard deviation.

NT: not tested.

7.3. Mechanism of action of PHMBs against non-enveloped virus

The results suggested a possible mechanism of action for PHMBs against non-enveloped viruses (Figure 7-3).

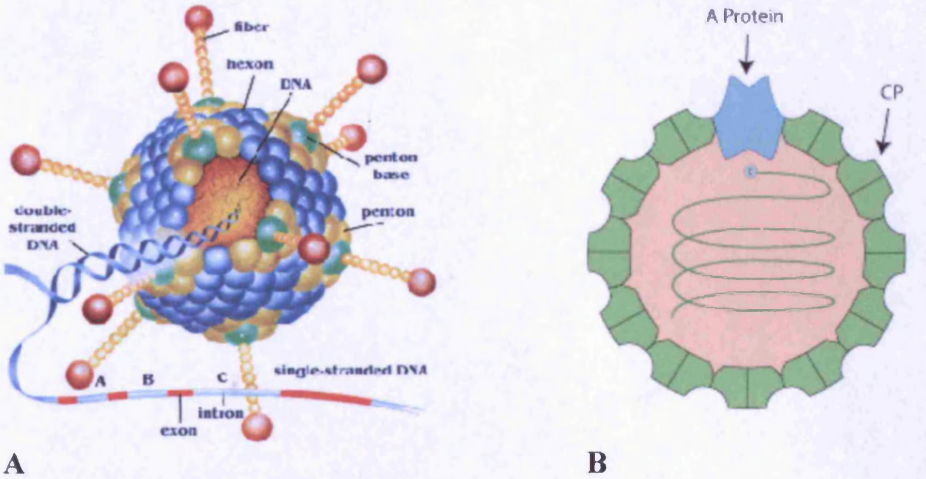
PHMB molecules are polycationic compounds derived from repeated biguanide units separated by hydrophobic hydrocarbon chains of variable length. MS2 (Vega *et al.*, 2005) and Ad5 (Favier *et al.*, 2004) have pI of 3.9 and between 5 and 5.89 respectively; therefore both viruses are negatively charged during the treatment at pH 7-8 (Figure 7-3, Phase I). As the PHMB and the viral particles interact, the opposite charges are attracted, and the net charge on viral capsid progressively decreases (Figure 7-3, Phase II). The PHMB hydrocarbon chains function as a bridge between particles, inducing viruses and biocide molecules to form aggregates (Figure 7-3, Phase III).

Therefore after an initial electrostatic interaction, hydrophobic forces begin to dominate. At this stage, different actions arise between the two viruses. The MS2 inactivation is temperature-dependent; after aggregates are formed, slight conformational changes may have occurred (only quaternary and tertiary structure) and caused a small reduction in titre (Figure 7-3, Phase IV). As the temperature increases, the higher thermal energy (thus high kinetic energy) causes bacteriophages to disperse uniformly and helps the PHMB to interact with the individual viral particles, increasing the virus susceptibility due to conformational changes (Figure 7-3, Phase V and VI). A different mechanism was observed when PHMBs interact with the more complex Ad5 (Figure 7-2, Table 7-3). After the formation of aggregates, PHMB interacts with the minor coat protein VI, causing a precise breakage of the capsid (Figure 7-3, Phase IV). The DNA is then released into the outer environment (Figure 7-3, Phase V). Temperature has only a small influence on this action, only slightly enhancing the viral inactivation. Two factors may contribute chiefly to such result: firstly, the structural complexity of the human virus. As Figure 7-2 and Table 7-3 summarised, Ad5 has 11 structural proteins compared to the 2 of the coliphage with its genome encoding 10 times more proteins than MS2. Such greater complexity could increase the forces involved (hydrophobic, electrostatic and Van der Waals) in the formation of aggregates and in the viral particle itself. Secondly the RNA of MS2 contains several coat protein-binding sites, supporting the hypothesis that its genome is closely bound to the capsid (Koning *et al.*, 2003). Therefore, any breakage to the capsid caused by the PHMB could damage the MS2 nucleic acid. In addition, even though PHMB does not cause major damage to the nucleic acid, as shown in the F116 DNA analysis [Chapter 4, section 4.4.4], it has to be considered that the Ad5 genome is known to be highly infective (Seth, 1999) and can be repaired by the DNA repair machinery of the host cell (Eischeid *et al.*, 2009).

Overall, electrostatic and then hydrophobic forces seem to have an important role in the attachment of PHMB molecules to the virus. Such association will lead to viral aggregate formation, which inhibits the action of the active compound. PHMB is able to react with proteins to various

extents, altering their structure. No relevant interactions were observed with the DNA.

Figure 7-2. Structure of the human adenovirus (A) and of the bacteriophage MS2(B).



A **B**

CP: coat protein.

From ICTV (International Committee on Taxonomy of Viruses, 2002).

Table 7-3. Summary of MS2 and Ad5 features.

	MW	Size	Shape	Envelope	Genome	Protein coding	Structural proteins	pI
MS2	$3.6x10^6$ Da	25 nm	Icosahedral	no	ssRNA linear 3569 bp	4¥	2¥¥	3.9
Ad5	$150x10^6$ Da	100 nm	Icosahedral	no	dsDNA linear 35938 bp	45*	11**	5/5.9

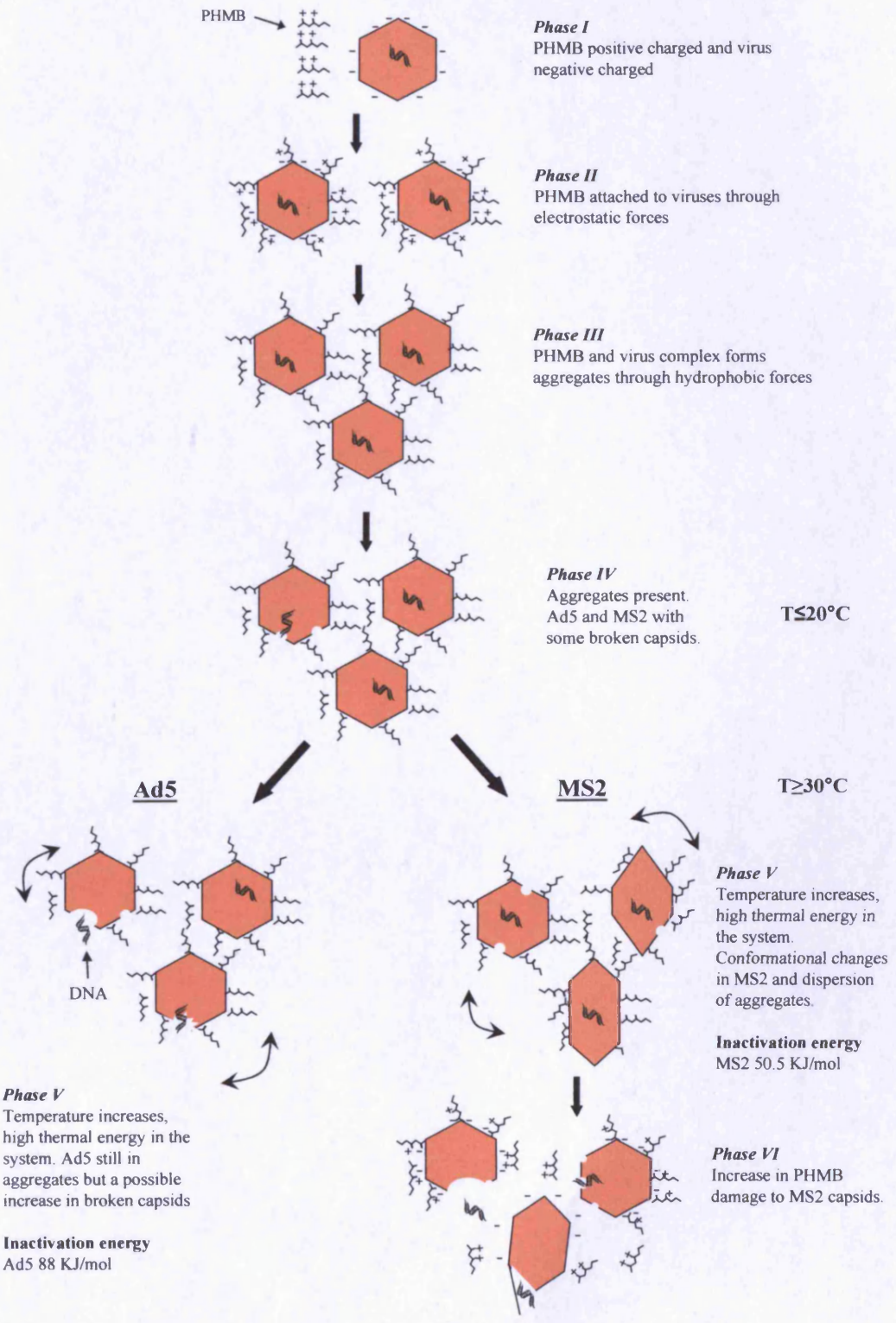
*(Cusack, 2005)

**(Rux & Burnett, 2004)

¥(van den Worm *et al.*, 2006)

¥¥(Kuzmanovic *et al.*, 2006)

Figure 7-3. Proposed mechanism of action of PHMB against adenovirus type 5 and MS2. Phase I to III are similar for the two viruses, then different mechanisms are involved for the bacteriophage and the human virus.



7.4. Public health implications and practical applications

PHMBs have been widely used as biocides and an antiseptic for the past 40 years. PHMB has a broad spectrum activity, a low toxicity (skin irritancy and hypersensitivity), and it is stable for long-term storage. Such features have led to its use as a swimming pool sanitizer (Goeres *et al.*, 2004), wound dressing (Salas Campos *et al.*, 2006), antiseptic, and contact lens cleaning solution (Bruinsma *et al.*, 2006). Currently, there is no evidence to suggest any PHMB resistance development by nosocomial pathogens (Gilbert & Moore, 2005). PHMBs are active against bacteria (Broxton *et al.*, 1983; Allen *et al.*, 2006; Oule *et al.*, 2008) and *Acanthamoeba* keratinitis (Khunkitti *et al.*, 1998; Hsin-Chiung *et al.*, 2009). Regarding viruses, there is some knowledge about the activity of PHMBs against the enveloped ones, such HIV (Krebs *et al.*, 2005) and herpes simplex virus (Valluri *et al.*, 1997), but nothing on non-enveloped viruses. The latter category comprises important human pathogens, causing, for instance, the common gastrointestinal and respiratory infections. Therefore the discovery of undocumented activity of a biocide against non-enveloped viruses would be of particular relevance. Here, results showed that PHMB attached to the virus and interacted with the capsid, causing viral inactivation to various extents. Therefore, PHMBs have the potential to be virucidal compounds. Clearly this is a starting point, and further investigations are needed to better understand the mechanisms of action and to possibly improve the PHMB formulations already present.

Another important feature of this project is the use of phages as surrogate viruses.

Some human pathogens are difficult if not impossible to grow and dangerous to handle, therefore using surrogates in some instances is necessary. There are no officially recognized surrogates for testing biocides against viruses (Sattar *et al.*, 2003), since the difficulty is to find a pattern of viral resistance in order to group in categories of susceptibility different species or families. The only attempt was performed be Klein and Deforest (1965), who found that the presence of the lipidic envelope was responsible for the lower resistance. Generally, similar morphology and comparable susceptibility to biocides are the factors used to choose the appropriate

surrogate (Eterpi *et al.*, 2009). In this study, F116 proved to be more susceptible to the PHMB compound, thus not suitable as surrogate; whereas the coliphage MS2 acted in a manner similar to the human adenovirus after being in contact with the biocide and the reductions were comparable at 20°C. Differences were observed when the treatment was performed at higher temperatures (≥30°C), possibly due to the nature of the genome and the higher Ad5 structural complexity. Therefore, results support the use of MS2 as a surrogate for human viruses for testing biocides used at 20°C. Considering the nature of the genome, which apparently could influence the virucidal activity, it is considered more appropriate to use MS2 as a model virus specifically for RNA non-enveloped viruses, such as coxsackievirus, norovirus and astrovirus reinforcing what has been already suggested elsewhere (Havelaar *et al.*, 1993; Dawson *et al.*, 2005; Koivunen & Heinonen-Tanski, 2005). Another practical application of MS2 emerged from this project. In addition to using the coliphage as a surrogate organism to test new compounds, MS2 could also be used to design and model the whole set of experiments. Such experiments have been performed with the human virus Ad5. This use of MS2 is extremely important, especially when working with unsafe and complex viruses. In fact, MS2 is easy to handle, cheap, grows quickly (20/24h) and is harmless. Therefore it can be considered an appropriate organism to design experiments which need to be developed or modified in order to work with human viruses.

7.5. General conclusions

This research project clarified the virucidal properties and the mechanisms of action of two PHMB-based biocides, VANTOCIL™ TG and COSMOCIL™ CQ, against the two bacteriophages F116 and MS2, used as surrogates, and a non-enveloped human virus, adenovirus type 5 (Ad5). Suspension tests at 20°C showed that the PHMB at 800 ppm (0.08% v/v) reduced MS2 and Ad5 by 90%. Higher reductions were achieved against F116 (99%). Times of exposure did not affect the activity, and non-linear disinfection kinetics were observed. Temperature, instead, showed great influence on the ability to kill MS2; in fact at 30°C 99.99% of the

bacteriophage was inactivated after 10 min exposure time. The activity against Ad5 was also enhanced, but to a minor extent (from 90% to 99% reduction). Aggregation seemed to have a key role in such low reductions, especially for MS2. Regarding the mechanisms of action, the binding of PHMB molecules to the MS2 and Ad5 capsid was observed to be highly cooperative (aggregate formation) and occurred through first electrostatic and then hydrophobic forces. Since damage to capsids was found, it is likely that this caused the viral inactivation. However, the hypothesis of domain formation by PHMB molecules and thus the inhibition of the virus-host cell receptor interaction could be considered.

Concluding, PHMB-based biocides have modest potential virucidal activity against non-enveloped viruses and warrant further investigations.

7.6. Limitations and future works

Results from this project could help improve PHMB-based biocides, but some limitations need to be acknowledged. The method used to test the efficacy of the biocide was the suspension test. This test is known to be less challenging for microorganisms, often overestimating the inactivation factor. However, it is essential as a first screening of the efficacy of a new or untested biocide, such as PHMB against non-enveloped virus. Moreover, the project was mainly focused on understanding the mechanisms of virucidal actions of the biocide so that only a simple assessment was sufficient to gain a basic knowledge regarding the ability to reduce the viral titre.

This three-year project is an essential starting point for future investigations aiming at improving the efficacy of PHMB-based biocides not only against bacteria but also viruses.

The first stage in the mechanism of action was identified, but further clarification about the factors (physical, chemical and biological) involved in the attachment of PHMB to the capsid will provide important information in order to apply appropriate changes to the biocide formulations. This

could be achieved by testing the biocide in the absence of either electrostatic or hydrophobic forces, and the analysis of the virucidal activity and the aggregation state of the viruses. Working at pHs similar to the viral pI value would eliminate any surface charges, and therefore no electrostatic force will be involved. Moreover, mixing a surfactant with the virus suspension would exclude any hydrophobic interactions.

Another important research step would be testing the PHMB-based biocides in the presence of organic load. This would better mimic realistic conditions of contamination. Furthermore, factors such as organic load alter the surface properties of viruses, possibly affecting the initial interaction between PHMB and viral particles.

Studies on the permeability of virus to the biocides might explain further the higher virucidal activity as the temperature increases. Transmission electron microscopy could be a useful method for such assessment (Nelson *et al.*, 2008a). In addition, testing whether the temperature influences the susceptibility of the viral capsid would be considered. A variety of methods can be considered, such as susceptibility to proteolysis, dynamic light scattering, and accessibility to the genome (e.g. TOTO-1).

Main research questions might include a detailed understanding of the interaction of PHMB with the capsid, specifically:

1. Does the PHMB target a precise protein or amino acids?
2. Does PHMB inhibit the infection by blocking the virus-host cell receptor?

Analysis through SDS-PAGE, Western blot, immunochromatography, and viral infection inhibition assay would help to answer such questions.

The genome is the last possible biocide target. Here a difference in inactivation between a DNA and a RNA virus was noted. It would be interesting to understand how PHMBs interact with RNA to compare with that found for the DNA and to determine whether the genome is still infective after the inactivation.

Testing the PHMB biocides against human RNA viruses would be appropriate. In fact, comparing the activity and the mechanisms of action with what was observed with MS2, it should be possible to confirm the use

of this coliphage as an appropriate surrogate and therefore to develop an appropriate testing method against viruses.

8. REFERENCES

ABAD, F. X., PINTO, R. M. & BOSCH, A., 1994. Survival of enteric viruses on environmental fomites. *Applied And Environmental Microbiology,* 60(10), pp.3704-3710.

ABAD, F. X., PINTO, R. M. & BOSCH, A., 1997. Disinfection of human enteric viruses on fomites. *FEMS Microbiology Letters,* 156(1), pp.107.

ACKERMANN, H. W., 2003. Bacteriophage observations and evolution. *Research in Microbiology,* 154(4), pp.245-251.

ADOLFSSON-ERICI, M., PETTERSSON, M., PARKKONEN, J. & STURVE, J., 2002. Triclosan, a commonly used bactericide found in human milk and in the aquatic environment in Sweden. *Chemosphere,* 46(9-10), pp.1485-1489.

AFNOR. NFT72-180, 1989. Antiseptiques et désinfectants utilisés à l' état liquide, miscibles à l' eau. Détermination de l' activité virucide vis-à-vis des virus de vertébrés.

AITKEN, C. & JEFFRIES, D. J., 2001. Nosocomial spread of viral disease. *Clinical Microbiology Reviews,* 14(3), pp.528-546.

ALBERTS, B., JOHNSON, F., LEWIS, J., RAFF, M., ROBERTS, K. & WALTER, P., 2002. *Molecular biology of cell.* 4th ed. NY: Garland Science.

ALLEN, M. J., WHITE, G. F. & MORBY, A. P., 2006. The response of *Escherichia coli* to exposure to the biocide polyhexamethylene biguanide. *Microbiology,* 152, pp.989-1000.

ALPERT, G., CHARNEY, E., FEE, M. & PLOTKIN, S. A., 1986. Outbreak of fatal adenoviral type 7a respiratory disease in a children's long-term care inpatient facility. *American Journal of Infection Control,* 14(4), pp.188-190.

ALVAREZ, M. E. & OBRIEN, R. T., 1982. Mechanisms of inactivation of poliovirus by chlorine dioxide and iodine. *Applied and Environmental Microbiology,* 44(5), pp.1064-1071.

AMERICAN SOCIETY FOR TESTING AND MATERIALS (ASTM). Designation 2011-99, 1999. Standard test method for evaluation of hand-washing formulation for virus-eliminating activity using the entire hand.

ANDERS, R. & CHRYSIKOPOULOS, C. V., 2006. Evaluation of the factors controlling the time-dependent inactivation rate coefficients of bacteriophage MS2 and PRD1. *Environmental Science & Technology,* 40(10), pp.3237-3242.

ANONYMOUS, 1986a. PhastSystem separation technique file No.110. In: *Pharmacia AB.* pp.

ANONYMOUS, 1986b. Silver staining technique file No. 210. In: *Pharmacia AB.* pp.

ANONYMOUS. Designation 2011-99, 1999. Standard test method for evaluation of hand-washing formulation for virus-eliminating activity using the entire hand.

ANONYMOUS. prEN 14476, 2002. Chemical disinfectants and antiseptics - Virucidal quantitative suspension test for chemical disinfectants and antiseptics used in human medicine - Test method and requirements (phase 2/step 1).

ARAP, M. A., 2005. Phage display technology - Applications and innovations. *Genetics and Molecular Biology,* 28(1), pp.1-9.

ASTM. E 1052-85, 1989a. Standard test method for efficacy of virucidal agents intended for inanimate environmental surfaces.

ASTM. E 1052-85, 1989b. Standard test method for efficacy of virucidal agents intended for special applications.

AUSAR, S. F., FOUBERT, T. R., HUDSON, M. H., VEDVICK, T. S. & MIDDAUGH, C. R., 2006. Conformational stability and disassembly of Norwalk virus-like particles - Effect of pH and temperature. *Journal of Biological Chemistry,* 281(28), pp.19478-19488.

BAILLY, J. L., CHAMBON, M., PEIGUE-LAFEUILLE, H., LAVERAN, H., DE CHAMPS, C. & BEYTOUT, D., 1991. Activity of glutaraldehyde at low concentrations (less than 2%) against poliovirus and its relevance to gastrointestinal endoscope disinfection procedures. *Applied and Environmental Microbiology,* 57(4), pp.1156-1160.

BAQUI, A., KELLEY, J. I., JABRA-RIZK, M. A., DEPAOLA, L. G., FALKLER, W. A. & MEILLER, T. F., 2001. *In vitro* effect of oral antiseptics on human immunodeficiency virus-1 and herpes simplex virus type 1. *Journal of Clinical Periodontology,* 28(7), pp.610-616.

BARKER, J., STEVENS, D. & BLOOMFIELD, S. F., 2001. Spread and prevention of some common viral infections in community facilities

and domestic homes. *Journal of Applied Microbiology,* 91(1), pp.7-21.

BARON, S., PEAKE, R. C., JAMES, D. A., SUSMAN, M., KENNEDY, C. A., SINGLETON, M. J. D. & SCHUENKE, S., 1996. *Medical Microbiology.* 4th ed. Texas: The University of Texas Medical Branch at Galveston.

BATES, R. C., SHAFFER, P. T. B. & SUTHERLAND, S. M., 1977. Development of poliovirus having increased resistance to chlorine inactivation. *Applied and Environmental Microbiology,* 34(6), pp.849-853.

BAXTER, C. S., HOFMANN, R., TEMPLETON, M. R., BROWN, M. & ANDREWS, R. C., 2007. Inactivation of adenovirus types 2, 5, and 41 in drinking water by UV light, free chlorine, and monochloramine. *Journal of Environmental Engineering,* 133(1), pp.95-103.

BEAN, H. S., 1967. Types and characteristics of disinfectants. *Journal of Applied Bacteriology,* 30(1), pp.6-16.

BELLAMY, K., 1995. A review of the test methods used to establish virucidal activity. *Journal of Hospital Infection,* 30(389-396.

BERCHIERI, A., LOVELL, M. A. & BARROW, P. A., 1991. The Activity in the Chicken Alimentary-Tract of Bacteriophages Lytic for Salmonella-Typhimurium. *Research in Microbiology,* 142(5), pp.541-549.

BERNE, B. J. & PECORA, R., 1976. *Dynamic light scattering : with applications to chemistry, biology and physics.* 1st ed. New York: Wiley.

BEWIG, B. & SCHMIDT, W. E., 2000. Accelerated titering of adenoviruses. *Biotechniques,* 28(5), pp.870-873.

BICHAI, F. & BARBEAU, B., 2006. Assessing the disinfecting power of chlorite in drinking water. *Water Quality Research Journal of Canada,* 41(4), pp.375-382.

BLUMENTHAL, T. & CARMICHAEL, G. G., 1979. RNA replication - Function and structure of beta-replicase. *Annual Review of Biochemistry,* 48, pp.525-548.

BONT, L., 2009. Nosocomial RSV infection control and outbreak management. *Paediatric Respiratory Reviews,* 10(Supplement 1), pp.16-17.

BOONE, S. A. & GERBA, C. P., 2007. Significance of fomites in the spread of respiratory and enteric viral disease. *Applied and Environmental Microbiology,* 73(6), pp.1687-1696.

BRION, G. M. & SILVERSTEIN, J., 1999. Iodine disinfection of a model bacteriophage, MS2, demonstrating apparent rebound. *Water Research,* 33(1), pp.169-179.

BRITISH PHARMACOPOEIA, 2008. *Appendix XVIII Methods of Sterilisation (Methods of Preparation of Sterile Products).* [Online] Available at: http://www.pharmacopoeia.co.uk/cgi-bin/bp.cgi?id=823&ca=display&WINDNKp2p794

BROXTON, P., WOODCOCK, P. M. & GILBERT, P., 1983. A Study of the antibacterial activity of some polyhexamethylene biguanides towards *Escherichia coli* ATCC 8739. *Journal of Applied Bacteriology,* 54(3), pp.345-353.

BROXTON, P., WOODCOCK, P. M., HEATLEY, F. & GILBERT, P., 1984. Interaction of some polyhexamethylene biguanides and membrane phospholipids in *Escherichia coli. Journal of Applied Bacteriology,* 57(1), pp.115-124.

BRUCKOVA, M., WADELL, G., SUNDELL, G., SYRUCEK, L. & KUNZOVA, L., 1980. An outbreak of respiratory disease due to a type-5 Adenovirus identified as genome type 5a. *Acta Virologica,* 24(3), pp.161-165.

BRUINSMA, G. M., RUSTEMA-ABBING, M., VAN DER MEI, H. C., LAKKIS, C. & BUSSCHER, H. J., 2006. Resistance to a polyquaternium-1 lens care solution and isoelectric points of *Pseudomonas aeruginosa* strains. *Journal of Antimicrobial Chemotheraphy,* 57(4), pp.764-766.

BURLESON, G. R., MURRAY, T. M. & POLLARD, M., 1975. Inactivation of viruses and bacteria by ozone, with and without sonication. *Applied and Enviromental Microbiology,* 29(3), pp.340-344.

BUTZ, A. M., FOSARELLI, P., DICK, J., CUSACK, T. & YOLKEN, R., 1993. Prevalence of rotavirus on high-risk fomites in day-care facilities. *Pediatrics,* 92(2), pp.202-205.

BYRNE, M. & KROPINSKI, A. M., 2005. The genome of the *Pseudomonas aeruginosa* generalized transducing bacteriophage F116. *Gene,* 346, pp.187-194.

CEN. prEN 13610, 2002a. 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. EN 14476+A1, 2002b. Chemical disinfectants and antiseptics - Virucidal quantitative suspension test for chemical disinfectants and antiseptics used in human medicine - Test method and requirements (phase 2, step 1).

CENTRAL VETERINARY LABORATORY, 1970. Protocol of test for approval of disinfectants for use against fowl pest (Newcastle disease virus, Fowl plague virus). MAFF CENTRAL VETERINARY LABORATORY, Ministry of Agriculture Fisheries and Food.

CHAMBON, M., ARCHIMBAUD, C., BAILLY, J.-L., GOURGAND, J.-M., CHARBONNE, F. & PEIGUE-LAFEUILLE, H., 2004. Virucidal efficacy of glutaraldehyde against enteroviruses is related to the location of lysine residues in exposed structures of the VP1 capsid protein. *Applied and Environmental Microbiology,* 70(3), pp.1717-1722.

CHAMBON, M., BAILLY, J. L. & 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,* 58(11), pp.3517-3521.

CHAMBON, M., BAILLY, J. L. & PEIGUELAFEUILLE, H., 1994. Comparative sensitivity of the echovirus type 25 jv-4 prototype strain and two recent isolates to glutaraldehyde at low concentrations. *Applied and Environmental Microbiology,* 60(2), pp.387-392.

CHARREL, R. N., DE CHESSE, R., DECAUDIN, A., DE MICCO, P. & DE LAMBALLERIE, X., 2001. Evaluation of disinfectant efficacy against hepatitis C virus using a RT-PCR-based method. *Journal of Hospital Infection,* 49(2), pp.129.

CHATTERJEE, P. K., VAYDA, M. E. & FLINT, S. J., 1985. Interactions among the 3 adenovirus core proteins. *Journal of Virology,* 55(2), pp.379-386.

CHAWNER, J. A. & GILBERT, P., 1989a. A comparative study of the bactericidal and growth inhibitory activities of the bisbiguanides alexidine and chlorhexidine. *Journal of Applied Bacteriology,* 66(3), pp.243-252.

CHAWNER, J. A. & GILBERT, P., 1989b. Interaction of the bisbiguanides chlorhexedine and alexedine with phospholipid vescicles: evidence

for separate modes of action. *Journal of Applied Bacteriology,* 66(3), pp.253-258.

CHEESBROUGH, J. S., BARKESS-JONES, L. & BROWN, D. W., 1997. Possible prolonged environmental survival of small round structured viruses. *Journal of Hospital Infection,* 35(4), pp.325-326.

CHICHON, F. J., RODRIGUEZ, M. J., RISCO, C., FRAILE-RAMOS, A., FERNANDEZ, J. J., ESTEBAN, M. & CARRASCOSA, J. L., 2009. Membrane remodelling during vaccinia virus morphogenesis. *Biology of the Cell,* 101(7), pp.401-414.

CHICK, H., 1908. An investigation of the laws of disinfection. *Journal of Hygiene,* 8(1), pp.92-158.

CHICK, H., 1913. Factors conditioning the velocity of disinfection. *Chemical Abstracts,* 7, pp.2273.

CHURN, C. C., BOARDMAN, G. D. & BATES, R. C., 1984. The inactivation kinetics of H-1 parvovirus by chlorine. *Water Research,* 18(2), pp.195-203.

CLEVENGER, T., WU, Y., DEGRUSON, E., BRAZOS, B. & BANERJI, S., 2007. Comparison of the inactivation of *Bacillus subtilis* spores and MS2 bacteriophage by MIOX, ClorTec and hypochlorite. *Journal of Applied Microbiology,* 103(6), pp.2285-2290.

COX, N. J. & FUKUDA, K., 1998. Influenza. *Infectious Disease Clinics of North America,* 12(1), pp.27-38.

CRITCHLEY, M. & BENTHAM, R., 2009. The efficacy of biocides and other chemical additives in cooling water systems in the control of amoebae. *Journal of Applied Microbiology,* 106(3), pp.784-789.

CURIALE, M. 2004. MPN Calculator [Online]
Available at: http://www.i2workout.com/mcuriale/mpn/index.html
[1/09/2009]

CUSACK, S., 2005. Adenovirus complex structures. *Current Opinion in Structural Biology,* 15(2), pp.237-243.

DAVIES, B. D., DULBECCO, R., EISEN, H. N. & GINSBERG, H. S., 2004. *Microbiology.* ed. Maryland: Harper & Row, Publishers Inc.

DAVIES, J. G., BABB, J. R., BRADLEY, C. R. & AYLIFFE, G. A. J., 1993. Preliminary study of test methods to assess the virucidal activity of skin disinfectants using poliovirus and bacteriophages. *Journal of Hospital Infection,* 25(2), pp.125-131.

DAWSON, D. J., PAISH, A., STAFFELL, L. M., SEYMOUR, I. J. & APPLETON, H., 2005. Survival of viruses on fresh produce, using MS2 as a surrogate for norovirus. *Journal of Applied Microbiology,* 98(1), pp.203-209.

DENYER, S. P., HANLON, G. W. & DAVIES, M. C., 1993. Mechanisms of microbial adherence. In: S.P., D., ed. *Microbial biofilms: formation and control.* Oxford: Blackwell Scientific Publications. pp. 13-28.

DETTENKOFER, M. & BLOCK, C., 2005. Hospital disinfection: efficacy and safety issues. *Current Opinion in Infectious Diseases,* 18(4), pp.320-325.

DETTENKOFER, M., WENZLER, S., AMTHOR, S., ANTES, G., MOTSCHALL, E. & DASCHNER, F. D., 2004. Does disinfection of environmental surfaces influence nosocomial infection rates? A systematic review. *American Journal of Infection Control,* 32(2), pp.84-89.

DUCKWORTH, D. H. & GULIG, P. A., 2002. Bacteriophages: potential treatment for bacterial infections. *BioDrugs: clinical immunotherapeutics, biopharmaceuticals and gene theraphy,* 16(1), pp.57-62.

DUIZER, E., BIJKERK, P., ROCKX, B., DE GROOT, A., TWISK, F. & KOOPMANS, M., 2004. Inactivation of Caliciviruses. *Applied and Environmental Microbiology,* 70(8), pp.4538-4543.

DULBECCO, R., 1980. The nature of Viruses. In: 3rd. *Microbiology.* Maryland: Harper & Row. pp. 853-883.

ECHAVARRIA, M., 2008. Adenoviruses in immunocompromised hosts. *Clinical Microbiology Reviews,* 21(4), pp.704-715.

EISCHEID, A. C., MEYER, J. N. & LINDEN, K. G., 2009. UV disinfection of adenoviruses: Molecular indications of DNA damage efficiency. *Applied and Environmental Microbiology,* 75(1), pp.23-28.

EISERLING, F. A. & BLACK, L. W., 1994. Pathways in T4 morphogenesis. In: KARAN, J. D., ed. 1st. *Molecular Biology of bacteriophage T4.* Washinton DC: American Society of Microbiology. pp. 209-212.

ETERPI, M., MCDONNELL, G. & THOMAS, V., 2009. Disinfection efficacy against parvoviruses compared with reference viruses. *Journal of Hospital Infection,* 73(1), pp.64-70.

EUROPEAN COMMITTEE FOR STANDARDIZATION. prEN 14476, 1996. European standard of virucidal suspension test for chemical

disinfectants and instrument disinfectants and antiseptics used in human medicine.

FACHAUSSCHUSS "VIRUSDESINFEKTION" DER DEUTSCHEN VEREINIGUNG ZUR BEKAMPFUNG DER VIRUSKRANKHEITEN UND DES ROBERT KOCH-INSTITUTS, 2003. Richtlinie des Bundesgesundheitsamtes und der Deutschen Vereinigung zur Bekampfung der Viruskrankheiten zur Prufung von chemischen Desinfektionsmitteln auf Wirksamkeit gegen Viren. *Bundesgesundheitsbl Gesundheitsforsch Gesundheitsschutz,* 46(7), pp.619.

FARA, G. M., LUGARO, G., GALLI, M. G. & GIANNATT.G, 1974. Antiviral activity of selected biguanide derivatives. *Pharmacological Research Communications,* 6(2), pp.117-126.

FAVIER, A.-L., BURMEISTER, W. P. & CHROBOCZEK, J., 2004. Unique physicochemical properties of human enteric Ad41 responsible for its survival and replication in the gastrointestinal tract. *Virology,* 322(1), pp.93-104.

FERRIER, A., GARIN, D. & CRANCE, J. M., 2004. Rapid inactivation of vaccinia virus in suspension and dried on surfaces. *Journal of Hospital Infection,* 57(1), pp.73.

FLINT, S. J., ENQUIST, L. W., RACANIELLO, V. R. & SKALKA, A. M., 2004a. Patterns of infection. In: FLINT, S. J., ENQUIST, L. W., RACANIELLO, V. R. & SKALKA, A. M., ed. 2nd. *Principles of virology. Molecular biology, pathogenesis, and control of animal viruses.* Washington: ASM Press. pp. 596-621.

FLINT, S. J., ENQUIST, L. W., RACANIELLO, V. R. & SKALKA, A. M., 2004b. Prevention and control of viral diseases. In: FLINT, S. J., ENQUIST, L. W., RACANIELLO, V. R. & SKALKA, A. M., ed. 2nd. *Principles of virology. Molecular biology, pathogenesis, and control of animal viruses.* Washington: ASM Press. pp. 596-621.

FLINT, S. J., ENQUIST, L. W., RACANIELLO, V. R. & SKALKA, A. M., 2004c. The science of Virology - Foundations. In: FLINT, S. J., ENQUIST, L. W., RACANIELLO, V. R. & SKALKA, A. M., ed. 2nd. *Principles of virology. Molecular biology, pathogenesis, and control of animal viruses.* Washington: ASM Press. pp. 2-24.

FLINT, S. J., ENQUIST, L. W., RACANIELLO, V. R. & SKALKA, A. M., 2004d. Structure. In: FLINT, S. J., ENQUIST, L. W., RACANIELLO, V. R. & SKALKA, A. M., ed. 2nd. *Principles of virology. Molecular biology, pathogenesis, and control of animal viruses.* Washington: ASM Press. pp. 83-125.

FLOYD, R. & SHARP, D. G., 1977. Aggregation of poliovirus and reovirus by dilution in water. *Applied and Environmental Microbiology,* 33(1), pp.159-167.

FLOYD, R. & SHARP, D. G., 1979. Viral aggregation: buffer effects in the aggregation of Poliovirus and Reovirus at low and high pH. *Applied and Environmental Microbiology,* 38(3), pp.395-401.

FLYVHOLM, M. A., 2005. Preservatives in registered chemical products. *Contact Dermatitis,* 53(1), pp.27-32.

FRAISE, A. P., 2004. Historical introduction. In: FRAISE, A. P., LAMBERT, P. A. & MAILLARD, J.-Y., ed. 4th. *Disinfection Preservation & Sterilization.* Oxford: Blackwell Publishing. pp. 3-7.

GANCZAK, M. & BARSS, P., 2008. Nosocomial HIV infection: Epidemiology and prevention - A global perspective. *Aids Reviews,* 10(1), pp.47-61.

GARCIA-DE-LOMAS, J., LERMA, M., CEBRIAN, L., ESTEBAN, E., GIMENEZ, M. J., AGUILAR, L., DOMINGUEZ, V. & RANDEZ, J. J., 2008. Evaluation of the *in vitro* -cidal activity and toxicity of a novel peroxygen biocide: 2-butanone peroxide. *Journal of Hospital Infection,* 68(3), pp.248-254.

GARCIA, P., MADERA, C., MARTINEZ, B. & RODRIGUEZ, A., 2007. Biocontrol of Staphylococcus aureus in curd manufacturing processes using bacteriophages. *International Dairy Journal,* 17(10), pp.1232-1239.

GASSILLOUD, B. & GANTZER, C., 2005. Adhesion - Aggregation and inactivation of Poliovirus 1 in groundwater stored in a hydrophobic container. *Applied and Environmental Microbiology,* 71(2), pp.912-920.

GAUSTAD, J. W., MCDUFF, C. R. & HATCHER, H. J., 1974. Test method for evaluation of virucidal efficacy of three common liquid surface disinfectants on a simulated environmental surface. *Applied Microbiology,* 28(5), pp.748-752.

GE HEALTHCARE. 2009. Sephadex G-25 media [Online]
Available at:
http://www4.gelifesciences.com/aptrix/upp01077.nsf/Content/uk_homepage
[20/09/2009]

GELLER, C., FONTANAY, S., FINANCE, C. & DUVAL, R. E., 2009. A new Sephadex™-based method for removing microbicidal and cytotoxic residues when testing antiseptics against viruses:

Experiments with a human coronavirus as a model. *Journal of Virological Methods,* 159(2), pp.217-226.

GERMAN ASSOCIATION FOR THE CONTROL OF VIRUS DISEASES, 1990. Guidelines of Bundesgesundheitsamt (BGA; German Federal Health Office) and Deutsche Vereinigung zur Bekampfung der Viruskrankheiten e.V. (DVV; German association for the control of virus diseases) for testing the effectiveness of chemical disinfectants against viruses. *ZENTRALBLATT FUR HYGIENE UND UMWELTMEDIZIN,* 189(6), pp.554-562.

GILBERT, P. & MOORE, L. E., 2005. Cationic antiseptics: diversity of action under a common epithet. *Journal of Applied Microbiology,* 99(4), pp.703-715.

GOERES, D. M., PALYS, T., SANDEL, B. B. & GEIGER, J., 2004. Evaluation of disinfectant efficacy against biofilm and suspended bacteria in a laboratory swimming pool model. *Water Research,* 38(13), pp.3103-3109.

GORDON, V., PARRY, S., BELLAMY, K. & OSBORNE, R., 1993. Assessment of chemical disinfectants against human immunodeficiency virus: Overcoming the problem of cytotoxicity and the evaluation of selected actives. *Journal of Virological Methods,* 45(3), pp.247-257.

GRABOW, W. O. K., 2001. Bacteriophages: Update on application as models for viruses in water. *Water South African,* 27(2), pp.251-268.

GROSSGEBAUER, K., 1970. Virus disinfection. In: BENARDE, M. A., ed. 1st. *Disinfection.* New York: Marcell Dekker INC. pp. 103-148.

GYUREK, L. L. & FINCH, G. R., 1998. Modeling water treatment chemical disinfection kinetics. *Journal of Environmental Engineering-Asce,* 124(9), pp.783-793.

HALL, C. B., DOUGLAS, R. G. & GEIMAN, J. M., 1980. Possible transmission by fomites of respiratory syncytial virus. *Journal of Infectious Diseases,* 141(1), pp.98-102.

HAMES, B. D., 2002. Gel electrophoresis of proteins: a practical approach. In: HAMES, B. D., ed. 3rd. *The Practical Approach Series.* Oxford: Oxford University Press. pp. 1-50.

HANSEN, S., STAMM-BALDERJAHN, S., ZUSCHNEID, I., BEHNKE, M., RUDEN, H., VONBERG, R. P. & GASTMEIER, P., 2007. Closure of medical departments during nosocomial outbreaks: data from a systematic analysis of the literature. *Journal of Hospital Infection,* 65(4), pp.348-353.

HARAKEH, M. S., 1984. Inactivation of enteroviruses, rotaviruses and bacteriophages by peracetic acid in a municipal sewage effluent. *Fems Microbiology Letters,* 23(1), pp.27-30.

HAVELAAR, A. H., VANOLPHEN, M. & DROST, Y. C., 1993. F-specific RNA bacteriophages are adequate model organisms for enteric viruses in freshwater. *Applied and Environmental Microbiology,* 59(9), pp.2956-2962.

HAYNIE, D. T., 2001. *Biological thermodynamics.* 1st ed. Cambridge: Cambridge University Press.

HELGE, M., ELLING, U. & BIRGITTA, Å., 2009. The hepatitis C virus enigma. *APMIS,* 117(5-6), pp.427-439.

HILMER, J. K., ZLOTNICK, A. & BOTHNER, B., 2008. Conformational equilibria and rates of localized motion within hepatitis B virus capsids. *Journal of Molecular Biology,* 375(2), pp.581-594.

HINDLEY, J., 1973. Molecular structure and function in RNA phages. *British Medical Bulletin,* 29(3), pp.236-240.

HOFF, J. (2002) Inactivation of microbial agents by chemical disinfectants. Washington, D.C., U.S. Environmental Protection Agency.

HOFF, J. C. & AKIN, E. W., 1986. Microbial resistance to disinfectants - Mechanisms and significance. *Environmental Health Perspectives,* 69, pp.7-13.

HOHN, T., 1976. Packaging of genomes in bacteriophages - A comparison of ssRNA bacteriophages and dsDNA bacteriophages. *Philosophical Transactions of the Royal Society of London Series B-Biological Sciences,* 276(943), pp.143-150.

HOLLOWAY, B. W., EGAN, J. B. & MONK, M., 1960. Lysogeny in *Pseudomonas aeruginosa. Australian Journal of Experimental Biology and Medicine,* 38(4), pp.321-330.

HONG, J. Y., LEE, H. J., PIEDRA, P. A., CHOI, E. H., PARK, K. H., KOH, Y. Y. & KIM, W. S., 2001. Lower respiratory tract infections due to adenovirus in hospitalized Korean children: Epidemiology, clinical features, and prognosis. *Clinical Infectious Diseases,* 32(10), pp.1423-1429.

HOTA, B., 2004. Contamination, Disinfection, and Cross-Colonization: Are Hospital Surfaces Reservoirs for Nosocomial Infection? *Clinical Infectious Diseases,* 39(8), pp.1182-1189.

HSIN-CHIUNG, L., CHING-HSI, H., DAVID HUI-KANG, M., LUNG-KUN, Y., HSIN-YUAN, T., MENG-YIN, L. & SAMUEL CHAO-

MING, H., 2009. Medical treatment for combined *Fusarium* and *Acanthamoeba* keratitis. *Acta Ophthalmologica,* 87(2), pp.199-203.

HUDSON, J. B., SHARMA, M. & PETRIC, M., 2007. Inactivation of Norovirus by ozone gas in conditions relevant to healthcare. *Journal of Hospital Infection,* 66(1), pp.40-45.

HUTCHINSON, A. K., COATS, D. K., LANGDALE, L. M., STEED, L. L., DEMMLER, G. & SAUNDERS, R. A., 2000. Disinfection of eyelid specula with chlorhexidine gluconate (Hibiclens) after examinations for retinopathy of prematurity. *Archives of Ophthalmology,* 118(6), pp.786-789.

HYGIENE AND ENVIRONMENTAL MEDICINE CHARITÉ – UNIVERSITY MEDICINE BERLIN, 2008. *Outbreak database - Worldwide database for nosocomial outbreaks.* [Online] (13\03\2009)
Available at: http: outbreak-database.charite.de Outbreak Home.aspx
[4\06\09]

INTERNATIONAL COMMITTEE ON TAXONOMY OF VIRUSES, 2002. *The universal virus database.* [Online] (07/09/2008)
Available at: http: www.ncbi.nlm.nih.gov ICTVdb index.htm
[06/09/2009]

INTERNATIONAL UNION OF MICROBIOLOGIST SOCIETIES, 2002. *International Commitee on Taxonomy of Viruses (ICTV).* [Online] Available at: http: www.ncbi.nlm.nih.gov ICTVdb index.htm
[6/1/2009]

JENSEN, H., THOMAS, K. & SHARP, D. G., 1980. Inactivation of coxsackie-viruses B3 and B5 in water by chlorine. *Applied and Environmental Microbiology,* 40(3), pp.633-640.

JIMENEZ, L. & CHIANG, M., 2006. Virucidal activity of a quaternary ammonium compound disinfectant against feline calicivirus: A surrogate for norovirus. *American Journal of Infection Control,* 34(5), pp.269-273.

JONES, M. V., BELLAMY, K., ALCOCK, R. & HUDSON, R., 1991. The Use Of Bacteriophage-Ms2 As A Model System To Evaluate Virucidal Hand Disinfectants. *Journal Of Hospital Infection,* 17(4), pp.279-285.

JURSCH, C. A., GERLICH, W. H., GLEBE, D., SCHAEFER, S., MARIE, O. & THRAENHART, O., 2002. Molecular approaches to validate disinfectants against human hepatitis B virus. *Medical Microbiology and Immunology,* 190(4), pp.189-197.

KA-WAI HUI, E., 2006. Reasons for the increase in emerging and re-emerging viral infectious diseases. *Microbes and Infection,* 8(3), pp.905-916.

KEYSTONE, J. S. & HERSHEY, J. H., 2008. The underestimated risk of hepatitis A and hepatitis B: benefits of an accelerated vaccination schedule. *International Journal of Infectious Diseases,* 12(1), pp.3-11.

KHUNKITTI, HANN, LLOYD, FURR & RUSSELL, 1998. Biguanide induced changes in *Acanthamoeba castellanii*: an electron microscopic study. *Journal of Applied Microbiology,* 84(1), pp.53-62.

KHUNKITTI, W., LLOYD, D., FURR, J. R. & RUSSELL, A. D., 1997. Aspects of the mechanisms of action of biguanides on trophozoites and cysts of *Acanthamoeba castellanii. Journal of Applied Microbiology,* 82(1), pp.107-114.

KIM, C. K., GENTILE, D. M. & SPROUL, O. J., 1980. Mechanism of ozone inactivation of bacteriophage F2. *Applied and Environmental Microbiology,* 39(1), pp.210-218.

KISSMANN, J., AUSAR, S. F., FOUBERT, T. R., BROCK, J., SWITZER, M. H., DETZI, E. J., VEDVICK, T. S. & MIDDAUGH, C. R., 2008. Physical stabilization of norwalk virus-like particles. *Journal of Pharmaceutical Sciences,* 97(10), pp.4208-4218.

KLEIN, M. & DEFOREST, A., 1965. Chemical inactivation of viruses. *Federation Proceedings,* 24(2P1), pp.319-&.

KLEIN, M. & DEFOREST, A., 1983. Principles of viral inactivation. In: BLOCK, S. S., ed. Third edition. *Disinfection, Sterilization and Preservation.* Philadelphia: Lea & Febiger. pp. 422-434.

KOIVUNEN, J. & HEINONEN-TANSKI, H., 2005. Inactivation of enteric microorganisms with chemical disinfectants, UV irradiation and combined chemical/UV treatments. *Water Research,* 39(8), pp.1519-1526.

KOK, T., WATI, S., BAYLY, B., DEVONSHIRE-GILL, D. & HIGGINS, G., 2000. Comparison of six nucleic acid extraction methods for detection of viral DNA or RNA sequences in four different non-serum specimen types. *Journal of Clinical Virology,* 16(1), pp.59-63.

KONING, R., VAN DEN WORM, S., PLAISIER, J. R., VAN DUIN, J., ABRAHAMS, J. P. & KOERTEN, H., 2003. Visualization by cryo-electron microscopy of genomic RNA that binds to the protein capsid inside bacteriophage MS2. *Journal of Molecular Biology,* 332(2), pp.415-422.

KOOPMANS, M. & DUIZER, E., 2004. Foodborne viruses: an emerging problem. *International Journal of Food Microbiology,* 90(1), pp.23-41.

KRAMER, A., GALABOV, A. S., SATTAR, S. A., DOHNER, L., PIVERT, A., PAYAN, C., WOLFF, M. H., YILMAZ, A. & STEINMANN, J., 2006a. Virucidal activity of a new hand disinfectant with reduced ethanol content: comparison with other alcohol-based formulations. *Journal of Hospital Infection,* 62(1), pp.98.

KRAMER, A., SCHWEBKE, I. & KAMPF, G., 2006b. How long do nosocomial pathogens persist on inanimate surfaces? A systematic review. *Bmc Infectious Diseases,* 6, pp.130-138.

KREBS, F. C., MILLER, S. R., FERGUSON, M. L., LABIB, M., RANDO, R. F. & WGDAHL, B., 2005. Polybiguanides, particularly polyethylene hexamethylene biguanide, have activity against immunodeficiency virus type 1. *Biomedicine & Pharmacotheraphy,* 59(8), pp.438-445.

KUIKEN, T., FOUCHIER, R., RIMMELZWAAN, G. & OSTERHAUS, A., 2003. Emerging viral infections in a rapidly changing world. *Current Opinion in Biotechnology,* 14(6), pp.641-646.

KUZMANOVIC, D. A., ELASHVILI, I., WICK, C., O'CONNELL, C. & KRUEGER, S., 2003. Bacteriophage MS2: Molecular weight and spatial distribution of the protein and RNA components by small-angle neutron scattering and virus counting. *Structure,* 11(11), pp.1339-1348.

KUZMANOVIC, D. A., ELASHVILI, I., WICK, C., O'CONNELL, C. & KRUEGER, S., 2006. The MS2 coat protein shell is likely assembled under tension: A novel role for the MS2 bacteriophage A protein as revealed by small-angle neutron scattering. *Journal of Molecular Biology,* 355(5), pp.1095-1111.

LAEMMLI, U. K., 1970. Cleavage of structural proteins during assembly of head of bacteriophage T4. *Nature,* 227(5259), pp.680-&.

LAMBERT, P. A., 2004. Mechanisms of action of biocides. In: FRAISE A.P., LAMBERT P.A. & J.Y., M., ed. 4th. *Principles and practice of Disinfection, Preservation & Sterilization.* Oxford: Blackwell Publishing. pp. 139-153.

LAMBERT, R. J. W., 2003. Evaluation of antimicrobial efficacy. In: ADAM FRAISE, A., LAMBERT, P. A. & MAILLARD, J. Y., ed. 4th. *Principles and practices of disinfection, preservation & sterilization.* Oxford: Wiley-Blackwell Publisher. pp. 345-360.

LEVY, S. B., 2002. Active efflux, a common mechanism for biocide and antibiotic resistance. *Journal of Applied Microbiology,* 92(S1), pp.65S-71S.

LI, J. W., XIN, Z. T., WANG, X. W., ZHENG, J. L. & CHAO, F. H., 2004. Mechanisms of inactivation of hepatitis A virus in water by chlorine dioxide. *Water Research,* 38(6), pp.1514-1519.

LIU, R., LIN, Q., SUN, Y., LU, X., QIU, Y., LI, Y. & GUO, X., 2009. Expression, purification, and characterization of Hepatitis B virus surface antigens (HBsAg) in yeast *Pichia pastoris*. *Applied Biochemistry and Biotechnology,* 158(2), pp.432-444.

LOMBARDI, M. E., LADMAN, B. S., ALPHIN, R. L. & BENSON, E. R., 2008. Inactivation of Avian Influenza virus using common detergents and chemicals. *Avian Diseases,* 52(1), pp.118-123.

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, R. J., 1951. Protein measurement with the folin phenol reagent. *Journal of Biological Chemistry,* 193, pp.265-275.

LURIA, S. E., 1947. Reactivation of irradiated bacteriophage by transfer of self-reproducing units. *Proceeding of the National Academy of Sciences U.S.A.,* 33(9), pp.253-264.

MAGULSKI, T., PAULMANN, D., BISCHOFF, B., BECKER, B., STEINMANN, E., STEINMANN, J., GORONCY-BERMES, P. & STEINMANN, J., 2009. Inactivation of murine norovirus by chemical biocides on stainless steel. *Bmc Infectious Diseases,* 9(

MAHESHWARI, G., JANNAT, R., MCCORMICK, L. & HSU, D., 2004. Thermal inactivation of adenovirus type 5. *Journal of Virological Methods,* 118(2), pp.141-146.

MAILLARD, J. Y., 2001. Virus susceptibility to biocides: an understanding. *Reviews in Medical Microbiology,* 12(2), pp.63-74.

MAILLARD, J. Y., HANN, A. C., BEGGS, T. S., DAY, M. J., HUDSON, R. A. & RUSSELL, A. D., 1995. Electron - microscopic investigation of the effects of biocides on *Pseudomonas aeruginosa* PA0 bacteriophage F116. *Journal of Medical Microbiology,* 42(6), pp.415-420.

MANDEL, M. & HIGA, A., 1970. Calcium-dependent bacteriophage DNA infection. *Journal of Molecular Biology,* 53, pp.159-162.

MARTIN, H., LE POTIER, M. F. & MARIS, P., 2008. Virucidal efficacy of nine commercial disinfectants against porcine circovirus type 2. *Veterinary Journal,* 177(3), pp.388-393.

MBITHI, J. N., SPRINGTHORPE, V. S., BOULET, J. R. & SATTAR, S. A., 1992. Survival of hepatitis A virus on human hands and its transfer on contact with animate and inanimate surfaces. *Journal of Clinical Microbiology,* 30(4), pp.757-763.

MCDONNEL, G., 2007. Biocides: modes of actions and mechanisms of resistance. In: MANIVANNAN, G., ed. 1st. *Disinfection and decontamination: principles, applications and related issues.* CRC Press. pp. 87-124.

MCDONNELL, G. & RUSSELL, A. D., 1999. Antiseptics and disinfectants: Activity, action, and resistance. *Clinical Microbiology Reviews,* 12(1), pp.147-179.

MILES, A. A. & MISRA, S. S., 1938. The estimation of the bactericidal power of the blood. *Journal of Hygiene,* 38, pp.732-749.

MONTEFIORI, D. C., ROBINSON, W. E., MODLISZEWSKI, A. & MITCHELL, W. M., 1990. Effective inactivation of Human immunodeficiency virus with chlorhexidine antiseptics containing detergents and alcohol. *Journal of Hospital Infection,* 15(3), pp.279-282.

MOYA, A., HOLMES, E. C. & GONZALEZ-CANDELAS, F., 2004. The population genetics and evolutionary epidemiology of RNA viruses. *Nature Reviews Microbiology,* 2(4), pp.279-288.

MUELLER, S. W. & KREBSBACH, L. E., 2008. Impact of an antimicrobial-impregnated gauze dressing on surgical site infections including methicillin-resistant *Staphylococcus aureus* infections. *American Journal of Infection Control,* 36(9), pp.651-655.

MURR, A. S. & GOLDMAN, J. M., 2005. Twenty-week exposures to the drinking water disinfection by-product dibromoacetic acid: reproductive cyclicity and steroid concentrations in the female Sprague-Dawley rat. *Reproductive Toxicology,* 20(1), pp.73-80.

MURRAY, B. K., OHMINE, S., TOMER, D. P., JENSEN, K. J., JOHNSON, F. B., KIRSI, J. J., ROBISON, R. A. & O'NEILL, K. L., 2008. Virion disruption by ozone-mediated reactive oxygen species. *Journal of Virological Methods,* 153(1), pp.74-77.

NCBI, 2009. *GenBank database.* [Online] Available at: http://www.ncbi.nlm.nih.gov/sites/entrez?db=nucleotide [8/1/08]

NELSON, C. D. S., MINKKINEN, E., BERGKVIST, M., HOELZER, K., FISHER, M., BOTHNER, B. & PARRISH, C. R., 2008a. Detecting

small changes and additional peptides in the Canine parvovirus capsid structure. *Journal of Virology,* 82(21), pp.10397-10407.

NELSON, E. A. S., BRESEE, J. S., PARASHAR, U. D., WIDDOWSON, M. A. & GLASS, R. I., 2008b. Rotavirus epidemiology: The Asian Rotavirus Surveillance Network. *Vaccine,* 26(26), pp.3192-3196.

NEWMAN, M., SUK, F. M., CAJIMAT, M., CHUA, P. M. & SHIH, C., 2003. Stability and morphology comparisons of self-assembled virus-like particles from wild-type and mutant human hepatitis B virus capsid proteins. *Journal of Virology,* 77(24), pp.12950-12960.

NICOLETTI, G., BOGHOSSIAN, V., GUREVITCH, F., BORLAND, R. & MORGENROTH, P., 1993. The antimicrobial activity in-vitro of chlorhexidine, a mixture of isothiazolinones (KATHON CG) and cetyl trimethyl ammonium bromide (CTAB). *Journal Of Hospital Infection,* 23(2), pp.87-111.

O'BRIEN, R. T. & NEWMAN, J., 1979. Structural and compositional changes associated with chlorine inactivation of polioviruses. *Applied and Environmental Microbiology,* 38(6), pp.1034-1039.

OGORZALY, L. & GANTZER, C., 2006. Development of real-time RT-PCR methods for specific detection of F-specific RNA bacteriophage genogroups: Application to urban raw wastewater. *Journal of Virological Methods,* 138(1-2), pp.131-139.

OSBOURN, M., MCPHIE, K. A., RATNAMOHAN, V. M., DWYER, D. E. & DURRHEIM, D. N., 2009. Outbreak of human metapneumovirus infection in a residential aged care facility. *Communicable Diseases Intelligence,* 33(1), pp.38-40.

OULE, M. K., AZINWI, R., BERNIER, A. M., KABLAN, T., MAUPERTUIS, A. M., MAULER, S., NEVRY, R. K., DEMBELE, K., FORBES, L. & DIOP, L., 2008. Polyhexamethylene guanidine hydrochloride-based disinfectant: a novel tool to fight meticillin-resistant Staphylococcus aureus and nosocomial infections. *Journal of Medical Microbiology,* 57(12), pp.1523-1528.

PARISA, R., NATALIA, M., ANITA, S. & BILAL, I. J., 2009. HIV vaccines under study. *Dermatologic Therapy,* 22(2), pp.158-167.

PARK, G. W., BOSTON, D. M., KASE, J. A., SAMPSON, M. N. & SOBSEY, M. D., 2007. Evaluation of liquid- and fog-based application of sterilox hy ochlorous acid solution for surface inactivation of human norovirus. *Applied and Environmental Microbiology,* 73(14), pp.4463-4468.

PEDAHZUR, R., KATZENELSON, D., BARNEA, N., LEV, O., SHUVAL, H. I., FATTAL, B. & ULITZUR, S., 2000. The efficacy

of long-lasting residual drinking water disinfectants based on hydrogen peroxide and silver. *Water Science and Technology,* 42(1-2), pp.293-298.

PILCHER, K. S., SOIKE, K. F. & TROSPER, F., 1961. Studies of chemical inhibitors of influenza virus moltiplication: I. Biguanides and related compounds. *Antimicrobial Agents and Chemotherapy,* 11(6), pp.381-389.

PLOWMAN, R., GRAVES, N., GRIFFIN, M., ROBERTS, J. & SWAN, A., 1999. *The socio-economic burden of hospital acquired infection.* 1st ed. London: Public Health Laboratory Service.

POSCHETTO, L. F., IKE, A., PAPP, T., MOHN, U., BOHM, R. & MARSCHANG, R. E., 2007. Comparison of the sensitivities of noroviruses and feline calicivirus to chemical disinfection under field-like conditions. *Applied and Environmental Microbiology,* 73(17), pp.5494-5500.

PRINCE, D. L., PRINCE, H. N., THRAENHART, O., MUCHMORE, E., BONDER, E. & PUGH, J., 1993. Methodological approaches to disinfection of human hepatitis B virus. *Journal of Clinical Microbiology,* 31(12), pp.3296-3304.

PRINCE, H. N. & PRINCE, D. L., 2001. Principles of viral control and transmission. In: BLOCK, S. S., ed. 5th. *Disinfection, sterilization, and preservation.* Philadelphia: Lippincott Williams & Wilkins. pp.

RAFFO, P., SALLIEZ, A. C., COLLIGNON, C. & CLEMENTI, M., 2007. Antimicrobial activity of a formulation for the low temperature disinfection of critical and semi-critical medical equipment and surfaces. *New Microbiologica,* 30(4), pp.463-469.

RAVIS, S. M., SHAFFER, M. P., SHAFFER, C. L., DEHKHAGHANI, S. & BELSITO, D. V., 2003. Glutaraldehyde-induced and formaldehyde-induced allergic contact dermatitis among dental hygienists and assistants. *Journal of the American Dental Association,* 134(8), pp.1072-1078.

REXROAD, J., EVANS, R. K. & MIDDAUGH, C. R., 2006. Effect of pH and ionic strength on the physical stability of adenovirus type 5. *Journal of Pharmaceutical Sciences,* 95(2), pp.237-247.

REXROAD, J., WIETHOFF, C. M., GREEN, A. P., KIERSTEAD, T. D., SCOTT, M. O. & MIDDAUGH, C. R., 2003. Structural stability of adenovirus type 5. *Journal of Pharmaceutical Sciences,* 92(3), pp.665-678.

ROSS, R. S., VIAZOV, S., KHUDYAKOV, Y. E., XIA, G. L., LIN, Y., HOLZMANN, H., SEBESTA, C., ROGGENDORF, M. &

JANATA, O., 2009. Transmission of hepatitis C virus in an orthopedic hospital ward. *Journal of Medical Virology,* 81(2), pp.249-257.

ROY, D., WONG, P. K. Y., ENGELBRECHT, R. S. & CHIAN, E. S. K., 1981. Mechanism of enteroviral inactivation by ozone. *Applied and Environmental Microbiology,* 41(3), pp.718-723.

RUSSELL, A. D., 2003. Biocide use and antibiotic resistance: the relevance of laboratory findings to clinical and environmental situations. *Lancet Infectious Diseases,* 3(12), pp.794-803.

RUSSELL, A. D., 2004. Factors influencing the efficacy of antimicrobial agents. In: FRAISE, A. P., LAMBERT, P. A. & MAILLARD, J. Y., ed. 4th. *Principles and practice of disinfection, preservation & sterilization.* Oxford: Blackwell Publishing. pp. 98-127.

RUSSELL, A. D. & MCDONNELL, G., 2000. Concentration: a major factor in studying biocidal action. *Journal Of Hospital Infection,* 44(1), pp.1-3.

RUSSELL, W. C., 2009. Adenoviruses: update on structure and function. *Journal of General Virology,* 90, pp.1-20.

RUTALA, W. A., PEACOCK, J. E., GERGEN, M. F., SOBSEY, M. D. & WEBER, D. J., 2006. Efficacy of hospital germicides against adenovirus 8, a common cause of epidemic keratoconjunctivitis in health care facilities. *Antimicrobial Agents and Chemotherapy,* 50(4), pp.1419-1424.

RUX, J. J. & BURNETT, R. M., 2004. Adenovirus structure. *Human Gene Therapy,* 15(12), pp.1167-1176.

SALAS CAMPOS, L., GOMEZ FERRERO, O., ESTUDILLO PEREZ, V. & FERNANDEZ MANSILLA, M., 2006. Preventing nosocomial infections. Dressings soaked in polyhexamethylene biguanide (PHMB). *Revistas de Enfermagen,* 29(6), pp.43-8.

SAMBROCK, J. & RUSSEL, D. W., 2001. Bacteriophage lamda and its vectors. In: SAMBROCK, J. & RUSSEL, D. W., ed. 3rd. *Molecular cloning: A laboratory manual.* Cold Spring Harbor, NY: Cold Spring Harbor Press. pp. 34-51.

SATTAR, S. A., 2006. Allen Denver Russell Memorial Lecture - The use of microbiocides in infection control: a critical look at safety, testing and applications. *Journal of Applied Microbiology,* 101(4), pp.743-753.

SATTAR, S. A. & ANSARI, S. A., 2002. The fingerpad protocol to assess hygienic hand antiseptics against viruses. *Journal of Virological Methods,* 103(2), pp.171-181.

SATTAR, S. A., JASON, T., BIDAWID, S. & FARBER, J., 2000. Foodborne spread of hepatitis A: Recent studies on virus survival, transfer and inactivation. *Canadian Journal of Infection Disease,* 11(3), pp.159-63.

SATTAR, S. A., KARIM, Y. G., SPRINGTHORPE, V. S. & JOHNSONLUSSENBURG, C. M., 1987. Survival of human rhinovirus type 14 dried onto non-porous inanimate surfaces - Effect of relative humidity and suspending medium. *Canadian Journal of Microbiology,* 33(9), pp.802-806.

SATTAR, S. A., RAPHAEL, R. A., LOCHNAN, H. & SPRINGTHORPE, V. S., 1983. Rotavirus inactivation by chemical disinfectants and antiseptics used in hospitals. *Canadian Journal of Microbiology,* 29(10), pp.1464-1469.

SATTAR, S. A. & SPRINGTHORPE, V. S., 2001. Methods of testing the virucidal activity of chemicals. In: LAWRENCE, C. A. & BLOCK, S. S., ed. *Disinfection, sterilization, and preservation.* Philadelphia: Lea and Febiger. pp. 1391-1412.

SATTAR, S. A., SPRINGTHORPE, V. S., ADEGBUNRIN, O., ZAFER, A. A. & BUSA, M., 2003. A disc-based quantitative carrier test method to assess the virucidal activity of chemical germicides. *Journal of Virological Methods,* 112(1-2), pp.3-12.

SAUERBREI, A., EICHHORN, U., SCHEIBENZUBER, M. & WUTZLER, P., 2007. Hexon denaturation of human adenoviruses by different groups of biocides. *Journal of Hospital Infection,* 65(3), pp.264-270.

SAUERBREI, A., SCHACKE, M., GLUCK, B., EGERER, R. & WUTZLER, P., 2006. Validation of biocides against duck hepatitis B virus as a surrogate virus for human hepatitis B virus. *Journal of Hospital Infection,* 64(4), pp.358-365.

SAUERBREI, A., SEHR, K., BRANDSTADT, A., HEIM, A., REIMER, K. & WUTZLER, P., 2004a. Sensitivity of human adenoviruses to different groups of chemical biocides. *Journal of Hospital Infection,* 57(1), pp.59-66.

SAUERBREI, A., SEHR, K., EICHHORN, U., REIMER, K. & WUTZLER, P., 2004b. Inactivation of human adenovirus genome by different groups of disinfectants. *Journal of Hospital Infection,* 57(1), pp.67-72.

SCHABRUN, S. & CHIPCHASE, L., 2006. Healthcare equipment as a source of nosocomial infection: a systematic review. *Journal of Hospital Infection,* 63(3), pp.239-245.

SCHERBERICH, J. E., FISCHER, P., BIGALKE, A., STANGL, P., WOLF, G. B., HAIMERL, M. & SCHOEPPE, W., 1989. Routine diagnosis with Phastsystem compared to conventional electrophoresis - Automated sodium dodecyl sulfate polyacrylamide gel-electrophoresis, silver staining and western blotting of urinary proteins. *Electrophoresis,* 10(1), pp.58-62.

SCHIFF, G. M., STEFANOVIC, B. Y. & PENNEKAMP, J. K., 1984. Minimum human infectious dose of enteric viruses (echovirus-12) in drinking water. In: MELNICK, J. L., ed. 1st. *Enteric viruses in water.* Basel: Karger. pp.

SETH, P., 1999. *Adenoviruses: basic biology to gene therapy.* 1st ed. Austin: Landes Bioscience.

SHAPIRO, C. N. & MARGOLIS, H. S., 1993. Worldwide epidemiology of hepatitis A virus infection. *Journal of Hepatology,* 18(S11-S14.

SHARP, D. G., FLOYD, R. & JOHNSON, J. D., 1975. Nature of the surviving plaque-forming unit of reovirus in water containing bromine. *Applied Microbiology,* 29(1), pp.94-101.

SHARP, D. G. & KIM, K. S., 1966. Multiplicity reactivation and radiation survival of aggregated vaccinia virus. Calculation of plaque titer based on MR and particle aggregation seen in electron microscope. *Virology,* 29(3), pp.359-366.

SHARP, D. G. & LEONG, J., 1980. Inactivation of poliovirus-I (Brunhilde) single particles by chlorine in water. *Applied and Environmental Microbiology,* 40(2), pp.381-385.

SICKBERT-BENNETT, E. E., WEBER, D. J., GERGEN-TEAGUE, M. F., SOBSEY, M. D., SAMSA, G. P. & RUTALA, W. A., 2005. Comparative efficacy of hand hygiene agents in the reduction of bacteria and viruses. *American Journal of Infection Control,* 33(2), pp.67-77.

SIMONET, J. & GANTZER, C., 2006. Degradation of the Poliovirus 1 genome by chlorine dioxide. *Journal of Applied Microbiology,* 100(4), pp.862-870.

SIYIKANCHANA, K., SHISLER, J. L. & MARINAS, B. J., 2008. Inactivation kinetics of adenovirus serotype 2 with monochloramine. *Water Research,* 42(6-7), pp.1467-1474.

SMITH, J. M. B., 2004. Laboratory evaluation of antimicrobial agents. In: HUGO, W. B., DENYER, S. P., HODGES, N. A., GORMAN, S. P. & RUSSELL, A. D., ed. 7th. *Pharmaceutical microbiology.* Oxford: Wiley-Blackwell Publisher. pp. 187-201.

SOBSEY, M. D., OLDHAM, C. E. & MCCALL, D. E., 1991. Comparative inactivation of hepatitis A virus and other enteroviruses in water by iodine. *Water Science and Technology,* 24(2), pp.331-337.

SPRINGTHORPE, V. S., GRENIER, J. L., LLOYDEVANS, N. & SATTAR, S. A., 1986. Chemical disinfection of human rotaviruses - Efficacy of commercially available products in suspension tests. *Journal of Hygiene,* 97(1), pp.139-161.

STEINMANN, J., 2001. Some principles of virucidal testing. *Journal of Hospital Infection,* 48(Supplement 1), pp.S15-S17.

STEINMANN, J., 2004. Surrogate viruses for testing virucidal efficacy of chemical disinfectants. *Journal of Hospital Infection,* 56(Supplement 2), pp.49.

SVENSSON, U. & PERSSON, R., 1984. Entry of adenovirus 2 into Hela cells. *Journal of Virology,* 51(3), pp.687-694.

TERPSTRA, F. G., VAN DEN BLINK, A. E., BOS, L. M., BOOTS, A. G. C., BRINKHUIS, F. H. M., GIJSEN, E., VAN REMMERDEN, Y., SCHUITEMAKER, H. & VAN 'T WOUT, A. B., 2007. Resistance of surface-dried virus to common disinfection procedures. *Journal of Hospital Infection,* 66(4), pp.332-338.

THAKKAR, N., PIRRONE, V., PASSIC, S., ZHU, W., KHOLODOVYCH, V., WELSH, W., RANDO, R. F., LABIB, M. E., WIGDAHL, B. & KREBS, F. C., 2009. Specific interactions between the viral coreceptor CXCR4 and the biguanide-based compound NB325 mediate inhibition of human immunodeficiency virus type 1 infection. *Antimicrobial Agents and Chemotherapy,* 53(2), pp.631-638.

THURMAN, R. B. & GERBA, C. P., 1988a. Molecular mechanisms of viral inactivation by water disinfectants. *Advances in Applied Microbiology,* 33, pp.75-105.

THURMAN, R. B. & GERBA, C. P., 1988b. Viral inactivation by water disinfectants. *Advances in Applied Microbiology,* 33, pp.75-105.

THURSTON-ENRIQUEZ, J. A., HAAS, C. N., JACANGELO, J. & GERBA, C. P., 2003a. Chlorine inactivation of adenovirus type 40 and feline calicivirus. *Applied and Environmental Microbiology,* 69(7), pp.3979-3985.

THURSTON-ENRIQUEZ, J. A., HAAS, C. N., JACANGELO, J., RILEY, K. & GERBA, C. P., 2003b. Inactivation of feline calicivirus and adenovirus type 40 by UV radiation. *Applied and Environmental Microbiology,* 69(1), pp.577-582.

TIWARI, A., PATNAYAK, D. P., CHANDER, Y., PARSAD, M. & GOYAL, S. M., 2006. Survival of two avian respiratory viruses on porous and nonporous surfaces. *Avian Diseases,* 50(2), pp.284-287.

UK DEPARTMENT OF HEALTH, 2007. *Health clearance for tuberculosis, hepatitis B, hepatitis C and HIV: New healthcare workers.* [Online] Available at: http://www.dh.gov.uk/en/Publicationsandstatistics/Publications/PublicationsPolicyAndGuidance/DH_073132

VALENTI, W. M., MENEGUS, M. A., HALL, C. B., PINCUS, P. H. & DOUGLAS, R. G., 1980. Nosocomial viral infections .1. Epidemiology and significance. *Infection Control and Hospital Epidemiology,* 1(1), pp.33-37.

VALLURI, S., FLEMING, T. P., LAYCOCK, K. A., TARLE, I. S., GOLDBERG, M. A., GARCIAFERRER, F. J., ESSARY, L. R. & PEPOSE, J. S., 1997. *In vitro* and *in vivo* effects of polyhexamethylene biguanide against herpes simplex virus infection. *Cornea,* 16(5), pp.556-559.

VAN DEN WORM, S. H. E., KONING, R. I., WARMENHOVEN, H. J., KOERTEN, H. K. & VAN DUIN, J., 2006. Cryo electron microscopy reconstructions of the Leviviridae unveil the densest icosahedral RNA packing possible. *Journal of Molecular Biology,* 363(4), pp.558-565.

VEGA, E., SMITH, J., GARLAND, J., MATOS, A. & PILLAI, S. D., 2005. Variability of virus attachment patterns to butterhead lettuce. *Journal of Food Protection,* 68(10), pp.2112-2117.

VICINI, P., ZANI, F., COZZINI, P. & DOYTCHINOVA, I., 2002. Hydrazones of 1,2-benzisothiazole hydrazides: synthesis, antimicrobial activity and QSAR investigations. *European Journal of Medicinal Chemistry,* 37(7), pp.553.

VIEIRA, O. V., HARTMANN, D. O., CARDOSO, C. M. P., OBERDOERFER, D., BAPTISTA, M., SANTOS, M. A. S., ALMEIDA, L., RAMALHO-SANTOS, J. & VAZ, W. L. C., 2008. Surfactants as microbicides and contraceptive agents: A systematic *in vitro* study. *PLoS ONE,* 3(8), pp.e2913.

VINCZE, T., POSFAI, J. & ROBERTS, R. J., 2003. NEBcutter: a program to cleave DNA with restriction enzymes. *Nucleic Acids Research,* 31, pp.3688-3691.

VON RHEINBABEN, F., SCHUNEMANN, S., GROSS, T. & WOLFF, M. H., 2000. Transmission of viruses via contact in a household setting: experiments using bacteriophage phi X174 as a model virus. *Journal of Hospital Infection,* 46(1), pp.61-66.

VONBERG, R. P. & GASTMEIER, P., 2007. Hospital-acquired infections related to contaminated substances. *Journal of Hospital Infection,* 65(1), pp.15-23.

VRIJSEN, R., ROMBAUT, B. & BOEYE, A., 1983. Ph-dependent aggregation and electrofocusing of poliovirus. *Journal of General Virology,* 64(OCT), pp.2339-2342.

WALLBANK, A. M., DRULAK, M., POFFENROTH, L., BARNES, C., KAY, C. & LEBTAG, I., 1978. Wescodyne - Lack of activity against poliovirus in presence of organic matter. *Health Laboratory Science,* 15(3), pp.133-137.

WANG, X. W., LI, J. S., JIN, M., ZHEN, B., KONG, Q. X., SONG, N., XIAO, W. J., YIN, J., WEI, W., WANG, G. J., SI, B. Y., GUOB, B. Z., LIU, C., OU, G. R., WANG, M. N., FANG, T. Y., CHAO, F. H. & LI, J. W., 2005. Study on the resistance of severe acute respiratory syndrome-associated coronavirus. *Journal of Virological Methods,* 126(1-2), pp.171-177.

WEBER, D. J., BARBEE, S. L., SOBSEY, M. D. & RUTALA, W. A., 1999. The effect of blood on the antiviral activity of sodium hypochlorite, a phenolic, and a quaternary ammonium compound. *Infection Control and Hospital Epidemiology,* 20(12), pp.821-827.

WEBER, T. P. & STILIANAKIS, N. I., 2008. Inactivation of influenza A viruses in the environment and modes of transmission: A critical review. *Journal of Infection,* 57(5), pp.361-373.

WEISSMAN, C., BILLETER, M. A., GOODMAN, H. M., HINDLEY, J. & WEBER, H., 1973. Structure and function of phage RNA. *Annual Review of Biochemistry,* 42, pp.303-328.

WHO, 2009a. *Influeanza A (H1N1) - Update 42.* [Online] Available at: http://www.who.int/csr/don/2009_06_05a/en/index.html [02/06/2009]

WHO, 2009b. *Influenza A(H1N1).* [Online] Available at: http://www.who.int/csr/disease/swineflu/en/index.html [18/06/2009]

WICHELHAUS, A., BADER, F., SANDER, F., KRIEGER, D. & MERTENS, T., 2006. Effective disinfection of orthodontic pliers. *Journal of Orofacial Orthopedics/Fortschritte der Kieferorthopädie,* 67(5), pp.316-336.

WOOD, A. & PAYNE, D., 1998. The action of three antiseptics/disinfectants against enveloped and non-enveloped viruses. *Journal of Hospital Infection,* 38(4), pp.283-295.

WOOLWINE, J. D. & GERBERDING, J. L., 1995. Effect of testing method on apparent activities of antiviral disinfectants and antiseptics. *Antimicrobial Agents and Chemotherapy,* 39(4), pp.921-923.

WUTZLER, P. & SAUERBREI, A., 2000. Virucidal efficacy of a combination of 0.2% peracetic acid and 80% (v/v) ethanol (PAA-ethanol) as a potential hand disinfectant. *Journal of Hospital Infection,* 46(4), pp.304-308.

ZAFAR, A., HABIB, F., HADWANI, R., EJAZ, M., KHOWAJA, K., KHOWAJA, R. & IRFAN, S., 2009. Impact of infection control activities on the rate of needle stick injuries at a tertiary care hospital of Pakistan over a period of six years: an observational study. *Bmc Infectious Diseases,* 9, pp.78-82.

ZHANG, L. Y., FU, Y. P., XIE, J. T., JIANG, D. H., LI, G. Q. & YI, X. H., 2009. A novel virus that infecting hypovirulent strain XG36-1 of plant fungal pathogen Sclerotinia sclerotiorum. *Virology Journal,* 6(96), pp.111-117.

ZHANG, W. & ARCOS, R., 2005. Interaction of the adenovirus major core protein precursor, pVII, with the viral DNA packaging machinery. *Virology,* 334(2), pp.194-202.

ZHANG, W., LOW, J. A., CHRISTENSEN, J. B. & IMPERIALE, M. J., 2001. Role for the Adenovirus IVa2 protein in packaging of viral DNA. *Journal of Virology,* 75(21), pp.10446-10454.

9. APPENDIX

Published version of chapters 3, 4, 5.

- Pinto F., Maillard J-Y. and Denyer S., 2010. Effect of surfactants, temperature and sonication on the virucidal activity of polyhexamethylene biguanide (PHMB). *Journal of American Infection Control.* In Press.
- Pinto F. Maillard J-Y., Denyer S. and McGeechan P., 2010. PHMB microbicide exposure leads to viral aggregation. *Journal of Applied Microbiology.* In Press.

Journal of
Applied Microbiology

Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

Polyhexamethylene biguanide exposure leads to viral aggregation

F. Pinto[1], J.-Y. Maillard[1], S.P. Denyer[1] and P. McGeechan[2]

1 Welsh School of Pharmacy, Cardiff University, Cardiff, UK
2 Arch Chemicals, Hexagon Tower, Blackley, Manchester, UK

Keywords
activity, mechanisms of action, PHMB, virus.

Correspondence
Jean-Yves Maillard, Welsh School of Pharmacy, Cardiff University, King Edward VII avenue, Cardiff CF10 3NB, UK.
E-mail: maillardj@cardiff.ac.uk

2009/1220: received 8 July 2009, revised 18 September 2009 and accepted 28 September 2009

doi:10.1111/j.1365-2672.2009.04596.x

Abstract

Aims: This study reports the activity of two biguanides against MS2 bacteriophage used as a surrogate virus for nonenveloped mammalian viruses and provides an explanation as to their apparent limited efficacy.
Methods and Results: When tested in a standard suspension test, two polyhexamethylene biguanides (PHMB), VANTOCIL™ TG and COSMOCIL™ CQ, reduced the viability of MS2 by only 1–2 $\log_{10}$ PFU ml^{-1}. Exposure time up to 30 min did not affect the activity of the biguanides, although both PHMB were shown to strongly interact with MS2 proteins.
Conclusions: Inactivation kinetics and change in virus hydrophobicity suggested that PHMB induces the formation of viral aggregates. This hypothesis was supported using dynamic light scattering that showed an increase in viral aggregates sizes (up to 500 nm) in a concentration-dependent manner.
Significance and Impact of the Study: It has been reported that viral aggregation is responsible for virus survival to the biocide exposure. Here, this might be the case, because the virucidal activity of the biguanides was modest and viral aggregation important. The formation of viral aggregates during virus exposure to PHMB was unlikely to overestimate the virucidal potential of the biguanides.

Introduction

The use of biocides is crucial for the control of microbial contamination and infection in the health care environment (Maillard 2005). Although the number and use of products containing a biocide are increasing, there is generally a lack of understanding of their mechanisms of action. When viruses are concerned, the paucity of information deepened. A number of biocides (e.g. quaternary ammonium compounds, biguanides, phenolics) have been described to have a virucidal activity against enveloped viruses, but there is a general lack of efficacy against certain nonenveloped ones (Broxton *et al.* 1984; Krebs *et al.* 2005; Sauerbrei *et al.* 2006). Empirically, the explanation for this difference in activity has been based on both the interaction of these agents against the envelope of these viruses and their lack of activity against viral capsid proteins (Maillard 2001). Very few studies have explored the development of viral resistance during or following treatment. One of the described forms of resistance is viral aggregation whereby viral particles within the aggregate are somewhat protected from the deleterious effect of biocides (Sharp *et al.* 1975; Jensen *et al.* 1980).

Polyhexamethylene biguanide (PHMB) is a cationic antimicrobials agent used over 40 years in the health care environment, food and water industries. Current applications include impregnation of fabrics to prevent microbial growth, water treatment and disinfection of various solid surfaces. Its composition is a mixtures of polymeric biguanides of structure $[-(CH_2)_6.NH.C(=NH).NH.C.NH-]_n$, where n can vary from 2 to 40, resulting in a molecular mass range of 400–8000. Its antibacterial activity is directly correlated with the number of polymers, inducing anionic phospholipids phase separation and causing lost of membrane function with precipitation of intracellular

constituents in bacteria (Broxton *et al.* 1984; Allen *et al.* 2006). VANTOCIL™ TG, which is a commercial bactericide for use in industrial, institutional and domestic applications, causes domain formation of the acidic phospholipids of the cytoplasmic membrane, bringing permeability changes and altering enzymes function in bacteria (Chawner and Gilbert 1989). Although its interaction with viruses has not been described, PHMB has been found active against HIV-1 (Krebs *et al.* 2005), and other biguanide derivatives were shown to be effective against vaccinia and influenza viruses (Pilcher *et al.* 1961; Fara *et al.*, 1974). Such information indicates that the viral envelope is an important susceptible target site to these biocides, providing a justification for their preferential activity against enveloped viruses.

This study presents findings on the virucidal activity of PHMB against nonenveloped viruses and provides evidence that the biocide-induced viral aggregation in a concentration-dependent manner.

Materials and methods

Bacteriophage MS2 suspension and enumeration

The bacteriophage MS2 (NCIMB 10108; ATCC 15597-B1) was used as a surrogate for nonenveloped mammalian viruses and notably poliovirus (Jones 1995; Brion and Silverstein 1999; Koivunen and Heinonen-Tanski 2005; Sickbert-Bennett *et al.* 2005). MS2 was propagated in *Escherichia coli* (NCIMB 9481) following the protocol described by Woolwine and Gerberding (1995).

MS2 stock suspension was obtained by pipetting 5 ml of SM buffer (5% v/v 1 mol l^{-1} Tris/HCl pH 7·4, 0·2% w/v $MgSO_4$, and 0·5% w/v gelatine) onto a plaque assay plate showing at least 50 plaques. The top agar layer was scraped and poured into a centrifuge tube and centrifuged for 15 min at 11 000 *g* at 4°C. The supernatant containing phages was filtered through a 0·22-μm membrane filter and stored at 4–5°C until used. To enumerate the phage stock, a plaque assay was performed as described by Adams (1959).

Biocides and suspension test

PHMB-based biocides were VANTOCIL™ TG (MW 2678; Arch Chemicals, Manchester, UK) and cosmocil™ CQ (MW 3016; Arch Chemicals). Both PHMB were used at two concentrations, 200 and 800 ppm, which reflect the range of concentrations used in commercial products. They were prepared monthly in a sterile bottle using sterile deionized water.

The suspension test used here was based on the European Standard prEN13610 (Anon 2001). The efficacy of the PHMB was tested at four different contact times (1, 5, 10 and 30 min) at room temperature. Briefly, 1 ml of phage suspension adjusted to *c.* 10^{10} PFU ml^{-1} was added to 9 ml of PHMB. After the appropriate exposure time, 1 ml of the test mixture was added to 9 ml of neutralizer (6% w/v Tween 80; 0·45% w/v lecithin and 0·1% w/v L-histidine). After 2-min contact in the neutralizer, a sample was taken, serially diluted (tenfold) and surviving bacteriophages enumerated with the plaque assay (Adams 1959). In the control, PHMB was replaced with sterile distilled deionized water. The toxicity of the neutralizer towards MS2, and its host was validated together with the efficacy of the neutralizer to quench the activity of the biocide as follows: one ml of the overnight host cell suspension (*c.* 10^9 CFU ml^{-1}) was mixed with 9 ml of neutraliser. After 10-min exposure time, 1 ml of sample was serially diluted and plated using the Miles–Misra method (Miles and Misra 1938). After 24-h incubation at 37°C, colonies were counted, and the reduction factor (RF) was calculated as $\log_{10}$ (CFU ml^{-1} initial inoculum) − $\log_{10}$ (CFU ml^{-1} after exposure). The neutraliser toxicity test was similar with MS2. One millilitre of phage suspension (*c.* 10^9 PFU ml^{-1}) and 9 ml of neutraliser were mixed together for 10 min. Then, 1 ml of sample was withdrawn and serially diluted, and surviving bacteriophages enumerated with the plaque assay (Adams 1959). The RF was calculated as $\log_{10}$ (PFU ml^{-1} initial inoculum) − $\log_{10}$ (PFU ml^{-1} after exposure).

The efficacy of the neutraliser to quench effectively PHMB was performed with both the host bacteria and MS2. Two different ratios (1/1 and 2/1) between the biocide and the neutraliser were tested, as different amounts of disinfectant and neutraliser were used in this investigation. One millilitre of the overnight host cell (*c.* 10^9 CFU ml^{-1}) or phage suspension (*c.* 10^9 PFU ml^{-1}) was added to a disinfectant/neutraliser mixture and left for 10 min and serially diluted. Bacterial survival and phage survival following exposure were measured as described previously.

Precipitation and purification of MS2

The protocol to precipitate and purify the bacteriophage was based on Sambrook and Russell (2001) with the following modifications: PEG 8000 and NaCl were added to the MS2 suspension (*c.* 10^{10} PFU ml^{-1}) simultaneously. The CsCl equilibrium gradient was prepared with the three densities ρ = 1·35 g cm^{-3}, ρ = 1·45 g cm^{-3}, ρ = 1·70 g cm^{-3} in a 13·5-ml centrifuge tube, layering 2·6 ml of the three steps gradient. The three densities were chosen because of MS2 density of 1·38 g cm^{-3} (Kuzmanovic *et al.* 2003) The virus (5·2 ml) was layered on top of the gradient and then centrifuged at 363 000 *g* (Beckman Coulter;

Fullerton, CA, USA) for 8 h at 20°C. Purified bacteriophages were harvested, dialysed overnight against SM buffer and then filtered through 0·22-μm filter.

Hydrophobicity tests

Two different tests were performed: the adsorption to lipid and the MATH tests. The biocide concentrations tested were 2, 20, 200 and 800 ppm.

Adsorption to cholesterol was based on a modified protocol for bacteriophages described by Klein and Deforest (1983). A 5% w/v cholesterol suspension was prepared adding 0·01% w/v of Tween 80 to increase the lipid solubility. The suspension was kept at 4°C and used at room temperature during the experiments. One millilitre of MS2 suspension (10^{10} PFU ml^{-1}) was mixed with 9 ml of PHMB biocide at different concentrations (2, 20, 200 and 800 ppm). Deionized water was used instead of PHMB as a control. After 5-min exposure time, 1 ml of the text mixture was mixed with 4 ml of the cholesterol solution. The mixture was constantly agitated at 150 rev min^{-1} for 30 min at room temperature and then centrifuged twice for 30 min at 5000 *g* at 20°C. The number of phage present in the aqueous supernatant was enumerated according to Adams (1959).

Adsorption to hexadecane (MATH method) was described by Denyer *et al.* (1993). One ml of MS2 suspension (10^{10} PFU ml^{-1}) was mixed with 9 ml of PHMB biocide at different concentrations (2, 20, 200 and 800 ppm). Deionized water was used instead of PHMB as a control. After 5-min exposure time, 1 ml of the text mixture was mixed with 0·2 ml of hexadecane at room temperature for 10 min. After 60-s vortexing, the two phases were allowed to separate at room temperature for 7 min. The number of phages in the aqueous phase was measured as described earlier.

The percentage of MS2 partitioned to the lipidic-hydrocarbon phase was calculated as: (PFU ml^{-1} inoculated − PFU ml^{-1} in the aqueous phase)/PFU ml^{-1} inoculated × 100.

Dynamic light scattering

Dynamic light scattering measured with N4plus (Beckman Coulter) was used to measure the size of the aggregates of MS2 before and after the treatment with PHMB. Purified virus suspension (10^{11} PFU ml^{-1}) was dialysed overnight against KCl (0·250 mol l^{-1}) and then filtered through 0·22-μm filter. The sample concentration was adjusted to ensure that the count per second fell in the range 5×10^4–1×10^6. MS2 suspension (10^{11} PFU ml^{-1}) was then exposed to PHMB (2, 20, 200 and 800 ppm) for 2 min. Light scattering measurements ran for 240 s using a laser angle of 90° and were made in triplicate. To analyse the results effectively, the percentage of particles of a different size were pulled together into arbitrary size categories.

Protein analysis

MS2 exposure to biocide prior to protein analysis was performed at two different PHMB concentrations, 200 and 800 ppm. Controls were tested against deionized water.

A volume of the purified MS2 (0·375 ml; 10^{11} PFU ml^{-1}) was mixed with 0·125 ml of biocide. After 10-min contact time, a cold acetone precipitation of proteins was performed mixing 0·3 ml of the sample with 1·2 ml of cold acetone. After incubation at −20°C for 3 h, the sample was centrifuged at 15 000 *g* for 15 min at 4°C. The supernatant was discharged, and the pellet was air-dried for 5–10 min to drive off the acetone.

The pellet was then resuspended with 20 μl of 2× SDS sample buffer (15·14% w/v Tris–HCl pH 6·8, 20% v/v glycerol, 4% w/v SDS, 0·2% w/v 2-mercaptoethanol (2-ME) and 0·001% w/v bromophenol blue) and boiled for 3 min. The sample was allowed to cool at room temperature and a quick centrifugation (8000 *g* for 10 s at 15°C) was carried to remove any insoluble materials. Ten microlitres of sample (*c.* 0·5 mg ml^{-1} of proteins) was loaded onto a polyacrylamide 20% homogeneous gel (Amersham Pharmacia Biotech AB, Buckinghamshire, UK). Gels were developed by silver staining (Phast-System separation technique file no. 110; Pharmacia AB) using the automated developing chamber of the Phast-System (Amersham Pharmacia Biotech AB). The concentration of the proteins was measured according to Gerhart *et al.* (1994).

Attachment of PHMB to phage proteins was measured qualitatively with a colorimetric assay based on the property of PHMB to change the pK of certain indicator, such as Eosin Y (Gilbert *et al.* 1990). The proteins separation was run in duplicate gels. One gel was silver stained, whereas the other was dyed with Eosin Y. The gel was first fixed in 7% v/v acetic acid for 10 min and then for another 20 min in 50% v/v methanol. A washing step was performed for 20 min. The gel was finally soaked in 10% w/v sodium acetate (100 ml), 0·024% w/v Eosin y (250 ml) and water (150 ml), until pink bands were visible (1 h). A last step of washing was performed to wash away all the dye not attached to the biocide. Controls, which consisted of PHMB mixed with deionized water (final concentration 200 and 800 ppm) and processed through the cold acetone precipitation were used to assess the presence of the biocide in solution after the SDS-PAGE protocol.

Electron microscopy

One millilitre of concentrated phage stock (10^{11} PFU ml^{-1}) was treated with 1 ml of biocide. After 10-min exposure, 20 μl of sample was mixed with 30 μl of 2% methylamine tungstate for 10 min. A drop of the negatively stained phage was placed on a nickel- or copper-coated grid and left drying for 20 min. The excess of liquid was removed with filter paper. The grid was examined in a Philips EM 400T electron microscope operating at 80 kV accelerating voltage at magnification of ×28 000–44 000. Control consisted of MS2 treated in with distilled deionized water instead of PHMB.

Statistical analysis

Suspension tests were performed five times in duplicates. All data were transformed in $\log_{10}$ to assure normal distribution. RF was calculated as $\log_{10}$ (PFU ml^{-1} initial inoculum) − $\log_{10}$ (PFU ml^{-1} after exposure). The other tests described earlier (protein analysis and hydrophobicity) were performed on three separate occasions in duplicate. Any differences among results were analysed by parametric test, one-way ANOVA test. If the test resulted significant, *post hoc* Tukey test was used to underline which couple of variables resulted different. The dynamic light scattering results were analysed using the χ^2 test.

Results

Controls

The neutralizer (6% w/v Tween 80; 0·45% w/v lecithin and 0·1% w/v L-histidine) showed no toxicity against the host cell or MS2 (Table 1). Furthermore, it quenched effectively the biguanides (Table 1). A < 0·33 $\log_{10}$ reduction in PFU ml^{-1} was observed with MS2, and a < 0·52 $\log_{10}$ reduction in CFU ml^{-1} was measured with the host cell, both after treatment with vantocil™ TG and COSMOCIL™ CQ. Such a low reduction in number was deemed not to be significant.

A neutralizer was not used for the protein analysis and electron microscopical studies in order not to dilute the phage suspension. For these studies, the overall damage caused by PHMB was of interest. It was not the purpose of this specific investigation to link damage to time point.

Virucidal activity of PHMB

The maximum $\log_{10}$ reduction in number observed with VANTOCIL™ TG and COSMOCIL™ CQ at a concentration of 800 ppm was 1·02 ± 0·41 and 1·07 ± 0·30 $\log_{10}$ within 30-min exposure (Table 2). The activity of the biocide at concentrations of 200 or 800 ppm was independent of contact time for VANTOCIL™ TG (ANOVA; $F_{3,16} = 0·70$; $P > 0·05$) or COSMOCIL™ CQ (ANOVA;

Table 1 Neutraliser toxicity and neutraliser efficacy to quench the activity of VANTOCIL™ TG and COSMOCIL™ CQ. Data are expressed as $\log_{10}$ reduction in number (CFU ml^{-1} or PFU ml^{-1})

		Neutraliser efficacy to quench PHMB at different ratio			
		VANTOCIL™ TG		COSMOCIL™ CQ	
	Neutraliser toxicity	1/1	2/1	1/1	2/1
Escherichia coli	0·00*	0·28 ± 0·16	0·52 ± 0·36	0·41 ± 0·23	0·44 ± 0·18
MS2	0·01 ± 0·28	0·01 ± 0·28	0·33 ± 0·32	0·31 ± 0·31	0·18 ± 0·04

PHMB, polyhexamethylene biguanide.
*No reduction in number was measured.

Table 2 Suspension test results. VANTOCIL™ TG and COSMOCIL™ CQ at concentrations of 200 and 800 ppm against MS2. Data are expressed as $\log_{10}$

		$\log_{10}$ reduction in PFU ± SD			
Contact time (min)	pH	VANTOCIL™ TG 200 ppm	VANTOCIL™ TG 800 ppm	COSMOCIL™ CQ 200 ppm	COSMOCIL™ CQ 800 ppm
1	7·61 ± 0·18	1·06 ± 0·61	0·44 ± 0·67	0·00*	0·54 ± 0·36
5	7·64 ± 0·21	1·15 ± 0·53	0·61 ± 0·59	0·00*	0·76 ± 0·45
10	7·64 ± 0·23	1·12 ± 0·55	0·77 ± 0·85	0·00*	0·77 ± 0·40
30	7·64 ± 0·22	1·15 ± 0·64	1·02 ± 0·43	0·83 ± 0·02	1·07 ± 0·30

*No reduction in number was measured.

$F_{3,16} = 1{\cdot}61$; $P > 0{\cdot}05$), except for the latter after 30-min contact where a significant (ANOVA; $F_{3,16} = 11{\cdot}8$; $P < 0{\cdot}05$) higher reduction in MS2 was observed at a concentration of 200 ppm. The activity of VANTOCIL™ TG at 200 and 800 ppm did not differ significantly (*t*-test, $P > 0{\cdot}05$), even though RFs at 200 ppm appeared higher than at 800 ppm.

Hydrophobicity tests

MS2 was shown to be hydrophilic with 92% of viral particles partitioned in the aqueous phase when mixed with cholesterol (data not shown). The number of phage recovered in the aqueous phase was taken into account when the effect of the biguanides on hydrophobicity was tested. For comparison purpose, the sensitivity of the enumeration method was taken into account to calculate the number of phage in the aqueous phase. As a result, the number of phage counted in the aqueous phase where no pretreatment occurred was adjusted to 100% (Fig. 1). VANTOCIL™ TG and COSMOCIL™ CQ clearly modified the surface properties of the phage resulting in a statistically significant increase in hydrophobicity (Cholesterol test: VANTOCIL™ TG ANOVA; $F_{1,8} = 7{\cdot}4$; $P < 0{\cdot}05$; COSMOCIL™ CQ, ANOVA; $F_{1,8} = 162{\cdot}1$; $P < 0{\cdot}05$; MATH test: VANTOCIL™ TG, ANOVA; $F_{1,8} = 146{\cdot}4$; $P < 0{\cdot}05$; COSMOCIL™ CQ, ANOVA; $F_{1,8} = 32{\cdot}6$; $P < 0{\cdot}05$) (Fig. 1). Indeed, MS2 became so hydrophobic that no phage particles were recovered from the aqueous phase. Generally hydrophobicity increased with increasing concentrations of PHMB (Fig. 1). The negative results observed with lower concentrations of PHMB, might be explain with the addition of Tween 80 in the cholesterol suspension. Tween 80 is a surfactant which might help desegregation of clumps and produce higher count.

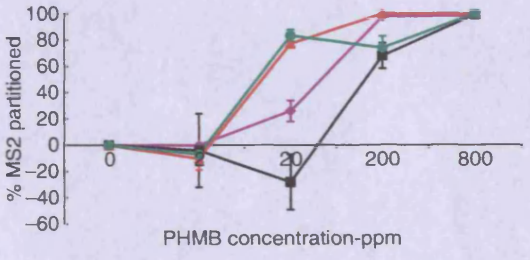


Figure 1 Hydrophobicity test results. Values expressed the % of the phage partitioned in the nonaqueous phase. Violet lozenge: VANTOCIL™ TG tested with MATH; black square; COSMOCIL™ CQ tested with MATH; red triangle VANTOCIL™ TG tested using the cholesterol assay; green circle COSMOCIL™ CQ tested using the cholesterol assay.

Dynamic light scattering

Dynamic light scattering is a technique which determines the size distribution of particles in solution. When a beam of light passes through a suspension, particles scatter light which is detected by a device. The detector measures the time-dependent fluctuations of the scattered intensity. These fluctuations derive by the random thermal (Brownian) motion of the particles, and the distance among them is therefore constantly varying. The time-dependent fluctuations are then analysed to calculate the particle sizes distribution (Berne and Pecora 1976). Here, to simplify the readings, five arbitrary size range categories were used. The results were expressed as percentage of intensity which reflects the mean value of the number of viral particles/aggregates within the size range taken on three separate readings. Control (i.e. untreated) MS2 showed some degrees of aggregation which might be explained with high concentration of phage particles used in this test (Fig. 2). However, as the concentration of VANTOCIL™ TG (Fig. 2) or COSMOCIL™ CQ (Fig. 3) increased, the size of the particles/aggregates increased significantly (χ^2 test, $P < 0{\cdot}01$). At a concentration of

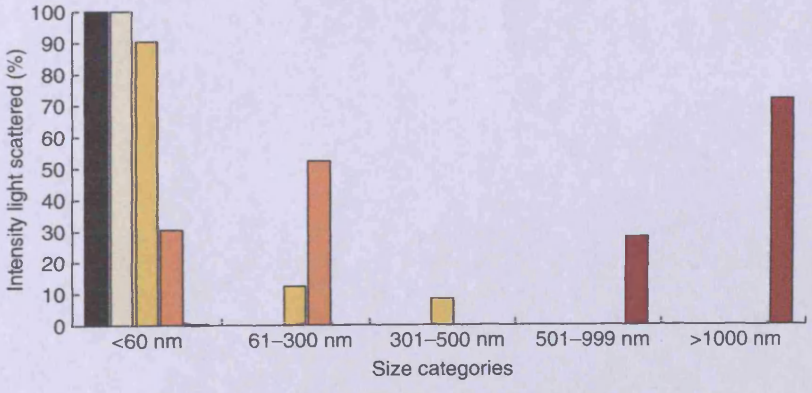


Figure 2 MS2 treated with VANTOCIL™ TG. Distribution of five size groups of MS2 aggregates in function of the % intensity of the light scattered. The magnitude of each histogram is proportional to the % of the total scattered intensity caused by the particles of the corresponding group size. MS2 exposure: Control (black); VANTOCIL™ TG at 2 ppm (light yellow) and at 20 ppm (yellow); VANTOCIL™ TG at 200 ppm (orange) and at 800 ppm (red).

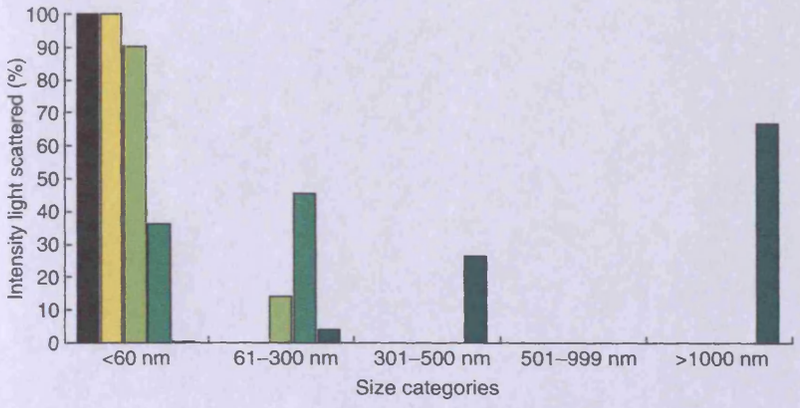


Figure 3 MS2 treated with COSMOCIL™ CQ. Distribution of five size groups of MS2 aggregates in function of the % intensity of the light scattered. The magnitude of each histogram is proportional to the % of the total scattered intensity caused by the particles of the corresponding group size. MS2 exposure: Control (black); COSMOCIL™ CQ at 2 ppm (yellow) and at 20 ppm (light green); COSMOCIL™ CQ at 200 ppm (green) and at 800 ppm (dark green).

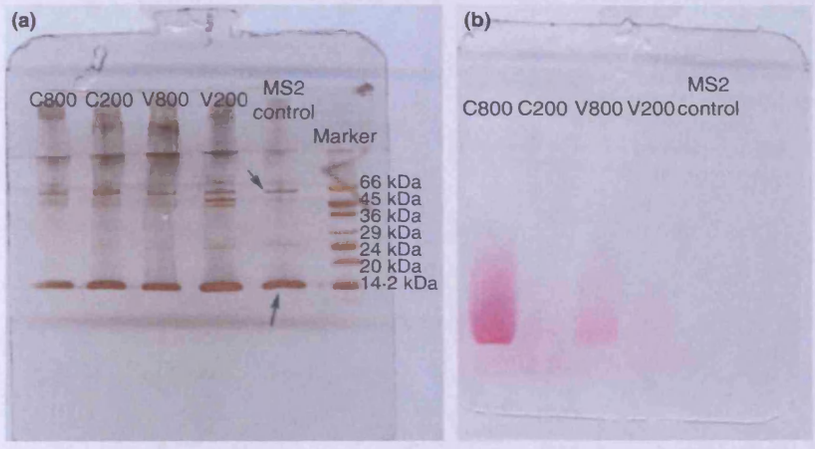


Figure 4 SDS-PAGE. (a) Silver-stained SDS-polyacrylamide gels of MS2 phages treated with VANTOCIL™ TG and COSMOCIL™ CQ. The arrows indicate the two proteins which formed MS2, coat protein (13·7 kDa) and A protein (44 kDa). (b) Eosin Y-stained gel. The pink staining indicates the presence of polyhexamethylene biguanide.

800 ppm, all MS2 particles were in aggregates of a size >300 nm (Figs 2 and 3).

Protein analysis

Protein analysis showed that PHMB did not alter MS2 protein profile (Fig. 4). The protein pattern of the untreated phage (control; Fig. 4) composed of three main protein bands was highly reproducible between the experiments ($n = 3$). The same amount of proteins was loaded on the gel pre and postphage treatment. The gel dyed with the Eosin Y showed the presence of the PHMB on the phage protein, and the intensity of the stain increased at 800 ppm.

Electron microscopy

Electron microscopy was used as a qualitative measurement of damage caused to the phage following treatment. Electron microscopy observations might not correlate with inactivation data (Maillard *et al.* 1995a), as viral inactivation might not necessarily be linked to gross-structural damage. Here, 30 separate fields of vision were observed. Untreated phage particles showed distinct icosahedral capsid of MS2 and did not present any alteration caused by the sample preparation for EM investigation. Treatment with either VANTOCIL™ TG 800 ppm or COSMOCIL™ CQ 800 ppm showed evidence of damage capsid (data not shown) when compared to the untreated phages. At the lower concentration of 200 ppm, evidence of broken capsid following treatment was also observed (data not shown).

Discussion

MS2 bacteriophage has been used as a virus model to test the efficacy of various biocides because of its structural similarity with enterovirus and comparable resistance profile (Jones *et al.* 1991; Davies *et al.* 1993; Koivunen and Heinonen-Tanski 2005; Sickbert-Bennett *et al.* 2005). In this study, VANTOCIL™ TG and COSMOCIL™ CQ only produced a 1 $\log_{10}$ reduction within 30-min exposure.

These results from a standard suspension test are in accordance with the literature, which reports a similar reduction in MS2 number following treatment with a range of biocides (Davies *et al.* 1993; Woolwine and Gerberding 1995). Notably, chlorhexidine, another biguanide, was reported to produce 90% reduction in MS2 in a suspension (Maillard *et al.* 1994) and carrier tests (Woolwine and Gerberding 1995). A more recent study (Sickbert-Bennett *et al.* 2005) on chlorhexidine hand wash reported a 2 $\log_{10}$ reduction in the bacteriophage.

Here, the biguanides were shown to attach to the phage proteins (Fig. 4b). Moreover, treated phage showed severe gross-structural damage, which was deemed to be related to biocide exposure rather than sample treatment for EM investigation, because the untreated phages showed no structural alteration. These observations are similar to the reported effect of chlorhexidine against F116 bacteriophage. The overall activity of chlorhexidine (1% w/v) against F116 was shown to be modest with <1 $\log_{10}$ reduction following a 20-min exposure (Maillard *et al.* 1993). Energy-dispersive analysis of X-ray investigation reported chlorhexidine not to bind the phage (Maillard *et al.* 1995b), although there was no gross alteration of the phage protein band profile following treatment with the biguanide (Maillard *et al.* 1996). However, gross but specific damage to the phage structure was identified (Maillard *et al.* 1995a), showing the deleterious effect of the biguanide against phage particles. One can note the similarity in the results from chlorhexidine-exposed phage with the data presented in this study on PHMB effect against MS2.

The lack of any increase in activity with increasing exposure time provides some useful information as to the interactions of the biguanides with the viral particles. Such inactivation kinetic is usually explained from either the depletion of the active biocide or the presence of 'resistant' organisms. The formation of viral aggregates has been shown to lead to the survival of viral particles following biocide exposure (Sharp *et al.* 1975, 1976; Espinosa *et al.* 2008), notably with chlorine (Jensen *et al.* 1980; Young and Sharp 1985). The propensity of particles to aggregates depends upon surface properties and notably hydrophobicity (Persson and Gekas 1994). Here, the results of two separate experiments provided a clear indication that the hydrophobicity of MS2 changed dramatically following exposure to high concentrations of PHMB (Fig. 1). An increase in hydrophobicity is likely to result in a decrease in polar surface on the viral capsid leading to aggregation. Generally, viruses remain dispersed in salt solutions near physiological strength and at high pH (Floyd and Sharp 1979). The degree of clumping is also related to the virus pI. MS2 has a pI of 3·9 and exhibited significant aggregation when $pH \leq pI$ (Langlet *et al.* 2008). In this study, the pH during PHMB exposure was $7{\cdot}6 \pm 0{\cdot}1$, hence the MS2 particles carried an overall negative charge on their surface which should keep the particles dispersed in the aqueous medium. However, exposure to PHMB long positively charge polymers decreases the polarity of the phage particles, leading to the formation of clumps in a polar medium such as the SM buffer and water. The formation of aggregates which increased in size with increasing concentrations of PHMB was demonstrated by dynamic light scattering. Viral aggregate size >300 nm was observed following exposure to VANTOCIL™ TG or COSMOCIL™ at a concentration of 800 ppm (Figs 2 and 3). Based on the evidence from other studies (Sharp *et al.* 1975, 1976; Jensen *et al.* 1980; Young and Sharp 1985; Espinosa *et al.* 2008) and our data demonstrating the formation of aggregates following exposure to PHMB, it would be tempting to associate the lack of activity of the biguanide to the formation of aggregates with the understanding that viral particles inside the clump are protected from the deleterious effect of the biguanide.

However, one has also to consider that the formation of viral aggregate will affect the measurement of phage survival by plaque assay. Indeed, an increase in the number of aggregates and/or the size of aggregates would effectively decrease the number of plaques observed after incubation with the host bacteria, and lead to an overestimation of the efficacy of the biguanide. In this study, the lack of increase in virucidal activity with increase PHMB concentration together with evidence of significant (χ^2 test, $P < 0{\cdot}01$) increase in aggregate size between exposure to 200 and 800 ppm of PHMB indicates that the presence of aggregate might not be linked with a decrease in plaque formation and a subsequent overestimation of activity. The use of Tween 80 in the neutralizing solution for the suspension test might have also helped in breaking up the aggregate.

This study aimed to understand the interactions between PHMB and viruses to improve their virucidal activity. We demonstrated that exposure to PHMB resulted in the formation of viral aggregates, likely as a result of change in phage surface hydrophobicity. We hypothesize that the formation of such clumps hindered dramatically the virucidal activity of these biguanides, although further evidence of the role of viral aggregates as an explanation for the modest virucidal efficacy of PHMB is needed. Other protocols based on the detection of antigens or viral genome rather than the formation of plaques (infectivity study) might not be affected by the formation of viral aggregates, although the use of molecular techniques suffers from some drawbacks (Sattar *et al.* 1989; Richards 1999).

Acknowledgements

The authors are grateful to Arch Chemicals, which contributed to the financial support of F. Pinto's studentship.

References

Adams, M.H. (1959) *Bacteriophages*. New York, NY: Interscience Publishers, pp. 450–456.

Allen, M.J., White, G.F. and Morby, A.P. (2006) The response of *Escherichia coli* to exposure to the biocide polyhexamethylene biguanide. *Microbiology* **152**, 989–1000.

Anon. (2001). *European Standard prEN13610. 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)*. Brussels: European Committee for Standardization.

Berne, B.J. and Pecora, R. (1976) *Dynamic Light Scattering: With Applications to Chemistry, Biology and Physics*. New York, NY: Wiley.

Brion, G.M. and Silverstein, J. (1999) Iodine disinfection of a model bacteriophage, MS2, demonstrating apparent rebound. *Water Res* **33**, 169–179.

Broxton, P., Woodcock, P.M., Heatley, F. and Gilbert, P. (1984) Interaction of some polyhexamethylene biguanides and membrane phospholipids in *Escherichia coli*. *J Appl Bacteriol* **57**, 115–124.

Chawner, J.A. and Gilbert, P. (1989) Interaction of the bisbiguanides chlorhexidine and alexidine with phospholipid vesicles: evidence for separate modes of action. *J Appl Bacteriol* **66**, 253–258.

Davies, J.G., Babb, J.R., Bradley, C.R. and Ayliffe, G.A.J. (1993) Preliminary-study of test methods to assess the virucidal activity of skin disinfectants using poliovirus and bacteriophages. *J Hosp Infect* **25**, 125–131.

Denyer, S.P., Hanlon, G.W. and Davies, M.C. (1993) Mechanisms of microbial adherence. In *Microbial Biofilms: Formation and Control* ed. Denyer, S.P. Oxford, UK: Blackwell Scientific Publications.

Espinosa, A.C., Mazari-Hiriart, M., Espinosa, R., Maruri-Avidal, L., Mendez, E. and Arias, C.F. (2008) Infectivity and genome persistence of rotavirus and astrovirus in groundwater and surface water. *Water Res* **42**, 2618–2628.

Fara, G.M., Lugaro, G., Galli, M.G. and Giannatt, G. (1974) Antiviral activity of selected biguanide derivatives. *Pharmacol Res Commun* **6**, 117–126.

Floyd, R. and Sharp, D.G. (1979) Viral aggregation: buffer effects in the aggregation of Poliovirus and Reovirus at low and high pH. *Appl Environ Microbiol* **38**, 395–401.

Gerhart, P., Murray, R.G.E., Wood, W.A. and Krieg, N.G. (1994) *Methods for General and Molecular Bacteriology*. Washington, DC: ASM Press.

Gilbert, P., Pemberton, D. and Wilkinson, D.E. (1990) Barrier properties of the Gram-negative cell envelope towards high molecular weight polyhexamethylene biguanides. *J Appl Microbiol* **69**, 585–592.

Jensen, H., Thomas, K. and Sharp, D.G. (1980) Inactivation of Coxsackie-viruses B3 and B5 in water by chlorine. *Appl Environ Microbiol* **40**, 633–640.

Jones, D.S. (1995) Preliminary investigations concerning the anti-adherence properties of polyhexamethylene biguanide (VANTOCIL IB™). *Int J Pharm* **117**, 123–126.

Jones, M.V., Bellamy, L., Alcock, R. and Hudson, R. (1991) The use of bacteriophage-MS2 as a model system to evaluate virucidal hand disinfectants. *J Hosp Infect* **17**, 279–285.

Klein, M. and Deforest, A. (1983) Principles in viral inactivity. In *Disinfection, Sterilization and Preservation*, 3rd edn ed. Block, S.S. pp. 422–434. Philadelphia, USA: Lea and Febiger.

Koivunen, J. and Heinonen-Tanski, H. (2005) Inactivation of enteric microorganisms with chemical disinfectants, UV irradiation and combined chemical/UV treatments. *Water Res* **39**, 1519–1526.

Krebs, F.C., Miller, S.R., Ferguson, M.L., Labib, M., Rando, R.F. and Wgdahl, B. (2005) Polybiguanides, particularly polyethylene hexamethylene biguanide, have activity against immunodeficiency virus type 1. *Biomed Pharmacother* **50**, 438–445.

Kuzmanovic, D.A., Elashvili, I., Wick, C., O'Connell, C. and Krueger, S. (2003) Bacteriophage MS2: molecular weight and spatial distribution of the protein and RNA components by small-angle neutron scattering and virus counting. *Structure* **11**, 1339–1348.

Langlet, J., Gaboriaud, F., Duval, J.-F.L. and Gantzer, C. (2008) Aggregation and surface properties of F-specific RNA phages: implication for membrane filtration processes. *Water Res* **42**, 2769–2777.

Maillard, J.-Y. (2001) Virus susceptibility to biocides: an understanding. *Rev Med Microbiol* **12**, 63–74.

Maillard, J.-Y. (2005) Usage of antimicrobial biocides and products in the healthcare environment: efficacy, policies, management and perceived problems. *Ther Clin Risk Manag* **1**, 340–370.

Maillard, J.-Y., Beggs, T.S., Day, M.J., Hudson, R.A. and Russell, A.D. (1993) Effect of biocides on *Pseudomonas aeruginosa* phage F116. *Lett Appl Microbiol* **17**, 167–170.

Maillard, J.-Y., Beggs, T.S., Day, M.J., Hudson, R.A. and Russell, A.D. (1994) Effect of biocides on MS2-coliphage and K-coliphage. *Appl Environ Microbiol* **60**, 2205–2206.

Maillard, J.-Y., Hann, A.C., Beggs, T.S., Day, M.J., Hudson, R.A. and Russell, A.D. (1995a) Electron-microscopic investigation of the effects of biocides on *Pseudomonas aeruginosa* PA0 bacteriophage F116. *J Med Microbiol* **42**, 415–420.

Maillard, J.-Y., Hann, A.C., Beggs, T.S., Day, M.J., Hudson, R.A. and Russell, 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. *Lett Appl Microbiol* **20**, 357–360.

Maillard, J.-Y., Beggs, T.S., Day, M.J., Hudson, R.A. and Russell, A.D. (1996) The effect of biocides on proteins of

Pseudomonas aeruginosa PAO bacteriophage F116. *J Appl Bacteriol* **80**, 291–295.

Miles, A.A. and Misra, S.S. (1938) The estimation of the bactericidal power of the blood. *J Hyg* **38**, 732–749.

Persson, K.M. and Gekas, V. (1994) Factors influencing aggregation of macromolecules in solution. *Process Biochem* **29**, 89–98.

Pilcher, K.S., Soikem, K.F. and Trosper, F. (1961) Studies of chemical inhibitors of influenza virus multiplication: I. Biguanides and related compounds. *Antimicrob Agents Chemother* **11**, 381–389.

Richards, G.P. (1999) Limitations of molecular biological techniques for assessing the virological safety of foods. *J Food Prot* **62**, 691–697.

Sambrook, J. and Russell, D.W. (2001) *Molecular Cloning: A Laboratory Manual*, 3rd edn, vol. **1**, 3rd edn. Cold Spring Harbor, NY: Cold Spring Harbor Press.

Sattar, S.A., Springthorpe, V.S., Karim, Y. and Loro, P. (1989) Chemical disinfection of non-porous inanimate surfaces experimentally contaminated with four human pathogenic viruses. *Epidemiol Infect* **102**, 493–505.

Sauerbrei, A., Schacke, M., Gluck, B., Egerer, R. and Wutzler, P. (2006) Validation of biocides against duck hepatitis B virus as a surrogate virus for human hepatitis B virus. *J Hosp Infect* **64**, 358–365.

Sharp, D.G., Floyd, R. and Johnson, J.D. (1975) Nature of the surviving plaque-forming unit of reovirus in water containing bromine. *Appl Microbiol* **29**, 94–101.

Sharp, D.G., Floyd, R. and Johnson, J.D. (1976) Initial fast reaction of bromine on reovirus in turbulent flowing water. *Appl Environ Microbiol* **31**, 173–181.

Sickbert-Bennett, E.E., Weber, D.J., Gergen-Teague, M.F., Sobsey, M.D., Samsa, G.P. and Rutala, W.A. (2005) Comparative efficacy of hand hygiene agents in the reduction of bacteria and viruses. *Am J Infect Control* **33**, 67–77.

Woolwine, J.D. and Gerberding, J.L. (1995) Effect of testing method on apparent activities of antiviral disinfectants and antiseptics. *Antimicrob Agents Chemother* **39**, 921–923.

Young, D.C. and Sharp, D.G. (1985) Virion conformational forms and the complex inactivation kinetics of echovirus by chlorine in water. *Appl Environ Microbiol* **49**, 359–364.

Effect of surfactants, temperature, and sonication on the virucidal activity of polyhexamethylene biguanide against the bacteriophage MS2

Federica Pinto, MSc, Jean-Yves Maillard, PhD, BSc, and Stephen P. Denyer, FRPharmS
Cardiff, United Kingdom

Background: Virucidal compounds are essential in preventing the transmission of viral infection in the health care environment. Understanding their mechanisms of action is necessary to improve their efficacy. Inactivation of viruses is less documented than that of bacteria notably because different types of virus have diverse response to microbicides, making difficult to establish an inactivation pattern.
Methods: The effect of viral aggregates on the virucidal activity of polyhexamethylene biguanide-based microbicide VANTOCIL™ TG (Arch Chemicals, Manchester, UK) against the bacteriophage MS2 was investigated by using in combination a standard suspension efficacy test under different conditions and dynamic light scattering measuring the presence and size of aggregates.
Results: Temperature had a key role in increasing significantly the virucidal activity of VANTOCIL™ TG, reducing virus concentration by 4-$\log_{10}$ within 10 minutes at 40°C. The high temperature was linked to a reduction of viral aggregates despite the exposure to the biguanide. In addition, the viral inactivation kinetic became significantly more linear at 30°C and 40°C. Such results were also observed with sonication during treatment, where a first-order kinetic was observed. However, the addition of surfactants, even though there was evidence of a decrease in viral clumps, did not enhance the virucidal activity of polyhexamethylene biguanide.
Conclusion: The presence of viral aggregates was an important factor in the virucidal efficacy of the biguanide as demonstrated by the correlation among high temperature, decrease in aggregates, and increase in activity, although it is possible that high temperatures might also cause conformational changes of the viral capsid, increasing the sensitivity of virions to the microbicide.
Key Words: Polyhexamethylene biguanide; naked virus; temperature; aggregation.

(Am J Infect Control 2010;■:1-6.)

Adequate and efficient disinfection procedures minimize the incidence of surface contamination and the transmission of viral infection in the health care environment.[1] The enhancement of disinfection procedures with the use of physical agents and of appropriate cleaning/disinfectant formulations has been studied in various fields.[2-4] The inactivation of nonenveloped viruses by microbicides is usually more difficult to accomplish than the inactivation of bacteria, probably because of the lack of metabolic processes and to the simplicity of their structure.[5]

From the Welsh School of Pharmacy, Cardiff University, Cardiff, United Kingdom.

Address correspondence to Jean-Yves Maillard, PhD, BSc, Welsh School of Pharmacy, Cardiff University, King Edward VII Ave, Cardiff, CF10 3NB, UK. E-mail: maillardj@cardiff.ac.uk.

Conflicts of interest: None to report.

Supported by funding from Arch Chemicals toward a PhD studentship (Federica Pinto).

0196-6553/$36.00

doi:10.1016/j.ajic.2009.08.012

Viral inactivation kinetics may theoretically exhibit a first-order reaction where the concentration of the virus decreases linearly with time.[5,6] However, environmental variables, such as concentration, contact time, organic load, temperature and pH,[7] and intrinsic virus properties (mostly absence of an envelope)[8] can alter the kinetics of disinfection, causing a deviation from the ideal linear inactivation. Viruses can also form aggregates in solution notably because of the hydrophobic properties of their capsid. The formation of such clumps has been found to impede the appropriate contact with microbicides.[9-11]

Polyhexamethylene biguanide (PHMB) is a broad-spectrum microbicide, commonly used as a disinfectant in hospitals, swimming pools, and food processing. The compound is active against bacteria interacting with the cytoplasmic membrane and causing alterations of membrane integrity.[12] PHMB was also found effective against enveloped viruses, specifically against HIV type 1 and herpes simplex virus,[13,14] but its activity against naked viruses has not been well established. To understand further the mechanisms of action of PHMB against naked virus and the role of viral aggregates, we measured the effects of temperatures, sonication, and surfactants on both aggregates formation and on the efficacy of the microbicide.

METHODS

Phage propagation

The bacteriophage MS2 (NCIMB 10108; ATCC 15597-B1) was used as a surrogate for nonenveloped mammalian viruses. MS2 has been considered an appropriate model virus because of its structural and behavioral similarity to enteroviruses when challenged with microbicides.[15,16] MS2 was propagated in *Escherichia coli* (NCIMB 9481) following the protocol described by Woolwine et al.[17]

MS2 stock suspension was obtained by pipetting 5 mL of phage buffer (5% vol/vol 1 mol/L Tris/HCl, pH 7.4, 0.2% wt/vol $MgSO_4$, and 0.5% wt/vol gelatine) onto a plaque assay plate showing at least 50 plaques. The top agar layer was scraped and centrifuged for 15 minutes at 11,000*g* at 4°C. The supernatant was filtered through a 0.22-μm membrane filter and stored between 4°C and 5°C until used.

Suspension tests under different conditions

The microbicide VANTOCIL™ TG (MW 2678) (Arch Chemicals, Manchester, UK) is an aqueous solution of 20% PHMB and was tested at 800 ppm active ingredient, which is a typical concentration used in commercially available hard surface liquid disinfectant products.

The suspension test was based on prEN13610,[18] and 4 contact times (1, 5, 10, and 30 minutes) were investigated. Briefly, 1 mL MS2 (10^9 plaque-forming units [PFU]/mL) was mixed with 9 mL the microbicide. After the appropriate contact time, 1 mL mixture was neutralized with 9 mL Tween 80 (60 g/L), lecithin (4.5 g/L), and L-histidine (1 g/L) for 2 minutes. Next, a plaque assay count[16] was used to measure the concentration of surviving phages. Controls consisted of replacing the microbicide with deionized water.

The suspension tests were performed at different temperatures: 10°C, 20°C, 30°C, 40°C, and 50°C, using a water bath to keep the temperature constant during the whole treatment. Solutions used were equilibrated at the appropriate temperature before use. Phage survival was also investigated for each studied temperature without the presence of microbicide.

The sonication treatment was applied to the suspension test for the whole contact time. It was performed in a Soniprep 150 sonicator (MSE, London, UK) at 23 kHz and at wave amplitude of 10 μm. Briefly, the sonicator probe was lowered down to the test vessels containing the phage suspension and the microbicide, and sonication was performed for the appropriate contact time. In addition, sonication was performed at 2 different temperatures: 10°C where the test vessel was kept in a water bath, and at room temperature. However, in this case, the temperature increased in the vessel to 30°C between 1 and 5 minutes and to 40°C between 10 and 30 minutes. Control tests were performed to check phage survival under such conditions without the presence of microbicide.

Finally, 3 formulations composed of VANTOCIL™ TG (800 ppm PHMB) and a range of surfactants at different concentrations were tested. These formulations, labeled F1, F2, and F3 (Arch Chemicals, Manchester, UK), were as follows: F1 (DI water, 67.37%; VANTOCIL™ TG, 25%; Armeen 2MCD, 5%; 32% HCl, 2.63%; pH 4.20), F2 (DI water, 70%; VANTOCIL™ TG, 25%; Neodol 23-6.5, 5%; pH 5.70), and F3 (DI water, 70%; VANTOCIL™ TG, 25%; Synperonic 91/6 5%; pH 5.70).

Neutralizer-toxicity and efficacy tests were performed before the suspension test on both phage and host cell. Briefly, 1 mL overnight host cell suspension (approximately 10^9 colony-forming units [CFU]/mL) was mixed with 9 mL neutralizer. After 10 minutes exposure time, 1 mL sample was serially diluted and plated, and colonies were counted after 24-hour incubation at 37°C. The neutralizer toxicity test was similar with MS2; 1 mL phage suspension (approximately 10^9 PFU/mL) and 9 mL neutralizer were mixed together for 10 minutes. Next, 1 mL sample was serially diluted, and surviving bacteriophages were enumerated as described above.

The efficacy of the neutralizer to quench effectively PHMB was performed with both the host bacteria and MS2. One milliliter overnight host cell (approximately 10^9 CFU/mL) or phage suspension (approximately 10^9 PFU/mL) was added to a disinfectant/neutralizer mixture (1/1 ratio) and left for 10 minutes and serially diluted. Bacterial and phage survival following exposure were measured as described above.

Analysis of viral aggregates

The size of aggregates after phage treatment at 10°C and 40°C was measured using dynamic light scattering with N4plus (Beckman Coulter; Fullerton, CA). Purified virus suspension (10^{11} PFU/mL) was dialyzed overnight against KCl (0.250 mol/L) and then filtered through 0.22 μm filter. The sample concentration was adjusted to ensure that the count per second fell in the range 5×10^4 to 1×10^6. MS2 suspension (10^{11} PFU/mL) was then exposed to PHMB (2 ppm, 20 ppm, 200 ppm, and 800 ppm) for 2 minutes at 10°C and at 40°C. Light scattering measurements ran at 10°C and 40°C for 200 seconds using a laser angle of 90° and were made in triplicate. To analyze the results effectively, the percentage of particles of a different size was pulled together into arbitrary size categories.

Statistical analysis

The phage concentration measured in the controls was used to calculate the $\log_{10}$ reduction after treatment.

Each suspension test was repeated 3 times in duplicate. pH and temperature were measured in the different test vessel. Any differences among results were analyzed by parametric test, 1-way ANOVA test. Where analysis showed significant differences, post hoc Tukey test was used to underline which couple of variables produced the difference. Light scattering results were analyzed by the χ^2 test.

RESULTS

The neutralizer was shown to have no toxicity against MS2 and the host cell and to quench effectively PHMB (data not shown). Rise in temperature, and sonication treatment at a constant temperature or with increasing temperatures (between 30°C and 40°C), increased the number of plaques observed. No significant increase was observed at 10°C (0.13- ± 0.14-$\log_{10}$ PFU/mL) or 20°C (0.21- ± 0.37-$\log_{10}$ PFU/mL), although a modest but significant increase (ANOVA test, $P \leq .05$) at 30°C (0.43- ± 0.19-$\log_{10}$ PFU/mL) and 40°C (0.28- ± 0.15-$\log_{10}$ PFU/mL) without sonication was found. Likewise, a significant increase (ANOVA test, $P \leq .05$) was obtained when samples were sonicated at 10°C (0.55- ± 0.29-$\log_{10}$ PFU/mL) and between 30°C and 40°C (0.52- ± 0.09-$\log_{10}$ PFU/mL). These unexpected results can be explained by the presence of a small number of viral aggregates in the phage suspension used for the suspension test, although large clumps were not observed (Figs 1 and 2). Following an increase in temperature or sonication, it is possible that these small clumps are broken down, resulting in a small increase in PFU observed. As a result, these results were taken into account when the reduction factors were calculated when phages were exposed to PHMB under these conditions.

Changes in temperature had a profound effect on the virucidal activity of VANTOCIL™ TG. Treatments from 30°C and above increased significantly (ANOVA test, $P \leq .05$) the virucidal efficacy of PHMB against MS2. The efficacy of PHMB increased significantly with time (ANOVA test, $P \leq .05$) at these temperatures (Fig 3). At 30°C, 2.76-$\log_{10}$ reduction was achieved after 30 minutes, whereas, at 40°C, 2.39-$\log_{10}$ was reached only after 5 minutes, and, at 50°C, VANTOCIL™ TG produced 5.30-$\log_{10}$ reduction in MS2 after 5 minutes (Table 1). At lower temperatures (10°C to 20°C), PHMB activity was low against MS2 and achieved less than 1-$\log_{10}$ reduction in number.

Sonication was applied to the test vessel at 2 different temperatures: a controlled low temperature (10°C) and at room temperature, in which case the temperature in the test vessel was raised to 30°C between 1 and 5 minutes and to 40°C between 10 and 30 minutes. Results from sonicated samples were compared

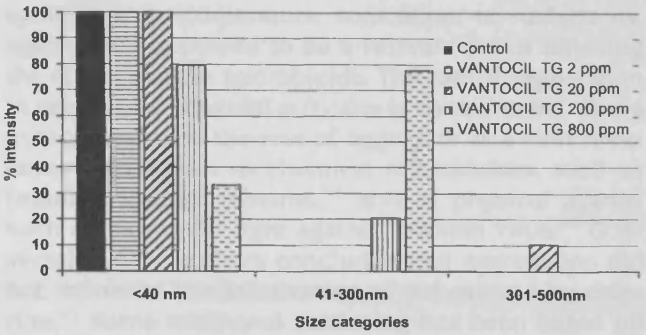


Fig 1. MS2 treated with VANTOCIL™ TG at 2 ppm, 20 ppm, 200 ppm, and 800 ppm active ingredient at 40°C. Distribution of 3 size groups of MS2 aggregates in function of the percent intensity of the light scattered. The magnitude of each histogram is proportional to the percentage of the total scattered intensity caused by the particles of the corresponding group size.

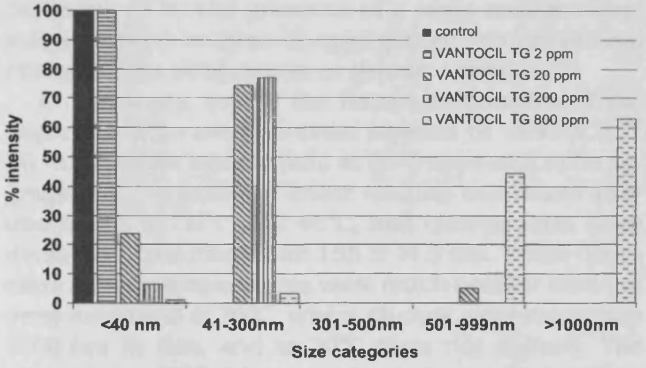


Fig 2. MS2 treated with VANTOCIL™ TG at 2 ppm, 20 ppm, 200 ppm, and 800 ppm active ingredient at 10°C. Distribution of 5 size groups of MS2 aggregates in function of the percent intensity of the light scattered. The magnitude of each histogram is proportional to the percentage of the total scattered intensity caused by the particles of the corresponding group size.

with the suspension tests performed at the same temperature without sonication. Specifically, the sonication treatment without refrigeration was compared with the suspension test at 30°C and at 40°C for 1 and 5 minutes and 10 and 30 minutes contact time, respectively.

PHMB was more efficient at the higher temperature tested (Table 1). At a low temperature, the addition of sonication significantly increased (ANOVA test, $P \leq .05$) the virucidal activity after 30 minutes exposure only, although the enhanced killing effect was only by 1 $\log_{10}$. At a higher temperature, sonication

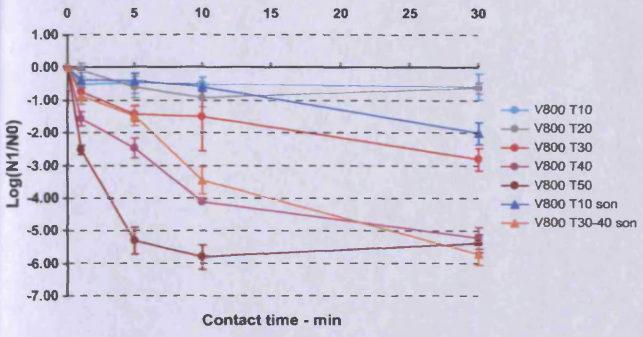


Fig 3. Kinetics curves of efficacy of VANTOCIL™ TG at 800 ppm against MS2 tested by suspension test. Grey lines represent treatments with sonication; black lines represent treatment without sonication at different temperatures (from 10°C to 50°C). V800: VANTOCIL™ TG at 800 ppm. Values are the mean of 3 experiments. Bars represent standard deviation.

did not improve significantly (ANOVA test, $P > .05$) the virucidal activity of VANTOCIL™ TG.

The 3 formulations (F1, F2, and F3) tested did not enhance the efficacy of VANTOCIL™ TG. They achieved similar reduction in MS2 number as with the PHMB tested alone at 20°C (Table 1).

Figure 3 showed the observed inactivation curves for the different treatments. Significant linear correlations were achieved at 30°C ($r = -0.93$, $P \leq .05$) and 40°C ($r = -0.88$, $P \leq .05$) and after treating MS2 with constant sonication (10°C: $r = -0.98$, $P \leq .05$; 30°C/40°C: $r = -0.96$, $P \leq .05$).

In the analysis of the size aggregates, 40 nm was considered the threshold over which aggregation happened because MS2 measures 26 nm. At 40°C small clumps (maximum sizes, 155 ± 11.5 nm) started to develop with PHMB concentrations of 200 ppm (χ^2 test, $P \leq .05$) (Fig 1). When the coliphage was treated at 10°C (Fig 2), the aggregates started to form at 2 ppm (χ^2 test, $P \leq .05$) with sizes of 51.6 ± 9.2 nm. As the PHMB concentration reached 800 ppm, the clumps measured over 1000 nm. The temperature influenced significantly the sizes of clumps (ANOVA, $F_{1,16} = 119.71$, $P < .05$).

DISCUSSION

Microbicides efficacy is dependent on environmental factors and on the type of viruses. This paper analyzed the relevance of aggregation in reducing the virucidal activity of a PHMB-based microbicide, VANTOCIL™ TG, against a nonenveloped virus. The presence of viral aggregates has been implicated in virus survival and persistence of infectivity after challenge with microbicides.[19] By testing the microbicide in synergy with temperature, sonication, or surfactants, aggregation appeared to be a relevant factor affecting the efficacy of the microbicide. The role of aggregation in preventing virucidal activities is controversial. Some authors reported the role of aggregate as a viral resistance mechanism to chemical microbicides, such as bromine against reovirus,[10] and to physical agents, such as ultraviolet light against vaccinia virus.[20] Conversely, other authors concluded that aggregation did not influence the inactivation of poliovirus-I by chlorine.[21] Some additional reasoning has been based on the use of disinfection kinetics, which has been described to be analogous to a chemical reaction.[22] Thus, it has been postulated that, to achieve an efficient inactivation of viruses, the reaction should follow a first-order kinetics. Differences in viral structure, microbicides, and environmental conditions will cause a deviation from the first-order kinetics. Observing the kinetics curves in Fig 3, a "tailing off" effect is visible, mostly after treatment at 10°C, 20°C, and 50°C. The "tailing off" shape is usually explained by the microbicide depletion or by the presence of a more resistant viral subpopulation because of aggregation, conformational change of the viral capsid, or genetic variation.[23]

In this study, one of the factors responsible of the departure from the first-order kinetics of VANTOCIL™ TG inactivation against MS2 at 20°C appeared to be aggregation. A significant linear relation was found after treatments at 30°C and 40°C, and clumps sizes were measured to be maximum 155 ± 11.5 nm. These diameters at high temperatures were much smaller than the ones measured at 10°C, where clumps were more than 1000 nm in size, and at 20°C (data not shown). The treatment at 50°C did not produce a first-order inactivation kinetic. However, it can be noted that the $\log_{10}$ reduction observed at this temperature was at the limit of detection of the assay.

Sonication increased significantly the reduction of the phage only at 10°C with an exposure time of 30 minutes. Furthermore, significant first-order kinetics occurred when the microbicide was tested in synergy with sonication, which has been used to enhance microbicides efficacy thanks to its capacity of breaking up clumps.[24]

Formulations are often used to enhance the efficacy of disinfection. The concept is based on a synergistic action.[25] Among surfactant properties, solubilization ability is important, as well as their ability to break up microbial clumps. Here, the 3 surfactant-containing formulations did not increase the virucidal activity of VANTOCIL™ TG.

Temperature had a profound effect on the activity of PHMB against a nonenveloped virus. In addition to dispersing the viruses uniformly, temperature could also alter the stability of viral capsid. Such properties have been extensively studied because of implications in

Table 1. Suspension test with and without sonication

		RF			
		Contact time (min)			
VANTOCIL™ TG 800ppm	T (°C)	1	5	10	30
Sonication	10	0.37 ± 0.24	0.36 ± 0.20	0.58 ± 0.05	1.89 ± 0.28
	20 (0-1 min) *	0.82 ± 0.06	1.43 ± 0.12	3.43 ± 0.21	5.20 ± 0.98
	30 (1-5 min)				
	40 (10-30 min)				
No sonication	10	0.43 ± 0.54	0.45 ± 0.28	0.50 ± 0.08	0.56 ± 0.36
	20	0.43 ± 0.34	0.52 ± 0.05	0.88 ± 0.17	0.50 ± 0.02
	30	0.64 ± 0.09	1.39 ± 0.09	1.46 ± 0.10	2.76 ± 0.34
	40	1.49 ± 0.05	2.39 ± 0.30	4.10 ± 0.02	5.19 ± 0.30
	50	2.49 ± 0.15	5.30 ± 0.42	5.79 ± 0.38	5.36 ± 0.28
Formulation	20	0.39 ± 0.14	0.99 ± 0.25	0.51 ± 0.11	0.27 ± 0.58
F1					
F2	20	0.10 ± 0.08	0.00 ± 0.15	0.87 ± 0.17	0.88 ± 0.06
F3	20	0†	0†	0.02 ± 0.29	0†

NOTE. Values are the mean of 3 experiments.
RF, reduction factor: calculated as $\log_{10}$ (PFU/mL initial inoculum) − $\log_{10}$ (PFU/mL after exposure); *T*, temperature.
*Temperature increased with duration of sonication.
†No reduction in phage number observed.

drugs design, inactivation strategies for vaccines development, and enhancement of antiviral activity.[26-29] For instance, temperatures induced changes in quaternary structure of Norwalk virus capsid, which were characterized by a loss of continuity in the icosahedral geometry.[26] Canine parvovirus capsid responded to increased temperature by higher DNA exposure and by an increased permeability to negative stain.[28] Here, high temperature did not affect phage survival. However, it is possible that an increase in capsid permeability enhanced the penetration of PHMB and its virucidal activity in a temperature-dependent manner.

In conclusion, aggregation is believed to play a part for the low virucidal activity of PHMB at 20°C. Temperature resulted to have a key role in the enhancement of the efficacy of the PHMB microbicide, helping virions dispersion, as shown by light scattering, and possibly increasing the virus susceptibility because of conformational changes. We tested the microbicide through a suspension test, which does not necessarily reflect conditions of disinfection practice of virus-contaminated surface. However, this study is a first screening of the potential activity of PHMB-based microbicides against naked virus. Further investigations are needed to understand specifically the mechanisms of action involved and thereafter improve the in-use efficacy of the biguanide.

The authors thank Paula McGeechan for her advice on PHMB.

References

1. Sehulster L, Chinn RYW. Guidelines for environmental infection control in health-care facilities. Recommendations of CDC and the Healthcare Infection Control Practices Advisory Committee (HICPAC). MMWR Recomm Rep 2003;52:1-42.
2. Capra ML, Quiberoni A, Reinheimer JA. Thermal and chemical resistance of *Lactobacillus casei* and *Lactobacillus paracasei* bacteriophages. Lett Appl Microbiol 2004;38:499-504.
3. De Benedictis P, Beato MS, Capua I. Inactivation of avian influenza viruses by chemical agents and physical conditions: a review. Zoonoses Public Health 2008;54:51-68.
4. Pratelli A. Canine coronavirus inactivation with physical and chemical agents. Vet J 2008;177:71-9.
5. Thurman RB, Gerba CP. Viral inactivation by water disinfectants. Adv Appl Microbiol 1988;33:75-105.
6. Churn CC, Boardman GD, Bates RC. The inactivation kinetics of H-1 parvovirus by chlorine. Water Res 1984;18:195-203.
7. Russell AD. Biocide use and antibiotic resistance: the relevance of laboratory findings to clinical and environmental situations. Lancet Infect Dis 2003;3:794-803.
8. Klein M, Deforest A. Principles of viral inactivation. In: Block SS, editor. Disinfection, sterilization and preservation. 3rd ed Philadelphia: Lea & Febiger; 1983. p. 422-34.
9. Sharp DG, Floyd R, Johnson JD. Initial fast reaction of bromine on reovirus in turbulent flowing water. Appl Environ Microbiol 1976;31:173-81.
10. Sharp DG, Floyd R, Johnson JD. Nature of the surviving plaque-forming unit of reovirus in water containing bromine. Appl Microbiol 1975;29:94-101.
11. Young DC, Sharp DG. Virion conformational forms and the complex inactivation kinetics of echovirus by chlorine in water. Appl Environ Microbiol 1985;49:359-64.
12. Allen MJ, White GF, Morby AP. The response of *Escherichia coli* to exposure to the biocide polyhexamethylene biguanide. Microbiology 2006;152:989-1000.
13. Krebs FC, Miller SR, Ferguson ML, Labib M, Rando RF, Wigdahl B. Polybiguanides, particularly polyethylene hexamethylene biguanide, have activity against immunodeficiency virus type 1. Biomedicine & Pharmacotherapy 2005;59:438-45.
14. Valluri S, Fleming TP, Laycock KA, Tarle IS, Goldberg MA, Garcia Ferrer FJ, et al. In vitro and in vivo effects of polyhexamethylene biguanide against herpes simplex virus infection. Cornea 1997;16:556-9.
15. Shin GA, Sobsey MD. Reduction of Norwalk virus, poliovirus 1, and bacteriophage MS2 by ozone disinfection of water. Appl Environ Microbiol 2003;69:3975-8.

16. Tree JA, Adams MR, Lees DN. Disinfection of feline calicivirus (a surrogate for Norovirus) in wastewaters. J Appl Microbiol 2005;98: 155-62.
17. Woolwine JD, Gerberding JL. Effect of testing method on apparent activities of antiviral disinfectants and antiseptics. Antimicrob Agents Chemother 1995;39:921-3.
18. European Standard prEN13610. 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). Brussels: European Committee for Standardization; 2001.
19. Maillard J-Y, Russell AD. Viricidal activity and mechanisms of action of biocides. Sci Prog 1997;80:287-315.
20. Sharp DG, Kim KS. Multiplicity reactivation and radiation survival of aggregated vaccinia virus: calculation of plaque titer based on MR and particle aggregation seen in electron microscope. Virology 1966; 29:359-66.
21. Sharp DG, Leong J. Inactivation of poliovirus-1 (Brunhilde) single particles by chlorine in water. Appl Environ Microbiol 1980;40:381-5.
22. Chick H. Factors conditioning the velocity of disinfection. Chem Abstracts 1913;7:2273.
23. Hoff JC, Akin EW. Microbial resistance to disinfectants: mechanisms and significance. Environ Health Perspect 1986;69:7-13.
24. Burleson GR, Murray TM, Pollard M. Inactivation of viruses and bacteria by ozone, with and without sonication. Appl Environ Microbiol 1975;29:340-4.
25. Jimenez L, Chiang M. Virucidal activity of a quaternary ammonium compound disinfectant against feline calicivirus: a surrogate for norovirus. Am J Infect Control 2006;34:269-73.
26. Ausar SF, Foubert TR, Hudson MH, Vedvick TS, Middaugh CR. Conformational stability and disassembly of Norwalk virus-like particles: effect of pH and temperature. J Biol Chem 2006;281:19478-88.
27. Hilmer JK, Zlotnick A, Bothner B. Conformational equilibria and rates of localized motion within hepatitis B virus capsids. J Mol Biol 2008; 375:581-94.
28. Nelson CDS, Minkkinen E, Bergkvist M, Hoelzer K, Fisher M, Bothner B, et al. Detecting small changes and additional peptides in the Canine parvovirus capsid structure. J Virol 2008;82:10397-407.
29. Newman M, Suk FM, Cajimat M, Chua PM, Shih C. Stability and morphology comparisons of self-assembled virus-like particles from wild-type and mutant human hepatitis B virus capsid proteins. J Virol 2003; 77:12950-60.