CRANFIELD UNIVERSITY

EKWU MARK AMEH

THE USE OF BACTERIOPHAGES AS NATURAL BIOCONTROL AGENTS AGAINST BACTERIAL PATHOGENS

SCHOOL OF ENERGY, ENVIRONMENT AND AGRIFOOD

CRANFIELD WATER SCIENCE INSTITUTE

PhD
2012 - 2015

Supervisors: Dr Andreas Nocker and Prof Sean Tyrrel
March 2016

CRANFIELD UNIVERSITY

SCHOOL OF ENERGY, ENVIRONMENT AND AGRIFOOD
CRANFIELD WATER SCIENCE INSTITUTE

PhD

2012 - 2015

EKWU MARK AMEH

The Use of Bacteriophages as Natural Biocontrol Agents against Bacterial Pathogens

Supervisors: Dr Andreas Nocker and Prof Sean Tyrrel
March 2016

This thesis is submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy

ABSTRACT

Bacteriophages are viruses that specifically infect bacteria. The bactericidal nature of lytic bacteriophages has been exploited by scientists for decades with the hope to utilise them in the fight against bacterial infections and antibiotic resistant bacteria in medical settings. More recently, the potential applications of bacteriophages for biocontrol in the agrifood and environmental sectors have been investigated in an attempt to develop 'natural' antimicrobial products. Bacteriophages have a couple of decisive advantages over conventional methods of controlling pathogenic bacteria, such as high host specificity, the ability to self-replicate, and the ability to evolve with their hosts. However, more research is needed to optimise the parameters for phage applications, including the impact of environmental conditions on lysis efficiency, multiplicity of infection, and to significantly minimise the emergence of bacterial resistance to phages.

Temperature plays a key role in every biological activity in nature. It is also assumed that temperature has an effect on phage lysis efficiency. A comprehensive study of it and how it affects both the host cells and their corresponding phages is crucial to ensure the efficient removal of bacterial pathogens. In this thesis, temperature (as a selected parameter) was investigated to determine its influence on the lysis effectiveness of the three different phages belonging to the family of the *Myoviridea* that were isolated and purified from a single water sample taken from a brook receiving treated wastewater. We used the multiplicity of infection of 1 in all of our study in this project. Temperature was found to have a significant impact on phage-mediated lysis efficiency. Both the temperature of incubation of the phage-bacteria mixture (incubation temperature) and the temperature history of bacterial hosts were found to have profound effects on plaque sizes as well as plaque numbers. Plaque size and number decreased with increasing

temperature. For the phages examined, bacterial lysis was more efficient at 20°C compared to 30 or 37°C. Phages were suggested to be well adapted to the environment where they were isolated from with general implications for use in biological disinfection. Furthermore, the temperature history of the bacteria (prior to phage encounter) was found to have a modulating effect on their susceptibility to lysis.

A second part of this study compared the performance of the three phages in regard to bacterial resistance. The emergence of bacterial resistance is a major obstacle to the success of bacteriophages applications. The use of multiple phages is typically recommended and has proven better than the use of a single phage. However, the bestway to perform phage treatment is still very unclear. This study therefore compared simultaneous addition of multiple phages (in form of a cocktail) with the sequential addition of the individual phages at different time points in trying to delay the emergence of bacterial resistance. The data obtained from this work suggest that lysis effectiveness can be adjusted to optimize any treatment goal. For fast initial bacterial clearance the use of a single phage with short time maximal lysis efficiency proved most efficient, while the simultaneous addition of phages in the form of a cocktail was most successful strategy in our study. Addition of selected phages sequentially can be normalized in such a way that is just as effective as a cocktail.

A third part of this thesis looked into the susceptibility of bacteria that had undergone sublethal disinfection. We addressed the question whether bacteria subjected to sublethal doses of chlorine and UV are still susceptible to phage-mediated lysis. The chlorine treatments indicated the development of a phage-insensitive phenotype for a critical chlorine dose in the transition zone between live and dead. The remaining live (and culturable) bacteria were shown insensitive to the selected phage. The lowest UV

exposure at 2.8 mJ/cm^{2}eliminated bacteria susceptibility to the phages. This phage-resistant phenotype may have serious consequences for the application of phages on foods or water that have previously undergone a weak disinfection regime.

Keywords:

Lytic phage, resistance, temperature, chlorine disinfection, UV disinfection, viable but non-culturable bacteria (VBNC), *Escherichia coli* (*E.coli*), multiplicity of infection (MOI), water, biological disinfection.

ACKNOWLEDGEMENTS

I would like to express my sincere and heartfelt appreciation to my supervisors Dr Andreas Nocker and Professor Sean Tyrrel, my subject adviser Professor Jim Harris, and thesis committee chairman Dr Ahmed Al-Ashaab for their guidance and encouragement during the course of the project. To my supervisors who painstakingly read this manuscript during it preparations, many thanks to you. Thanks to all the technicians and the administrative staff of Cranfield University and the Association of Commonwealth Universities (ACU) for their availability.

This project would have not been possible without the following support. Firstly, I would like to thank the Government of the United Kingdom who through the Commonwealth Scholarships funded my studies. Secondly, I am grateful to all my family members and in-laws for their moral support and encouragement. And lastly but not the least, special thanks to my lovely wife, who made all the difference and our dear boy, Gabriel Idoko who was born in the final stage of this study – a wonderful treasure to behold, and a delight to be around with.

Finally, thanks to my colleagues and friends from the Cranfield Water Science Institute and all the wonderful friends I have made throughout the course of this study, especially the Cranfield Catholic Students, it was uplifting to worship together at Masses on campus and the parish church of St. Mary's Woburn Sands.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

ABNC	Active but not culturable
AWWA	American Water Works Association
$CaCl_2$	Calcium chloride
CFU	Colony forming unit
DNA	Deoxyribonucleic acid
DVGW	German technical and scientific association for gas and water
FCM	Flow cytometer
ICC	Intact cells concentrations
ICTV	International committee on taxonomy of viruses
MLGA	Membrane lactose glucuronide agar
$MgCl_2$	Magnesium chloride
MOI	Multiplicity of infection
NaCl	Sodium chloride
OD_{600}	Optical density at 600 nm
PBS	Phosphate buffer saline
PFU	Plaque forming unit
RNA	Ribonucleic acid
TCMs	Turbidity causing materials
TEM	Transmission electron microscopy
TSA	Tryptone soya agar
TSB	Typtone soya broth
USEPA	United States Environmental Protection Agency
UV-LED	Ultraviolet light emitting diodes
VBNC	Viable but not culturable
V/V	Volume per volume
W/V	Weight per volume

CHAPTER 1 INTRODUCTION

1.1 PROJECT BACKGROUND

Bacteriophages (phages) also known as bacterial viruses are now considered to be the most prevalent and wide-ranging biologically entity on Earth today. They are found in every habitat where bacteria exist. The vast majority of all the phages described in published scientific literature are the double-stranded DNA (dsDNA), tailed phages and belong to the order *Caudovirales;* they are characterized by the presence of a polyhedral head being most frequently icosahedral. Bacteriophages are conceivable very primitive as a group, with some estimates placing their ancestors before the separation of the bacteria from the Archaea and Eukarya. Their tail functions like a molecular machine and is used during infection stage to recognise the host and ensure effective genome penetration of the host cell wall. The *Caudovirales* are classified on the basis of their structure into three distinct families: the *Myoviridae* (for example T4 phages, possessing a complex contractile tail), the *Podoviridae* (for example T7 and T3 phages bearing a short noncontractile tail) and the *Siphoviridae (*for example λ phages, T5 phages, and phi, all characterized by their long noncontractile tail). Nonetheless, there are other phages that occur in large numbers on planet Earth, phages with different virions, genomes and lifestyles (see figure 1-1 for details on ICTV classification of bacteriophages).

Bacteriophages (Greek: phagein = eat) are the viruses that specifically infect bacteria. Like the rest of the viruses they depend entirely on the host for survival. When not infective, their extracellular form (the virion) behaves as an unreactive particle that is made up of a nucleic acid (dsDNA or RNA) surrounded by a protein coat (the capsid). Many bacteriophages possessing dsDNA present an injection structure (the tail) to allow movement of the nucleic acid through the bacterial cell wall and plasma membrane.

Unlike animal viruses, enveloped bacteriophages are rare. Consequently, virions are able to remain in their surroundings for quite an extended period of time as they do not have any metabolism (Andreoletti et al., 2009). In general, bacteriophages are frequently an important cause of significant losses in the food and drug, and diary industries due to the contamination of the raw materials in the factory setting.

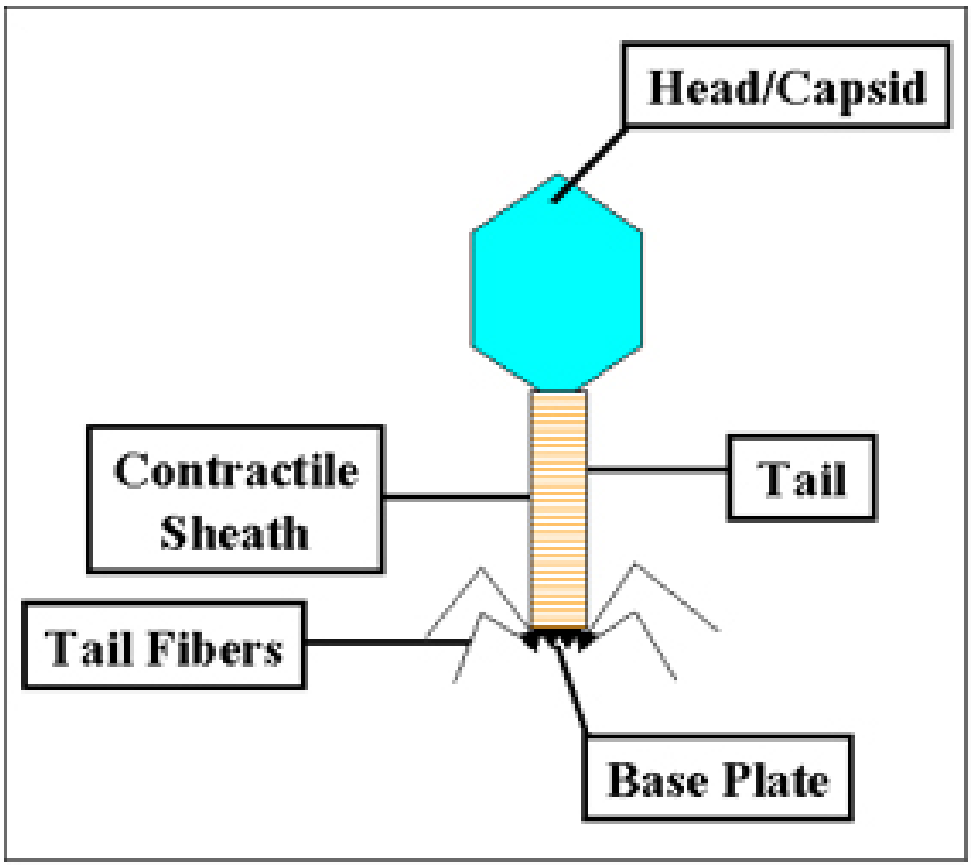


Figure 1-1 Structure of a T4 bacteriophage.

Source: www.microbiologybook.org/mayer/phages.htm. Accessed 21/09/2016

Bacteriophages were discovered almost a century ago by Frederick Twort in 1915 (Twort, 1915) and separately by Felix d'Herelle (d'Herelle, 1917). Since then they have played a key role in scientific research. Their ability to kill bacteria was first noticed by D'Herelle when using phages for experimental treatments. He observed at the start of his

studies that phages have the potential to kill bacteria that were responsible for diseases in humans as well as in livestock and plants. He initiated the use of phages as therapeutic agents before the discovery of antibiotics (Sulakvelidze et al., 2001). An abundance of bacteriophages is found in saltwater, freshwater, soil, plants and animals (Brussow and Kutter, 2005). Bacteriophages are also found in huge numbers in the human digestive and urinary systems as well as even on the skin (McGrath and van Sinderen 2007). Furthermore, they were used as curative agents almost since their discovery, in western countries up to the emergence of antibiotics. In many of the eastern countries of the former Soviet Union they are still being used for the treatment of wound infections without any consistent record of adverse effects attributed to their continuous use (McGrath and van Sinderen 2007).

The wide distribution and prevalence of phages in nature can havehas a profound impact on their bacterial hosts, including changes in bacterial physiology, competitive ability and virulence (Rohwer and Thurber, 2009; Day 2004; Brussow and Kutter, 2005). These impacts may follow logically from phage life cycles. For example, lytic phages have the potential to decrease host population density. However, they may also have unexpected consequences on individual bacteria and populations. For example, prophages can encode toxins or virulence factors that feedback to shape bacterial fitness. Aside from other predators of bacteria like the protozoans, phages by some estimates are accountable for up to 50% of bacterial death (Fuhrman and Noble, 1995), and in some environments phage predation can be even greater than grazing pressure by protists (Weinbauer and Peduzzi, 1995). The predator-prey relationship between phages and bacteria in addition, has a profound influence on the performance of microbial food chains/webs, biological, geochemical cycles (carbon, oxygen and nitrogen cycles),

nutrient cycling, biodiversity and species distribution and gene transfer (Weinbauer, 2004; Fuhrman, 1999, Suttle, 2005).

As such, this research project aims to further elucidate the possibility and potential of the use of lytic bacteriophages as environmental biocontrol agents against pathogenic bacteria in potable water systems and the study of the environmental conditions that would ensure the most efficient removal of these pathogens. Phage treatment, which targets specific bacteria, is a promising alternative to current disinfection strategies, which indiscriminately kill all bacteria, rather than just the pathogenic bacteria of interest.

The potential use of phages in pathogen removal has gained considerable attention over the recent few years. However, there are some research challenges that still need to be faced in order to realize the full potential of phage applications as environmental biocontrol agents against pathogens. One of these is to determine to what extent environmental parameters like temperature affect phage-mediated lysis efficiency? And how can bacterial lysis be rapidly monitored? Another challenge of importance is how best possible to suppress / delay the emergence of phage resistance in bacteria. These represent also the key challenges addressed in this research. Interest in phage applications as potential biocontrol agents is continuously increasing and is most likely to have a profound effect on the biotechnology and the environmental sector in general in the near future. As more phages are been discovered and characterized modern science may still has a lot to explore from the world's most abundant and genetically diverse entities nature has endowed mankind with. There is the general belief that phage research may be key in the battle against bacteria (Mc Grath and van Sinderen 2007).

ICTV classification of prokaryotic (bacteria and archaeal) viruses

Order	Family	Morphology	Nucleic acid	Example
	Myoviridae	Nonenveloped, contractile tail	Linear dsDNA	T4 phages, Mu, PBSX, P1Puna-like, P2,13, Bcep 1, Bcep 43, Bcep 78
Caudovirales	*Siphoviridae*	Nonenveloped, noncontractile tail (long)	Linear dsDNA	λ phages, T5phages, phi, C2, L5, HK97, N15
	podoviridae	Nonevenloped, noncontractile tail (short)	Linear dsDNA	T7 phages, T3 phages, Φ29, P22, P37
	Lipothrixviridae	Enveloped, rod-shaped	Linear dsDNA	Acidianus filamentus virus 1
Ligamenvirales	*Rudiviridae*	Nonenveloped, rod-shaped	Linear dsDNA	Sulfolobus islandicus rod-shaped virus 1
	Ampullaviridae	Enveloped, bottle-shaped	Linear dsDNA	
	Bicaudaviridae	Noneveloped, lemon-shaped	Circular dsDNA	
	Clavaviridae	Noneveloped, rod-shaped	Circular dsDNA	
	Corticoviridae	Nonenveloped, isometric	Circular dsDNA	
	Cystoviridae	Enveloped, spherical	Segmented dsRNA	
	Fuselloviridae	Nonenveloped, lemon-shaped	Circular dsDNA	
Unassigned	*Globuloviridae*	Enveloped, isometric	Linear DNA	
	Guttaviridae	Nonenveloped, ovoid	Circular dsDNA	
	Inoviridae	Nonenveloped, filamentous	Circular dsDNA	M13
	Leviviridae	Nonenveloped, isometric	Linear ssRNA	MS2, Qβ
	Microviridae	Nonenveloped, isometric	Circular ssDNA	ΦX174
	Plasmaviridae	Enveloped, pleomorphic	Circular dsDNA	
	Tectiviridae	Nonenveloped, isometric	Linear dsDNA	

Figure 1-2 ICTV classification of bacteriophages.

Source: Bacteriophage: Genetics amd Molecular Biology. 2007. In: McGrath and Van Sindereen (eds).

1.2 BACTRIOPHAGES BIOLOGY

1.2.1 Description and life cycle

The relationship between a phage and a bacteria is defined by a host-specific parasitic association in which the phage uses the energy and resources of a bacterium to produce more phages. Lysis of the host eventually takes place and the phage particles are released, which are in turn able to attack the surrounding bacteria and the cycle continues. The contact of the bacteriophage with its bacterial host follows the rules of statistical probability and is followed by the specific recognition and attachment on surface cell-receptors by the phage using it tail. This implies that bacteriophages can recognise the different structures present on the surface of the bacterial cell and they can sensitively differentiate sub-type of molecules. The implication of this is that they do have narrow host ranges, they only infect a strain or species or genus level for Gram positive and Gram negative bacteria respectively. However, they are unable to infect plants or animal cells (Calender, 2006).

The life cycle of bacteriophages may occur in three stages: (1) a mature phage that lives outside the bacterial cell and is metabolically stable and unreactive, like the spore state of bacteria, (2) a productive phage that lives inside the host after the attachment/adsorption process, (3) a temperate phage where genetic material is incorporated with the host cell leading to lysogeny. Lysogenic bacteria are non-infectious but capable of multiplying without being lysed; however, any spontaneous change such as exposure to stresses like UV radiation may lead to the host destruction (Adams 1959).

Bacteriophages entirely depend on bacteria for their replication and sustained survival. Depending on the type of infection, after the introduction of their genetic material inside the bacteria, phages either a) induce lysis of their bacterial host “lysis from

within" or b) they incorporate their nucleic acid into the host cell "lysis from without". Phages following the first route are referred to as lytic phages. Once inside the bacterial cell, lytic phages redirect/programme the bacteria's metabolism towards the production of virions, which are subsequently released upon lysis of the host cell. The number of released phage particles is designated as the 'burst size', (Thiel, 2004). The efficiency and coordination of the lytic cycle depends strongly on the viability status of the bacteria/host cell (Ceyssens and Lavigne 2010). In the case of lysogenic phages, the integrated genetic phage material resides either as a stable element called prophage inside the host cell or integrated into the host chromosome (temperate phages). A prophage is defined as a bacteriophage genome that is incorporated into the bacterial DNA chromosome or existing as an extrachromosomal plasmid. The latent form of a phage does cause any disturbance to the bacterial cell. A bacteria in this state could however, revert to the lytic cycle (where lysis occur) when subjected to stress in the surroundings environment such as starvation and exposure to chemicals (Hogg, 2005). The integration of the viral genome into the bacterium's is the reason why they are not used in phage therapy since they could incorporate, carry and transfer genes coding for undesirable elements such as the Shiga toxin from *Escherichia coli* (Skurnik and Strauch 2006, Monk et al., 2010).

1.2.2 Lytic life cycle

The stages of lytic (virulent) bacteriophage replication are as follows:

1. Attachment. The bacteriophage attaches/adsorbs to a specific structure/receptor on the cell of the host. Common receptors include surface proteins, parts of the lipopolysaccharide, pili and flagella.

2. Penetration. Phage nucleic acid is “introduced” in to the host cell. The capsid and other protein structures remain outside. In some tailed phages, the tail sheath contracts to induce the injection.
3. Synthesis of nucleic acid and protein. The phage DNA re-programmes the chemical and biological processes of the host to produce its own nucleic acids and proteins. The production of viral components begins after the phage DNA is injected into the host cytoplasm after which synthesis of viral nucleic acids and proteins occurs. The bacteria metabolic activity stops when the phage induces break down of host DNA, phage proteins interfere with genetic message processing of the host, and repression of translation occurs (Stone and Burton 1962; Cohen and Ennis 1966; Bautz et al. 1966; Hsu et al. 1967; Kennel 1970; Davis 2004). Bacteriophages use the host’s nucleotides and enzymes to produce copies of its DNA before synthesis of its macromolecules.
4. Assembly and maturation. The phage nucleic acid and protein products are assembled in a step by step process to form mature phage particles.
5. Release. The host cell wall is lysed by phage-encoded enzymes called lysins. Newly formed phage particles are released into the environment and subsequently infect other susceptible host cells (Madigan et al., 2006).

The lytic mechanism from an environmental/ecological point of view constitutes a predator/prey system, and from an epidemiological point of view a host-parasite model (Gorski and Weber-Dabrowska 2004). The phenomenon of transduction (transfer of bacterial DNA via phage) is rare in lytic phages (Monk et al., 2010).

1.2.3 Lysogenic life cycle

In the lysogenic or latent cycle, the temperate phage attaches to and penetrates the host cell in the same way as in the lytic cycle. However, once inside, the phage genome integrates with that of the host cell and becomes a dormant prophage (Hogg, 2005). The phage genome undergoes replication with every replication cycle of the bacterium. A bacterial host containing a prophage is called a lysogen, and is protected by the prophage from superinfection by another temperate phage. When the host is exposed to an environmental stressor such as ultraviolet light, the dormant prophage is induced to carry out the lytic cycle (Hogg, 2005). Although not suitable candidates for environmental biocontrol, temperate bacteriophages are very useful tools to the molecular biologist due to the role they play in transduction, recombination and lysogenic conversion (Prescott et al., 2005).

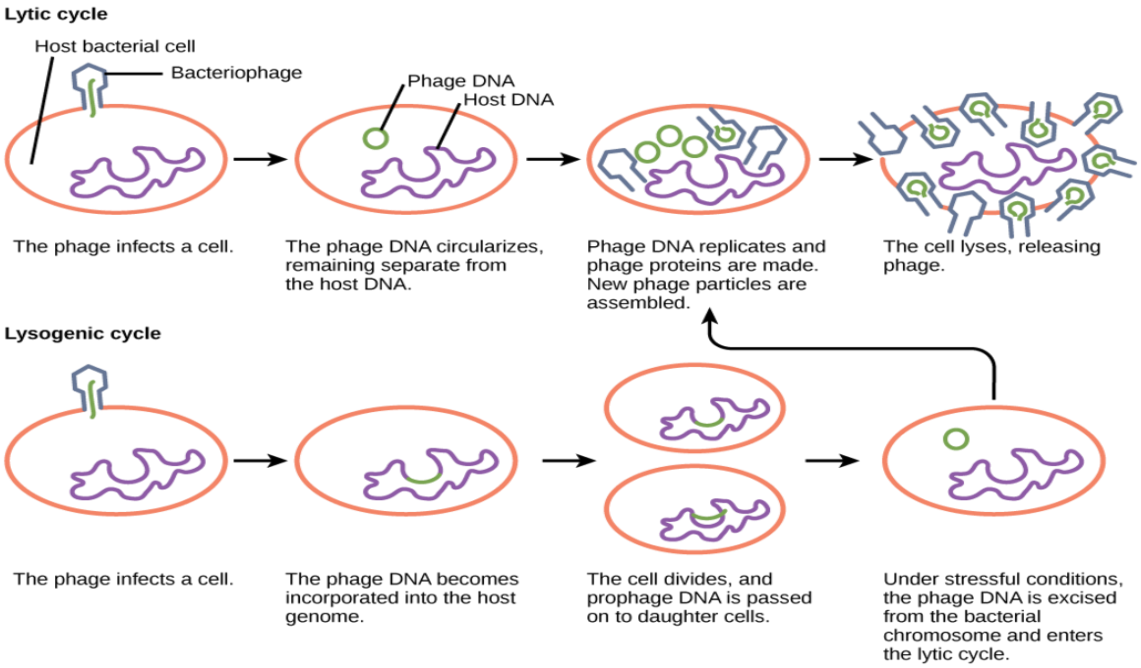


Figure 1-3 A temperate bacteriophage undergo lytic and lysogenic cycles. The lytic cycle involved lyses of the host cell. The phage DNA is incorporated in the host genome in the lysogenic cycle, where it is passed from generation to generation. The prophage can revert to the lytic cycle when exposued to environmental stressors such as starvation or the presence of toxic chemicals

Source: http://cnx.org/content/m44597/latest/figures_21-02-03.png Accessed 10/09/2016.

1.3 APPLICATIONSOF BACTERIOPHAGES AS BIOCONTROLAGENTS

1.3.1 Clinical/medical

For more than half a century since the discovery of antibiotics as “wonder drugs”, they have been used therapeutically to treat diseases caused by pathogenic bacteria. However, antimicrobials are becoming less effective because of it abuse in animal and crop production (Fischetti, 2008; Perisien et al., 2008). More recently, the emergence of

antibiotic resistance in bacteria has renewed the interest in bacteriophages as a viable natural alternative that has the potential to fight bacterial infections (Levin and Bull 2004). The application of lytic/virulent bacteriophages to treat bacterial infections in humans or animals is often termed phage therapy. Phages has some specific merits, which includes: (1) they are host-specific and therefore can infect only the target organism and not any other good microbial organisms, (2) they do not pose any threat to human cells, (3) phages reproduce very quickly, hence, a single dose can be sufficient to cure a bacterial infection, (4) phages can handle bacteria resistance better by evolving with their host, (5) production of bacteriophages is relatively cheap, (Barrow and Soothhill 1997; Barrow 2001; Summers 2001; Sulakvelidze et al. 2001; Merril et al. 2003; Levin and Bull 2004; Mastuzaki et al., 2005).The initial trials of phage therapy produced varied results which have been attributed to the limited knowledge of phage biology and lysogeny (Summers, 2001). Lysogenic phages are now known to be unstable for phage therapy, because of their ability to confer virulence and antibiotic resistance to bacterial hosts through phage-mediated gene transfer and their ability to remain in a dormant prophage state.

Despite its great potential, phage therapy was successfully pursued in Western countries after the 1940s, only some countries in the former Russian republic particularly at the Eliava Institute in Tbilisi, Georgia and Eastern Europe continued applying lytic phages as a therapeutic agent (Summers, 2001). Antibiotic-resistant pathogens constitute a serious global health problem and are now prevalent among many pathogens. Some of the microbes that have become resistant to conventional antibiotics includes: *Pseudomonas aeruginosa* (Ong et al., 2004), *Haemophilus influenza* (Bozdogan and Appelbaum, 2004), *Salmonella* (Fluit, 2005), *Mycobacterium tuberculosis* (Di Perri and

Bonora, 2004), *Acinetobacter* (Jain and Danziger, 2004). Others are; *Staphylococcus aureus* (Lowy, 2003), *Escherichia coli* (Saenz et al., 2004), *Streptococcus pneumonia* (Jacobs, 2004), *Campylobacter jejuni* (Lindmark et al., 2004), *Helicobacter pylori* (Megraud, 2004), and *Clostridium difficile* (Razavi et al., 2007). Due to the poor return on investment, multinational drug companies are less attracted in research into the discovery and production of new antibiotics. (Projan and Shlaes, 2004; Norrby et al., 2005).

Apart from the entire phage particles, purified phage-encoded enzymes are used to treat bacterial infections (Fischetti, 2008). It has been shown that isolated endolysins, the enzymes which help the phage to free the progeny phages after a complete life cycle, can be used to treat infections of Gram-positive bacteria (Fischetti, 2008). Jodo et al. (2003) used a phage encoded murein hydrolase to treat *Streptococcus pneumonia* infected mice. Such treated mice survived infections in contrast to untreated control mice. The authors suggested that phage lysins can protect animals from bacterial infections.

In summary, bacteriophages appear highly promising for the treatment of bacterial diseases. They are specific to their bacterial host and they do not cause any harm to humans or plants, they can multiply in large numbers as long as bacterial host are present, and they are self-limiting in that once they kill all of the target bacteria they no longer have a place to replicate (Summers, 2001; Clark and March, 2006). Therapeutic use of phage, or the use of bacteriophages to kill pathogenic bacteria of interest, represents a potentially important and natural biocontrol agent against bacterial pathogens.

1.3.2 Food safety

According to the Centers for Disease Control and Prevention (CDC), in the United State foodborne illness is a significant problem, causing 9.4 million cases of foodborne disease, nearly 56,000 hospitalizations, and more than 1,350 deaths every year (Scallan et al., 2011). Pathogenic bacteria are a serious threat to food safety/security worldwide. Food contamination resulting from microbes such as *Salmonella*, *Clostridium*, *Campylobacter*, *Escherichia coli*, (*E. coli* O157:H7 in particular), *Staphylococcus* and *Listeria* are associated with numerous illnesses and some deaths in both the US and EU (CDS and EFSA, 2011). Pathogenic bacteria can get access to food during harvesting/slaughtering, processing storage or packing, milking or fermentation. A reasonable numbers of foodborne microbes are becoming resistant to available antibiotics, a situation which can be partly attributed to the abuse of antibiotics as growth enhancers in animal and crop production (Martinez et al., 2008; Tabla et al., 2012; Bueno et al., 2012; Atterbury et al., 2003; Oliver, Jayarao and Almeida, 2000). Bacteriophages as natural biocontrol agents against bacterial pathogens can be employed in ensuring the safety of food products from production to consumption. Their safe usage, relatively easy handling, self-replicating and self-limiting nature and high specific antimicrobial activity make them a promising alternative to antibiotics.

The uses of bacteriophages in food safety have been reported by many researchers. Phage biocontrol has been considered effective in the reduction of the colonization of broiler chickens and chicken products by *Campylobacter and Salmonella* (Atterbury et al., 2003, 2005). Also Raya et al. (2006) reported the potential use of phages and phage cocktails to reduce *E. coli* 0157:H7 in sheep in pre-slaughter and post-slaughter intervention. Listeria is an important environmental pathogen due to its serious and often

vital consequences to susceptible individuals as well as its ability to survive and grow in foods stored under refrigeration conditions (Hudson et al., 2005). Vongkamjan et al., 2012 and 2013 report the potential application of listeriaphages for the biocontrol of *Listeria* in silage and smoked fish. Atterbury et al. (2007) isolated over 200 bacteriophages and selected three based on the most effective killing over the broadest host range. These phages were fed to chickens after inoculation of the birds with *Salmonella*. The three separate phages resulted in log reductions of 4.2, 2.19 and no effect even though all three showed high effectiveness in laboratory experiments. This suggests the importance not only of phage effectiveness *in vitro* but also its ability to remain viable *in vivo*.

Ready to eat fruits and vegetables are particularly vulnerable to contamination by pathogenic bacteria because they are often grown outdoors in soil and as such are exposed to many environmental pathogens either through direct contact or through contact with vectors harbouring bacteria. The irrigation of such crops with untreated sewage or fertilization with poorly composted manure may also contribute to the risk of contamination (Beuchat, 2002). Although fruits and vegetables possess skin or rinds which confer protection against contamination by pathogens, damage to this outer skin can allow penetration of bacteria which may be difficult to remove by chemical sanitisation. Fresh-cut produce is also susceptible to contamination if the skin is not thoroughly washed prior to slicing (Bowen et al., 2006). The two most common approaches to decontaminating fruits and vegetables involve washing with water and washing with sanitising agents such as trisodium phosphate or sodium hypochlorite. The routine use of the latter in food processing environments has led to various bacteria

developing resistance to these agents, causing a decline in their efficacy (Mokgatla, Brozel and Gouws, 1998).

The use of bacteriophage biocontrol of raw fruits and vegetables has been investigated as a potential method for reducing the risk of contamination and foodborne illness. Leverentz et al. (2001) examined bacteriophage as a biocontrol method for *Salmonella* on fresh cut melon and apple slices. In a similar study, Leverentz et al. (2003) investigated the use of bacteriophages in combination with nisin as a biocontrol treatment for Listeria monocytogenes on fresh-cut produce.

Given the successes recorded with the application of phages as natural biocontrol agents against bacterial pathogens, a number of bacteriophages applications are in the development process or are have already been marketed and is available to the general public. Among the products commercialized are the following: Agriphage™ (EPA registration number 67986-1) manufactured in USA by Omnilytic, this is a bacteriophage-based pesticide that is specific for the pathogen *Xanthomonas campestris* pv. *Vesicatoria* and *Pseudomonas syringae* pv tomato. Agriphage is used to protect tomato and pepper against bacterial disease (Balogh et al., 2010; Jones et al., 2007). Listex™ P100, produced in the Netherlands by Micreos is very effective against *Listeria monocytogenes* (Carton et al., 2005). The product is widely used in the EU and US after it has received a safe status by the FDA and USDA for use in cheese products (2006) and subsequently for use in all food materials (2007).

Bacteriophages can be used as an indicator of food contamination and as a tool for selective removal pathogens from food matter (biocontrol) to ensure food safety. Nevertheless, the future of phages in food safety in the West may have many hurdles to

cross as stringent regulations and more clinical trials is needed before any approval can be granted to any phage product. Farmer and the general public alike need to be enlightened about the safety and merit of phage usage (EFSA, 2009).

1.3.3 Water and Wastewater

Bacteriophages have been reported for potential application in water and wastewater treatment to remove bacterial pathogens of interest. Withey et al. (2005) reviewed the potential for bacteriophages biocontrol in wastewater treatment processes for the purpose of reducing pathogenic bacteria and improving effluent and sludge products. Sludge produced by biological wastewater treatment processes contain high numbers of microorganisms including bacteria and is often reused by application to agricultural lands. It has been highlighted that such use of sludge has the potential to transmit human diseases and so proper treatment and regulation is required, specifically regarding the required reduction in concentration of pathogens. The use of lytic bacteriophages to assist in the treatment process could reduce the costs associated with such treatment. Wastewater treatment facilities maintain a good balance of microbes, with many desirable microorganisms breaking down waste and preventing malodour at the plant. The aim is to remove target bacteria with minimal impact on beneficial bacteria. Using selected phages should not be a problem to the environmental or cause any health concern (Zhang and Hu, 2012).

The ability of bacteriophages to selectively remove the opportunistic pathogen *Pseudomonas aeruginosa* in drinking water filtration system was demonstrated by Zhang et al. (2013). Water and wastewater filtration systems usually contain pathogen bacteria, which must be removed to ensure potable water is supplied to the consumers. Biofilters and granular activated carbon (GAC) used in water treatment processes to improve water

quality, remove micro-pollutants, and odour can provide a good environment for the survival and growth of bacterial pathogens because of their porosity. The contamination of such biofilters might be difficult to treat with chlorine disinfection. The use of phages which target specific pathogenic bacteria is a promising alternative to chemical disinfection.

1.4 FACTORSHAMPERINGBACTERIOPHAGESAPPLICATIONS

1.4.1 Public perception of viruses

Phages are the most abundant and far-reaching biological entities on Earth. They are present all around us and impact on our daily activities and we live harmoniously with them (Sulakvalidze 2011).The public thinking of virus as very harmful can have a significant impact on the use of phage in our healthcare or as biological disinfectant in food and water for consumption. The general public perceives all viruses as dangerous and harmful, for example AIDS virus or polio virus. Therefore, there is the need for serious education to ease the acceptance of phages in all sectors of application by the general public. Like many now welcome the idea of "good bacteria" and purchase probiotic food like yoghurt, the public will accept phages as well if they work well, the public will accept them. Phages fit into the progressive appreciated trend that not all microbes are bad, and many are beneficial. Phages, if properly harnessed, can have a huge positive impact on many aspect of our lives, from food safety to environmental and health care.

1.4.2 Challenges to phage applications

The continuous attack on bacterial host cells by phages can be defended. Bacteria can mutate and cause the alteration of receptors used for attachment by phage and thus

become insensitive to bacteriophage infection (bacteriophage insensitive mutants, BIMs). This phenomena may results in loss, modification, or masking of the bacteriophage receptors located at the cell wall (Andreoletti et al., 2009).

According to Levin and Bull (2004) three mechanisms involved in the emergence of phage resistance in bacteria. 1) the first being the loss of receptors on bacterial wall as a result of sudden change in the gene or chromosome. Hence, phage cannot replicate in those bacteria. 2) the secretion of extracellular substance that enable bacterial cells to have partial resistance to phage infection. However, phage is capable of adsorbing to and replicating in these bacteria at minimal rate. 3) the third involves the disruption of the lytic cycle by the enzymes secreted by the bacteria to degrade the injected phage genome.

Since bacteriophages eliminate bacteria by attaching to specific cellular receptors on their external membranes, the potential exists for mutation. It has been mentioned in many of the phage studies that the presence of bacteriophage-resistant mutants were detected after use of bacteriophage (O'Flynn et. al., 2004; Tanji et. al., 2004). Both O'Flynn et. al. (2004) and Tanji et. al. (2004) conducted experiments to measure the formation of *E. coli* O157:H7 mutants that were resistant to bacteriophage. O'Flynn et. al. (2004) determined that while mutations did arise, the frequency was low and the mutants appeared to revert back to susceptible strains after multiple generations. A study by Fischer et al. (2004) found that because 15% of one particular strain of bacteria was resistant to a specific phage both phage and host could co-exist in continuous culture for days. They indicated the necessity for properly understanding phage-host interactions in a continuous culture before being able to harness phage for other forms of therapy or activity.

Bacteria however, develop some defence mechanism against phages, and evolution on one hand have aided phages to adapt to the changes in bacteria. Bacteria cannot be continuously resistance as a result of the fitness cost associated.

It is interesting to note, many phages have evolved to use receptors on the host cell which are important structures or virulence factors that cannot be lost without a fitness cost; this means that a host cell is less likely to become resistant to infection. However, mutations can arise that alter the receptors without loss of function, and it has been shown in many studies of phage-host ecology that co-evolution of both bacteria and phages occurs so that phage are selected that can still efficiently infect the bacterial cell present in a given environment (Scanlan et al., 2015). Therefore phage co-evolution is often cited as an advantage of phage biocontrol over conventional antibiotic application where the chemicals being used to treat the infection cannot change in response to the emergence of resistance. However, it is also seen that phages drive a faster mutation rate in the change of these key virulence traits used as receptors, resulting in cells with altered surface properties (O'Flynn, et al., 2004; Hudson et al., 2005). This raises the question whether co-evolution is a benefit or a threat, and this remains a significant topic regarding the development of phage biocontrol applications.

The cost of phage-resistance in bacteria may result in the development of less pathogenic bacterial specie as attachment sites/structures used for phage attack might be a virulence factors (Smith et al. 1987, Westwater et al. 2003).

1.4.3 Bacteria-phage interactions in natural environments

The interactions among microorganisms are now known to shape biological and geochemical processes in the biosphere at large. The historical path to this view, however,

has been very complicated and the study of microbes was traditionally much narrower in context, primarily with the germ theory of disease (Lederberg, 2000). Despite an early tradition of environmental microbiologist, the appreciation of microbes as fundamental parts of every ecosystem did not gain mainstream scientific acceptance until recently, when culture-independent methods of detecting microorganism developed (Hobbie; Daley and Jasper 1977), notably those based on nucleic acid and sequencing (Lane et al., 1985).

Bacteria, in particular, are conspicuous players in the microbial world and shape the water, carbon, nitrogen, oxygen and sulphur cycles that ultimately enabled the rise of eukaryotes (Karl et al., 1997). Bacteria serve as prey, fix nitrogen, generate oxygen, and may be involve in mass extinctions of animals (Rothman et al., 2006). The realization of the abundance of microbial life, originating mainly from environmental microbiology studies, has come full cycle to medical applications, as research into human microbiome has revealed that we humans depend on microbes for normal bodily functions, even prior to birth (Funkhouser and Bordenstein, 2013). Thus, the importance of microorganisms in shaping many of the global processes, and human health in particular, has led to a renewing interest in the microbial world.

More recently, attention has been drowned to the viruses of bacteria, the bacteriophages (Abedon, 2009). A sequence of scientific advancements in bacteriophage detection have provided evidence that phages are the most populous and more numerous and diverse than any other microbial entity (Engelhardt, Kallmeyer, Cypionka, and Engelen, 2014; Suttle, 2005, 2007; Williamson et al., 2013). However, we do not yet have a clear picture of the role that natural bacteriophages populations might play in shaping bacterial populations and communities as obligate parasite, vectors of horizontal gene

transfer, drivers of bacteria evolution, and mediators of competition among species. The reciprocal selection of bacteria on phage populations and phages on bacteria populations has been demonstrated in both lab and the field, and there is increasing evidence that theinteraction between bacteria and phages can maintain bacterial diversity, influence bacterial virulence, and shape the stability of ecosystems (Koskella and Brockhurst, 2014). Perhaps the interactions between bacteria and phages in the environment may have many questions that needs to be answered such as, which particular phage and bacteria will interact and how sustained are these interactions likely to be? Do phages impact bacteria population dynamics? Does phage-mediated bacteria selection influence bacterial diversity?

Although, the ability of phages killing their bacterial host cell was first discovered almost a century now, the impact that phages have on microbial populations and ecosystems in nature has not been given the due attention. A good number of studies have reveal that interaction between bacteria and their phage counterpart can shape the former genomes, drive and stain diversity within and among bacteria populations and stabilize microbial communities (Brüssow et al., 2004).

1.5 PROJECT DEVELOPMENT

1.5.1 Rationale

Access to safe and clean drinking water is fundamental to human health and wellbeing. Although safe drinking water is taken for granted by many people, sporadic outbreaks of waterborne disease sadly demonstrate that the control of pathogens is still a highly relevant (and increasingly important) topic. The death of several babies caused by infection with waterborne multidrug-resistant *Pseudomonas aeruginosa* in a Belfast hospital in 2012 or three British tourists dying of legionellosis in the Spanish tourist resort of Calpe in 2011 are just two out of the many examples, where disinfection regimes prove inadequate to control pathogens levels in engineered water supply systems.

This project aims at establishing an old, but nearly forgotten technology to combat bacterial pathogens using their natural predators: bacteriophages. The greatest benefit is that bacteriophages are highly specific for their bacterial host and do not pose any harm for other (beneficial) microorganisms or humans. However, the factors influencing their efficiency are poorly understood and there is little knowledge how the occurrence of resistant bacteria can be mitigated. Moreover the methods used to study the efficiency of lysis are time consuming and tedious.

1.6 Aim and objectives

The main aim of the project was to determine the overall efficiency of lytic bacteriophages as natural biocontrol agents against bacterial pathogens and to better understand factors that impact their lytic performance. It was hypothesised that bacteriophages from environmental sample can be used to control bacteria of concern (primarily pathogens).

The specific objectives are:

1. To isolate and purify lytic bacteriophages specific for *E. coli* from environmental water
2. To study the effects of temperature on host cells and phages in regards to lysis susceptibility/efficiency
3. To compare the lysis efficiencies of different phages
4. To establish more efficient diagnostic tools other than plaque counts to monitor phage-mediated bacterial lysis efficiency
5. To correlate lysis kinetics of distinct environmental phages with the plaque sizes they produce
6. To compare the effect of sequential or simultaneous addition of multiple distinct phages with different lysis kinetics
7. To study the lysis susceptibility of bacteria subjected to sublethal disinfection stress

1.7 Thesis plan

This thesis is presented as a series of chapters formatted as papers for publication. All papers forming this thesis were read, and edited by Dr Andreas Nocker and Prof Sean Tyrrel. All experimental work was designed, coordinated and completed by Ekwu Ameh, at Cranfield University (UK) with the contribution of Charlie Farrell in conducting the UV experiment. The *E.coli* cultures plates were periodically streaked aseptically and monitored to ensure active bacteria were used in all experiment in Cranfield University. In Chapter 2, part of the experiments to characterize the isolated bacteriophages by TEM were completed by Prof. Elena Orlova and her team at Birkbeck, University of London.

Chapter 2, Temperature dependence, focused on temperature as a key environmental parameter influencing the activity of bacteriophages. Paper 1, entitled *Effect of temperature on bacteriophage-mediated lysis efficiency with special focus on bacterial temperature history* by Ameh, E., Tyrrel, S., Harris, J., Orlova, E., Ignatious, A., and Nocker, A., submitted to Annals of Microbiology. This paper reports the experimental results relating to the temperature of incubation of the phage-bacteria mix and the temperature of growth of the bacteria prior to infection with the phages (temperature history). The paper considers how these two temperature-dependent processes play a key role in the lysis efficiency of the bacteriophages with implications on the their use as natural biocontrol agents

Chapter 3, Simultaneous and sequential applications of cocktail of bacteriophages, focuses on strategies aimed at delaying the emergence of phage resistance in bacteria. Paper 2, entitled *Lysis efficiencies of bacteriophages with different plaque sizes and comparison of lysis kinetics after simultaneous and sequential phage*

addition by Ameh, E., Tyrrel, S. and Nocker, A.to be submitted to Archive of Microbiology, evaluates the potential of increasing efficiency of lysis and suppressing the emergence of resistance with the use of cocktail of phages. It explores the use of different phages in both a simultaneous and sequential manner and concludes that the use of a cocktail of phages sequentially can be more sustainable, delaying the emergence of phage resistance in the host.

Chapter 4, Biological disinfection, the chapter entails the use of phages on substances as a secondary disinfectant such as water. Paper 3, entitled *Sustainability of the effect of chlorine and UV on E.coli growth suppression and impact of disinfection on the bacterial susceptibility to phage-mediated lysis* by Ameh, E., Tyrrel, S. and Nocker, A.to be submitted to Water Research. The hypothesis that bacteria that were previously subjected to sublethal disinfection are no longer susceptible to phage-mediated lysis efficiency was tested and the experimental results summarised in this paper. These results suggested that application of phages after sub-lethal disinfection greatly diminished the lytic efficiency. It also appeared that phages tend to prefer "healthy bacteria" to stressed ones, with implications for their use on food or water prior antimicrobial treatment.

Chapter 5, Discussion section synthesises and critically evaluates the key findings of the project and the implications of the study.

Chapter 6, Conclusions and future work, summarises the key results and suggests recommendations for future investigations on the potential of bacteriophages natural biological control agents of pathogenic bacteria in the environment.

1.8 REFERENCES

Abedon, S. T. 2009. Phage evolution and ecology. Advances in Applied Microbiology, 67, 1–45.

Adams, MH 1959. Bacteriophages. Interscience, New York, NY, USA.

Andreoletti, O., Budka, H., Buncic, S., Colin, P., Collins, J. D., De Koeijer, A., Griffin, J., Havelaar, A., Hope, J., Klein, G., Kruse, H., Magnino, S., Antonio, López, M., McLauchlin, J., Nyuyen-The, C., Noeckler, K., Noerrung, B., Maradona, M. P., Roberts, T., Vågsholm, I., and Vanopdenbosch, E. 2009. Scientific Opinion of the Panel on Biological Hazards on a request from European Commission on The use and mode of action of bacteriophages in food production. The EFSA Journal1076, 1-26

Atterbury, R. J., Van Bergen, M. A. P., Ortiz, F., Lovell, M. A. Harris, J. A., De Boer, A.,Wagenaar, J. A., Allen, V. M., and Barrow, P. A. 2007. Bacteriophage therapy to reduce *Salmonella* colonization of broiler chickens. Appl. Environ. Microbiol. 73:4543-4549

Atterbury, R. J., Dillon, E., Swift, C., Connerton, P. L., Frost, J. A., Dodd, C. E. R.,

Rees, C. E. D., and Connerton, I. F. 2005.Correlation of *Campylobacter* bacteriophage with reduced presence of hosts in broiler chicken ceca. Appl. Environ. Microbiol. 71: 4885-4887

Atterbury, R. J., Connerton, P. L., Dodd, C. E. R., Rees, C. E. D., and Connerton, I. F. 2003b. Isolation and characterization of *Campylobacter* bacteriophages from retail poultry. Appl Environ Microbiol. Vol.69, 4511–4518, ISSN 1462-2920

Balogh, B., 2010. Phage therapy for plant disease control, Curr Pharm Biotechnol, 11, 48 – 57

Barrow, P. A., Soothhill, J. S. 1997. Bacteriophage therapy and prophylaxis: rediscovery and renewed assessment of potential. Trend Microbiol 5:268–271

Barrow, P.A. 2001. Review the use of bacteriophages for treatment and prevention of bacterial disease in animals and animal models of human infection. J Chem Technol Biotechnol 76:677–682

Bautz, E. K, Kasai, T, Reilly, E., Bautz, F. A. 1966. Gene-specific mRNA. II Regulation of mRNA sysnthesis in *E. coli* after infection with bacteriophage T4. Proc Natl Acad Sci USA 55:1081–1088

Beuchat, L. 2002. Ecological factors influencing survival and growth of human pathogens on raw fruits and vegetables. Microb. Infect. 4, 413-423

Bohannan, B. J. M, Lenski, R. E. 2000. Linking genetic change to community evolution: insights from studies of bacteria and bacteriophage. Ecol Lett 3:362–377

Bowen, A., Fry, A., Richard, G., and Beauchat, L. 2006. Infections associated with cantaloupe consumption: a public health concern Epidemiol. Infect. 134, 675.

Bozdogan, B., and Appelbaum, P. C. 2004. Macrolide resistance in Streptococci and *Haemophilus influenza*. Clin Lab Med 24: 455-475

Brüssow, H., and Kutter, E. 2005. Genomics and evolution of tailed phages. In: Bacteriophages: Biology and applications, pp. 91-128

Brüssow, H., Dillmann, M., Bruttin, A., and Chibani-Chennoufi, S. 2004. Phage-Host Interaction: an Ecological Perspective. Journal of Bacteriology. Vol. 186 No. 12, 3677-3686

Bueno, E., García, P., Martínez, B., and Rodríguez, A. 2012. "Phage inactivation of *staphylococcus aureus* in fresh and hard type cheese", International journal of food microbiology, vol. 158, no. 1, 23-27

Calendar, R. 2006. The bacteriophages. New York, USA, Plenum Publishing Corporation

Carlton, R. M., Noordman, W. H., Biswas, B., de Meester, E. D., and Loessner, M. J. 2005. Bacteriophage P100 for control of *Listeria monocytogenes* in foods: genome sequence, bioinformatics analyses, oral toxicity study, and application, Regul Toxicol Pharmacol. 43, 301 – 12

CDC .2011. CDC Estimates of Foodborne Illness in the United States. Available at: www.cdc.gov/foodborneburden/2011-foodborne- estimates.html

Ceyssens, P. J., Lavigne, R. 2010. Introduction to bacteriophage biology and diversity. In: Sabour P, Griffiths M (eds). Bacteriophages in the control of food and waterborne pathogens. ASM Press. Washington, DC, USA, Pp 11-29.

Clark, J. R. and March, J. B. 2006. "Bacteriophages and Biotechnology: Vaccines, gene therapy and antibacterial" TRENDS in Biotechnology 24: 212-218

Cohen, P. S, Ennis, H.L. 1966. *In vivo* stability of bacteriophage T4 messenger ribonucleic acid. J Bacteriol 92:1345–1350

Curtright, A. J., and Abedon, S. T. 2011. Phage therapy: emergent property pharmacology. Journal of Bioanalysis and Biomedicine. Vol. S6, article 002

Day, M. 2004. Bacterial sensitivity to bacteriophages in the aquatic environment. Sci. Prog. 87, 179-191

Di Perri and Bonora, S. 2004. Which agent should be used for treatment of multidrug-resistant *Mycobacterium tuberculosis*? J Antimicrob Chemoth 54: 593-602

EFSA. 2011. The European Union Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents and Food- borne Outbreaks in 2009, EFSA Journal, 9

EFSA. 2009. "The use and mode of action of bacteriophages in food production. Scientific opinion of the panel on biological hazards," The European Food Safety Authority Journal, vol.1076, 1–26

Engelhardt, T., Kallmeyer, J., Cypionka, H., and Engelen, B. 2014. High virus-to-cell ratios indicate ongoing production of viruses in deep subsurface sediments. The ISME Journal, 8(7), 1503–1509

Fischer, C. R., M. Yoichi, H. Unno, and Y. Tanji. 2004. The coexistence of *Escherichia coli* serotype O157:H7 and its specific bacteriophage in continuous culture. FEMS Microbiol. Lett. 241:171-177.

Fischetti, V. A. 2008. Bacteriophage lysins as effective antibacterials. Current Opinion in Microbiology, Vol.11, pp 393 – 400, ISSN 1369-5274

Fluit, A. C. 2004. Towards more virulent and antibiotic-resistant *Salmonella*? FEMS Med Mic 43: 1-11

Fuhrman, J. A., and Noble, R. T. 19995. Viruses and protists cause similar bacterial mortality in coaster seawater. Limnology and Oceanography, 40, 1236-1242

Fuhrman, J. A., 1999. Marine viruses and their biogeochemical and ecological effects. Nature 399, 541-548

Funkhouser, L. J., and Bordenstein, S. R. 2013. Mom knows best: The universality of maternalmicrobial transmission. PLoS Biology, 11, e1001631

Gorski and Weber-Dabrowska, A. B. 2004. The potential role of endogenous bacteriophages in controlling invading pathogens. Cell Mol Life Sci62, 511-519

Hendrix, R. W., 2003. Bacteriophages genomics. Curr. Opin. Microbiol. 6, 506-511

Hudson, J.A., Billington, C., Carey-Smith, G. and Greening, G., 2005. Bacteriophages as biocontrol agents in food. J. Food Prot. 68 (2), 426-437.

Hsu, W., Foft, J. W, Weiss, S. B. 1967. Effect of bacteriophage infection on the sulphur–labeling of sRNA. Proc Natl Acad Sci USA 58:2028.

Hobbie, J. E., Daley, R. J., and Jasper, S. 1977. Use of nuclepore filters for counting bacteria by fluorescence microscopy. Applied and Environmental Microbiology, 33, 1225–1228

Jacobs, M. R. 2004. Building in efficacy: developing solutions to combat drug-resistant *S.pneumoniae*. Clin Microbiol Infec 10 (suppl 2): 18-27

Jain, R. and Danzinger, L. H. 2004. Multidrug-resistant acinetobacter infections: an emerging challenge to clinicians. Ann pharmacother 38: 1449-1459

Jason, J. G., and Paul Hyman. 2010. Phage choice, isolation, and preparation for phage therapy. Current pharmaceutical Biotechnology. Vol. 11, no. 1, 2-14

Jones, J. B., Jackson, L. E., Balogh, B., Obradovic, A., Iriarte, F. B., and Momol, M. T. 2007. Bacteriophages for plant disease control, Annu Rev Phytopathol, 45, 245 – 62

Karl, D., Letelier, R., Tupas, L., Dore, J., Christian, J., Hebel, D. 1997. The role of nitrogen fixation in biogeochemical cycling in the subtropical North Pacific Ocean. Nature, 388(6642), 533–538

Kennell, D .1970. Inhibition of host protein synthesis during infection of *Escherichia coli* by bacteriophage T4. II Indiction of host messenger ribonucleic acid and its exclusion from polysomes. J Virol 6:208–217

Koskella, B., and Brockhurst, M. A. 2014. Bacteria–phage coevolution as a driver of ecological and evolutionary processes in microbial communities. FEMS Microbiology Reviews.

Lane, D. J., Pace, B., Olsen, G. J., Stahl, D. A., Sogin, M. L., & Pace, N. R. (1985). Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. Proceedings of the National Academy of Sciences of the United States of America, 82, 6955–6959

Landmark, H. 2004. Genetic characterization and antibiotic resistant of *Campylobacter jejuni* isolated from meats, water and humans in Sweden. J Clin Microbiol 42: 700-706

Lederberg, J. 2000. Infectious history. Science, 288, 287–293

Lenski, R. E, Levin, B. R .1985. Constraints on the coevolution of bacteria and virulent phage: some experiments, and predictions from natural communities. Am. Nat. 125:585-602

Leverentz, B., Conway, W. S., Alavidze, Z., Janisiewicz, W. J., Fuchs, Y., Camp, M. J., Chighladze, E., and Sulakvelidze, A. 2001. Examination of bacteriophage as a

biocontrol method for *Salmonella* on fresh-cut fruit: a model study. J. Food Prot., 64, 1116-1121

Leverentz, B., Conway, W. S., Janisiewicz, W. J., Camp, M. J., Abuladze, T., Yang, M., Saftner, R., and Sulakvelidze, A. 2003. Biocontrol of *Listeria Monocytogenes* on fresh-cut produce by treatment with lytic bacteriophages and a bacteriocin Appl. Environ. Microbiol. 69, 4519-4526

Levin, B. R, and Bull, J. J .2004. Population and evolutionary dynamics of phage therapy. Nature Reviews Microbiology 2:166–173

Loc-Carrillo and Abedon, S. T., 2011. Pros and cons of phage therapy. Bacteriophages. Vol. 1. 111-114

Lowy, F. D. 2003. Antimicrobial resistance: the example of *Staphylococcus aureus*. J Clin Invest 111: 1265-1273

Martínez, B., Obeso, M. J., Ana Rodríguez, A., and García, P. 2008. "Nisin-bacteriophages cross-resistance in *Staphylococcus aureus*", International journal of food microbiology, vol. 122, no. 3, 253-258

Matsuzaki, S. Rashel, M., Uchiyama, J., Sakurai, S., Ujihara, T., Kuroda, M., Ikeuchi, M., Tani, T., Fujieda, M., Wakiguchi, H., and Imai, S. 2005. Bacteriophage therapy: a revitalized therapy against bacterial infectious diseases. J Infect Chemother 11:211–219

McGrath, S. and van Sinderen, D. 2007. Bacteriophages: Genetics and molecular biology. Norfolk, UK, Horizon Scientific Press

Megraud, F. 2004. *H. pylori* antibiotic resistance: prevalence, importance and advances in testing. Gut 53: 1934-1938

Megraud, F. 2004. *H. pylori* antibiotic resistance: prevalence, importance and advances in testing. Gut 53: 1934-1938

Merril, C.R., Scholl, D., and Adhya, S. L. 2003. The prospect for bacteriophage therapy in western medicine. Nat Rev Drug Discov 2:489–497

Mokgatla, R., Brozel, V. and Gouws, P. 1998. Isolation of Salmonella resistant to hypochlorous acid from a poultry abattoir Lett. Appl. Microbiol., 27, 379.

Monk, A. B.,Rees, C. D., Barrow, P., Hagens, S., and Harper, D. R. 2010. Bacteriophage applications: where are we now? Letters Appl Microbiol51, 363-369

Norrby, S. R., Nord, C. E., and Finch, R. 2005. Lack of development of new antimicrobial drugs: a potential serious threat to public health. Lancet Infect Dis 5: 115-119

O'Flynn, G., Ross, R.P., Fitzgerald, G.F. and Coffey, A., 2004. Evaluation of a cocktail of three bacteriophages for biocontrol of *Escherichia coli* O157:H7. Appl. Environ. Microbiol. 70 (6), 3417-3424

Oliver, S. P., Jayarao, B. M., and Almeida, R. A. 2005. “Foodborne pathogen in milk and the dairy farm environment: food safety and public health implications” Foodborne pathogen and disease, vol. 2, no. 2, 115-129

Ong, C. T., Kuti, J. L., Nightingale, C. H., and Nicolau, D. P. 2004. Emerging *Pseudomonas aeruginosa* resistance: implication in clinical practise. Conn Med 68: 11-15

Parisien, A., Allain, B., Zhang, J., Mandeville, R., and Lan, C. Q.2008. Novel alternatives to antibiotics: bacteriophages, bacterial cell wall hydrolases, and antimicrobial peptides. Journal of Applied Microbiology, Vol.104, pp 1 -13, ISSN 1365-2672

Park, S. C., Shimamura, I., Fukunaga, M., Mori, K. I., and Nakai, T. 2000. Isolation of bacteriophages specific to a fish pathogen, *Pseudomonas plecoglossicida*, as a candidate for disease control. Appl Environ Microbiol 66:1416–1422.

Projan, S. J., and Shlaes, D. M. 2004. Antibacterial drug discovery: is it all downhill from here? Clin Microbiol Infec 10 (suppl): 18-22

Raya, R. R., Varey, P., Oot, R. A., Dyen, M. R., Callaway, T. R., Edrington, T. S., Kutter, E. M., and Brabban, A. D. 2006. Isolation and characterization of a new T-even bacteriophage, CEV1, and determination of its potential to reduce Escherichia coli 0157:H7 levels in sheep. Appl. Environ. Microbiol. 72, 6405-6410

Razavi, B., Apisarnthanarak, A., and Mundy, L. M. 2007. Clostridium difficile: emergence of hypervirulence and fluoroquinolone resistance. Infection 35: 300-307

Rohwer, F., and Thurber, R. V. 2009. Viruses manipulate the marine environment. Nature, 459, 207-212

Sáenz, Y., Briñas, L., Domínguez, E., Ruiz, J., Zarazaga, M., Vila, J., and Torres, C. 2004. Mechanism of resistance in multiple-antibiotic-resistant *Escherichia coli* strains of human, animal, and food origins. Antimicrob Agents Ch 48: 3996-4001

Scallan, E, Hoekstra, R. M., Angulo, F. J., Tauxe, R. V., Widdowson, M. A., Roy, S. L., Jones, J. L., and Griffin, P. M. 2011 Foodborne illness acquired in the United

States—major pathogens. Emerg Infect Dis 17(1):7–17; http://dx.doi.org/10.3201/eid1701.P11101 (accessed February, 2016)

Scanlan, P. D., Buckling, A., and Hall, A. R. 2015. Experimental evolution and bacterial resistance: (co)evolutionary costs and trade-offs as opportunities in phage therapy research. Bacteriophage Vol. 5(2), e1050153

Skurnik, and Strauch, M. E. 2006. Phage therapy: facts and fiction. J.Med. Microbiol.296, 5-14

Smith, H. W, Huggins, M. B, Shaw, K. M. 1987. The control of experimental *Escherichia coli* diarrhea in calves by means of bacteriophage. J Gen Microbiol 133: 1111–1126

Stone, A. B., andBurton. 1962. Studies on the deoxyribonucleases of bacteriophage–infected *Escherichia coli*. Biochem J 85:600

Sulakvelidze A. 2011. Safety by nature: potential bacteriophage applications. Microbe 6(3):122–126 Available: http://goo.gl/mGmC3 [accessed 07 Jan 2016]

Summers, W.C. 2001. Bacteriophage therapy. Annu Rev Microbiol 55:437–451

Suttle, C. A., 2005. The viriosphere: the greatest biological diversity on Earth and driver of global processes. Environ. Microbiol. 7, 481-482

Suttle, C. A. 2007. Marine viruses—Major players in the global ecosystem. Nature Reviews. Microbiology, 5, 801–812

Tabla, R., Martínez, B., Rebollo, J. E., González, J., Ramírez, M. R., Roa, I., Rodríguez, A., and García, P. 2012. "Bacteriophages performance against *Staphylococcus aureus* in milk is improved by high hydrostatic pressure treatments", International journal of food microbiology, vol. 156, no. 3, 209-213

Tanji, Y., Shimada, T., Yoichi, M., Miyanaga, K., Hori, K., and Unno, H. 2004. Toward rational control of *Escherichia coli* O157:H7 by a phage cocktail. Appl. Microbiol. Biotechnol. 64:270-274.

Vongkamjan, K., Roof, S., Stasiewicz, M. J., and Wiedmann, M. 2013. Persistent Listeria monocytogenes subtypes isolated from smoked fish processing facility included both phage susceptible and resistant isolate. Food Microbiol. 35, 38-48

Vongkamjan, K., Switt, A. M., de Bakker, C. H., Fortes, E. D., and Wiedmann, M. 2012. Silage collected from dairy farms harbours an abundance of Listeriaphages with considerable host range and genome size diversity. Appl. Environ. Microbiol. Vol. 78, no. 24, 8666-8675

Weinbauer, M. G., 2004. Ecology of prokaryotic viruses. FEMS Microbiol. Rev. 28, 127-181

Weinbauer, M. G., and Peduzzi, P. 1995. Significance of viruses versus heterotrophic nanofiagellates for controlling bacteria abundance in the northern Adriatic Sea. Journal of Plankton Research, 17, 1851-1856

Westwater C, Kasman, L. M., Schofield, D. A., Werner, P. A., Dolan, J. W., Schmidt, M. G., and Norris, J. S. 2003. Use of genetically engineered phage to deliver antimicrobial agents to bacteria: an alternative therapy for treatment of bacterial infections. Antimicrob Agents Chemother 47:1301–1307

Williamson, K. E., Corzo, K. A., Drissi, C. L., Buckingham, J. M., Thompson, C. P., Helton, R. R. 2013. Estimates of viral abundance in soils are strongly influenced by extraction and enumeration methods. Biology and Fertility of Soils, 49, 857–869

Zhang, Y., and Hu, Z. 2013 Combined treatment of *Pseudomonas aeruginosa* biofilms with bacteriophages and chlorine. Biotechnol Bioeng 110(1):286–295 http://dx.doi.org/10.1002/bit.24630

CHAPTER 2 EFFECT OF TEMPERATURE ON BACTERIOPHAGE-MEDIATED BACTERIAL LYSIS EFFICIENCY WITH A SPECIAL EMPHASIS ON BACTERIAL TEMPERATURE HISTORY

Ekwu M Ameh[1], Sean Tyrrel[1], Jim A Harris[2], Elena V Orlova[3], Athanasios Ignatiou[3], Andreas Nocker[1,4]

[1] Cranfield Water Science Institute, School of Energy, Environment and Agrifood, Cranfield University, Cranfield, Bedfordshire, MK43 0AL, United Kingdom

[2] Cranfield Institute for Resilient Futures, School of Environment, Energy and Agrifood, Cranfield University, Cranfield, Bedfordshire, MK43 0AL, United Kingdom

[3]Institute for Structural and Molecular Biology, Department of Biological Sciences, Birkbeck, University of London, Malet Street, London WC1E 7HX, United Kingdom

[4]IWW Water Centre; Moritzstraße 26, 45476 Mülheim an der Ruhr, Germany

2.1 ABSTRACT

Despite the great potential of phages as biocontrol agents, there is much uncertainty about the environmental factors influencing lysis efficiency. In this study we investigated the effect of temperature using three distinct lytic *E. coli* phages that were isolated from a single environmental water sample. Whereas the optimal growth

temperature of *E. coli* is well known to be 37°C and exposure of phages to temperatures between 4 and 37°C (prior to mixing with bacteria) did not affect their infectivity, the phage host mix responded differently to temperature exposure: both plaque sizes and numbers greatly decreased with increasing incubation temperature (20°C, 30°C, 37°C). At 37°C, no visible plaques were observed. Results suggest that temperature sensitivity of the phage-host interaction is distinct from the temperature susceptibility of the two players (bacteria and phages) and corroborate previous reports that highest lysis rates are obtained at temperatures approximate with ambient conditions of the phage environment. Infectivity was however found not only to depend on the incubation temperature of the phage-host mix, but also on the bacterial temperature history. Moreover, exposure of bacteria to heat stress prior to phage challenge resulted in a phage-resistant phenotype raising the question whether bacterial pathogens that are shed from warm-blooded hosts might be less susceptible to phages adapted to environmental temperature conditions.

Key words: bacteriophage; temperature dependence; lysis efficiency; temperature sensitive

2.2 INTRODUCTION

Bacteriophages (herein abbreviated as phages) have the unique ability to eliminate bacteria with great specificity. It is estimated that phages in the environment are 5-10 fold more abundant than bacteria and are thus the most abundant biological entities on Earth (Brüssow and Hendrix, 2002; Suttle, 2007). They can be assumed to have a pronounced impact on microbial ecology (Clokie et al., 2011. Phages have furthermore long been recognized as biotherapeutic agents to combat bacterial infections (for example for

treating wounds; Kutter et al., 2010) and more recently for biological disinfection in food production (Hudson et al., 2005).

Although phage isolation and use seem straightforward, there are many pitfalls to successful application. One of the greatest obstacles is insufficient knowledge of parameters influencing phage lysis efficiency. This study aimed to shed more light on the temperature-dependent nature of phage-host interactions. A number of studies have revealed that temperature can be an important determinant affecting efficiency of bacterial removal. Most studies were linked to dairy research due to the economic damage potentially inflicted by phages on bacterial starter cultures and dairy production lines and the resulting strong interest to identify conditions where bacterial hosts were less susceptible (Hunter, 1943; Sozzi et al., 1978; Sanders and Klaenhammer, 1984). Both inhibition of phage-mediated lysis at low temperatures (relative to the optimal growth temperature of the bacterial host; Pearce, 1978; Sanders and Klaenhammer, 1980) and at high temperatures has been reported (Sozzi et al., 1978; Hunter 1943). Temperature dependence was later also reported for bacteriophages relevant for control of food pathogens like *Listeria* (Kim and Kathariou, 2009), *Salmonella* and *Campylobacter* (Goode et al., 2003). For coliphages Seeley and Primrose (1980) differentiated between three physiological types based on the effect of temperature on their 'plaquing' properties and introduced the terms of high temperature (HT; ≥25°C), mid-temperature (MT; 15-42°C) and low temperature (LT; ≤30°C) phages. Temperature profiles of bacteriophages from different habitats showed that phages isolated from faeces or sewage tended to belong to the LT or MT type, whereas phages isolated from a river tended to belong to the LT type. Plaque numbers of LT phages dropped sharply to zero at temperatures

between 33 and 37°C. More recently Koskella and Meadan, (2013), suggested in this context that phages are ‘locally adapted’ to their bacterial hosts.

Whereas Seeley and Primrose reported the ‘maximum and minimum plating temperatures to be stable properties of the phages that were not influenced by the growth temperature of the host’, Kim and Kathariou (2009) assigned the temperature-dependent susceptibility to the bacterial host. An epidemic *Listeria monocytogenes* strain was identified that was able to avoid phage-mediated lysis by broad-host-range phages when bacteria were grown at 37°C, but not when grown at 30°C. Resistance was regardless of the temperature during infection or subsequent incubation of bacteria-phage mixtures.

As different phages can respond in different ways to changing environmental parameters, it is essential to select the optimal conditions when isolating and applying environmental phages. The choice of the optimal temperature is particularly important in the case of human pathogens where the optimal growth temperature (typically the one encountered in the human host) is very different from the temperatures prevalent in the environmental lifecycle of those pathogens. Whereas phage screening at the optimal bacterial growth temperature makes sense for therapeutic applications, suboptimal results might be obtained for phage-based biological disinfection that is typically performed at ambient temperature. The screening of lytic bacteriophages for biocontrol applications is nevertheless typically performed at the optimal temperature of the host. This work tested the hypothesis whether efficient lysis could be obtained at suboptimal temperature of the host (thus addressing objectives 1, 2 and 3 of this research project).

This study was performed with three distinct coliphages that were isolated from a single brook water sample and that were chosen on the basis of different plaque sizes.

With all three of these exemplar environmental phages, a strong temperature impact on lysis activity was observed after mixing them with their bacterial host. We aimed to examine whether it is the temperature susceptibility of the phages or the bacterial host causing temperature sensitivity of the host-phage mixture and to which extent phage lysis activity depends on the temperature history of phages and bacteria.

2.3 MATERIALS AND METHODS

2.3.1 Bacteria and growth condition

The study was performed with *Escherichia coli* ATCC 25922, which is a clinical isolate. Bacteria were grown overnight on tryptone soya agar (TSA; CM0131; Oxoid Ltd Basingstoke, Hampshire, UK) at 30°C. This strain is referred to as 'host' as it enables the replication of the phages used in this study although it might not have been the 'host' in the environmental water where the phages were isolated from. Liquid cultures were obtained by inoculating 10 mL of tryptone soya broth (TSB; CM1016; Oxoid Ltd Basingstoke, Hampshire, UK) in 50 mL Falcon tubes followed by overnight incubation at 25^{o}C (or indicated temperature) at a 45^{o} angle at 250 rpm in a temperature-controlled mini shaker (cat. number 980151, VWR, USA). TSB broth and TSA plates were routinely amended with 5 mM $CaCl_2$ (added as $CaCl_2$*$7H_2O$) to allow for efficient phage adsorption. Cell density was measured in a spectrophotometer (JENWAY 6310, Camlab, England, UK) at 600 nm (OD_{600}) and adjusted to an OD_{600} = 1.0 by dilution with TSB equilibrated to room temperature. For experiments addressing temperature-dependent phage resistance, aliquots of 10 mL of overnight cultures and optical density-adjusted cultures were harvested by centrifugation (5,000 g, 5 min). Resulting cell pellets were re-suspended in phosphate buffered saline (PBS) and exposed to treatment temperatures for 24 hours using standard laboratory heat blocks or incubators. Cell aliquots were

subsequently equilibrated to room temperature, harvested by centrifugation and re-suspended in TSB. For experiments addressing effect of elongated temperature exposure on phage infectivity, 1 mL of phage stock (10^9 PFU/mL) was exposed to the indicated temperature in microcentrifuge tubes using standard heat blocks or incubators.

2.3.2 Sampling, enrichment and isolation of bacteriophages

Water samples were collected on 10 December 2012 from Chicheley brook flowing across the Cranfield University campus at a location where the brook water mixes with treated effluent discharge from the Cranfield University wastewater treatment plant (GPS coordinates 52.079622 N, 0.627883 W). The water temperature of the brook does not exceed 20˚C even in hot summer months and when there are low water levels. Samples were passed through a 0.22 µm membrane filter (Millex GP, Merck Millipore, Darmstadt, Germany) to remove bacteria and the filtrate containing phages was collected in a sterile 50 mL Falcon tube.

Bacteriophages specific for *E. coli* were subsequently enriched by mixing 10 mL of the filtrate with an equal volume of double strength TSB (supplemented with 5 mM $CaCl_2$) and inoculating the mixture with 0.4 mL of an overnight *E. coli* culture (grown as described previously) followed by overnight incubation (30ºC; 250 rpm). Incubation was performed until clearance indicated bacteriophages-mediated cell lysis (typically after 24 h). Chloroform was subsequently added to a final concentration of 2% (v/v) to eliminate remaining intact bacteria followed by centrifugation at 5,000 *g* for 5 min. The supernatant was filtered using a 0.22 µm membrane filter (Millex GP, Merck Millipore, Darmstadt, Germany). Appropriate dilutions of enriched sample were plated using the soft agar overlay method (Carey-Smith et al., 2006). Plates were examined for plaques after overnight incubation at 30ºC. Selected plaques were transferred into SM buffer (100 mM

NaCl, 8 mM $MgS0_4*7H_20$, 50 mM Tris-Cl, adjusted to pH 7.5), re-suspended and, serially 10-fold diluted for re-isolation.

2.3.3 Purification of bacteriophages

Bacteriophages were isolated three times using a modified soft agar overlay method to ensure purity. A mixture of 100 µL of viral concentrate serially 10-fold diluted in SM buffer and 150 µL of log-phase *E. coli* were added to 3 mL of molten 0.7% (w/v) TSB agar (maintained at 48ºC), mixed immediately by gentle vortexing and then distributed evenly over TSA agar plates supplemented with $CaCl_2$ to a final concentration of 5 mM. The soft agar was allowed to solidify for 20-30 min and plates were incubated overnight at 30ºC (or indicated temperature) to allow for plaque formation. Isolated plaques were picked using sterile wooden toothpicks to inoculate 5 mL log-phase *E. coli* cultures followed by incubation at 30ºC with shaking (250 rpm) for 8 hours. Lysate from single plaques were treated with chloroform to a final concentration of 2% (v/v), mixed and centrifuged at 5,000 g for 5 min. The phages were recovered from the upper phase suspension and passed through 0.22 µm filter (Millex GP, Merck Millipore, Darmstadt, Germany). Phage stocks were stored at 4ºC.

2.3.4 Morphological characterisation by transmission electron microscopy

All three phages produced for electron microscopy were high titre stocks of $\geq 10^8$ pfu $mL^{-1}$and were prepared in SM buffer as described above. For each phage preparation, 3 µL of sample was pipetted on to a negatively glow discharged 10 micron thick C-Flat™ carbon grid (400-mesh) with no dilution and allowed to sit for 1 minute. The sample droplet was then partially blotted with Whatman® qualitative filter paper, Grade 1. 3 µL of 2 % (w/v) uranyl acetate stain solution was then immediately applied to the remainder

of the sample. After 1 min of staining the solution was then fully blotted removing all excess fluid from the grid surface and allowed to air-dry. The sample on each grid was then imaged in low dose conditions on a FEI Tecnai 10 transmission electron microscope operating at 100 keV. Images were taken at 20 K magnification and captured using a Gatan Ultrascan 4000 4k × 4k CCD camera with an ultra-sensitive phosphor scintillator (Gatan, USA) to produce a final pixel sampling of 11 Angstroms per pixel.

2.3.5 Phage challenge test and monitoring of optical densities

Phage challenge experiments were performed in 50 mL conical centrifuge tubes (Fisherbrand, Fisher Scientific UK Ltd., Loughborough) by adding bacteriophage stock solution to 10 mL of bacterial suspension adjusted to an optical density OD_{600} of 1.0. In all experiments 10^9 PFU/mL were applied corresponding to a multiplicity of infection (MOI) of 1.

Temperature stress was performed by exposing six bacterial aliquots of 1 mL each (bacteria re-suspended in PBS, pH 7) to different temperatures (4, 20, 37, 41, 44 or 50°C) for 24 hours. Cells killed at 70°C for 15 min served as a control (to check viability). After stress exposure, cells were harvested by centrifugation (5,000 g for 5 min) washed in 1 mL TSB, and re-suspended in 9 mL TSB to obtain a final concentration of approximately 10^9 cells/ml. Temperature-exposed bacteria were mixed with phage as described earlier and shaken at 250 rpm at 20°C. Accordingly, phages were exposed to different temperatures (4, 20, 37, 44 or 50°C) for 24 hours after dilution in SM buffer to 10^9 pfu mL^{-1}. Phages were subsequently serially diluted in SM buffer in steps of 10-fold prior to spotting 1.5 µL aliquots on square culture plates (Greiner, 688102, Sigma-Aldrich, UK) containing TSA (supplemented with $CaCl_2$ to a final concentration of 5 mM) and with pre-spread *E. coli* (prepared by spreading a mixture of 9 mL 0.7% TSB agar, maintained

at 48ºC, and 450 µL of log-phase *E. coli).* Plates were incubated overnight at 20°C for plaques to develop.

Optical densities of cell suspensions were measured on a TECAN M200 Pro plate reader (Tecan UK Ltd, Reading, UK). Samples of 1 mL were transferred into transparent 48 well tissue culture plates (non-treated, flat bottom, cat. nr. TCP001048; Jet Biofil, Braine I'Alleud, Belgium) and absorbance at 600 nm was measured. Readings were typically taken directly after addition of phages and every hour after the challenge or after 6, 12, 24 and 36 hours (as indicated). All samples were measured in triplicate.

2.3.6 Adsorption experiment

To investigate a possible temperature effect on adsorption, *E. coli* and phage suspensions were equilibrated to 20, 30 or 37°C for 10 min prior to mixing in a MOI ratio of 1 (in the presence of 5 mM $CaCl_2$) and incubation for 30 min at the corresponding temperature to allow for adsorption. The mixtures were then added to liquid agar (maintained at 45-48°C in a heat block), mixed and spread on plates. After settling for 30 min, plates were incubated at 20°C for 24 hours for plaques to develop.

2.3.7 Statistical analysis

One-way analysis of variance (ANOVA) and Tukey-Kramer testing was performed following the Handbook of Biological Statistics by McDonald (2014; available at http://www.biostathandbook.com/onewayanova.html) to identify statistical differences between the conditions tested. Tests were conducted to a confidence interval of 95% and therefore a p value below 0.05 resulted in the rejection of the null hypothesis.

2.4 RESULTS

Lytic phages were isolated from a brook water sample on the basis of the plaque sizes produced when incubating bacteria-phage mixtures at ambient room temperature

(20°C; Fig. 2-1(a) and (b)). Distinct phages resulting in big, medium and small plaques were referred to as phage B, M, and S. The three phages were morphologically characterised by transmission electron microscopy and found to belong to the group of Myoviridae, which are double-stranded DNA phages with contractile tails. Phage M has a prolate head, while phages B and S both have icosahedral heads.

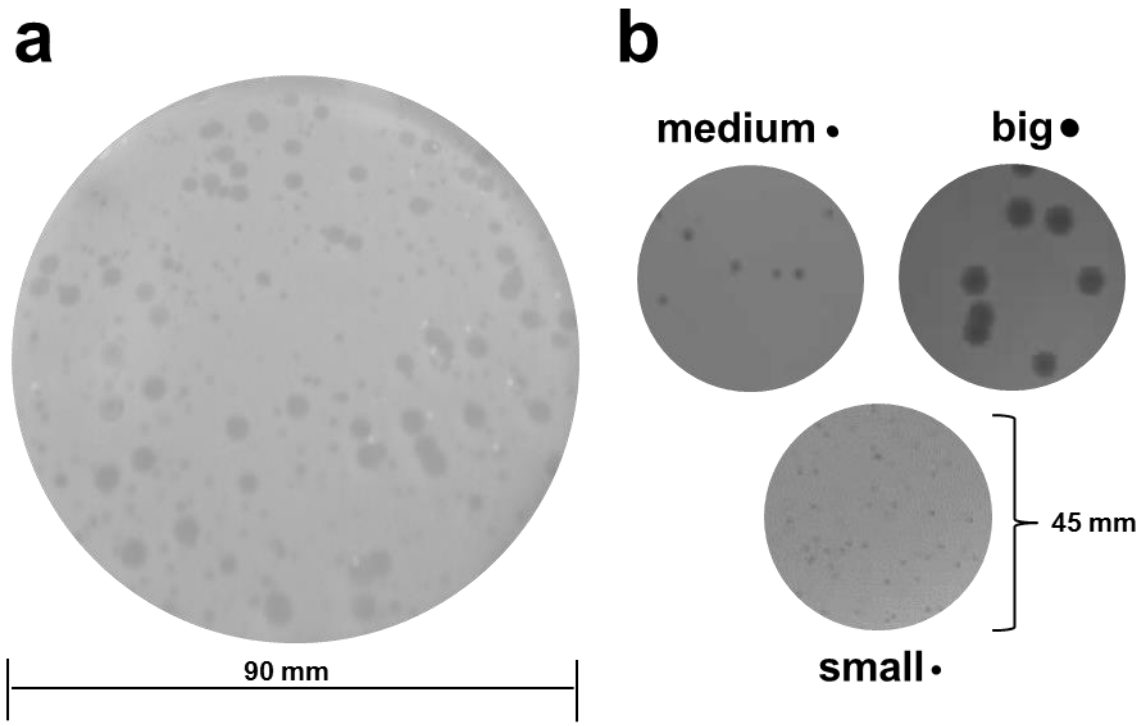


Figure 2-1 Plaque diversity of *E. coli* specific bacteriophages in brook water receiving treated wastewater effluent. (A) Plaque size diversity from a single isolation with plates being incubated at 20°C. The photo shows a standard Petri dish with a diameter of 90 mm (45 mm radius). (B) Plaques after multiple isolation rounds. Based on plaque sizes produced at this condition, corresponding phages were designated as: big (B), medium (M), and small (S).

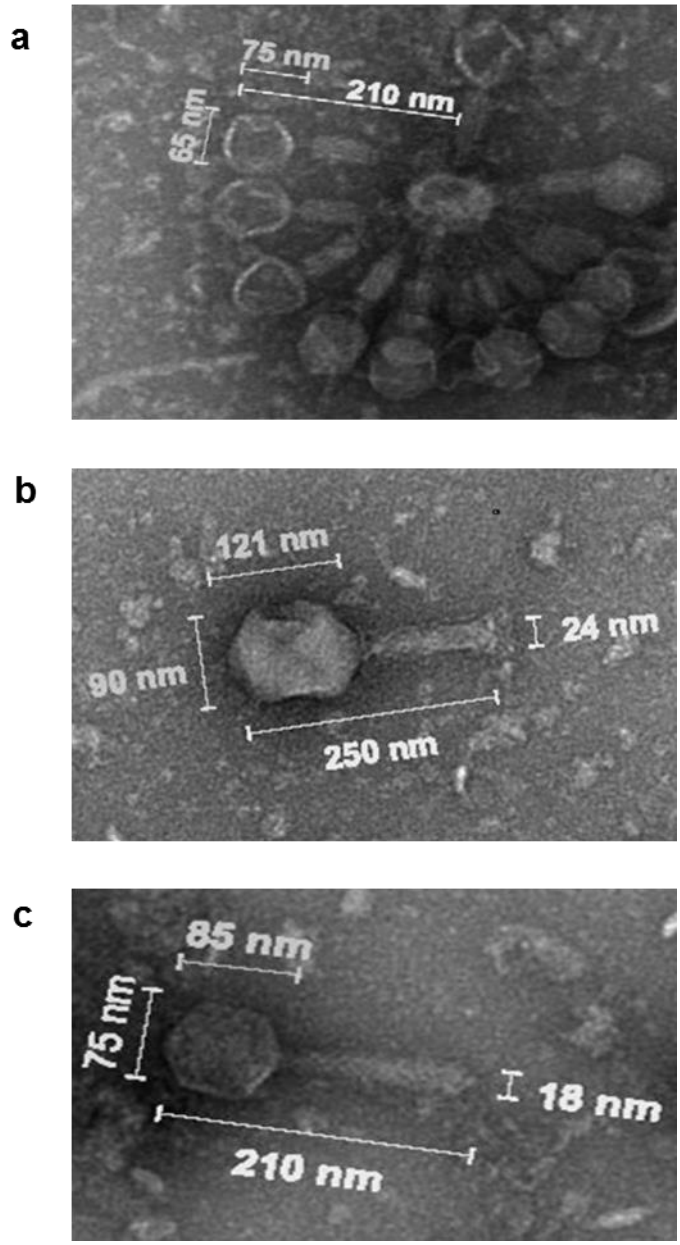


Figure 2-2 Electron micrographic pictures of *E.coli* specific bacteriophages (A) phage B, (B) phage M, and (C) phage S. Average dimensions of each phage are shown in nm.

2.4.1 Effect of incubation temperature on plaque sizes and numbers

Plaque sizes and numbers were compared when incubating bacteria-phage mixtures (embedded in soft overlay agar) at 20, 30 and 37°C. For all three phages, greatest plaque sizes were obtained when incubating plates at 20°C (Fig. 2-3). Plaque sizes decreased when raising the incubation temperatures to 30°C and plaques were not visible at all for phages B and M at 37˚C. In agreement with plaque sizes, plaque numbers decreased with increasing incubation temperatures with relative changes indicated in Fig. 2-3. Differences in plaque numbers between each temperature series were statistically significant ($p<0.05$).

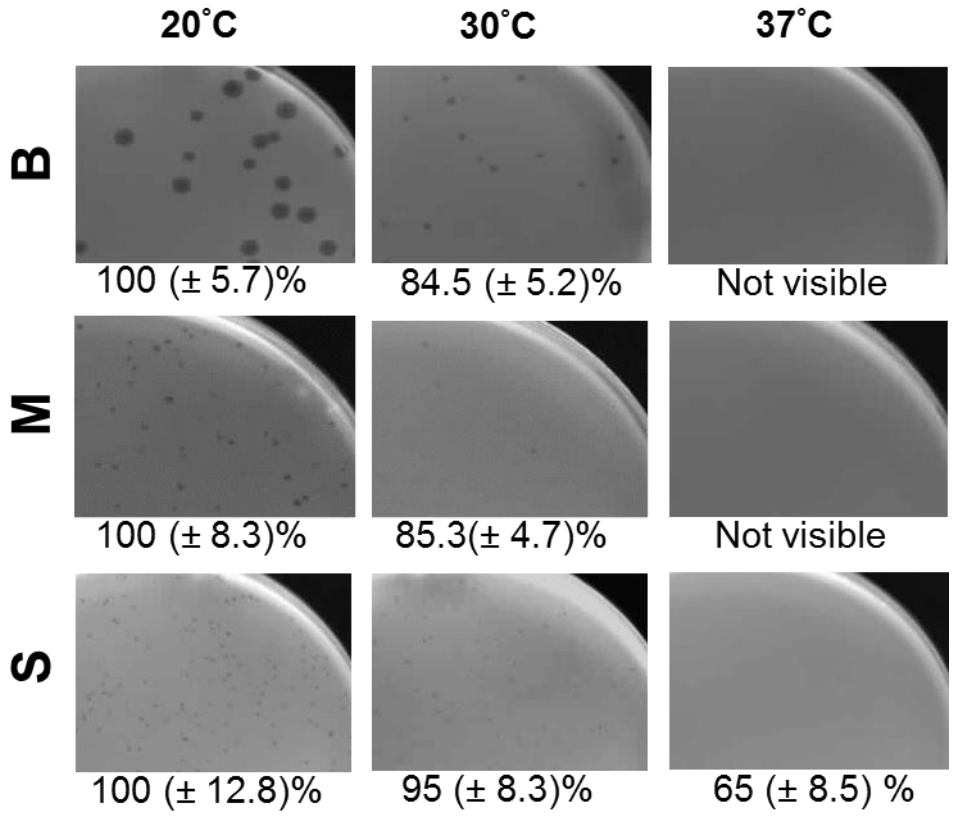


Figure 2-3 Effect of plate incubation temperature (20°C, 30°C, 37°C) on plaques sizes and numbers for the three *E. coli* phages (big, medium and small). *E. coli* was grown at 20°C prior to mixing with phages and overlaying mixtures on TSA. Plates

were incubated at indicated temperatures with representative pictures shown. Plaque numbers were normalized and are shown as percentages of the numbers obtained at 20°C. Standard deviations from three independent experiments are shown in brackets.

2.4.2 Temperature susceptibility of phage

In order to identify whether the temperature susceptibility of the phages or of the bacteria was responsible for the observed phenomenon, purified phage suspensions were exposed to different temperatures (4, 20, 30, 37, 44 or 50°C) for 24 hours. Serial 10-fold dilutions of such treated phage suspensions were subsequently spotted on agar plates with pre-aliquoted *E. coli* and incubated at 20°C (Fig. 2-4). For phage B, plaques generated by diluted phages were comparable for all temperature treatments suggesting that this phage was remarkably temperature-stable. The same held true for phages M and S for temperatures up to 37°C, whereas temperatures of 44 and 50°C resulted in a decrease in infectivity by approx. 1-2 log units.

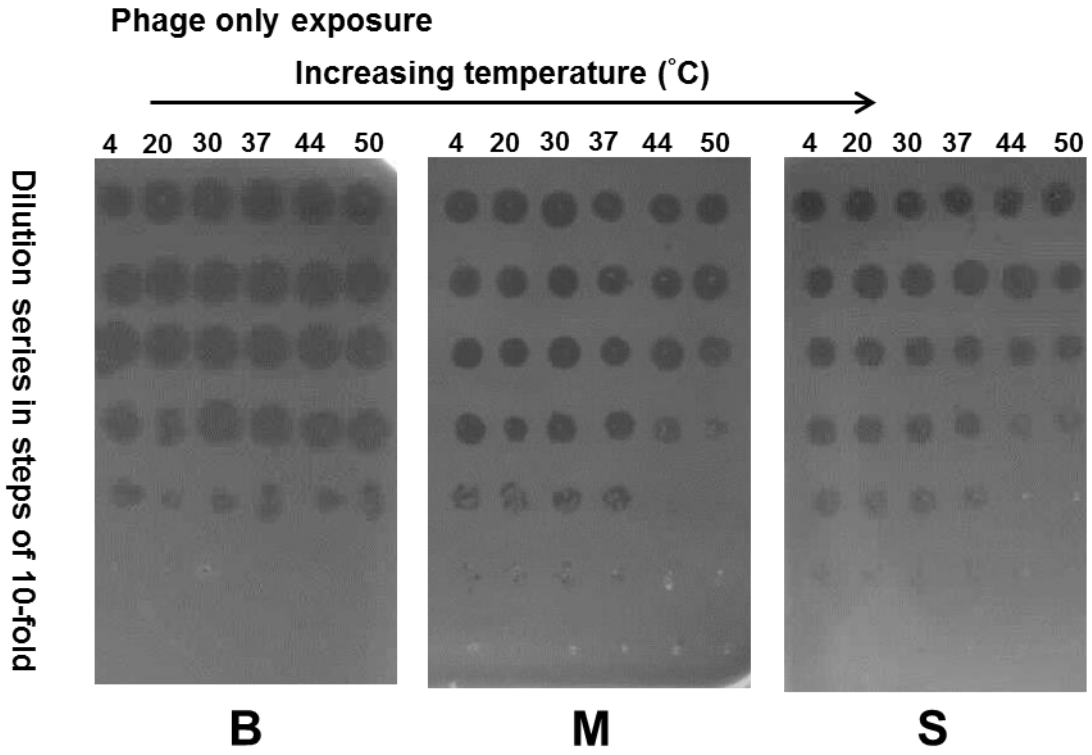


Figure 2-4 Effect of temperature on infectivity of phages B, M and S after 24 hours of incubation at 4˚C, 20˚C, 30˚C, 37˚C, 44˚C and 50˚C. Temperature-treated phage suspensions were serially 10-fold diluted and aliquots were spotted on tryptic soy agar with pre-plated *E. coli.* Agar plates were incubated at room temperature (20˚C) until plaque clearings had reached adequate sizes.

2.4.3 Effect of exposing fully grown *E. coli* to different temperatures prior to phage challenge

When exposing pre-grown bacterial host cells (grown at 20°C and suspended in physiological salt to prevent cell replication) to different temperatures (4, 20, 30, 37, 44 or 50°C) prior to phage challenge at 20°C, bacterial susceptibility to lysis greatly depended on their temperature history. Bacteria exposed to temperatures ≤ 37°C were all greatly susceptible to phage lysis reflected by a decrease in OD_{600} (Fig. 2-5 b-d).

Susceptibility was greatest when bacteria had been exposed to 4 and 20°C. Bacteria exposed to sublethal heat (41 and 44°C) on the other hand appeared resistant to phage lysis and increased in numbers. No change in optical density was observed for bacteria exposed to 50°C (this temperature can be considered lethal as no colonies were obtained after this treatment).

To assign the temperature effect to the phage susceptibility, bacterial suspensions with no phage added served as controls (Fig. 2-5a). Optical densities of cells exposed to 4, 20, 37°C increased rapidly once nutrients were available suggesting viable bacteria. Optical densities of cells exposed to 41 and 44°C also showed an increase (although more slowly) indicating that these stressed cells were still alive. The trends are reflected in a slope analysis showing the rates of change in optical densities (Fig. 2-6). For bacterial suspensions exposed to 4, 20, 37°C prior phage challenge, previously positive slopes (as seen in the control without phage) are turned negative by phage-mediated lysis resulting in a drop in optical densities from 0.6 nm to 0.2 nm. For suspensions held at 41 and 44°C, on the other hand, slopes are all slightly positive independent of whether a phage was added or not. The effect of the phage thus appeared very small for those bacteria exposed to sublethal heat.

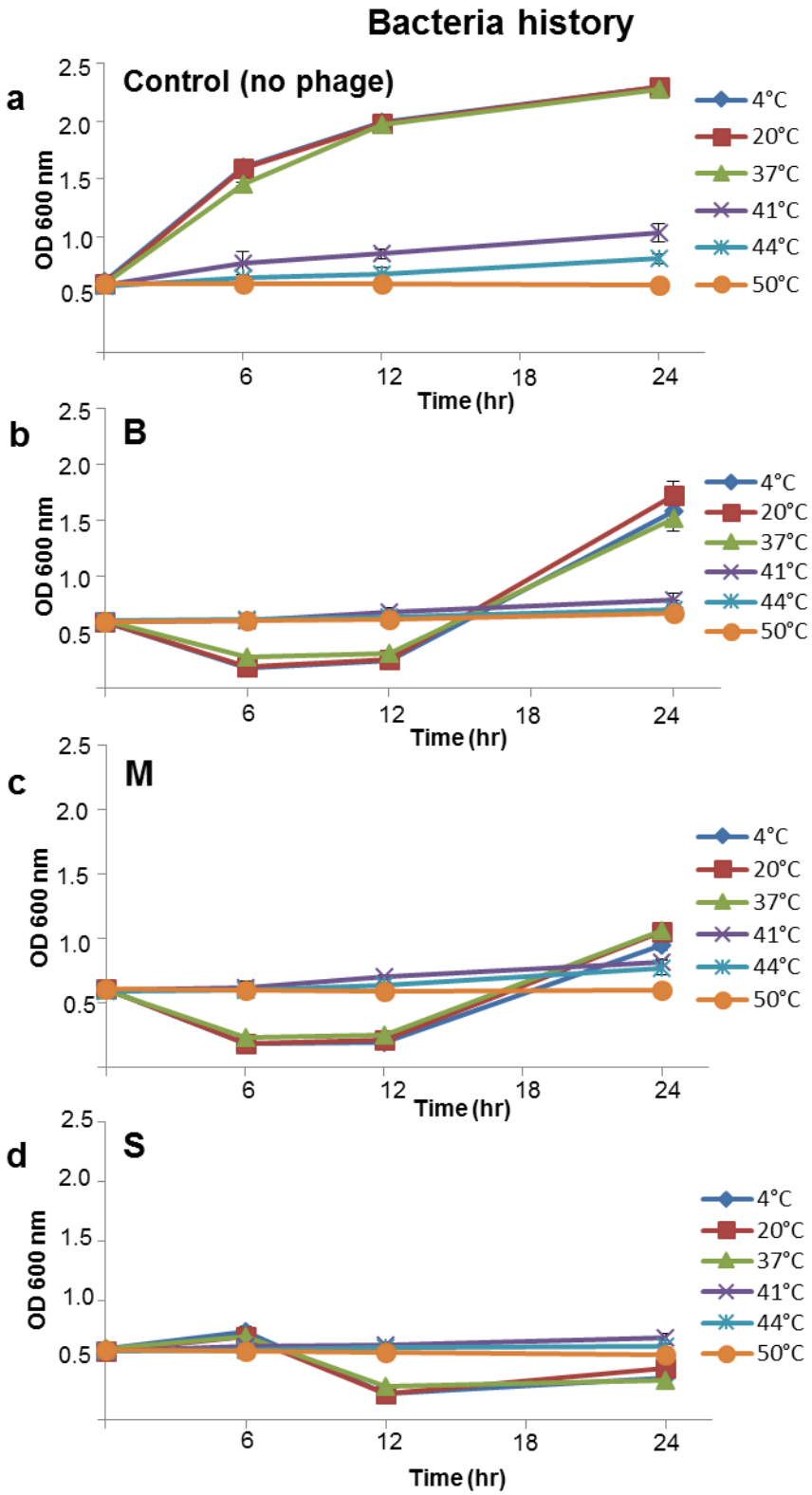


Figure 2-5 Effect of temperature history of fully grown *E. coli* on susceptibility to lysis by phage big (a), medium (b), small (c), and (d) control. Bacteria (grown at

20°C; OD_{600} = 1) were suspended in PBS and exposed to different temperatures (4, 20, 30, 37, 41, 44 or 50°C) for 24 h prior to resuspension in TSB and challenge with phages B, M or S (MOI = 1) at 20°C. Changes in optical densities were monitored using a microplate reader. Error bars show standard deviations from three independent experiments.

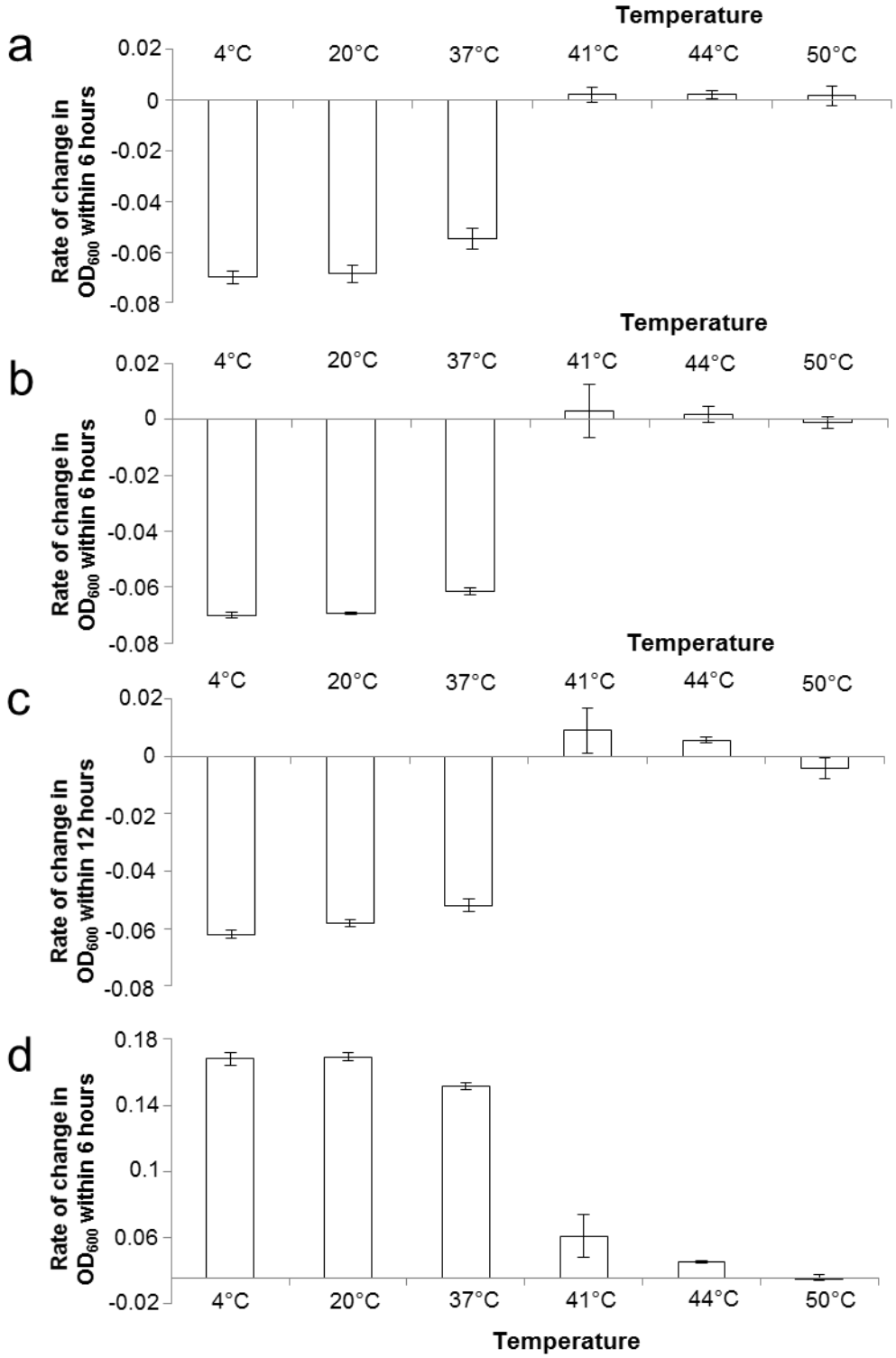


Figure 2-6 Rate of change (slope analysis) of OD_{600} of *E. coli* after exposure to different temperatures for 24 hours and subsequent mixing with phage big (a), medium (b), small (c) compared to a control without phage addition (d). Rates of change in OD_{600} refer to 6 hours after addition of phage B and M as well as for the

control without phage and to 12 hours after addition of phage S. After challenge of the *E. coli* suspensions with phages were shaken at 20°C, the same applied to the control without phage. Error bars indicate standard deviations as seen in three independent experiments.

2.4.4 Effect of *E. coli* growth temperature history on plaque size

Whereas the previous experiment investigated the effect of temperature on pre-grown *E. coli*, we were interested in the susceptibility of bacteria actively grown at different temperatures. For this purpose *E. coli* was cultivated either at 20 or 37°C prior to mixing with phage B, plating on soft agar and incubation of plates at either 20, 30 or 37°C. Resulting plaque sizes were greatest when host cells were grown at 20°C and plates were incubated at the same temperature. If bacteria on the other hand were grown at 37°C, equilibrated to room temperature prior to phage addition and the phage-host mix incubated at 20°C, resulting plaques were visibly smaller (Fig. 2-7). This result demonstrates that it is not only the incubation temperature of the phage-host mix that affects plaque size, but also the growth temperature history of the host cells. A similar trend was observed for the incubation temperature of 30°C, although plaque sizes were generally smaller making the difference less obvious. In agreement with previous data, no plaques were obtained when soft agar plates were incubated at 37°C.

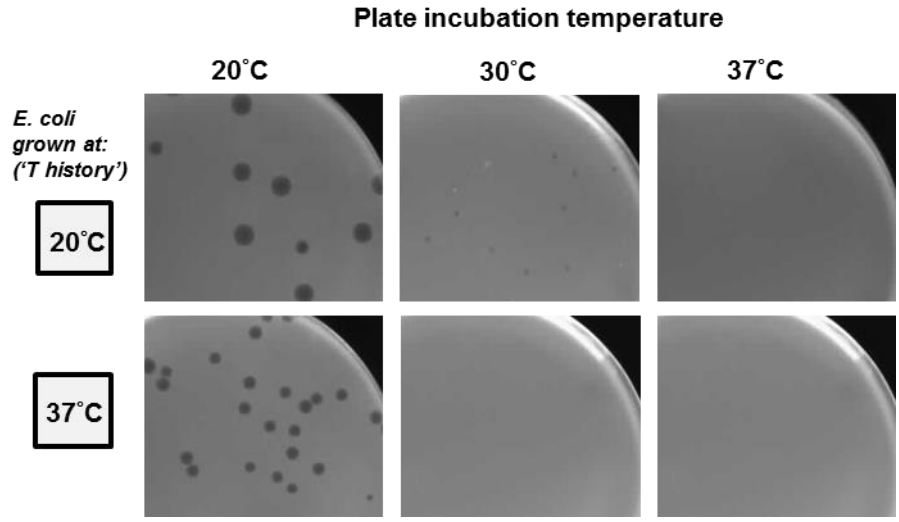


Figure 2-7 Effect of *E. coli* growth temperature on susceptibility to phage lysis. *E. coli* cells were grown overnight either at 20°C or 37°C, followed by addition of phage big (phage producing big plaque size) (MOI=1.0) and incubation of bacteria-phage mixtures at 20°C, 30°C or 37°C. Plaque sizes obtained after overnight incubation.

2.4.5 Adsorption temperature

The temperature of adsorption of bacteria-phage mixture before incubation at room temperature was verified. Host-phage suspension was subjected to temperature of adsorption of 20, 30 or 37°C for half an hour. The plaque size was not affected by the temperature of adsorption (fig. 2-8).

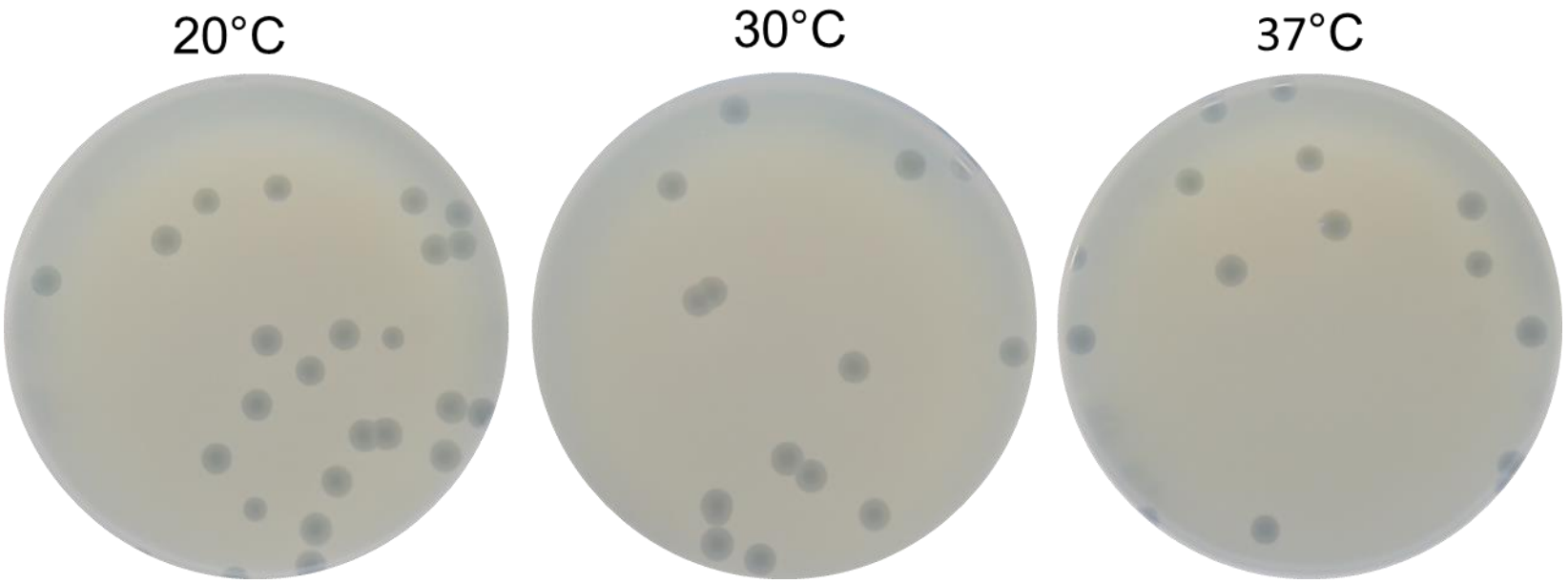


Figure 2-8 Effect of adsorption temperature on plaque sizes produced by phage big. Both bacterial and phage suspensions were equilibrated to the desired temperatures (20°C, 30°C or 37°C), mixed in a MOI ratio of 1 and incubated for 30 min at the corresponding temperatures to allow adsorption. Resulting plaques were obtained by the agar-overlay method. Photos shows standard Petri dishes with a diameter of 90 mm.

2.5 DISCUSSION

The study investigated the effect of temperature on the susceptibility of a clinical *E. coli* isolate to three exemplar environmental phages distinguishable by the plaque sizes they develop at room temperature. The fact that temperature-dependence of lysis efficiency was found for all three phages (isolated from a single water sample) suggests that this trait might be common. Phage-host mix incubation temperatures that were below the optimal growth temperature of the bacterial host tended to result in bigger plaque sizes and higher plaque numbers. Similar observations have been made previously with dairy starter strains where the optimal lysis temperature was lower than the optimal growth temperature of the bacterial host (Hunter, 1943; Sozzi and Klaenhammer, 1978). For example, a phage specific for *Lactobacillus casei* did not replicate above 40˚C although the bacterial host had a temperature optimum between 37 and 41°C and grew well at 43-44°C (Murata 1971). Sozzi et al. (1978) summarized findings that certain phages do not multiply at temperatures at which their bacterial hosts show active growth and Hunter (1943) concluded that 'in many cases the conditions for maximum activity of bacteria and phage are not identical, meaning that the optimal growth temperature of a bacterium does not necessarily indicate that this temperature will also be favourable for a phage which uses this bacterium as a host. Findings are furthermore in line with the before-mentioned existence of LT, MT and HT coliphages (Seeley and Primrose, 1980). Based on this classification, the phages isolated in our study would fall into the LT category as they fail to produce plaques when the host-phage mixture is incubated at 37°C (phages B and M) or showed reduced plaque numbers (phage S). The impact of incubation temperature on lysis corroborates the recommendation by Mohany et al. (2011) that a screening for

suitable phages should always be performed under the temperature conditions relevant for the subsequent application to avoid suboptimal results or complete failures.

Our findings suggest, however, that the host-phage interaction is not only dependent on the ambient conditions encountered after mixing bacteria with phages, but additionally by the bacterial temperature history prior to encountering the phages. As for the phages, the exposure to different temperatures prior to addition to bacteria did not affect lysis efficiency (probably as long as virus stability was maintained). Temperature history was unlikely to be an important factor for the phages, but predominantly for the host cells. The temperature history of fully grown cells produced remarkable differences in lysis susceptibility (Fig. 2-7). When *E. coli* cultures (grown at ambient temperature, approx. 20°C) were exposed to temperatures ≤37°C prior to being challenged with a phage at 20°C, they underwent a decline in optical density. Exposure to temperature stress in the sublethal range (41 and 44°C) on the other hand resulted in a lysis-resistant phenotype. One reason could lie in the temperature-dependent expression of surface antigens which can serve as phage attachment sites (Orskov et al., 1984). Also an involvement of the bacterial heat shock regulon could potentially play a role as suggested in a different context by Wiberg et al. (1988). Authors of this study found that, when *E. coli* were grown at 30°C for many generations, infected with phage (T2, T4, T6 or T7) and shifted to 42.8-44°C, low to negligible phage numbers were produced (compared to phage production at 30°C without temperature shift). If host cells were however adapted to the higher temperature for 8 min before challenge, production of all four phages increased between 3.4 and 267-fold. It needs further analysis to answer whether this observation holds true for more phage-host interactions and potentially to other stresses.

Apart from the temperature that pre-grown host cells were subjected to prior to phage challenge, the growth temperature of host cells was found to modulate lysis efficiency (Fig. 2-7). A role of bacterial growth temperature was also reported for *Listeria monocytogenes* (Kim and Kathariou, 2009) although with a reversed temperature effect. Broad host range phages produced plaques only on *Listeria* strain ECII when the bacteria were grown at 37°C, but not when they were grown at temperatures ≤30°C. Bacteria grown at lower temperatures were reported to be resistant regardless of the temperature during phage infection and plate incubation. For the phage-host combinations in our study, on the other hand, phages could still form plaques on *E. coli* grown at 37°C prior to phage challenge (which is a plate incubation temperature that did not permit lysis for the phage used) as long as phage-host mixtures were incubated at lower temperature. The largest plaques however resulted from cultures of *E. coli* grown at 20°C and then incubated at 20°C after phage challenge.

Whilst this study did not aim to elucidate the mechanism responsible for the reported observations, much research will be necessary to understand the underlying reasons. Whereas an effect of adsorption temperature could be excluded (figure 2-8), other parameters possibly affected by temperature include phage penetration and multiplication (Jonczyk et al., 2011). Phage performance is further strongly dependent on bacterial physiology and metabolism which in turn strongly depends on temperature (Carvalho et al., 2012). As phages do not have a metabolism and therefore no temperature-dependent expression of surface structures, factors determining lysis efficiency might be more limited than for the bacteria although phages seem to be highly adapted to the local conditions in their environment and might have an in-built temperature preference for reversible and irreversible adsorption and replication once

mixed with bacteria. As long as phages and their bacterial host co-exist in the same environment for a long time, this specialization appears adequate. The scenario is different however in the case of bacterial pathogens with two distinct life cycles. The temperature in a human or warm-blooded animal host (37°C) tends to be very different from the temperature encountered, in the environmental life cycle where water and food are important vehicles of transmission (Nocker *et al*., 2013). It is tempting to speculate that bacteria replicating at 37°C (as prevalent in humans and warm-blooded animals) might, after being released into the environment, be more resistant to phages that are adapted to lower temperatures. The observation that bacteria are not equally susceptible under all conditions would provide them with the possibility to escape predation and add to other defence mechanisms. A change in habitat might sometimes be all that is needed to escape a specific phage although every habitat can be assumed to have its own challenges.

2.6 CONCLUSIONS

We report here an example of three different coliphages isolated from a single environmental water sample. Temperature of incubation of the phage-host mix greatly influenced both plaque sizes and numbers. The susceptibility of the phage lysis efficiency was very distinct from the temperature stability of the two players suggesting that temperature classification of lysis has to refer to the phage-host mixture as an entity and not to the temperature preferences of phages or bacteria. Apart from the temperature at which the phage-host mixture is incubated and at which lysis occurs, the temperature history of the bacteria was found important. Both exposure of fully grown bacteria to different temperatures or growth at different temperatures prior to phage encounter was found to greatly determine their susceptibility to lysis. The results of these findings have

implications for ecological interactions of phages with bacterial pathogens that can spend different parts of their lifecycle at different temperatures. The elucidation of the mechanisms for attachment and phage growth, and bacterial escape warrant further investigation.

2.7 ACKNOWLEDGEMENTS

The authors would like to express their gratitude for the financial support of this project by Cranfield University and the Commonwealth Scholarship Scheme funded by the UK government.

2.8 REFERENCES

Brüssow, H., and Hendrix, R. W. 2002. Phage genomics: small is beautiful. Cell 108:13-16.

Carey-Smith, G. V., Billington, C., Cornelius, A. J.,Hudson, J. A., and Heinemann, J. A. 2006. Isolation and characterization of bacteriophages infecting *Salmonella spp.* FEMS Microbiol. Lett.258:182 –186.

Carvalho, C. M., Santos, S. B, Kropinski, A. M., Ferreira, E. C., and Azeredo, J.2012. Phages as therapeutic tools to control major foodborne pathogens: *Campylobacter* and *Salmonella*. In: Bacteriophages. I Kurtboke (ed.). InTech. Available from: http://www.intechopen.com/books/bacteriophages/phages-as-therapeutic-tools-to-control-the-major-foodborne-pathogens-campylobacter-and-salmonella

Clokie, M. R. J., Millard, A. D., Letarov, A. V., and Shaun Heaphy. 2011. Phages in nature. Bacteriophage, 1:1, 31-45, DOI: 10.4161/bact. 1. 1.14942

Fernandes, E., Martins, V. C., Nóbrega, C.,Carvalho, C. M., Cardoso, F. A., Cardoso, S., Dias, J., Deng, D., Kluskens, L. D., Freitas, P. P., Azeredo, J.2014. A bacteriophage

detection tool for viability assessment of *Salmonella* cells. Biosensors and Bioelectronics 52:239-246.

Goode, D., Allen, V. M., and Barrow, P. A. 2003. Reduction of experimental *Salmonella* and *Campylobacter* contamination of chicken skin by application of lytic bacteriophages. Appl. Environ. Microbiol. 69:5032-5036

Hudson, J. A., Billington, C., Carey-Smith, G.**,** and Greening, G.2005. Bacteriophages as biocontrol agents in food. J Food Prot. 68:426-437.

Hunter, G. J., 1943. Bacteriophages for *Streptococcus cremoris* phage development at various temperatures. J Dairy Res.13:136-145.

Jończyk, E., Kłak, M., Międzybrodzki, R.,and Górski, A.2011. The influence of external factors on bacteriophages—review. Folia Microbiol. 56**:**191–200.

Kim, J. W., and Kathariou, S. 2009. Temperature-dependent phage resistance of *Listeria monocytogenes* epidemic clone II. Appl. Environ. Microbiol.75:2433-2438.

Klein, W., Weber, M. H., and Marahiel, M. A. 1999. Cold shock response of *Bacillus subtilis:* iso-leucine branching pattern for membrane adaptation to low temperatures J. Bacteriol. 181:5341-5349.

Kokjohn, T. A., Schrader, J. O., Waller, J. J., and Schrader, H. S. 1994. Effects of stress on bacteriophage replication. Proceedings and Papers from the 1994 Risk, Assessment Research, Symposium. Chapter 4.

Koskella, B., and Meadan, S. 2013. Understanding bacteriophage sensitivity in natural microbial communities. Viruses. 5:806-823.

Kutter, E., De Vos, D., Gvasalia, G.,Alavidze, Z., Gogokhia, L., Kuhl, S., and Abedon, S. T.2010.Phage therapy in clinical practice: treatment of human infections. Curr. Pharm. Biotechnol. 11:68-86.

Mahony, J., McAuliffe, O., Ross, P. R., and van Sinderen, D.2011. Bacteriophages as biocontrol agents of food pathogens. Current Opin. Biotechnol. 22:157–163.

McDonald, J. H., Handbook of Biological Statistics (3rd ed.). 2014 p 145-156. Sparky House Publishing, Baltimore, Maryland.

Murata, A. 1971. Temperature-sensitive growth of wild-type phage J1 of *Lactobacillus casei*. Agric. and Biol. Chem.35:667-673.

Nocker, A., Burr, M., and Camper, A. 2013. Pathogens in water and biofilms. In: Microbiology of waterborne diseases: Microbiological aspects and risks. Eds. Percival S. et al.; Second Edition. 2013 p. 3-32. Elsevier.

Orskov, F., Sharma, V., and Orskov, I. 1984. Influence of growth temperature on the development of *Escherichia coli* polysaccharide K antigens. J. Gen. Microbiol. 130:2681-2684

Pearce, L. E.1978. The effect of host controlled modification on the replication of a lactic streptococcal bacteriophage. New Zealand J Dairy Sci. and Technol.13:166-171.

Said, M. B., Masahiro, O., and Hassen, A. 2010. Detection of viable but non cultivable Escherichia coli after UV irradiation using Qβ phage. Annu. Microbiol. 60:121-127

Sanders, M. E., and Klaenhammer, T. R. 1984. Phage resistance in a phage-insensitive strain of *Streptococcus lactis*: temperature-dependent phage development and host-controlled phage replication. Appl. Environ. Microbiol.47:979-985.

Sanders, M. R., and Klaenhammer, T. R. 1980. Restriction and modification of group N streptococci: effect of heat on development of modified lytic bacteriophage. Appl. Environ. Microbiol.40:500-506.

Seeley, N. D., and Primrose, S. B. 1980. The effect of temperature on the ecology of aquatic bacteriophages. J Gen Virol.46:87-95.

Sozzi, T., Poulin, J. M., and Klaenhammer, T.R. 1978. Effect of incubation temperature on the development of lactic acid bacteria and their phages. J Dairy Res.45:259-265.

Suttle, C. A. 2007. Marine viruses - major players in the global ecosystem. Nature Rev. Microbiol. 5:801-812.

Suutari, M., and Laakso, S. 1994. Microbial fatty acids and thermal adaptation. Crit. Rev. Microbiol.20:285-328.

Wiberg, J. S., Mowrey-McKee, M. F, and Stevens, E. J. 1988. Induction of the heat shock regulon of *Escherichia coli* markedly increases production of bacterial viruses at high temperatures. J. Virol.62:234-245.

CHAPTER 3 LYSIS EFFICIENCIES OF BACTERIOPHAGES WITH DIFFERENT PLAQUE SIZES AND CAMPARISON OF LYSIS KINETICS AFTER SIMULTANEOUS AND SEQUENTIAL PHAGE APPLICATIONS.

Ekwu M Ameh[1], Sean Tyrrel[1], Jim A Harris[2], Andreas Nocker[1,4]

[1] Cranfield Water Science Institute, School of Energy, Environment and Agrifood, Cranfield University, Cranfield, Bedfordshire, MK43 0AL, United Kingdom

[2] Cranfield Institute for Resilient Futures, School of Environment, Energy and Agrifood, Cranfield University, Cranfield, Bedfordshire, MK43 0AL, United Kingdom

[4]IWW Water Centre; Moritzstraße 26, 45476 Mülheim an der Ruhr, Germany

3.1 ABSTRACT

Although bacteriophages are promising natural agents for wound treatment and food (and potentially water) safety applications, there is still much uncertainty about strategies how to achieve most rapid and long lasting lytic activity. This study investigated the lysis kinetics of three phenotypically-distinct environmental coliphages reproducibly forming different plaque sizes (big, medium and small). Plaque size happened to correlate with lysis kinetics with phages producing big, medium and small plaques showing maximal lysis under the chosen conditions within 6, 12 and 18 hours. Lysis performance by individual phages was compared with the one obtained after simultaneous or sequential addition of all three phages. Lysis kinetics was monitored by density absorbance or by

flow cytometry with the latter having the advantage of providing higher sensitivity. Use of a phage cocktail (meaning all three phages were added simultaneously) resulted in slower initial lysis compared to the fastest lysing phage with greatest plaque size alone, but tended to suppress the emergence of bacterial resistance longer. In other words: simultaneous adition of phages thus delayed bacterial resistance compared to the addition of a single phage. Adding phages as cocktail nevertheless did not entirely eliminate resistance. When administering phages sequentially, overall lysis kinetics could be influenced by administration of distinct phages at different time points. It has to be noted that lysis rates and sustainability of the effect might be very different in real-world applications from laboratory batch settings with high densities of the target species and resulting rapid nutrient deprivation.

Key words: plaque size; resistance; lysis kinetics; cocktail; flow cytometry.

3.2 INTRODUCTION

The increasing resistance of bacterial pathogens to available antibiotics is a major threat to human health. Given the slow pace to develop new drugs there is the urgent need for alternative biocides that are safe for medical applications. Alternative biopreservatives are also in demand by the food industry with an emphasis on safety for human consumption. Bacteriophages (phages) are an attractive natural choice as they are highly specific for their bacterial host and do not pose threat to humans. Nature offers a seemingly unlimited diversity of these self-perpetuating entities which naturally evolve with their bacterial targets.

Despite their great promise, science is only beginning to understand how to use phages in the most efficient manner. Overshadowed by the attention that antibiotics received, the efficacy and kinetics of phages as biocidal agents have historically not undergone the required scrutiny (Merril et al., 2003). Given the natural diversity, great differences exist in the efficiency of the different phages to infect their bacterial targets (Forest Rohwer, 2003). The strongest focus is on obtaining fast and efficient clearance and on avoidance of the emergence of bacterial resistance. For this reason and to ensure a great host-spectrum, phage cocktails are typically used. Cocktails have been reported to be more effective in reducing bacterial mutation frequency (Gu et al., 2012).

Alternatively to phage cocktails, individual phages can be administered in a sequential manner. It is currently not clear whether it is best to apply phages simultaneously or sequentially (Hall et al., 2012). If bacteria targets are unlikely to develop multiresistance to different phages, simultaneous application may be considered better (Livermore, 2002; Musser, 1998) following a 'hit them hard' strategy. If multiresistance to individual phages however is likely to evolve relatively fast, it might be beneficial to add individual phages sequentially to control bacterial numbers for longer (Hall et al., 2012). In one of the few studies comparing the two different types of phage application, Hall et al. (2012) reported that simultaneous phage addition is most effective to clear bacterial infections as different phages target different bacterial receptors and therefore minimise the probability of cross-resistance. Sequential applications on the other hand are associated with a lower incidence of multiresistance (Hall et al., 2012). The current strategy to increase efficiency of phage-mediated lysis (before emergence of resistance) is therefor the simultaneous application of multiple phages in form of a "cocktail". This approach which is preferred to the use of a monophage however does not

prevent the development of resistance. This chapter is based on the hypothesis whether the application of multiphages in a widespread manner could better suppress the emergence of resistance. This addresses thesis objectives four, five and six.

In this study, we investigated strategies to achieve highest efficiency based on the use of three phages that reproducibly formed different plaque sizes. Phages giving rise to big, medium and small plaques were referred to as B, M and S with all three presenting ds phages belonging to the Myoviridae family (Chapter 2). Plaque sizes were correlated with lysis kinetics. Performance of individual phages was compared with that of a phage cocktail where all the three phages were applied simultaneously. The lysis kinetics of best individual performer and the cocktail were further compared with sequential additions of phages. In the latter case, individual phages with different lysis kinetics were added at different time points to compare lysis speed and sustainability of the overall effect. The overall aim of this *in vitro* study was to gain more insight into the efficiency of bacterial clearance and the sustainability of the effect when applying distinct phages with different lysis kinetics individually, simultaneously or sequentially.

3.3 MATERIALS AND METHODS

3.3.1 Bacteria and growth conditions

Escherichia coli ATCC 25922, a clinical isolate was used both to isolate phages and as target organism for studying lysis kinetics. Bacteria were grown overnight on tryptic soya agar (TSA; CM0131; Oxoid Ltd Basingstoke, Hampshire, UK) at 30ºC. In experiments running for up to 36 hours, selected samples were also plated on selective Membrane Lactose Glucuronide Agar (MLGA; Oxoid, Fischer Scientific, UK) for

verification purposes. Green colonies were considered to be *E. coli* colonies. Liquid cultures were obtained by inoculating 10 mL of tryptic soya broth (TSB; CM1016; Oxoid Ltd Basingstoke, Hampshire, UK) contained in 50 mL Falcon tubes followed by overnight incubation for approx. 18 h at 25^{o}C (or indicated temperature) at a 45^{o} angle at 250 rpm in a temperature-controlled mini shaker (cat. number 980151, VWR, USA). TSB broth and TSA plates were routinely amended with 5 mM $CaCl_2$ (added as $CaCl_2$*$6H_2O$, SIGMA-ALDRICH, Croatia) to allow for efficient phage adsorption. Cell density was measured in a spectrophotometer (JENWAY 6310, Camlab, England, UK) at 600 nm (OD_{600}) and adjusted to an OD_{600} = 1.0 by dilution with TSB equilibrated to room temperature.

3.3.2 Sampling, enrichment and isolation of bacteriophages.

The same as chapter 2.

3.3.3 Purification of bacteriophages.

See chapter 2 for details.

3.3.4 Propagation and determination of bacteriophage titres.

Phage suspensions were propagated further to obtain higher titre suspensions by infecting 10 mL in 50 mL capacity conical centrifuge tubes (Fisherbrand, Fisher Scientific UK Ltd., Loughborough) *E. coli* cultures with phage at a multiplicity of infection (MOI) of 1.0. The mixture was incubated at room temperature until complete lysis had occurred (typically within 6 – 8h), followed by addition of 8 – 10 drops of chloroform to get rid of remaining bacteria. It was further centrifuged in a 15 mL conical centrifuge tubes (Fisherbrand, Fisher Scientific UK Ltd., Loughborough) at 5,000g for 5 minutes. The resulting phage suspension was filtered through 0.22 µm filter (Millex GP,

Merck Millipore, Darmstadt, Germany) and serially diluted in sodium and magnesium (SM) buffer. (NaCl 5.8g; $MgSO_4.7H_2O$, 2g; Tris pH 50 mL; distilled H_2O, 950 mL). Selected dilutions were subjected to plaque assay using the soft agar overlay method (as described previously). After overnight incubation resulting plaques were counted and expressed as plaque forming unit per millilitre (PFU ml^{-1}). High titer phage stocks of above 10^9 PFU/mL were stored at 4°C for further experiments.

3.3.5 Phage challenge and monitoring of optical densities.

Phage challenge experiments were performed at room temperature in 50 mL conical centrifuge tubes (Fisherbrand, Fisher Scientific UK Ltd., Loughborough) by adding bacteriophage suspension to 10 mL of bacterial suspension (adjusted to an optical density OD_{600} of 1.0) at an MOI of 1. Changes in optical densities of cell suspensions (due to phage-induced lysis) were measured on a TECAN M200 Pro plate reader. Samples of 1 mL were aseptically transferred into transparent 48 well tissue culture plates (non-treated, flat bottom, cat. nr. TCP001048; Jet Biofil, Braine I'Alleud, Belgium) and absorbance at 600 nm was measured. Readings were typically taken directly after addition of phages and every hour after the challenge or after 6, 12, 24 and 36 hours (as indicated).Three replicates of each sample were measured.

3.3.6 Flow cytometric analysis

The flow cytometry method followed that of Lipphaus et al., (2014) with some slight adjustments to align with the use of the 96 well plate format; all samples were diluted in 0.1 μm filtered Evian mineral water to cell densities $\leq 10^6$ mL^{-1} to be within the detection range. In order to quantify intact cell counts (ICCs) a 10,000× stock of SYBR Green I (cat. S-7567; Life Technologies Ltd., Paisley, UK) was diluted with dimethyl

sulphoxide (Fisher Scientific, Fair Law, NJ) to a working 100 × concentration stock. A dye mixture was made of SYBR Green I and Propidium iodide (PI) (1 mg mL^{-1}, corresponding to 1.5mM; cat. P3566; Life Technologies Ltd, Paisley, UK) at a ratio of 5:1 respectively. A 2.4 μL volume of this mixed dye was then added to 200μL of sample to give a final SYBR Green I concentration of 1× and PI of 3 μM. Once the dyes and sample had been mixed they were incubated for 13 mins at 37°C in a shaking plate incubator (Grant instrument Ltd, Cambridge, UK). For the analysis a BD Accuri C6 flow cytometer with a 488 nm solid-state laser (Becton Dickinson UK. Ltd, Oxford, UK) was used. 25 μL of sample was analysed when determining the population of bacteria at a flow rate of 66 μL min-1. The green fluorescence was collected in the FL1 channel at 533nm and red florescence in the FL3 channel at 670nm with the trigger set on the green fluorescence at a value of 1000. For the analysis of ICC the fixed gate described previously (Gatza et al., 2013) was used as a template. In the case of sequential additions, phage 2 and 3 (either B, M or S) were always added at the time point of the maximal lysis of the previous phage. A detail experimental design of how the methodology were followed is shown in figure 3-4a. All experiments were carried out with three replicates per sample. All data processing was carried out using the accompanying Accuri C6 software and Microsoft Excel.

3.4 RESULTS

Experiments were performed with three distinct coliphages isolated from a single brook water sample. Phages were used in a previous study (Chapter 2) and differentiated on the basis of the relative plaque size they produce at room temperature. Phages producing the big plaques were designated B and those producing medium and small were designated M and S, respectively.

3.4.1 Correlation of plaque sizes with lysis kinetics.

Phages B, M and S were mixed with *E. coli* suspensions in a MOI ratio of 1 and lysis kinetics was monitored over a total time of 36 hours using plate reader absorbance measurements (Fig. 3-1a) and a flow cytometer (Fig. 3-2a). Absorbance measurements suggested that lysis kinetics correlated with plaque size with the fastest lysis being obtained with phage B, followed by phages M and S (Fig. 3-1a). The resulting curves were used to define time to maximal lysis (TML) as a rough indicator of lysis efficiency with TMLs being within 6, 12 and 18 hours for phages B, M and S, respectively (fig. 3-1b). Bacterial resistance (as measured by an increase in cell numbers) built up fastest for phage B with substantial recovery/growth after 24 hours. Recovered cells were confirmed to be *E.coli* by growth on selective MLGA growth agar. Recovery and growth of *E.coli* exposed to phages M and S followed with a time delay after 36 hours. Absorbance measurements were largely in consensus with flow cytometric quantification of intact cell numbers (being visible in the gated region, Fig. 3-2a). Intact cell concentrations as obtained by flow cytometry are numerically shown in Fig. 3-2b.

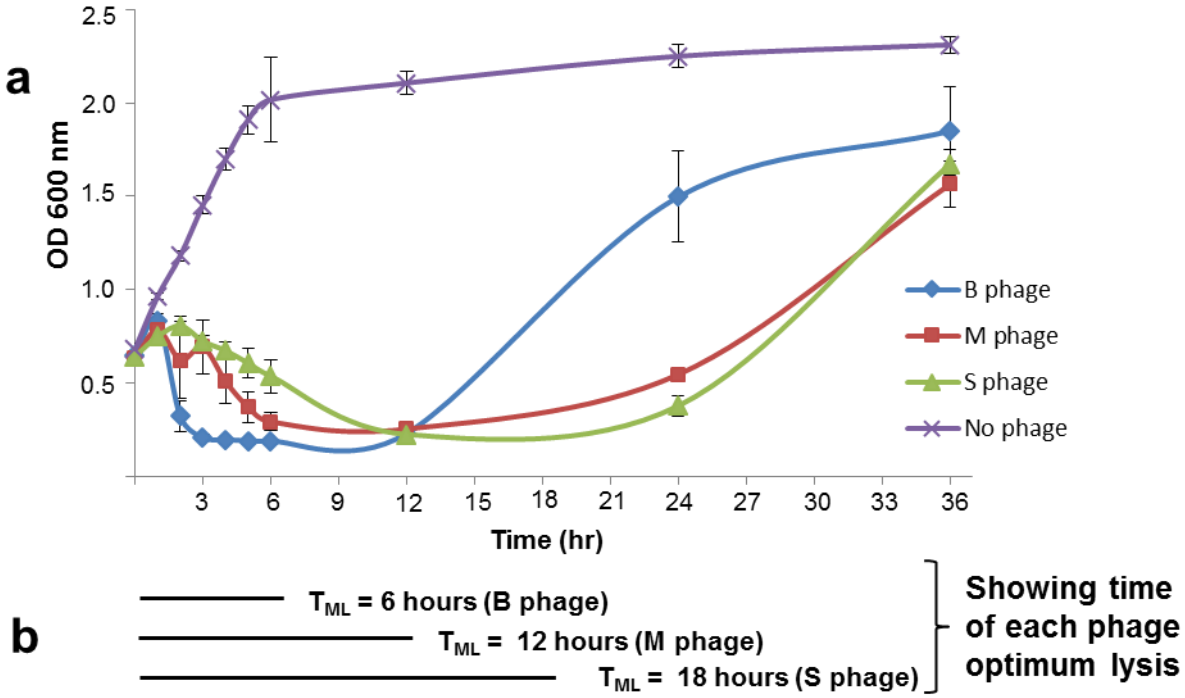
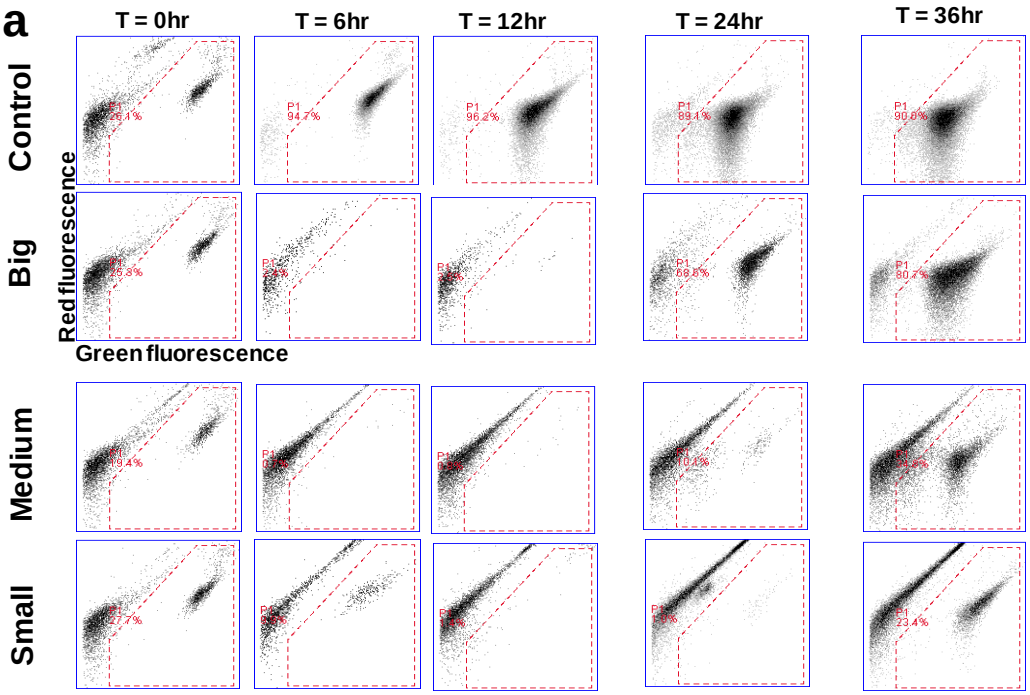


Figure 3-1 (A) Lysis kinetics of *E. coli* after exposure to phages B, M or S as measured on a plate reader at room temperature. (B) Times of maximal lysis (T_{ML}) indicate time intervals in which most efficient lysis occurs. Error bars are standard deviations of means from three repeated experiments.

a



b

Phage ID	T=0hr	T=6hr	T=12hr	T=24hr	T=36hr
BIG	100%	1.4 (±0.8)%	2.4 (±0.2)%	388.4 (±159.2)%	1072.9 (±90.5)%
MEDIUM	100%	2.5 (±0.6)%	3.1 (±0.9)%	106.8 (±8.5)%	372.8 (±83.4)%
SMALL	100%	22.7 (±2.7)%	7.2 (±2.1)%	9.7 (±2.3)%	254.9(±25.6)%
CONTROL (No phage)	100%	584.9 (±21.8)%	1375.7 (±162.2)	1783.4 (±250.8)%	1935.5 (±207.9)%

Figure 3-2 Comparison of bacterial lysis kinetics after exposure to three different *E. coli*- specific phages producing distinct plaques sizes at room temperature: Big (B), Medium (M), and Small (S). (A) Flow cytometric (FCM) dot plots showing intact *E. coli* cells in the gated area at different time points after infection. Samples were stained with SYBR Green I and PI. (B) Changes in bacterial numbers after phage exposure relative to numbers at time point zero (as measured by flow cytometry). Standard deviations from three independent experiments are shown in brackets.

3.4.2 Comparison of phage B with a three phage cocktail.

Lysis kinetics of the fastest lysing phage (B) was compared with the performance of a cocktail of all three phages (B, M, S) added simultaneously to an *E. coli* suspension (MOI of 0.33 for each of the three phages). Absorbance measurements suggested a comparable lysis efficiency of the single phage B and the BMS cocktail for the first 12 hours (Fig. 3-3a). After that time period regrowth was more strongly suppressed in the case of the cocktail whereas cell numbers strongly increased when using only phage B. A more detailed picture for the initial lysis efficiency was obtained by flow cytometry. Flow cytometry suggested greater initial lysis after 6 hours in the presence of only one single phage (phage B) compared to the phage cocktail (Fig. 3-3b). Faster regrowth of *E. coli* only exposed to phage B was confirmed also by flow cytometry similar to the results provided by the plate reader. Given the higher sensitivity of flow cytometry for low cell densities, only this analytical method was applied in the following experiment.

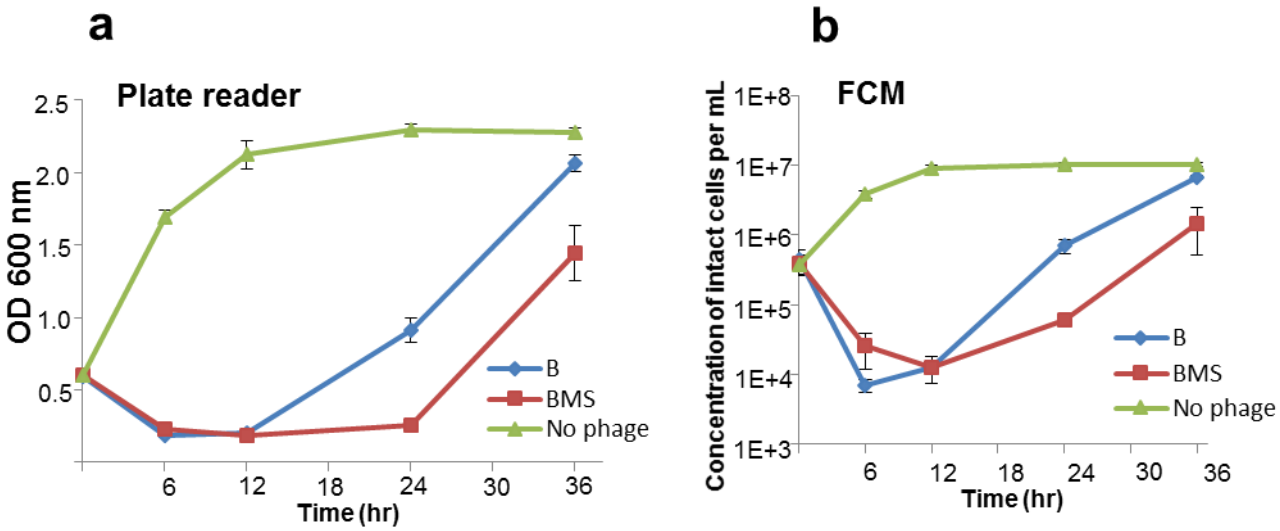


Figure 3-3 *E. coli* lysis kinetics obtained after infection with a single phage and after simultaneous addition of three different phages (B, M and S) in the form of a cocktail. (a and b) Comparison of two cultivation-independent methods based on (A) the measurement of the decline in optical density (using a microplate reader) and (B) fluorescent signal measurement (using flow cytometry) at different time points. Error bars indicate standard deviations as seen in three independent experiments.

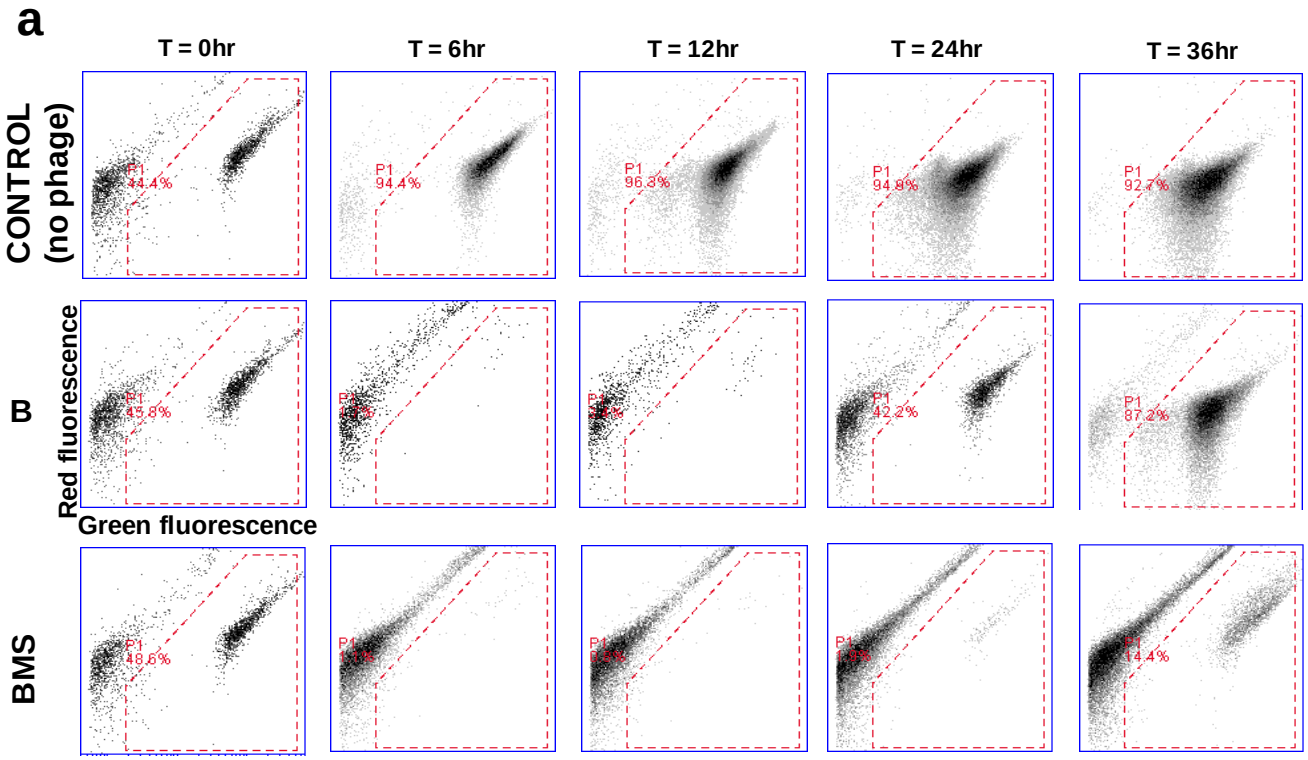


Figure 3-4 *E. coli* lysis kinetics obtained after infection with a single phage and after simultaneous addition of three different phages (B, M and S) in the form of a cocktail. (A) Flow cytometric dot blots of bacteria-phage mixtures after staining with SYBR Green I and PI. Intact cells appear within the gated area.

3.4.3 Comparison of lysis kinetics of simultaneous addition of multiple phages vs. sequential phage addition.

Bacterial lysis of *E. coli* was monitored after addition of a single phage (B), the simultaneous addition of all three phages (BMS) or the sequential addition of the individual phages at different time points to bacterial suspensions. In the latter case, an additional phage was always added at the time that represented the end of maximal lysis time of the previous phage. The time scale of phage addition is shown in Appendix B Fig. B-2. In agreement with the previous experiment, fastest initial lysis was obtained when only one phage (B) was mixed with the bacteria (Fig. 3-5). Also consistent with previous data, lysis kinetics was in the order of phage B > M > S for the mixtures receiving sequential phage addition. Whereas phage B alone led to the fastest lysis within the first 6 hours, all combinations with three phages (whether applied simultaneously or sequentially) performed better at a later stage (>24 hours) as bacterial regrowth was more efficiently suppressed. Comparing simultaneous and sequential phage addition, no generalizations were possible. The phage combination resulting in most sustainable suppression of *E. coli* was the sequential cocktail $S_0M_{18}B_{30}$, however this combination also led to slowest lysis in the first 12 hours. Simultaneous addition of all three phages (= the phage cocktail) showed an intermediate performance. Flow cytometry dot blots of end point bacterial populations obtained after 36 h are shown in Fig. 3-5.

Data from multiphage experiments suggest that the timing of phage addition (using the phages with different lysis kinetics) allows for a choice between fast initial and a longer lasting effect under the laboratory conditions chosen. For a fast effect the phage with the greatest plaque size proved best, whereas cocktails with slower lysing phages proved beneficial to obtain a more lasting effect. Addition of phage at a later time point

($S_0M_{18}B_{30}$) might not have the desired effect any more under laboratory conditions in our study. One possible explanation for this observation may be the depletion of nutrients which affect bacteria growth. This subsequently has a turnaround effect on the phage infectivity.

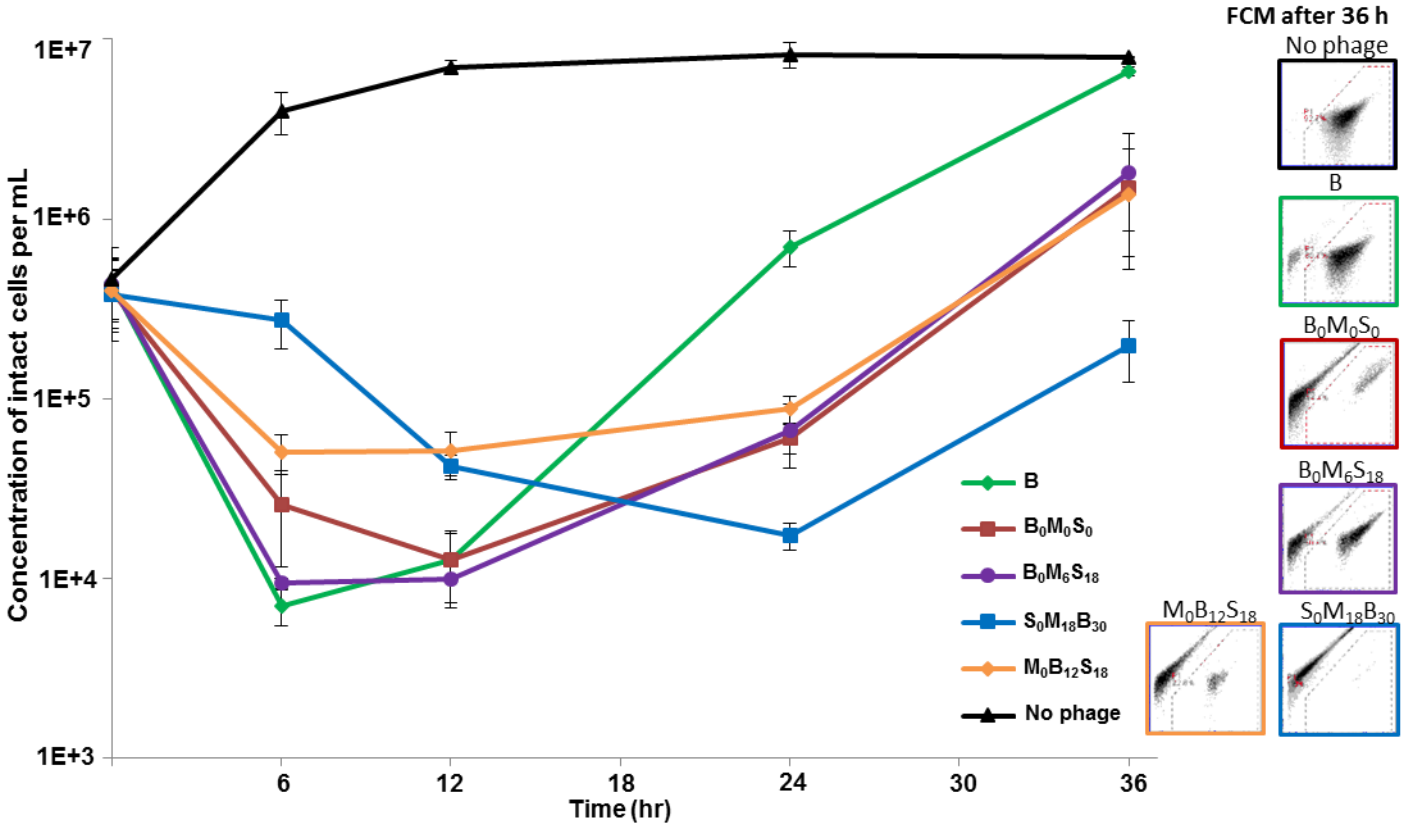


Figure 3-5 Effect of the time of phage addition on lysis kinetics and the emergence of bacterial resistance. Concentrations of intact *E. coli* cells after addition of different phages at different time points as measured by flow cytometry. A sample without phage addition served as a control. Error bars are standard deviations of means from three repeated experiments.

3.5 DISCUSSION

In this study we investigated the lytic performance of three distinct Myoviridae phages that gave rise to different plaque sizes. Phages were applied individually or in different combinations. For the isolated phages, plaque sizes on plates correlated with lysis rates in bacterial liquid suspensions. Phage B producing the largest plaques was able to lyse the bacteria at the fastest rate, followed by the phages producing medium and small plaques. This relationship is not surprising as among the factors determining plaque size (for an overview see Gallet et al. 2011) some are shared in common between a semi-solid and liquid phage propagation including adsorption rate, replication rate, burst size (given the identical bacterial host, temperature, and osmolarity). Diffusion rates, bacterial densities and bacterial metabolic rates (influenced by nutrient availability, cell density and oxygen availability) on the other hand can be very different between agar and well mixed broth (Abedon and Yin 2009), which might explain why this correlation is not always observed. When studying the effect of three different phages on *E. coli* 0157:H7 with two phages producing pinpoint plaques (0.5 mm) and one producing medium-sized plaques (3 mm), O'Flynne (2004) for example reported faster and more efficient lysis for the two phages producing smaller plaques. Despite plaque size being one of the most important phage traits, many questions still remain how this characteristic can be used for optimal choice of phages for biocontrol applications.

The central research questions of this study however was to compare the lytic performances of the three individual phages with the one of multiple phages that were applied either simultaneously in form of a cocktail or sequentially at different time points in regard to lysis efficiency and the sustainability of this effect. One of the first questions

was which diagnostic assay to apply to measure lytic activity. As the plaque assay is not compatible with high sample numbers and subject to variation (depending on the experimenter, timing and experimental conditions; Henry et al. 2014), both the commonly applied bacterial density measurements and flow cytometry (in combination with live-dead staining) were applied. Both methods are substantially faster than the tedious plaque assay which is not compatible with high sample numbers. Although grossly in agreement, a difference in sensitivity between the two methods became visible when comparing the lytic performance of phage B with the one of the cocktail containing all three phages. Whereas density measurements suggested a highly comparable performance up to 12 hours, flow cytometric measurements offered higher resolution and showed a greater decrease in the concentration of intact bacteria when exposed to phage B alone. Whereas the limit of detection of a plate reader in combination with the comparable LIVE/DEAD BacLight™ (comprising staining with Syto9 and propidium iodide) was reported to have a limit of detection in the range between 10^6 and 10^7 cells mL^{-1} (Kort et al. 2010), the detection limit of flow cytometry is around 100 cells mL^{-1} (depending on how clean the machine is kept, calibration on a regular basis is key to good performance). The better limit of detection of flow cytometry allowed the observation that the application of the best performing phage alone resulted in a greater decrease in intact cell concentrations than the cocktail within the first 6 hours after phage application (Fig. 3-3b). Both methods suggested on the other hand the faster upcoming of resistance in the monovalent case. Due to this superior resolution in the low cell density range, flow cytometry was used for subsequent experiments.

The finding that a single fast-lyzing phage can lead to more rapid bacterial clearance than a phage cocktail (containing the same number of the fast-lyzing phage, but

in addition to two other phages with slower lysis kinetics) suggests that the presence of other phages can impede initial bacterial clearance velocity. Superinfection exclusion mechanisms that prevent infection with multiple phages competing for the same host bacterium are well known (for reviews: Labrie et al. 2010 and Seed 2015) and include the modification of entry receptors (Cumby et al. 2012 http://www.ncbi.nlm.nih.gov/pubmed/22797755/), blocking of DNA translocation into the host cytoplasm (Lu and Henning 1994), inhibition of viral peptidoglycan degradation enzymes and expression of restriction enzymes (Tock and Dryden 2005). The interference caused by phages with slower kinetics makes us conclude that a cocktail is not necessarily the best choice, if the emphasis is on fast bacterial clearance.

Whereas the better performance of the single phage in regard to fast initial clearance is an example of interference where the use of multiple phages is worse than the best single phage, the multivalent approach can also produce better results than a monovalent phage (Schmerer et al. 2014). In our example simple changing of the snapshot time is sufficient to undermine this view as the single phage application resulted in fastest emergence of resistance and worse performance than the cocktail after 36 hours. Overall the finding is in agreement with Fischer et al. (2013) comparing the impact of a single phage and a cocktail of four phages in the biocontrol of *Campylobacter jejuni* in broilers. The application of the cocktail was reported to have only a small advantage over the single phage treatment in regard to bacterial lysis potential, however the cocktail delayed the emergence of phage resistance. The benefit of cocktails in regard to suppression of resistance was also reported by Tanji et al. (2005) for *Escherichia coli* O157:H7.

Apart from the before mentioned lower likelihood of the development of bacterial resistance, multiphage treatment is typically seen advantageous over monovalent

applications due to a broader host range (Kelly et al. 2011) and potentially faster killing of bacterial targets (Schmerer et al. 2014). As it is however currently unclear how multiple phages should be best applied and how the individual cocktail members should be selected, the outcome of phage applications have a poor predictability (Payne et al. 2000). One interesting study was the comparison of bacterial densities (*P. aeruginosa*) after addition of single phages and multiple phages applied in a simultaneous and sequential fashion (Hall et al., 2012). The efficiency of application was assessed *in vitro* (over 12 transfer cycles with aliquots being transferred every 24 h into fresh medium) and *in vivo* using wax moth larvae infected with *P. aeruginosa*. The outcome of the study was that use of multiple phages was more efficient than individual phages. Comparing the different strategies for multiple phages, simultaneous addition was equal or superior to sequential addition for clearing bacteria. Authors however pointed out that sequential approaches can be devised that are just as efficient as a cocktail and that sequential applications might be associated with a lower incidence of multiresistance. In our experiments, although not employing multigeneration transfers, a bespoke strategy was chosen for sequential applications by adding the second and third phage at time points that defined the end of maximal lysis of the previous phage. Looking at the performance after 36 hours, the best overall suppression in bacterial numbers was obtained by sequential addition of S, M and B phage ($S_0M_{18}B_{30}$; Fig. 3-5). Other sequential combinations performed comparably as the phage cocktail.

Comparing the lytic performance of the phage combinations in our study, one of the eminent conclusions is that fast initial lysis comes at a cost of relatively poor sustainability. If bacteria get lysed fast, also resistance emerges fast. If it takes longer to lyse the bacteria, then typically it also takes longer for resistance to emerge. We did not

succeed in our experimental setup to achieve fast initial lysis and at the same time a long-lasting suppression of bacterial growth. It has to be acknowledged that the experiments as performed by us and other researchers conducting this type of laboratory experiments are bound to be relatively artificial as they involve working with a single bacterial species at high cell densities in a closed system experiment. Typically resistance occurs fast under these laboratory conditions within 24 hours or less (O'Flynn et al., 2004; Turki et al 2012; Hall et al., 2012). It furthermore remains to be seen whether such observations also hold true at higher MOIs. A low MOI was chosen for these experiments to increase the likelihood of fast development of bacterial resistance. Another limitation of any *in vitro* research is the inability to project findings to *in vivo* and real world applications (Payne et al. 2000). In sharp contrast to laboratory experiments, typically a large abundance of different bacterial species in lower concentrations is given in the environment and the nutrient spectrum does not undergo such a dramatic change in composition and concentration such as in a closed vial.

3.6 CONCLUSIONS

The study performed with phages with different lysis kinetics provides an example for the following: (A) a single phage with the fastest lysis kinetics can outperform a phage cocktail in regard to the ability to lyse the bacteria at high efficiency within a short time although the cocktail is likely to achieve a more sustainable bacterial clearance, (B) all multivalent applications had a more long-lasting effect than the fastest single phage and (C) certain sequential phage applications (which were $B_0M_6S_{18}$ and $S_0M_{18}B_{30}$) can in some cases produce a more sustainable result in terms of clearance of bacteria than the cocktail. Overall the appropriate choice of phages depends on whether the focus is on fast clearance or sustainability of the effect. We did not succeed in our

experimental setup to achieve both fast initial lysis and a long-lasting suppression of bacterial growth, much more knowledge is required. It remains to be seen whether a smart choice of sequentially added phages in a real-world scenario could meet both requirements of rapid clearance of the bacterial target and a sustained regrowth suppression of surviving cells. Depending on the application, the 'optimal' phage combination might be able to be determined in the future by computer programmes allowing a tailored solution.

3.7 ACKNOWLEDGEMENTS

This study was part of a PhD research funded by the Commonwealth Scholarships and supported by the British government which is gratefully acknowledged.

3.8 REFERENCES

Abedon, S. T., and Yin, J. 2009. Bacteriophage plaques: theory and analysis. Methods Mol. Biol. 501:161-174. http://www.ncbi.nlm.nih.gov/pubmed/19066821 (accessed March, 2016)

Adams, H. M. 1959. Bacteriophages. New York, USA, Interscience Publishers

Alisky, J., Ichzkowski, K., Rapoport, A., and Troitsky, N. 1998. Bacteriophages show promise as antimicrobial agents. J. Infect. 36:5–15.

Ameh, et al., 2016. Effect of temperature on bacteriophage-mediated bacterial lysis efficiency with a special emphasis on bacterial temperature history. Submitted for publication in Annals of Microbiology.

Arias, C.A., Murray, B.E., 2009. Antibiotic-resistant bugg in the 21st Century – A clinical super-challenge. New Engl. J. Med. 360, 439-443

Benetti, A. G. and Reinheimer, J. A., 2000. Thermal and chemical inactivation of indigenous *streptococcus thermophiles* bacteriophages isolated from Argentinian diary plants. J. Food Prot. 63 (4), 509-515

Betts, A, Vasse, M., Kaltz, O., and Hochberg, E. M. 2013. Back to the future: evolving bacteriophages to increase their effectiveness against the pathogen *Pseudomonas aeruginosa* PAO1. Evol Appl. 6:1054-63; PMID: 24187587

Carey-Smith, G.V., Billington, C., Cornelius, A.J., Hudson, J.A. and Heinemann, J.A. 2006. Isolation and characterization of bacteriophages infecting *Salmonella spp*. FEMS Microbiol Lett 258, 182-186

Carlton, R.M. 1999. Phage therapy: past history and future prospects. Arch Immunol Ther Exp 47(5), 267-274

Cumby, N., Edwards, A. M., and Davidson, A. R. 2012. The bacteriophage HK97 gp15 moron element encodes a novel superinfection exclusion protein. Journal of Bacteriology. Vol. 194, No. 18, 5012-5019.

Fischer, S., Kittler, S., Klein, G., and Glünder, G. 2013. Impact of a Single Phage and a Phage Cocktail Application in Broilers on Reduction of *Campylobacter jejuni* and the Development of Resistance. PLos ONE 8(10): e78543. Doi:10.1371/journal.pone.0078543

Forest Rohwer. 2003. Global phage diversity. Cell Vol., 113 2, 141

Gatza, E., Hammes, F. and Prest, E. 2013. Assessing water quality with BD C6 flow cytometer. White paper BD Biosciences

Gu, J., Liu, X., Li, Y., Han, W., Lei, L. et al. 2012. A method for generation of phage cocktail with great therapeutic potential. PLos ONE 7(3):e31698

Hall, A. R., De Vos, D., Friman, Ville-Petri., Pirnay, Jean-Paul., and Buckling, A. 2012. Effects of sequential and simultaneous applications of bacteriophages on populations of Pseudomonas aeruginosa in vitro and wax moth larvae. Appl. Environ. Microbiol 78:5646-5652

Hill, C. 1993. Bacteriophage and bacteriophage resistance in lactic acid bacteria. FEMS Microbiol. Rev. 12:87-108

Hudson, J. A., Billington, C., Carey-Smith, G. and Greening, G., 2005. Bacteriophages as biocontrol agents in food. J. Food Prot. 68 (2), 426-437

Hyman, P., and Abedon, S.T., 2010. Bacteriophage host range and bacterial resistance. Adv. Appl. Microbiol. 70, 217-248

Inal J.M. 2003. Phage therapy: a reappraisal of bacteriophage as antibiotics. Arch Immunol Ther Exp 51, 237-244

Jaiswal, A., Koley, H., Ghosh, A., Palit, A., and Sarkar, B. 2013. Efficacy of cocktail phage therapy in treating Vibrio choleae infection in rabbit model. Microbes Infect 15: 152-156. http:/dx.doi.org/10.1016/j.micinf.2012.11.002 (accessed Feb., 2016).

Kahrstom, C.T. 2013. Entering a post-antibiotic era? Nature Reviews. Microbiology, 11(3), 146-147

Kelly, D., McAuliffe, O., O'Mahony, J., and Coffey, A. 2011. Development of a broad-host-range phage cocktail for biocontrol. Bioeng. Bugs 2(1), 31-37

Knoll, B.M., and Mylonakis, E. 2014. Antibacterial bioagents based on principle of bacteriophage biology: An overview. Clinical infectious Diseases, 58(4), 528-534

Kort, R., Nocker, A., Angelino-Bart, A., van Veen, S., Verheij, H., Schuren, F., and Montijn, R. 2010. Real-time detection of viable microorganisms by intracellular phototautomerism. BMC Biotechnology. 10:45

http://www.ncbi.nlm.nih.gov/pubmed/20565844 (accessed March, 2016)

Kudva, I. T., Jelacic, S., Tarr, P. I., Youderian, P., and Hovde, C. J. 1999. Biocontrol of *Escherichia coli* O157 with O157-specific bacteriophages. Appl. Environ. Microbiol. 65:3767-3773

Labrie, J., Samson, E., and Moineau, S. 2010. Bacteriophage resistance mechanisms. Nature Reviews Microbiology. Vol. 8, 317-327 http://www.nature.com/nrmicro/journal/v8/n5/execsumm/nrmicro2315.html (accessed March, 2016)

Livermore, D. M., 2002. Multiple mechanisms of antimicrobial resistance in *Pseudomonas aureginosa*: our worst nightmare? Clin. Infcet. Dis. 34:634-640

Lu, M. J., and Henning, U. 1994. Superinfection exclusion by T-even-type coliphages. Trends Microbiol. 2(4):137-139

Merril, C. R., Scholl, D., and Adhya, S. L., 2003. The prospect for bacteriophage therapy in Western medicine. Nat. Rev. Drug Discov 2:489-497. http://www.nature.com/nrd/journal/v2/n6/full/nrd111.htm (accessed Feb., 2016)

O'Flynn, G., Ross, P. R., Fitzgerald, G. F., and Coffey, A.2004. Evaluation of a cocktail of three bacteriophages for biocontrol of *Escherichia coli* O157:H7. Appl. Environ. Microbiol. 70:3417–3424.

Olivier, A., Budka, H., Buncic, S., Colin, P., Collins, J. D., De Koeijer, A., Griffin, J., Havelaar, A., Hope, J., Klein, G., Kruse, H., Magnino, S., Antonio, López, M., McLauchlin, J., Nyuyen-The, C., Noeckler, K., Noerrung, B., Maradona, M. P., Roberts, T., Vågsholm, I., and Vanopdenbosch, E. 2009. The use and mode of action of bacteriophages in food production. Scientific Opinion of the Panel on Biological Hazards. The European Food Safety Authority Journal 1076, 1-26

Payne, R.J., Phil, D., Jansen, V.A. 2000. Phage therapy: the peculiar kinetics of self-replicating pharmaceuticals. Clin. Pharmacol. Ther. 68, 225-230

Pirnay, J.P., De Vos, D., Verbeken, G., Merabishvili, M., Chanishvili, N., Vaneechoutte, M., Zizi, M., Laire, G., Lavigne, R., Huys, I., Mooter, G. V., Buckling, A., Debarbieux, L., Pouillot, F., Azeredo, J., Kutter, E., Dulanchet, A., Górski, A., and Adamia, R. 2011. The phage therapy paradigm: pret-a-porter or sur-mesure? Pharm. Res. 28, 934-937. http://dx.doi.org/10.1007/s11095-010-0313-5

Ramaswamy, S., and Musser, J. M., 1998. Molecular genetic basis of antimicrobial agent resistance in Mycobacterium tuberculosis: 1998 update. Tubercle Lung Dis. 73:3-29

Ronner, A. B., and Cliver, D. O. 1990. Isolation and characterization of a coliphage specific for *Escherichia coli* O157:H7. Journal Food Prot. 53:994-947

Seed, K. D. 2015. Battling phages: How bacterial defend against viral attack. PLoS Pathogens 11(6):e1004847. http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4465916 (accessed Feb., 2016)

Smith, H. W., and Huggins, M. B**.** 1982. Successful treatment of experimental *Escherichia coli* infections in mice using phage: its general superiority over antibiotics. J. Gen. Microbiol. 128:307–318

Suarez, V. B. and Reinheimer, J. A., 2002. Effectiveness of thermal treatments and biocides in the inactivation of Agentinian Lactococcus lactis phages. J. Food Prot. 65 (11) 1756-1759

Sulakvelidze, A., Kutter, E. 2005. Bacteriophage therapy in humans. In: Kutter, E. Sulakvelidze, A. (eds) Bacteriophages: biology and application. CRC press, Boca Raton, pp 381-436

Summers, A., O**.** 2002. Generally overlooked fundamentals of bacterial genetics and ecology. Clin. Infect. Dis. 34:S85–S92.

Tanji, Y., Shimada, T., Fukudomi, H., Miyanaga, K., et al. 2005. Therapeutic use of phage for controlling Escherichia coli 0157:H7 in gastrointestinal tract of mice. J Biosc Bioeng 100, 280-287

Torres-Barceló, C., Arias-Sachez, F. I., Vasse, M., Ramsayer, J., and Kaltz, O. 2014. A window of opportunity to control the bacteria pathogen *Pseudomonas aeruginosa*combining antibiotics and phages. PLoS ONE 9(9): e106628, doi:10.1371/journal.pone.0106628

Turki, Y., Ouzari, H., Mehri, I., Ben Ammar, A., and Hassen, A. 2012. Evaluation of a cocktail of three bacteriophages for the biocontrol of *Salmonella* of wastewater. Food Research International 45: 1099-1105

Tock, M. R, and Dryden, D. T 2005 The biology of restriction and anti-restriction.

Curr Opin Microbiol. 8(4):466-72

Gallet, R., Kannoly, S., and Ing-Nang Wang. 2011. Effects of bacteriophage traits on plaque formation. BMC Microbiology. 11:181.

http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3176204/pdf/1471-2180-11-181.(accessed March, 2016)

Henry, M., Biswas, B., Vincent, L., Mokashi, V., Schuch, R., Bishop-Lilly, K. A., and Sozhamannan, S. 2012. Development of a high throughput assay for indirect measuring phage growth using the OmniLog™ system. Landes Bioscience. Research paper Bacteriophages 2:3, 159-167

CHAPTER 4 SUSTAINABILITY OF THE EFFECT OF CHLORINE AND UV ON *E. COLI* GROWTH SUPPRESSION AND IMPACT OF DISINFECTION ON THE BACTERIAL SUSCEPTIBILITY TO PHAGE-MEDIATED LYSIS

Alternative: Fate of chlorine or UV exposed *Escherichia coli* in the absence or presence of a coliphage

Ekwu M Ameh[1], Sean Tyrrel[1], Jim A Harris[2], Andreas Nocker[1,4]

[1] Cranfield Water Science Institute, School of Environment, Energy and Agrifood, Cranfield University, Cranfield, Bedfordshire, MK43 0AL, United Kingdom

[2] Cranfield Institute for Resilient Futures, School of Environment, Energy and Agrifood, Cranfield University, Cranfield, Bedfordshire, MK43 0AL, United Kingdom

[4]IWW Water Centre; Moritzstraße 26, 45476 Mülheim an der Ruhr, Germany

4.1 ABSTRACT

Both chemical and photochemical disinfection procedures aim at maximal inactivation of target organisms and the sustainable suppression of their regrowth. Whereas both chlorine and UV disinfection are well known to achieve efficient inactivation when applied under appropriate conditions, there are questions about the sustainability of this effect. One of these questions refers to the exposed bacteria themselves, whether they might recover and possibly regain the ability to grow. In an environmental context another question is how amenable surviving bacteria are to

predation by bacteriophages which are omnipresent and exceed bacterial numbers by a factor of ten. Previous data suggested that bacteria that were subjected to sublethal heat stress developed a phage-resistant phenotype. We addressed in this study whether the same holds true for bacteria exposed to different chlorine and UV disinfection strengths ranging from sublethal to lethal. Data underlined different dose-response relationships with chlorine causing strong bacterial inactivation when exceeding a critical concentration threshold, whereas inactivation by UV is rather linear over the applied UV gradient. Whereas bacteria exposed to low sublethal chlorine doses still underwent phage-mediated lysis, the critical chlorine Ct of 0. 5 mg L min^{-1} eliminated this susceptibility and induced phage resistance in the cells that survived chlorination. In the case of UV, even the minimal dose of 2.8 mJ cm^{-2} abolished phage lysis. Results lead to the hypothesis that bacteria surviving disinfection by UV might have higher environmental survival chances compared to non-treated cells. A potential reason could lie in their compromised metabolism that is essential for viral replication.

Key words: UV; viable but non-culturable; disinfection; chlorine gradient; sublethal.

4.2 INTRODUCTION

Disinfection with chlorine and ultraviolet light in the UV-C range represent two of the most common disinfection procedures for a wide variety of applications including water treatment (Wang et al., 2005; Clancy et al., 1998; Kruithof et al., 1992; Said et al., 2010; Bolton and Linden, 2003; Zhang et al., 2015; Deborde and Gunten, 2008; Hijen, Beerendonk and Medema, 2006), disinfection of surfaces (Salvesen and Vadstein, 1995; Beuchat, 1992; Zhang and Farber, 1996) or (in case of bleach) household cleaning.

Whereas the aim of every disinfection procedure typically is to maximally reduce counts of viable microorganisms, there are (in contrast to sterilization that utilizes harsher killing conditions) always surviving microbes (referred to as 'persisters' in a biofilm context). Factors that can reduce the efficiency of chlorination include performance of disinfection at suboptimal pH (Ward, Wolf and Olson, 1984; Wattie and Butterfield, 1944; Morris, 1967), the presence of chlorine demand (Hejkal et al., 1979; Health Canada, 2009; Haas and Karra, 1984; LeChevallier, 1981) or cold temperature (Haas, 1980; Le Dantec et al., 2002). Factors known to reduce the efficiency of UV-C disinfection include insufficient transmissivity of the irradiated liquid (AWWA, 1999; Clarke and Bettin, 2006; Shin et al., 2001), shielding effects by particulates (Emerick et al., 1999, 2000; Hofmann et al., 2004; Ormeci and Linden, 2002; Mamane-Gravetz and Linden, 2004) or the presence of substances with UV absorbing properties (Templeton, Andrew and Hofmann, 2004; USEPA, 2003).

Microbiological questions that are associated with any disinfection procedure refer to the following points: (1) immediate efficiency of the treatment and (2) the sustainability of the disinfection effect. Whereas the first depends on the treatment conditions chosen, the nature of the treated matrix and the target organism and can be determined relatively easily, there is more uncertainty associated with the latter due to the fact that it cannot be answered by a diagnostic snapshot directly after treatment. The sustainability of disinfection depends both on the innate ability of the specific organism to repair and to recover and the impact of disinfection on the organism's susceptibility to physical and biological environmental factors. For bacteria, an important biological factor consists in their susceptibility to bacteriophages which represent (together with protozoan grazers) the most important group of predators in nature (Clokie et al., 2011; Murray and

Eldridge, 1994; Proctor and Fuhrman, 1990, 1992; Heldal and Bratbak, 1991). Phage numbers in the environment are estimated to exceed the ones of bacteria by up to 10-fold (Labrie, Samson and Moineau, 2010; Brüssow and Hendrix, 2002). Bacteria are however not equally susceptible to phage attack in every stage of their life cycle (Mc Grath and van Sinderen 2007). We could show in a previous study that bacteria exposed to heat stress were less susceptible to phage-mediated lysis (Chapter 2). The research question to be addressed here was whether a phage-resistant phenotype can also originate from other stresses such as those as induced by common disinfection procedures. Chlorine and UV treatment were chosen as common disinfection procedures with different bacterial inactivation mechanisms. This work addressed the hypothesis whether bacteria subjected to sublethal disinfection doses of chlorine and UV are still amenable to phage-mediated lysis (thus addressing the objective seven of this research work).

In this study we investigated the change in bacterial numbers up to 4 days following disinfection both in absence and presence of bacteriophages. *E. coli* was chosen as a test organism. A lytic coliphage that was isolated from an environmental brook and was found to induce efficient lysis (chapter 3) of *E. coli* with a time to maximal lysis of approx. 6 hours was used as an exemplary phage.

4.3 MATERIALS AND METHODS

4.3.1 Bacteria growth and conditions

A clinical isolate of *Escherichia coli,* ATCC 25922 was streaked from glycerol stock onto tryptone soya agar (TSA; CM0131; Oxoid Ltd Basingstoke, Hampshire, UK) and grown for 24 h at 30ºC. Single colonies were subsequently transferred into 10 mL of 10% tryptone soya broth (TSB; CM1016; Oxoid Ltd Basingstoke, Hampshire, UK)

contained in 50 mL Falcon tubes followed by overnight incubation at 25°C (or indicated temperature) at a 45° angle at 250 rpm in a temperature-controlled mini shaker (cat. number 980151, VWR, USA). Cell density was measured in a spectrophotometer (JENWAY 6310, Camlab, England, UK) at 600 nm (OD_{600}) and adjusted to an OD_{600} = 1.0 by dilution with 10% TSB (equilibrated to room temperature). Aliquots of 1 mL of the density adjusted bacterial culture were then transfer aseptically to a sterile 1.5 mL microcentrifuge tube and spun at 5,000 g for 5 minutes to. The supernatant was removed carefully using a tip and the cell pellet was resuspended in an equal volume of 0.1 µm filtered Evian mineral water (Evian-les-basin, France), Nescerecta et al., 2015; Prest et al., 2013; Lipphaus et al., 2013. This washing step was repeated three times to remove organic traces from the culture broth. Cells were subsequently further diluted to obtain a concentration of 10^5 CFU/mL for disinfection experiments.

4.3.2 Chlorine demand-free glassware

To obtain chlorine demand-free glassware for chlorination experiments, organics were removed following the method described by Charnock and Kjønnø (2000). Borosilicate glass beakers (Fischer Scientific, UK) were initially machine washed at high temperature with detergent. Thereafter it was rinsed first with normal tap water and subsequently three times with ultrapure water. Beakers were filled to the neck with 0.2N hydrochloric acid (HCl), covered with aluminium foil and left to stand overnight to hydrolyze organics. After this acid wash, glassware was rinsed three times with ultrapure water, air-dried and capped with aluminium foil. Removal of residual carbon was achieved by heating to 550°C for at least six hour in a Muffle furnace (Muffle Furnace

1400, Pave Testing Ltd, Hertfordshire, UK). Glassware was stored in a dry place until use.

4.3.3 Disinfection by chlorination

A chlorine solution was prepared by diluting 95 µL of sodium hypochlorite stock solution (10-15% available chlorine, SIGMA-ALDRICH, USA) in 50 mL of 0.22 µm filtered Evian mineral water of 100 mL volume flask to a final concentration of approximately 200 mg L^{-1}. This solution was further diluted to the desired working concentrations. 0.5, 1.25, 5, 12.5, 25, 50, and 125 µL of chlorine was added to 50 mL aliquots of bacterial suspensions (10^5 CFU/mL in Evian water, prepared as described earlier) and stirred at 150 rpm in chlorine demand-free beakers to obtain the following final chlorine concentrations: 0.002, 0.005, 0.02, 0.05, 0.1, 0.2, and 0.5 mg L^{-1}. Cells were exposed to chlorine for 10 min translating to a Ct value range between 0.02-5 mg L^{-1} min. Chlorine disinfection was stopped by addition of 250 µL of 0.1N sodium thiosulphate ($Na_2S_2O_3*5H_2O$) (Acros Organics, Geel, Belgium). A bacterial suspension without added chlorine served as a control. After letting the samples stand for 10 min, TSB was supplemented to a final concentration of 10% TSB. To one series of samples a small volume of concentrated phage stock (typical titre $\geq 10^9$ PFU/mL) to obtain a phage concentration of 10^5 PFU/mL (corresponding to a multiplicity of infection (MOI) of 1. All experiments were carried out in triplicate.

4.3.4 Disinfection by LED UV

UV disinfection was performed using a UVC-LED system consisting of a UVCLEAN lamp with multi-chip collections of UV LEDs enclosed in a metal and glass casing from Sensor Electronic Technology (Columbia, South Carolina). UV light emitted

by the LEDs was at 256 nm with a power output of 15 mW. Samples were placed at a distance of 1 cm to the UV source. The delivered dose was verified using a UVC radiometer (VLX-3W from Vilber Lourmat) and determined to be 20 W/m^2. Bacterial suspensions (10^5 CFU/mL in Evian water, prepared as described earlier) were transferred in 20 mL aliquots into a 25 mL Pyrex petri dish (Fisher Scientific, UK). During UV exposure suspensions were stirred constantly using a magnetic stir bar (Fisher Scientific, UK) on a stirrer set to 400 rpm. Bacteria were exposed to the following UV doses: 2.8, 4.8, 10.5, 15.4, 20.3, and 39.9 mJ/cm^2. A bacterial suspension that was treated identically, but not exposed to UV served as a control. Following UV exposure, suspensions were supplemented with TSB to a final concentration of 10% to provide nutrients for the cells and bacteriophages. To one series of samples phages were added (as described for the chlorine experiments) to achieve a multiplicity of infection (MOI) of 1. The experiments were carried out at room temperature, 20 °C. All experiments were carried out with three replicates per sample.

4.3.5 Bacteria quantification/plate counting

For enumeration of *E.coli*, samples were plated on membrane lactose glucuronide agar (MLGA; Oxoid, Fischer Scientific, UK) using the membrane filtration method. MLGA gives rise to green *E. coli* colonies. Filtered Evian (~ 3 mL) was poured into the filter manifold (Combisart, Sartorius, UK) prior to addition of 100 µL of sample (10^5 CFU/mL) to ensure homogeneous distribution of bacteria on the filter. Samples were filtered onto 0.45 µm cellulose filters (47 mm white gridded, Cat. No. HAWG047S6 Fisher Scientific, UK) and placed on 55 mm Petri dishes with MLGA. Plates were

incubated at 35°C for 24 hours before enumeration using a colony counter (SC6, Bibby Sterilin Ltd. Staffordshire, UK). Three replicates of each sample were treated.

4.3.6 Flow cytometry analysis

Bacterial lysis was measured using an Accuri C6 flow cytometer (Becton Dickinson UK. Ltd, Oxford, UK) equipped with a 488nm solid-state laser and an auto-sampler. A 10,000× solution of SYBR Green I (cat. S-7567; Life Technologies Ltd., Paisley, UK) was diluted with dimethyl sulphoxide (DMSO, Fisher Scientific, UK) to a working 100× solution. This 100x SYBR Green I solution was mixed with 1.5 mM propidium iodide (PI, corresponding to1 mg mL^{-1}; cat. P3566; Life Technologies Ltd, Paisley, UK) in a volumetric ratio of 5:1. Aliquots of 2.4 μL of this fluorescent dye mixture were then aliquoted into the wells of a 96-well microtiter plate (untreated, flat bottom Cat. No. TCP001096; Jet Biofil, Braine I'Alleud, Belgium), followed by addition of 200 μL of sample using a multichannel pipette. The final dye concentrations were 1x for SYBR Green I and 3 μM for PI. Once the dyes and sample had been mixed by pipetting up and down several times, they were incubated for 13 mins at 37°C (following the recommendations by Lipphaus et al., 2014; SLMB 2012) in a shaking plate incubator (Grant-bio-Thermo-Shaker, PHMP, No. 010119-1404-0160, Cambridgeshire, England). Sample volumes 25 μL were analysed with the trigger on FL-1 and a FL-1 threshold of 2, 500 units at a flow rate of 66 μL min^{-1}. Green fluorescence was collected in the FL-1 channel at 533nm and red florescence in the FL-3 channel at 670nm. No compensation was used. For the quantification of intact cells a fixed gate described previously (Gatza et al., 2013) was used as a template with slight adjustments on the low green end. All data processing was carried out using the Accuri C6 software and Microsoft Excel. All

experiments were performed in three independent repeats and each sample were measured in duplicate on the flow cytometer.

4.4 RESULTS

E. coli was subjected to two disinfection gradients of increasing intensity with bacterial aliquots not undergoing treatment serving as controls. Whereas exposure to increasing chlorine concentrations resulted in increasing damage to the cell membrane (as measured by increasing influx of the integrity stain propidium iodide; Fig. 4-1a), the killing by UV followed a different mechanism with no effect on cell envelope (Fig. 4-1b). In a next experiment we assessed the effect of the two disinfection gradients on the culturability of *E. coli* in the absence or presence of a coliphage for up to 4 days.

4.4.1 Fate of *E. coli* after chlorine disinfection

A bacterial suspension was exposed to chlorine concentrations up to 0.2 mg L^{-1} for 10 min. After neutralization of the disinfectant by addition of thiosulphate, we assessed the ability of the cells to grow on selective agar. Independent of whether a phage was added after neutralization of the disinfectant, strong growth was detected for bacteria exposed to chlorine concentrations up to 0.02 mg L^{-1} (Fig. 4-2). Exposure to a disinfectant concentration of 0.05 mg L^{-1} resulted in a substantial decrease in colonies on the filters, whereas higher chlorine concentrations did not allow any growth. In the absence of phage the bacterial density on filters increased over time for all samples where growth was initially seen directly after disinfection. For bacteria exposed to the two highest chlorine concentrations, the effect was sustainable with no growth detected even after 96 hours.

The outcome was different when a phage was added directly after disinfection. Bacterial densities were visibly reduced within 6 hours for samples exposed to chlorine concentrations up to 0.02 mg L^{-1}. The lytic activity of the phage seemed strongest for samples with no chlorine or with exposure to the lowest chlorine concentration. The density of bacteria exposed to 0.05 mg L^{-1} on the other hand did not decrease and bacterial numbers on the filters were comparable to the corresponding sample where no phage had been added. Also comparable to the samples without phage, the two highest chlorine concentrations sustainably eliminated all *E. coli* growth. Data suggest that cells that were exposed to 0.05 mg L^{-1} of chlorine were not lysed by the phage, but rather directly increased in numbers.

4.4.2 Fate of *E.coli* after UV disinfection

When exposing *E. coli* to UV-C radiation up to approx. 40 mJ cm^{-1} (which is the dose recommended for disinfection of drinking water, DVGW, 2006), bacterial densities on filters steadily decreased with increasing radiation intensity (both for samples where phage was absent or present). No colony was visible in the sample experiencing the highest UV dose. In the absence of a phage, bacterial densities on filters increased over time for all samples, even the one which did not show any growth directly after UV disinfection. The latter might be due to repair of UV damage over time or the replication of bacteria that survived UV treatment for example due to shading effects during stirring.

The outcome was identical for samples where a phage had been added after UV disinfection, with the control samples as the only exception. Whereas all bacterial suspensions experiencing UV treatment showed increasing colony numbers (equal to the

samples without phage), only the control sample that was not UV disinfected, showed a decrease in bacterial numbers in the first hours after phage addition. Results suggested that only bacteria that were not UV-treated could be lysed by the phage, whereas even low UV doses prevented phage-mediated lysis.

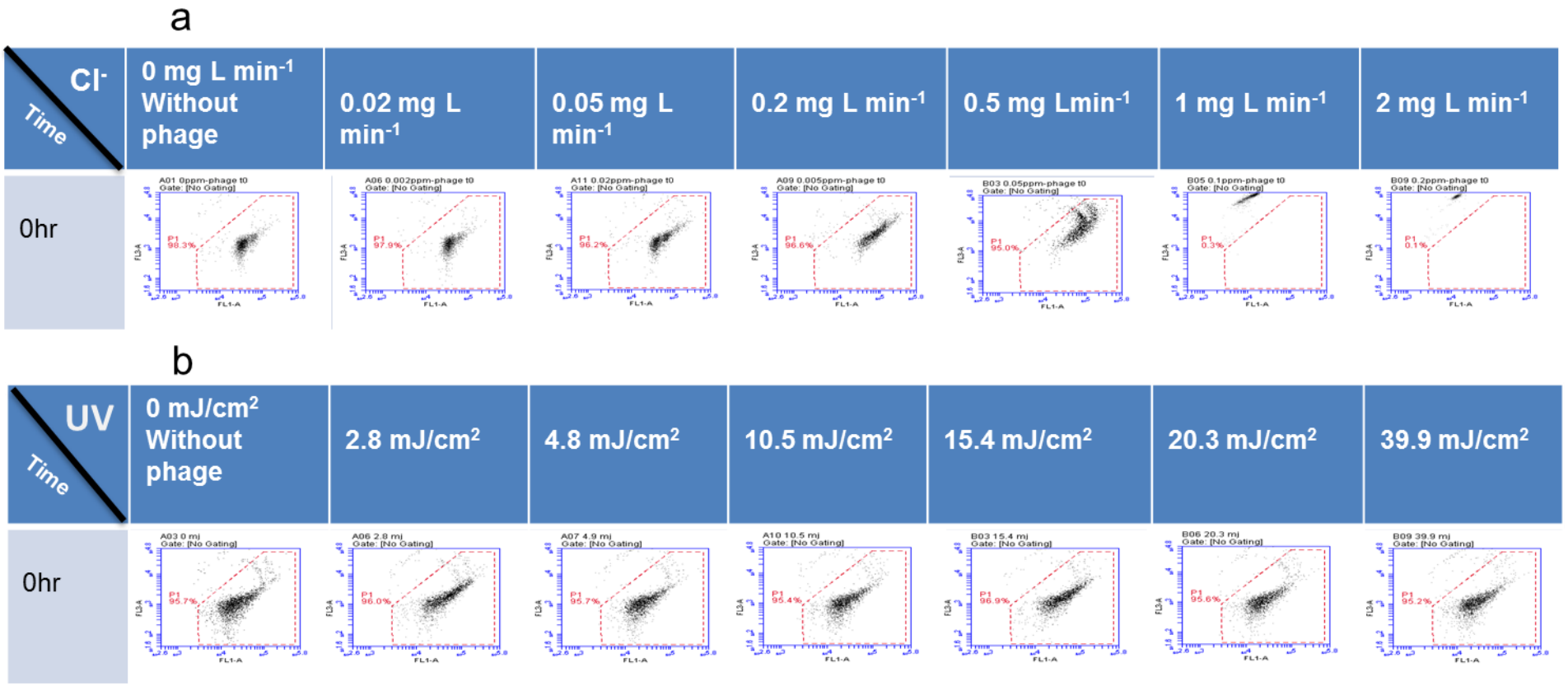


Figure 4-1 Flow cytometric dot blots showing images of (a) the susceptibility of *E. coli* (10^5 per mL) subjected to indicated chlorine concentrations followed by dechlorination and incubation at room temperature and (b) UV-LED exposed cell in the absence of bacteriophages at 0 h.

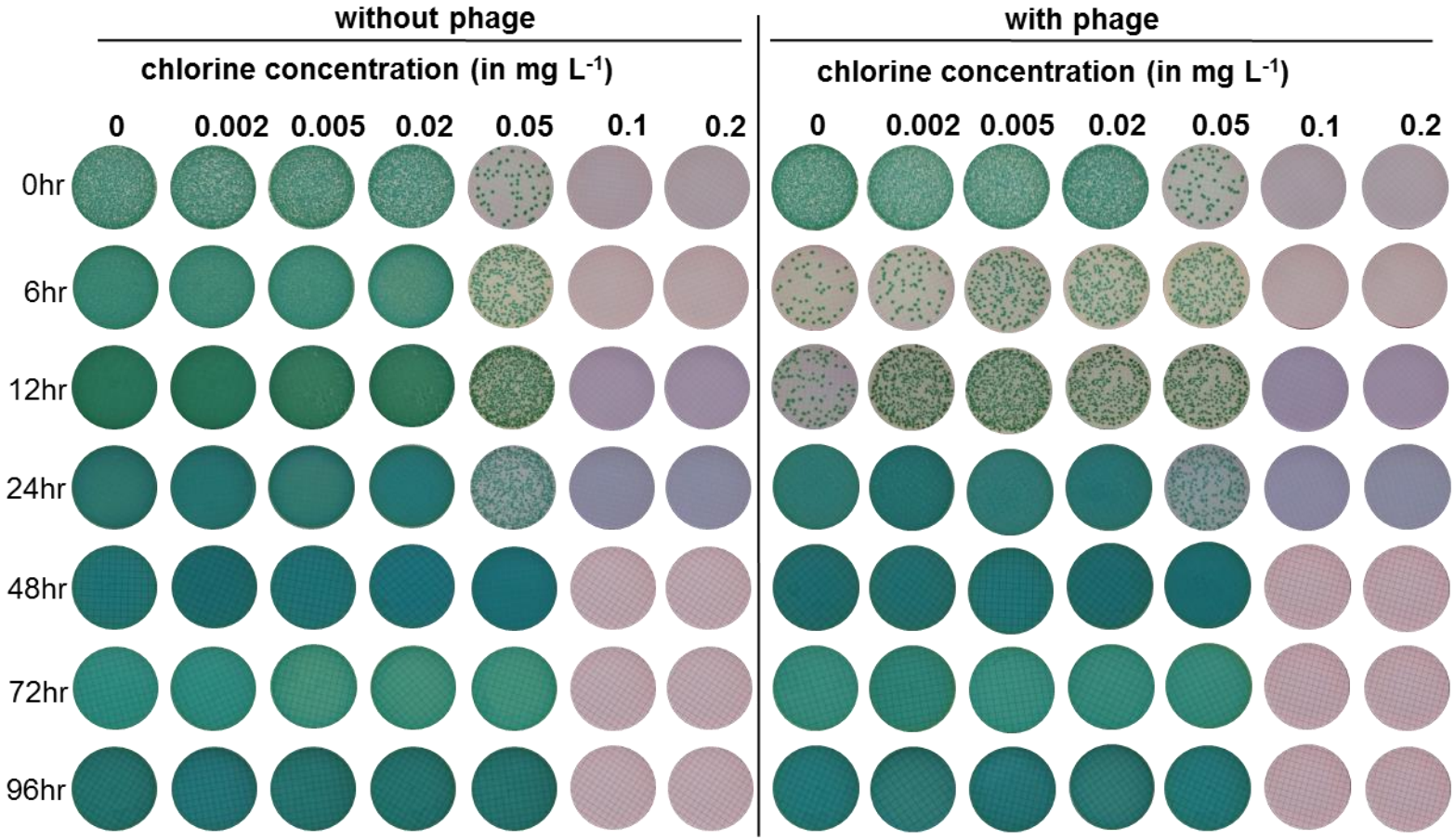


Figure 4-2 Culturability of *E.coli* exposed to different chlorine concentrations on MLGA plates in the absence and/or presence of bacteriophages. All plates were incubated at 35 ° C for 24 h and monitored for 96 h. *E. coli* exposed to no chlorine were strongly lysed within 6 h.

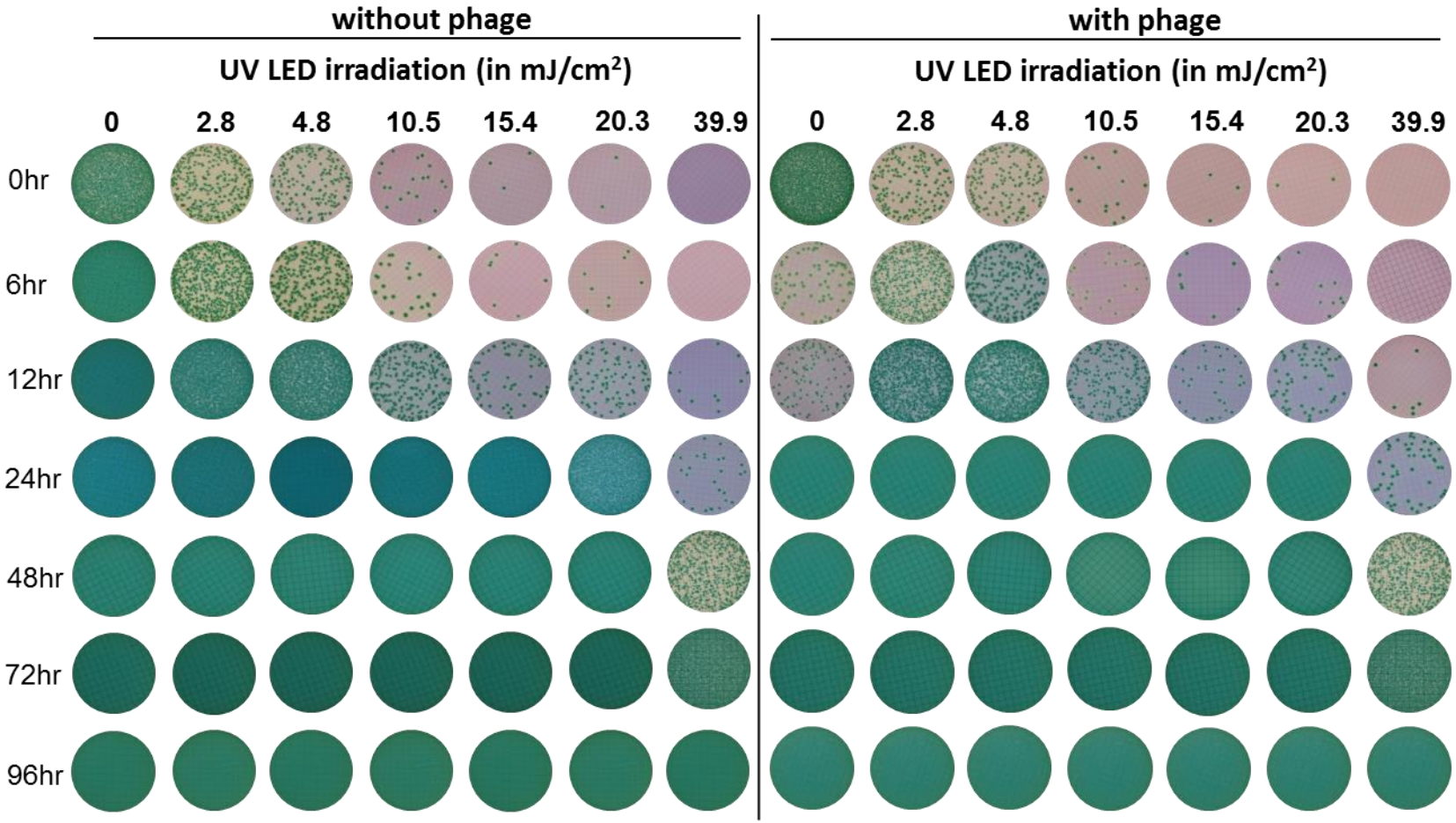


Figure 4-3 Culturability of *E.coli* exposed to different UV-LED doses in the absence and/or presence of bacteriophages on MLGA plates after incubation at 35 ° C for 24 h and monitored for 96 hours. Lysis was observed mainly on none UV exposed cells.

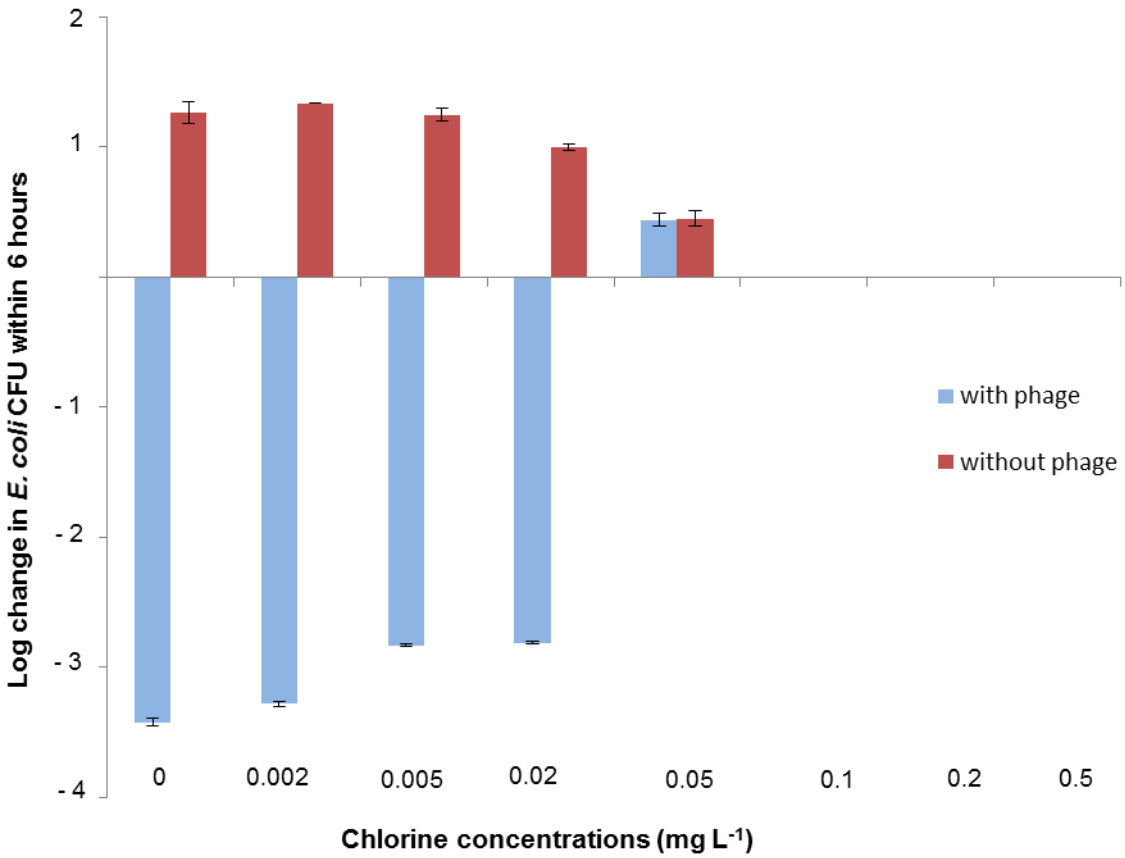


Figure 4-4 Log reduction of *E.coli* within 6 h after subjection to chlorine disinfection stress in the absence and presence of phage. A strong reduction in colony forming units (PFUs) was observed for samples that were not chlorinated in the presence of phage. Error bars are standard deviations from three independent repeats.

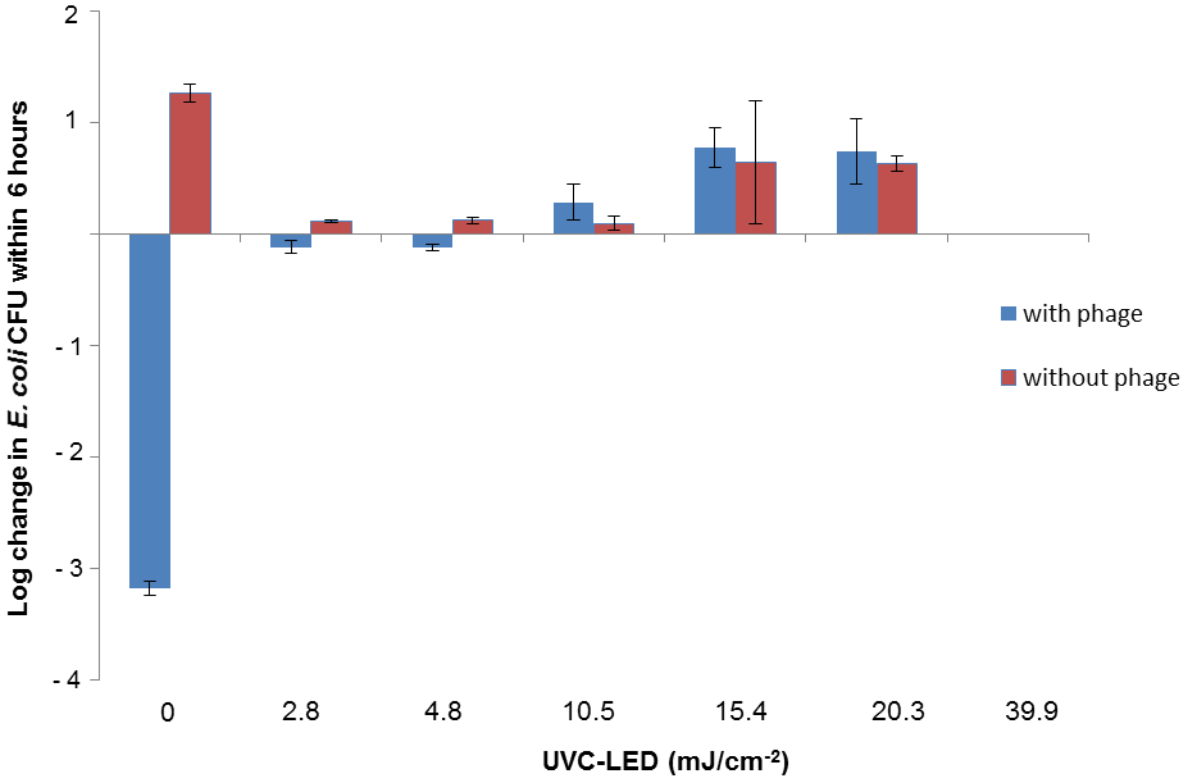


Figure 4-5 Log reduction of *E.coli* within 6 h after subjection to UV irradiation stress in the absence and presence of phage. A 3 log reduction in PFUs was observed for all samples not exposed to UV in the presence of phage. Error bars show standard deviations of three independent repeats.

4.4.3 Numerical change in bacterial cell numbers in absence and presence of phage

Numbers of bacteria in diluted samples were counted to calculate the change in colony forming units (CFU) within 6 hours representing the time to maximal lysis of the phage that was used. In the case of chlorine disinfection, the phage diminished bacterial numbers in samples exposed to chlorine concentrations up to 0.02 mg L^{-1} (Fig. 4-4). Bacteria in samples where no phage was added, on the other hand, increased in numbers due to cellular recovery and regrowth. The log change in CFU in the sample exposed to 0.05 mg L-1 was identical for the two experimental series (independent of whether a phage was present or not) and increased slightly. The result numerically suggests that the addition of a phage did not produce any difference, apparently due to the inability of the phage to lyse the cells.

In the case of UV disinfection, the log changes in bacterial numbers were comparable for all samples exposed to radiation (Fig. 4-5). The only sample where phage-mediated lysis occurred was the one that did not undergo disinfection. As in the case of the chlorine experiment, the phage could reduce bacterial numbers by slightly more than 3 log units. Already the exposure of the bacteria with a UV dose of 2.8 mJ cm^{-2} extinguished this effect with bacterial numbers being only slightly under the ones where no phage was added.

4.5 DISCUSSION

In this study we investigated how exposure of *E. coli* to disinfection gradients of increasing intensities affected its susceptibility to a selected lytic bacteriophage that has a time to maximal lysis of 6 hours (chapter 3). Effects on bacterial viability were shown

both visibly (in form of growth on filters) and numerically (after counting colonies). Chlorine and UV were chosen not only because they are commonly used in water treatment, but also because they have different mechanisms of action. Whereas chlorine as an oxidant causes damage to any cellular component it reacts with (including the cell envelope, enzymes, and nucleic acid (Camper and McFeters, 1979; du Preez et al., 1995), UV inactivates cells by inflicting damage to their nucleic acids (Clarke and Bettin, 2006; Chang et al., 1985; Vilhunen, Sarkka and Sillanpaa, 2009).

Independent of any phage, results of the two disinfection procedures clearly demonstrate a principal difference in the dose-response relationships between chlorine and UV. Although the chlorine concentration and the UV dose are not directly comparable, both disinfection strength gradients covered the range between sublethal to lethal. For chlorine, no dramatic effect was visible up to 0.02 mg L^{-1}. When however exceeding this concentration, a sharp decrease in culturability could be observed at 0.05 mg L^{-1} chlorine. A doubling of the critical concentration completely and sustainably suppressed growth. Chlorine thus produces a sharp decrease in bacterial viability, when exceeding a critical concentration. This is shared with other chemical disinfectants/biocides (Hoff and Geldreich, 1981; Benarde et al., 1965; Wilczak et al., 1996). For UV, the impact on viability was strongest at 0-4.8 mJ/cm^2 (the beginning of the gradient), followed by a more gradual decline in colony numbers with increasing UV doses. This inactivation behaviour is in consensus with modified first order kinetics described previously, Brahmi et al., (2010).

Another phage-independent difference between the two disinfection methods was the sustainability of the effect. Whereas no regrowth was observed in samples subjected to lethal chlorine concentrations (Fig. 4-4), samples exposed to a UV dose of 40 mJ cm^{-2}

showed a re-appearance of colonies after 12 hours until full regrowth was obtained after 72 hours (Fig. 4-5). In the case of chlorine, this finding suggests that once *E. coli* suffers lethal damage (at a Ct value of 1 mg L^{-1} min under the experimental conditions chosen), no recovery was possible over time under the chosen conditions. Cell numbers in samples with surviving *E. coli* directly after disinfection on the hand showed an increase, although they certainly must have suffered some degree of injury. This is in line with a study looking at recovery of chlorine-exposed *E. coli* in estuarine microcosms (Bolster et al. 2005; http://www.ncbi.nlm.nih.gov/pubmed/15926556). Authors concluded that when exposing *E. coli* to chlorine concentrations that cause cell injury (rather than death), numbers of culturable *E. coli* may subsequently increase. Cells that were exposed to a lethal chlorine dose on the other hand did not recover. As both studies did not employ single cell techniques, it cannot be said whether the increase in cell numbers originates from surviving cells (that were culturable directly after disinfection) or whether some of the regrowing cells were initially not culturable but rather recovered from injury. In the case of regrowing *E. coli* after UV disinfection, no conclusion is possible. Although regrowth is observed even in the sample where no colonies were observed directly after disinfection, growth might have derived from cells that were protected from the UV by shielding effects of the stirrer (Hejkal et al., 1979; Craik et al., 2002; Emerick et al., 1999; Qualls et al., 1983) and were not captured initially rather than by recovery of injured cells.

The main focus of this study was however, the susceptibility of bacteria after being exposed to stress. Consistent with a previous study of this research project (Chapter 2), where we reported an acquisition of a phage-resistant phenotype when exposing *E. coli* to temperature stress in the sublethal range (41 and 44°C), we identified conditions also with chlorine and UV, where bacteria did not undergo lysis. In the case of chlorine,

this applied to cells exposed to the critical chlorine Ct value of 0.5 mg L^{-1} min, in the case of UV even very low doses of radiation prevented cell lysis. This observation could be explained by different hypotheses. One reason for the loss in susceptibility to phage-mediated lysis might lie in the destruction of phage receptors on the bacterial surface as a result of the disinfection. This possibility especially applies to bacteria exposed to chlorine as oxidation might have damaged surface proteins. In the case of UV exposure, this explanation appears less likely as membrane integrity was not compromised (Fig. 4-1b). A more likely hypothesis is that the metabolic and biosynthetic capabilities of the UV-irradiated cells might be compromised to an extent where phage replication is no longer possible within the host cell. This is a result of the degeneration effect of the UV on the DNA proteins (Zimmer and Slawson, 2002).

The viability of the bacterial host has been reported previously to be an important determinant for the success of phage propagation. Awais et al. (2006) reported for *E. coli* 0157:H7 that only culturable cells allowed phage propagation, whereas nonculturable VBNC cells only allowed phage adsorption, but not proliferation. The VBNC state was induced by starvation of the cells in artificial seawater and sterilized distilled water for 48 days at 4 °C (http://www.ncbi.nlm.nih.gov/pubmed/16739971). This observation was upheld by a subsequent results by Fernandes et al. (2014; http://www.ncbi.nlm.nih.gov/pubmed/24055938) for *Salmonella*. When introducing a phage-based system to assess the viability of *Salmonella*. VBNC cells (produced by treatment with sodium hypochlorite, stock concentration of 5% (w/v) at 0.006% (w/v) working concentrations) were reported to be recognized by the phage, but no lysis was induced. Heat-killed cells in contrast were recognized only to a minor extent suggesting

a different underlying mechanism with phage receptors on the cell envelope possibly being destroyed by the heat.

For UV irradiation, the success of phage replication was not limited to culturability, but rather attributed to the presence of activity. Said et al. (2010; http://www.ncbi.nlm.nih.gov/pubmed/20351763) found that only active *E. coli* cells with metabolic activity support phage replication independent of whether they were culturable or not. Non-culturable cells that still show activity are often referred to as active but not culturable (ABNC; Hoefel et al., 2003; Kell et al., 1998). Cells without activity, on the other hand, allowed adsorption, but do not support lysis. We acknowledge for our study that only an overall effect on bacterial growth and recovery was tested. As pointed out by Said et al. (2010), a bacterial population that is subjected to disinfection stress presents a mixture of dead bacteria, culturable bacteria and viable but not culturable (VBNC) bacteria. Currently we cannot comment whether regrowth in the presence of phage originates from cells that were culturable also directly after disinfection or whether from cells that possibly underwent repair and regained culturability. In broad consent with the other authors, we conclude that phages sense the viability or vitality of their bacterial host, which in turn determines their bacteria's susceptibility and fate. Results lead to the question whether stressed bacteria that survive disinfection and show less susceptibility to phages might have an increased chance of survival in the environment where phages are one of the major causes of bacterial mortality.

4.6 CONCLUSIONS

We report here that chlorine and UV stress can modulate the bacteria's susceptibility to phage-mediated lysis. Whereas regrowth of untreated *E. coli* or cells that were exposed to low concentrations of chlorine disinfection was suppressed by the

presence of a lytic coliphage, regrowth rates of cell suspensions that were exposed to UV or a critical chlorine concentration were independent of the presence of phage.

4.7 ACKNOWLEDGEMENTS

This research was supported financially by the Commonwealth Scholarship scheme of the British government.

4.8 REFERENCES

Ameh, E. M., Tyrrel, S., Harris, J. A., Orlova, E. V., Ignatious, A., and Nocker, A. 2016. Effect of temperature on bacteriophage-mediated bacterial lysis efficiency with a special emphasis on bacterial temperature history (submitted)

American Water Works Association, 1999. Water quality and treatment. A handbook of community water supplies fifth edition. McGraw-Hill, Inc. New York, NY

Awais, R.,Fukudomi, H., Miyanaga, K., Unno, H., and Tanji, Y. 2006. A recombinant bacteriophage-based assay for the discriminative detection of culturable and viable but nonculturable *Escherichia coli* O157:H7. Biotechnol. Prog. 22, 853-859

Benarde, M. A., Israel, B. M., Olivieri, V. P., and Granstrom, M. I. 1965. Efficiency of chlorine dioxide as a bactericide. Applied Microbiology. Vol. 13, No. 5 776-780

Beuchat, L. R. 1992. Surface disinfection of raw produce. Dairy, Food, and Environmental Sanitation, 12, 6–9

Bolton, J., and Linden, K. 2003. Standardization of methods for fluence (UV dose) determination in bench scale UV experiments. J. Environ. Eng. 129:3, 209-215

Brahmi, M., Belhadi, N.H., Hamdi, H., Hassen, A. 2010. Modelling of secondary treated wastewater disinfection by UV irradiation: effects of suspended solids content. Journal of Environmental Science,22:1218–1224

Brussow, H., and Hendrix, R. W. 2002. Phage genomics: small is beautiful. Cell 108, 13-16

Chang, J. C., Ossoff, S. F., Lobe, D. C., Dorfman, M. H., Dumais, C. M., Qualls, R. G., and Johnson, J. D. 1985. UV inactivation of pathogenic and indicator organisms. Applied Environmental Microbiology, Vol. 49, No. 6, 1361-1365.

Charnock, C. & Kjønnø, O. (2000). Assimilable organic carbon and biodegradable dissolved organic carbon in Norwegian raw and drinking waters. *Water Research*. 34 (10). pp. 2629–2642. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0043135400000075.

Clancy, J. L., Hargy, T. M., Marshall, M. M., and Dyksen, J. E. 1998. UV light inactivation of Cryptosporidium oocysts. J. Am. Water Works Assoc. 90 (9), 92–102.

Clarke, S., Bettin, W. 2006. "Ultraviolet Light Disinfection in the Use of Individual Water Purification Devices", Defense Technical Information Center, http://www.dtic.mil/dtic/index.html

Clokie, M. R. J., Millard, A. D., Letarov, A. V., and Heaphy, S. 2011. Phages in nature. Landes Bioscience Review. Bacteriophages 1:1, 31-45

Craik, S. A., Amoah, D., and Smith, D. W. 2002. The impact of turbidity on Cryptosporidium and Giardia inactivation by ultraviolet light. Water Quality Technology Conference, American Water Works Association

Deborde, M., Gunten, U.V. 2008. Reactions of chlorine with inorganic and organic compounds during water treatment kinetics and mechanisms: a critical review. Water Res. 42 (1), 13-51.

DVGW (German Technical and Scientific Association for Gas an Water). 2006. UV device for the disinfection of the water supply. German standard W294

Emerick, R. W., Loge, F. J., Ginn, T., and Darby, J. L. 2000. Modelling the inactivation of coliform bacteria associated with particles. Water Environ Res .72:432-438

Emerick, R. W., Loge, F. J., Thompson, D., and Darby, J. L. 1999. Factors influencing ultraviolet disinfection performance—Part II - association of coliform bacteria with wastewater particles. Water Environ Res. 71:1178-1187

Fernandes, E., Martins, V. C., Nóbrega, C., Carvalho, C. M., Cardoso, F. A., Cardoso, S., Dias, J., Deng, D., Kluskens, L. D., Freitas, P. P., and Azeredo, J. 2014. A bacteriophage detection tool for viabiliity assessment for *Salmonella* cells. Biosensors and Bioelectronics. 52:239-246

Gatza, E., Hammes, F., Prest, E., 2013. Assessing water quality with the BD Accuri™ C6 flow cytometer. White paper BD Biosciences

Haas, C. N.1980. A mechanistic kinetic model for chlorine disinfection. Env. Sci. Technol. 14:339-340.

Haas, C. N., Karra, S.B. 1984. Kinetics of microbial inactivation by chlorine—II Kinetics in the presence of chlorine demand. Water Res. 18, 1451–1454.

Health Canada, 2009. Guidelines for Canadian Drinking Water Quality: Chlorine Guideline.

Heldal, M., and Bratbak.G. 1991. Production and decay of viruses in aquatic environments. Mar. Ecol. Prog. Ser.,72, 205-212

Hejkal, T. W., Wellings, F. M., LaRock, P. A., and Lewis, A. L. 1979. Survival of Poliovirus within organic Solids during chlorination. Applied and Environ Microbiol. 38(1):114-118

Hijnen, W.A.M., Beerendonk, E. F., Medema, G. J. 2006. Inactivation credit of UV radiation for viruses, bacteria and protozoa (oo)cysts in water: A review. Water research, 40:3-22

Hoefel, D. Grooby, W. L., Monis, P. T., Andrew, S., and Saint, C. P. 2003. Enumeration of water-borne bacteria using viability assays and flow cytometry: a comparison to culture-based techniques. Journal of Microbiological Methods, 55:585-597

Hoff, J. C., and Geldreich, E. E. 1981. Comparison of the biocidal efficiency of alternative disinfectants. J. Am. Water Works Assoc. 73:40-44

Hofmann, R., Andrews, B., and Lachmaniuk, P. 2004. Guidelines for ultraviolet disinfection of drinking water: consideration for Ontario. Journal of Toxicology and Environmental Health, Part A, 67, 1805-1812

Kell, D. B., Kaprelyants, A. S., Weichart, D. H., Harwood, C. R., and Barer, M. R. 1998. Viability and activity in readily culturable bacteria: a review and discussion of the practical issues. Antonie Van Leeuwenhoek 73, 169-187

Kruithof, J.C., Van der Leer, R.C., and Hijnen, W.A.M., 1992. Practical experiences with UV disinfection in The Netherlands. J Water SRT-Aqua 41 (2), 88–94

Labrie, J., Samson, E., and Moineau, S. 2010. Bacteriophage resistance mechanisms. Nature Reviews Microbiology. Vol. 8, 317-327 http://www.nature.com/nrmicro/journal/v8/n5/execsumm/nrmicro2315.html

LeChevallier, M.W., Evans, T.M., Seidler, R.J. 1981. Effect of turbidity on chlorination efficiency and bacterial persistence in drinking water. Appl. Environ. Microbiol. 42, 159-167

Le Dantec, C., Duguet, J. P., Montiel, A., Dumoutier, N., Dubrou, S., and Vincent, V. 2002. Chlorine disinfection of atypical mycobacteria isolated from a water distribution system. Appl. Environ. Microbiol. Vol. 68, 3:1025-1032

Lipphaus, P., Hammes, F., Kötzsch, S., Green, J., Gillespie, S., and Nocker, A.2014. Microbiological tap water profile of a medium-sized building and effect of water stagnation. Environmental Technology. 35 (5). 620–628

Mamane-Gravetz, H., Linden, K.G., 2004. Impact of particle aggregated microbes on UV disinfection. In: Proceedings of the American Water Works Association Water Quality Technology Conference, November 14–18, San Antonio, USA, 2004.

Morris, J., C. 1967. Kinetics of reactions between aqueous chlorine and nitrogen compounds, p. 23-53. In S. D. Faust and J. V. Hunter (ed.), Principles and application of water chemistry. John Wiley & Sons, New York

Murray, G. A., Eldridge, M. P. 1994. Marine viral ecology: incorporation of bacteriophages into the microbial planktonic food web paradigm. Journal of Plankton Research. Vol. 16, 6:627-641

Nescerecka, A., Rubulis, J., Vita, M., Juhna, T., Hammes, F. 2014. Biological instability in a chlorinated drinking water distribution network. PloS ONE 9(5): e96354. doi: 10.1371/journal.pone.0096354

du Preez, M., Kfir, R. and Coubrough, P. 1995. Investigation of injury of coliforms after chlorination. Water Sci Tech 31, 115-118

Prest, E. I., Hammes, F., Kötzsch, S., van Loosdrecht, M. C. M., Vrouwenvelder, J. S. 2013. Monitoring microbiological changes in drinking water systems using a fast and reproducible flow cytometric method. Water Research 47, 7131 - 7142

Proctor, L. M., and Fuhrman, J. A. 1990. Viral mortality of marine bacteria and cyanobacteria. Nature,343, 60-62

Proctor, L. M., and Fuhrman, J. A. 1992. Mortality of marine bacteria in response to enrichment of the virus size fraction from seawater. Mar. Ecol. Prog. Ser.,87, 283-293.

Örmeci, B., Linden, K.G. 2002. Comparison of UV and chlorine inactivation of particle and non-particle associated coliform, in: Water Science and Technology: Water Supply. 403-410.

Qualls, R. G., Flynn, M. P, and Johnson, J. D. 1983. The role of suspended particles in ultraviolet disinfection. J Water Pollu Control Fed. 55:1280-1285

Said, M. B., Masahiro, O., Hassan, A. 2010. Detection of viable but noncultiviable Escherichia coli after UV irradiation using a lytic Qβ phage. Ann Microbiol 60: 121- 127

Salvesen and Vadstein, O. 1995. Surface disinfection of eggs from marine fish: evaluation of four chemicals. Aquaculture international 3, 155-171

Shin, G., Linden, K. G., Arrowood, M. J., and Sobsey, M. D. 2001. Low-Pressure UV inactivation and DNA repair potential of Crystosporidium parvum Oocysts. Appl. Environ. Microbiol. 67(7), 3029-3032

SLMB, 2012. Determining the Total Cell Count and Ratios of High and Low Nucleic Acid Content Cells in Freshwater Using Flow Cytometry. Analysis Method 333.1, the Swiss Food Book (Schweizerische Lebensmittelbuch). Federal Office of Public Health, Switzerland

Sommer, R., Cabaj, A., Pribil, W., and Haider, T. 1997. Influence of lamp intensity and water transmittance on the UV disinfection of water. Water Sci. Technol. 35 (11-12), 113–118.

Templeton, M., Andrews, R. C., and Hofmann, R. 2004. Particle characteristics influencing the UV disinfection of drinking water. Water quality technology conference, American Water Works Association (AWWA)

United State Environmental Protection Agency, Office of Water. 2003. Ultraviolet disinfection guidance manual. EPA 815-D-03-007. Washington, D.C

Vilhunen, S., Sarkka, H., Sillanpaa, M. 2009. Ultraviolet light-emitting diodes in water disinfection. Environmental Science and Pollution Research International, Vol. 16, No. 4, 439-442.

Wang, L.K., Hung, Y.T., Shammas, N.K., 2005. Halogenation and disinfection. In: Wang, L.K., Yuan, P.C., Hung, Y.T. (Eds.), Handbook of Environmental Engineering, Physicochemical Treatment Processes, vol. 3. Humana Press Inc, Totowa, NJ, USA, 288-293.

Ward, R., Wolf, R., and Olson, B. 1984. Effect of pH, application ytechnique, and chlorine-nitrogen ratio on disinfection activity of inorganic chloramines with pure culture bacteria. Appl. Environ. Microbiol. Vol. 48:3, 508-514

Wattie, E., and C. T. Butterfield. 1944. Relative resistance of *Escherichia coli* and *Eberthellatyphosa* to chlorine and chloramines. Public Health Rep. 59:1661-1671.

Wilczak, A., Jacangelo, J. G., Marcinko, J. P., Odell, L. H., Kirmeyer, G. J., and Wolfe, R. L. 1996. Occurrence of nitrification in chloraminated distribution systems. J. Am. Water Works Assoc. 88:74-85

Zhang, X., Li, W., Blatchley, E. R., Wang, X., and Ren, P. 2015. UV/chlorine process for ammonia removal and disinfection by-product reduction: Comparison with chlorine. Water research, 68:804-811

Zhang, S., Ye, C., Lin, H., Lv, L., and Yu, X. 2015. UV disinfection induces a Vbnc state in *Escherichia coli* and *Pseudomonas aeruginosa*. Environmental Science and Technology, 49, 1721-1728

Zhang, S. and Farber, J. M. 1996. The effects of various disinfectants against *Listeria monocytogenes* on fresh-cut vegetables. Food Microbiology, 13, 311–321

Zimmer, J. L., Slawson, R. M. 2002. Potential repair of Escherichia coli DNA following exposure radiation from both medium and low UV sources in drinking water treatment. Appl. Environ. Microbiol. 68: 2393-2399

CHAPTER 5 THESIS DISCUSSION

The potential advantages of the use of bacteriophages as versatile and natural antimicrobial agents are numerous, (Loc-Carrillo and Abedon 2011; Curtright and Abedon 2011). Above all they are safe for humans and are environmentally friendly when compared with conventional infection-control agents. (Gill and Hyman 2010). Overall bacteriophages are highly effective, relatively cost effective (affordable) antibacterial agents that can be easily obtained and can be used both in clinical (phage therapy) and environmental (biocontrol) applications. The use for biocontrol purposes was the main focus of this project. Phages producing big, medium and small plaques were referred to as B, M and S with all three presenting dsDNA phages belonging to the order *Caudovirale* and *Myoviridae* family (Chapter 2, Table B 1). These phages were used in this research.

The research involved determining the efficiency and potential use of lytic bacteriophages as biocontrol agents against bacteria of interest. Although, the research was performed with a clinical isolate of *E. coli* (ATCC 25922 which is mainly for studies on antibiotics resistance), no pathogens were utilized for this study for safety reasons. Nevertheless, the results obtained might hold true for real pathogens. The project aimed at addressing three obvious research gaps: the impact of environmental factors on phage performance (with temperature been chosen as example), the comparison of a monovalent and multivalent phage application and the question whether bacteria subjected to sublethal disinfection are still susceptible to phage-mediated lysis. Thus, the present project was carried out in a series of phases to address the overall objectives.

In regard to the diagnostic methods employed to answer the research questions, the conventional plaque overlay method (which is time consuming and laborious) was

supplemented with rapid measurement of cell densities with a plate reader and cultivation-independent flow cytometer (FCM). Especially FCM proved useful because of its low detection limit and high degree of sensitivity (approx. 100 cells per mL) compared to the microplate reader.

In chapter 2, the research question was how does temperature (as selected environmental parameter) influences phage-mediated lysis efficiency? Whereas temperature is well accepted to strongly impact bacterial growth and metabolic activities, its influence on phage-host interactions still has unsolved questions. The aim of our study was to improve our knowledge of how temperature affects phage lysis. For all three isolated phages used in this study the plate incubation temperature affected both plaque size and numbers and thus the effectiveness of bacteriophage performance as stated in chapter 2 of this thesis. Efficient phage performance was surprisingly found at temperatures below that of the host's optimal growth temperature. Our findings agree with similar observations made previously with dairy starter strains where the optimal lysis temperature was lower than the optimal growth temperature of the bacterial host (Hunter, 1943; Sanders and Klaenhammer, 1980; Murata, 1971; Zehren and Whitehead, 1954). The screening for suitable phages should always be performed below the temperature conditions relevant for the subsequent application to avoid suboptimal results or complete failures (Mohany *et al*., 2011).

Whereas the previous observations refer to the incubation temperature of the phage-host mixture, this study looked also into the effect of exposure of the two individual players (phage and bacteria) to different temperatures prior to mixing. Exposure of the phages to different temperatures before addition to bacteria did not affect lysis efficiency (probably as long as virus stability was maintained). The temperature history of fully

grown host (prior to phage encounter) on the other hand, was found to have a substantial impact on the lysis performance of the phages. Optimal lysis was observed when the host was grown at room temperature and incubation of the phage-host mix also at room temperature. A further experiment on the effect of temperature on the fully grown host indicated that exposure to temperature stress in the sublethal range (41 and 44°C) resulted in a phage-resistant phenotype. One reason that could be attributed to the observation lies in the temperature-dependent expression of surface antigens (Orskov, Sharmat and Orskov, 1984) which can serve as phage attachment sites. Also an involvement of the bacterial heat shock regulon could potentially play a role as suggested in a different context by Wiberg et al., 1988. Unfortunately time constraints did not allow us to look further into this aspect. Further research will be necessary to find answers to the effect of heat shock on the expression of *E. coli* surface structures needed for phages attachment. It will furthermore need more experimental data whether this observation also hold true for other phage-host interactions than those studied here and potentially to other stresses (like nutrient starvation, pH and osmotic pressure).

Temperature has been reported to affect phage adsorption, penetration and, multiplication (Jonczyk *et al*., 2011) and phage performance is strongly dependent on bacterial physiology and metabolism which in turn depends on temperature (Carvalho *et al*., 2012).As phages do not have a metabolism of their own and therefore no temperature-dependent expression of surface structures, factors determining lysis efficiency might be more limited than for the bacteria although phages seem to be highly adapted to the local conditions in their environment (Vos et al., 2009; Koskella et al., 2011), phages might have an in-built temperature preference for reversible and irreversible adsorption and replication once mixed with bacteria.

Chapter 3 of this study looked at the lytic performance of individual phages and combinations thereof with the goal of determining the best possible approach to delay the emergence of bacteria resistance. Bacterial resistance to phages might be considered the greatest hurdle in the success of phage biocontrol application in the environment. The lytic efficiency of the three phages producing big, medium and small plaques on solid medium was found to be reflected also in the liquid suspension and found to be in the order $B \geq M \geq S$. The lytic efficiency of the individual phages was further compared with the one of multiple phages that were applied either simultaneously in form of a cocktail or sequentially at different time points in regard to lysis rate and the sustainability of this effect.

Our results suggest that for the phages used in this study the application of multiple phages simultaneously in a cocktail fashion was more effective in delaying the emergence of bacterial resistance than the use of a single phage, thus confirming the work of other authors (O'Flynn et al., 2004; Gu et al., 2012; Kelly et al., 2011; Chan, Abedon and Loc-Carrillo, 2013; Jaiswal et al., 2013; Pirnay et al., 2011; Betts et al., 2013). However, also the multiphage approach does not eliminate bacterial resistance entirely which is also in agreement with many previous researchers (Hill, 1993; Hall et al., 2012; Summers, 2002, O'Flynn et al., 2004; Smith and Huggin, 1982; Alisky et al., 1998; Fischer et al., 2013; Turki et al., 2012). Although, our work did not study the defence mechanism in bacteria, Labrie et al. (2010) reviewed in detail the viral resistance mechanisms in bacteria under the headings; 1) preventing phage adsorption (change of surface attachment site structure, production of extracellular matrix and production of competitive inhibitors) 2) preventing viral DNA entry. Further studies will be needed to engineer cocktail with the highest possibility of suppressing bacterial resistance.

Alternative to adding multiple phages simultaneously in the form of a cocktail, different phages can be added in a sequential manner (Hall et al., 2012). Some of the sequential combinations in our study had both an advantage in regard to initial bacterial clearance and the effect was more long-lasting compared to the performance of the phage cocktail, which showed an intermediate performance in regard to fast initial clearance and sustainability of this effect. According to our study, to obtain a favourable results in the application of a multiphage approach the following criteria may be necessary; 1) selection of suitable individual phage to be used, 2) knowledge of the different phage lysis kinetics (time to maximum lysis efficiency), 3) proper timing of the application in line with their time to maximum lysis rate. One of the most important conclusions of our experimental data is that fast initial lysis comes at a cost of relatively poor sustainability. If bacteria get lysed fast, also resistance emerges fast. If it takes longer to lyse the bacteria, then typically it also takes longer for resistance to emerge. While our study did not work further with resistant bacteria populations, several studies have indicated that natural phages are well-adapted to their environment and local bacterial population (Vos et al., 2009; Koskella et al., 2011) and that bacteria in turn adapt to resist their local phages (Kunin et al., 2008; Koskella, 2013). Other authors see the phage resistant trait as transient and not permanent as a result of the fitness cost (manifested as reduced growth or virulence) it incurred on the bacteria, that such bacteria can become susceptible to phage lysis in the future (Scanlan et al., 2015).

In contrast to many other studies, we based our study on a bacteria to phage ratio (= multiplicity of infection) of 1. This choice was done with the reasoning that high MOIs tend to result in greater lysis and suppress bacterial resistance more effectively, which in turn maybe counterproductive when examining the influence of different parameters on

exactly those traits. Whereas the choice of such suboptimal conditions suggested to serve this research purpose better, it may on the other hand have limited our development of understanding of phage performance at other MOIs under the otherwise same conditions. This especially applies to the emergence of bacterial resistance. In the assessment of three bacteriophages for the biocontrol of *Salmonella* of wastewater in the form of a cocktail, Turki et al. (2012) reported that the emergence of a resistant bacterial population was observed when the bacteria were infected with a mixture of phages at lower MOIs of 1 or 2. The authors went further to report that a higher MOI a sustained reduction of *Salmonella* was obtained as also agreed by O'Flynn et al. (2004), who studied the evaluation of a cocktail of three bacteriophages for the biocontrol of *Escherichia coli* O157:H7. It will be increasingly crucial to thoroughly understand the interactions between phages and their bacterial hosts in order to fully exploit their antimicrobial potential in the environmental settings as well as other fields of application. For example, in the laboratory typically one strain of high bacterial density and high nutrient availability is present, whereas in the real-world is opposite is highly likely. While it must also be stated that some laboratory conditions can be successfully implemented in the real environment, for real applications it is important to go beyond the laboratory and try to understand why a phage infects one strain but not the other.

Chapter 4 of this research focused on the question was whether bacteria subjected to sublethal disinfection were still amenable to phage-mediated lysis. For this purpose, *E. coli* were exposed to chlorine or UV disinfection gradients of increasing strength to determine their susceptibility to the best performing phage in this study (phage B showing a time to maximum lysis of 6 hours). Effects on bacterial viability were shown by cultivation on MLGA plates and colony counting. These disinfection methods were

chosen for two reasons: 1) their mode of action are different, chlorine inactivates the cells by membrane damage while UV damages the DNA (Chang, et al., 1985). 2) they are commonly used as disinfectants in the water and food industries.

For the chlorine disinfection a dose of 1 mg L^{-1} completely inhibited culturability (cells were completely inactivated or viability is lost). For the UV disinfection, the impact on viability decreased with time (first order kinetics). This behaviour corroborates results of Brahmi et al., 2010.

E. coli subjected to disinfection of critical chlorine Ct value of 0.5 mg L min^{-1} manifested a phage-resistant phenotype, while in the case of UV disinfection already minimal doses prevented cell lysis. The emergence of a phage-resistant phenotype upon exposure to sublethal chlorine and UV doses confirmed our previous study that exposure of bacteria to sublethal heat stress at 41 or 44 °C equally resulted in phage resistance (Chapter 2). The 'full' viability of bacteria therefore seems crucial for the successful applications of phage biocontrol. This observation is also validated by Awais et al. (2006). The authors studied a recombinant bacteriophage-based assay for the discriminative detection of culturable and viable and nonculturable *Escherichia coli* O157:H7. They reported that for nonculturable cells (VBNC and dead), phages were able to adsorb to them, but lysis was not induced. They went further to state that VBNC and dead cells were differentiable based on nutrient uptake over a period of prolong incubation (21 days). They concluded that VBNC cells were able to grow in size by becoming elongated and easily recognizable from dead cells. One limitation of our study was the fact that we did not try to identify the VBNC cells as reported by Said et al. (2010). The author and colleagues reported that VBNC cells are present in UV treated water together with dead cells and viable cells. The same authors went further to state that VBNC cells are still

susceptible to phage-mediated lysis. Fernandes et al. (2014) who induced a VBNC state in *Salmonella* cells on the other hand reported that phages adsorbed to VBNC *Salmonella* cells but did not induce lysis. A similar observation was also made by Awais et al., 2006 on *E. coli* O157:H7. The correlation of bacterial viability and the susceptibility to phages will requird much further research.

Phage application for environmental biocontrol aimed at eliminating pathogenic bacteria is gaining importance. The abundance of phages in the environment makes it a relatively simple task to isolate and purify phages against any given pathogen. Currently many pathogenic bacteria have acquired multidrug resistance posing a serious medical problem. Phages, when properly and carefully selected, offer the most cost-effective alternative to antibiotics. Nevertheless some hurdles remain to be overcome including public perception of viruses and regulations regarding safety and efficacy of phage applications. Many experts are of the opinion that applications of phages for environmental biocontrol will find a place in the future.

5.1 REFERENCES

Alisky, J., Ichzkowski, K., Rapoport, A., and Troitsky, N. 1998. Bacteriophages show promise as antimicrobial agents. J. Infect. 36:5–15.

Awais, R.,Fukudomi, H., Miyanaga, K., Unno, H., and Tanji, Y. 2006. A recombinant bacteriophage-based assay for the discriminative detection of culturable and viable but nonculturable *Escherichia coli* O157:H7. Biotechnol. Prog. 22, 853-859.

Betts, A, Vasse, M., Kaltz, O., and Hochberg, E. M. 2013. Back to the future: evolving bacteriophages to increase their effectiveness against the pathogen *Pseudomonas aeruginosa* PAO1. Evol Appl. 6:1054-63; PMID: 24187587

Carvalho, C. M., Santos, S. B, Kropinski, A. M., Ferreira, E. C., and Azeredo, J. 2012. Phages as therapeutic tools to control major foodborne pathogens: *Campylobacter* and *Salmonella.* In: Bacteriophages. I Kurtboke (ed.). InTech. Available from: http://www.intechopen.com/books/bacteriophages/phages-as-therapeutic-tools-to-control-the-major-foodborne-pathogens-campylobacter-and-salmonella.

Chan, B. K., Abedon, S. T., Loc-Carrillo, C. 2013. Phage cocktails and the future of phage therapy. Review Future Microbiol. Vol 8(6), 769-783

Chang, J. C., Ossoff, S. F., Lobe, D. C., Dorfman, M. H., Dumais, C. M., Qualls, R. G., and Johnson, J. D. 1985. UV inactivation of pathogenic and indicator organisms. Applied Environmental Microbiology, Vol. 49, No. 6, 1361-1365.

Curtright, A. J., and Abedon, S. T. 2011. Phage therapy: Emergent property pharmacology. Journal Bioanalysis and Biomedicine. S6: 002.

Fernandes, E., Martins, V. C., Nóbrega, C.,Carvalho, C. M., Cardoso, F. A., Cardoso, S., Dias, J., Deng, D., Kluskens, L. D., Freitas, P. P., Azeredo, J. 2014. A bacteriophage detection tool for viability assessment of *Salmonella* cells. Biosensors and Bioelectronics 52:239-246.

Fischer, S., Kittler, S., Klein, G., and Glünder, G. 2013. Impact of a Single Phage and a Phage Cocktail Application in Broilers on Reduction of *Campylobacter jejuni* and

the Development of Resistance. PLos ONE 8(10): e78543. Doi:10.1371/journal.pone.0078543

Gill, J. J., and Hyman, P. 2010. Phage choice, isolation, preparation for phage therapy. Curr. Pharm. Boitechnol. 11, 2-14

Gu, J., Liu, X., Li, Y., Han, W., Lei, L. et al. 2012. A method for generation of phage cocktail with great therapeutic potential. PLos ONE 7(3):e31698

Hall, A. R., De Vos, D., Friman, Ville-Petri., Pirnay, Jean-Paul., and Buckling, A. 2012. Effects of sequential and simultaneous applications of bacteriophages on populations of Pseudomonas aeruginosa in vitro and wax moth larvae. Appl. Environ. Microbiol 78:5646-5652

Hill, C. 1993. Bacteriophage and bacteriophage resistance in lactic acid bacteria. FEMS Microbiol. Rev. 12:87-108

Hunter, G. J., 1943. Bacteriophages for *Streptococcus cremoris* phage development at various temperatures. J Dairy Res. 13:136-145.

Jaiswal, A., Koley, H., Ghosh, A., Palit, A., and Sarkar, B. 2013. Efficacy of cocktail phage therapy in treating Vibrio choleae infection in rabbit model. Microbes Infect 15: 152-156. http:/dx.doi.org/10.1016/j.micinf.2012.11.002 (accessed Feb., 2016).

Jończyk, E., Kłak, M., Międzybrodzki, R., and Górski, A. 2011. The influence of external factors on bacteriophages—review. Folia Microbiol. 56**:**191–200.

Kelly, D., McAuliffe, O., O'Mahony, J., and Coffey, A. 2011. Development of a broad-host-range phage cocktail for biocontrol. Bioeng. Bugs 2(1), 31-37

Koskella, B. 2013. Phage-mediated selection on microbiota of a long-lived host. Curr. Biol. 23, 1256-1260.

Koskella, B., Thompson, J. N., Preston, G. M., and Buckling, A. 2011. Local biotic environment shapes the spatial scale of bacteriophage adaptation to bacteria. Am. Nat. 177, 440-451. Doi: 10.1086/658991

Kunin, V., He, S., Warnecke, F., Peterson, S. B., Garcia Martin, H., Haynes, M., et al. 2008. A bacteria metapopulation adapts locally to phage predation despite global dispersal. Genome Res. 18, 293-297

Loc-Carrillo, C., and Abedon, S. T. 2011. Pros and cons of phage therapy. Bacteriophage, 1:2, 111-114, DOI: 10.4161/bact.1.2.14590

Mahony, J., McAuliffe, O., Ross, P. R., and van Sinderen, D. 2011. Bacteriophages as biocontrol agents of food pathogens. Current Opin. Biotechnol. 22:157–163.

Murata, A. 1971. Temperature-sensitive growth of wild-type phage J1 of *Lactobacillus casei*. Agric. and Biol. Chem.35:667

O'Flynn, G., Ross, P. R., Fitzgerald, G. F., and Coffey, A.2004. Evaluation of a cocktail of three bacteriophages for biocontrol of *Escherichia coli* O157:H7. Appl. Environ. Microbiol. 70:3417–3424.

Orskov, F., Sharmat, V., and Orskov, I. 1984. Influence of Growth Temperature on the Development of *Escherichia coli* Polysaccharide K Antigens. Journal of General Microbiology. 130, 2681-2684

Pirnay, J.P., De Vos, D., Verbeken, G., Merabishvili, M., Chanishvili, N., Vaneechoutte, M., Zizi, M., Laire, G., Lavigne, R., Huys, I., Mooter, G. V., Buckling, A.,

Debarbieux, L., Pouillot, F., Azeredo, J., Kutter, E., Dulanchet, A., Górski, A., and Adamia, R. 2011. The phage therapy paradigm: pret-a-porter or sur-mesure? Pharm. Res. 28, 934-937. http://dx.doi.org/10.1007/s11095-010-0313-5

Said, M. B., Masahiro, O., Hassan, A. 2010. Detection of viable but noncultiviable Escherichia coli after UV irradiation using a lytic Qβ phage. Ann Microbiol 60: 121- 127

Sanders, M. R., and Klaenhammer, T. R. 1980. Restriction and modification of group N streptococci: effect of heat on development of modified lytic bacteriophage. Appl. Environ. Microbiol. 40:500-506.

Scanlan, P. D., Buckling, A., and Hall, A. R. 2015. Experimental evolution and bacterial resistance: (co)evolutionary costs and trade-offs as oppurtunities in phage therapy research. Bacteriophage Vol. 5(2), e1050153

Smith, H. W., and Huggins, M. B**.** 1982. Successful treatment of experimental *Escherichia coli* infections in mice using phage: its general superiority over antibiotics. J. Gen. Microbiol. 128:307–318

Summers, A., O**.** 2002. Generally overlooked fundamentals of bacterial genetics and ecology. Clin. Infect. Dis. 34:S85–S92.

Turki, Y., Ouzari, H., Mehri, I., Ben Ammar, A., and Hassen, A. 2012. Evaluation of a cocktail of three bacteriophages for the biocontrol of *Salmonella* of wastewater. Food Research International 45: 1099-1105

Vos, M., Birkett, P. J., Birch, E., Griffiths, R. I., and Buckling, A. 2009. Local adaptation of bacteriophages to their bacterial hosts in soil. Science. Vol.325, 833

Wiberg, J. S., Mowrey-McKee, M. F, and Stevens, E. J. 1988. Induction of the heat shock regulon of *Escherichia coli* markedly increases production of bacterial viruses at high temperatures. J. Virol. 62:234-245.

Zehren, V. L., Whitehead, H. R. 1954. Growth characteristics of streptococcal phages in relation to cheese manufacture. Journal of Dairy Science. Vol. 37(2), 209-219

CHAPTER 6 OVERALL CONCLUSIONS

On the whole, the present work has met all its set out objectives. The use of bacteriophages as natural biocontrol agents has been demonstrated using *Escherichia coli* ATCC 25922 as a model organism. The major findings/conclusions are provided in relation to the objectives stated in the section 1.6 AIM AND OBJECTIVES.

Objective 1: To isolate and purify lytic bacteriophages specific for *E. coli* from environmental water

- Three different bacteriophages were isolated and purified from surface water receiving treated wastewater using the double agar overlay method
- Plaque size varied in phages from a treated wastewater

Objective 2: To study the effects of temperature on host cell and phages in regards to lysis susceptibility/efficiency

- Plaque sizes were affected by the incubation temperature of the phage-host mix and the temperature history of host cells (pre-incubation)
- Temperature stress in the sublethal range resulted in a phage-resistant phenotype

Objective 3: To compare the lysis efficiency of different phages

- Lysis efficiency was correlated with plaque size
- The lytic efficiency was in the order $B \geq M \geq S$

Objective 4: To establish more efficient diagnostic tools for other than plaque counts to monitor phage-mediated lysis efficiency

- Both the FCM and microplate reader were used as rapid monitoring tools for following phage lysis kinetics

- The FCM had a high degree of reliability in monitoring phage lysis efficiency thus reducing the time required to obtain a result.

Objective 5: To correlate kinetics of distinct environmental phages with the plaque sizes they produce

- Fast lysis rates were recorded by the big size producing phages, this was followed by the medium size and lastly the small size plaque producing phage
- All the three purified phages have different time to attain their maximal lysis. The big plaque producing phage take approximately 6 hours, the medium size plaque producing phage take 12 hours, while the small plaque size producing phage take about 18 hours

Objective 6: To compare the effect of sequential or simultaneous addition of multiple distinct with different lysis kinetics

- Adding phages as cocktail did not totally eliminate bacterial resistance to phages
- Some sequential phage applications for example $B_0M_6S_{18}$ and $S_0M_{18}B_{30}$ produced a more sustainable suppression of resistance than the cocktail

Objective 7: To study the lysis susceptibility of bacteria subjected to sublethal disinfection stress

- Bacteria subjected to a critical concentration threshold of chlorine of 0.5 mg L min^{-1} resulted in a phage-resistant phenotype
- A minimal dose of UV eliminated host susceptibility to phage lysis

6.1 FUTURE RECOMMENDATIONS

All of the evidence presented in this thesis strongly shows that the objectives of our study have been achieved, and that further experiments may be recommended using our environmentally isolated phages. Below are the specific future recommendations.

- Host range determination. Further testing should be carried out on other strains of *E. coli* and other enterobacteria to determine if the phages can infect more than one single strain of *E. coli* or whether they have a broad spectrum of lytic activity.
- Another area of possible applications of the phages for further studies is the potential utilization of phage in the context of water system against a range of pathogen of interest. Especially those that are known to easily form biofilms such as *Pseudomonas aeruginosa* and *Legionella*. These organisms (which are opportunistic) can be found in drinking water supply and are known to be resistance to chemical disinfection (Anaissie et al., 2002).
- Morphologically our isolated phages have been characterized by the plaque size they produced, and TEM show that they all belong to the family of *Myoviridae*. Further characterization of their genetic material may be necessary to determine whether their genomic size is responsible for the features exhibited by the phages. The genetic characterization can be carried out as described by Maniatis et al. (1982).
- All our experiments were carried out based on the host/phage ratio of 1. A variation of the multiplicity of infection (MOI) in future subsequent studies is highly recommended.
- One area of further research to combat the threat of resistance may be to use the phage protein that is responsible for bursting open the bacteria releasing it content

and new phages (phage progeny). This phage protein is called “lysin or endolysin”, the idea is that instead of lysing the cell from the inside as is the case following infection of bacterial cells phages, lysins can be applied to the outside of the host cells. This “unnatural” form of attack may be so new that bacteria may be unable to build a defence against it. If tested and proven, the prospect of lysin as antimicrobial agent will be boosted.

- A further investigation may be needed to study the response of *E.coli* receptors used by phages to heat shocks.

6.2 REFERENCES

Maniatis, T. J., Sambrook, and Fritsch, E. F. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Habor, N. Y

Anaissie, E. J., Penzak, S. R., and Dignani, M. C. 2002. The hospital water supply as a source of nosocomial infections: a plea for action. Archives of Internal Medicine 162(13), 1483-1492

APPENDICES

Appendix A Bacteriophage isolation protocol

Background

Basically all types of cell are vulnerable to virus infection; virus diseases in plants and animals can at most time result in death. Viruses that infect bacteria are known as *bacteriophages*, or simply *phages (Greek: phagein = eat)*, because they generate a *plaque*, on a lawn of susceptible bacteria. When bacteria lyse, a great number of newly produced phages are released. Bacteriophages consist of nucleic acid genome (RNA or DNA) protected by a protein structure called capsid.

This protocol describes the isolation of lytic bacteriophages from environmental samples, their propagation and storage.

Required materials:

Isolation:

EQUIPMENT:	**SUPPLIES:**
Sterile 50 mL Falcon tubes, wire loop, Bunsen burner,	Double strength TSB (2X TSB), 1M $CaCl_2$ or $MgCl_2$, fresh overnight culture of bacteria (such as Pseudomonas aeruginosa) Pre-warmed (30°C) nutrient agar plates (TSA plates supplemented with 3 mM $CaCl_2$)

Propagation:

EQUIPMENT:	**SUPPLIES:**
Sterile 50 mL Falcon tubes, wire loop, hot block (set at 45 °C to 48°C), vortex, Bunsen burner, Pasteur pipettes, glass culture tubes with 3 mL of pe-aliquoted top agar (0.75%)	○ SMG buffer (saline-Mg-gelatine) or SM buffer ○ 1 M $CaCl_2$ ○ TSA top agar with 3 mM $CaCl_2$ and 3 mM $MgCl_2$; prepare 3 mM aliquots ○ TSA petri dishes supplemented with 3 mM $CaCl_2$ and 3 mM of $MgCl_2$ (add 3 mL of 1 M stock solutions to 1 L of TSA before autoclavation)

Pre-preparations

TSA plates with supplements (bottom agar)

Prepare 1 L of TSA following the manufacturer's instructions. Let cool down to approx., 60˚C and add 3 mL of sterile 1M $CaCl_2$ and 3 mL of sterile 1 M $MgCl_2$. Final concentrations: 3 mM of $CaCl_2$ and $MgCl_2$. Mix thoroughly and pour immediately. Dispense approx. 20 mL of medium per plate (to completely cover the plate). Let cool and dry. As the humidity content is very important, plates should not be too dry, but also not too humid. If not used immediately, store at 4˚C (plates can be stored for 2-3 months).

Top agar with supplements

As the top agar has a different agar concentration from the bottom agar, it is convenient to prepare the media from broth formulations rather than agar formulations. Agar is added to the desired concentration (0.4-0.6%).

Prepare 100 mL of TSB following the manufacturer's instructions. Stir until completely dissolved. Add 0.5 g of agar while stirring. Autoclave and let cool down a little. Add 0.3 mL of sterile 1M $CaCl_2$ and 0.3 mL of sterile 1 M $MgCl_2$. Final concentrations: 3 mM of $CaCl_2$ and $MgCl_2$.

Mix thoroughly and aliquot in a sterile manner into glass culture tubes (3 mL aliquots). Cap to avoid contamination.

Let solidify. Vials should be stored at 4˚C (stored for approx. 2 months).

SMG buffer (saline-Mg-gelatine)

Recipe per L:

NaCl (MW = 58), 5.8g	final concentration: 100 mM
$MgSO_4 .7H_2O$ (MW 246.47), 2g	final concentration: 8.1 mM
Tris-HCl (1 M, pH 7.5), 50 ml	final concentration: 50 mM
Gelatine, 0.1 g	final concentration: 0.01%

Add water to 1 L, autoclave and store at room temperature

1. Phage isolation from environmental water

Sample collection: Environmental water is collected in a 50 mL Falcon tube (or any other suitable flask or bottle) and stored on ice when not processed on the same day. The collection bottle does not necessarily have to be sterile (as the sample is not sterile…).

Removal of bacteria: The water is filtered through a 0.22 µm membrane filter. The flow-through is collected in a sterile 50 mL Falcon tube.

The flow-through should be free of bacteria and other microorganisms and should only contain bacteriophages.

Note: bacterial culture and phage enrichment are prepared according to standard microbiological methods.

Prepare in advance:

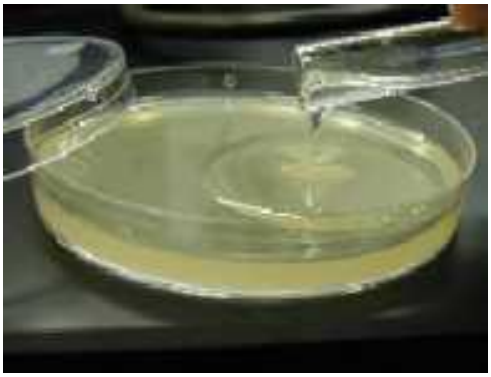
- Remove the required number of **top agar tubes** (3 mL aliquots) from the cold room. Melt agar (microwave?) and place in a water bath or heat block set at 46-48˚C.
- Pre-warm bottom agar plates before use. When plates are cold: place at room temperature for 20 min before warming them up to 30˚C for > 20 min before use. Make sure that the plates are completely dry without condensation, but not overly dry.

Phage adsorption to cells:

- Place 7 sterile 1.5 ml microcentrifuge tube in a rack. Label the lid of the tubes "Adsorption"
- Add 100 µl of phage suspensions (from above) and 100 µl of bacteria (overnight culture) to each tube. Total volume: 200 µl.
- Add 1 µl of 1M $CaCl_2$ stock solution to obtain a final concentration of 5 mM. The presence ofdivalent cations enables the adsorption of phages to bacteria.
- Mix thoroughly by gently flicking the tube
- Incubate for 15-20 minutes: during this time the phages adsorb to the bacteria.

Overlay technique:

- Use a P1000 pipette to aseptically transfer the phage/bacteria mixture (200 µl) to a 3 mL soft agar aliquot (46 – 48^{o}C)
- Mix immediately by modest vortexing.
- Pour the soft agar containing phages and bacteria onto the hard agar plate and spread evenly by gentle shaking.
- Put the plate on a level surface until the soft agar solidifies (this usually takes 30 minutes).
- Incubate plate overnight at 30^{o}C. Invert plates for incubation (to allow nutrient flow by gravity).



Example of pouring of soft agar

3. Isolation of bacteriophages for purification

- Use a Pasteur pipette to pierce the agar surrounding the plaque and transfer the "plague" into a microfuge tube
- Add 1 ml of SMG buffer and one or two drops of chloroform to separate phages and remaining bacteria (and to lyse bacteria)
- Centrifuge this at 2,000 RPM for 5 minutes (low speed is used to avoid damage to the phages). Most of the remaining bacterial debris will be pelleted. The top portion suspension caintain the phages.
- Increase volume with SMG buffer to 5 mL.

- Make a 5 ml storage tube for the collection of phage supernatant as it is filtered. Then pipette the supernatant into a 5-10ml syringe fitted with a 0.45 µm membrane filter. Carefully slide the plunger, allowing the flow-through to drip into the storage tube. This process get rid of the remaining bacteria from the phage suspension. The resulting solution is now the phage stock.

4. Re-isolation of bacteriophages

Background:

When isolating phages from environmental water or soil samples, it is crucial to realize that the phage numbers may consist of several phage variants with a common characteristic; they all produced clearing on the host used in the double agar overlay technique. Therefore, it significant to purify phages since a plaque may consist of more than one type of phage. Purity can be obtained via multiple re-isolation.

For re-isolation repeat steps 2-3 of the above protocol. The phage suspension from isolated plaques should be diluted 10-fold prior processing. To ensure purity, re-isolation should be performed 2 – 3 times.

5. Storage

After isolation as above the phage suspension can be stored at 4^{o}C or less and is available for further experiments. Phage suspensions can alternatively be frozen for long-term storage.

Typical storage times:

4^{o}C: 2 – 3 months

-20^{o}C: 5 years

-80^{o}C: 20 years

6. Determination of titre

The phage titre can be determined by diluting a phage solution in SM buffer or SMG. Appropriate dilution is spotted on a top agar plate with the appropriate host. After incubation for 12 to 15 hours at 20 or 30ºC the plaques derived from a single phage is counted and the plaque forming unit (PFU) per ml can be calculated. For example if the 10^{-9} dilution spot resulted in the formation of 10 plaques and 100 µl was used. The calculation will be as follow:

10 PFU/100 µl x 1000 µl/ 1 ml x 10^{9} = 1.0 x 10^{11} PFU/ml

Appendix B Protocol for phage stocks

Background:

A glycerol stock of microorganisms in microbiology is a way of long-term preservation of microbes considered relevant for science education and research training.The conservation of stock is necessary for genetic resources and providing the foundation for many biotechnological advancement. The objective is to maintain microorganism and to prevent genetic drift that occurs when the organism is maintained indefinitely in an actively growing state. The following protocol is a general guideline to on how to prepare and maintain a phage stock.

A: picking a single plaque

B: confluent lysis (elution)

Required materials:

a. sterile 50 ml and 15 ml Falcon tubes
b. sterile toothpicks and forceps
c. Bunsen burner
d. Chloroform
e. Sterile glycerol
f. incubator shaker
g. phage buffer
h. TSA plates

A: Picking a single plaque

Steps:

1. Grow 10 mL overnight culture of the bacterial host at an appropriate temperature (approx. 15 hours). For environmental phages grow bacteria for example at 25°C.
2. Inoculate bacterial suspension with phage: carefully remove an isolated plaque using a sterile toothpick. Use flamed forceps to handle the toothpick. Drop the toothpick with attached phage material into the bacterial culture from step 1.
3. Shake at 25°C in an incubator. The culture should completely lyse in 12 hours although it might still be slightly turbid due to cell debris.
4. Add 10 drops of chloroform and mix thoroughly to kill remaining bacteria and to denature bacterial debris.
5. Transfer volume into 15 mL Falcon tubes (as no centrifuge is available for 50 mL tubes).
6. Spin at 5,000 rpm for 5 minutes to remove cell debris. If lysate is of very high titer (10^{10} – 10^{12}) it should appear slightly “milky”.
7. Decant the phage rich supernatant into new Falcon tube. Be careful not to transfer chloroform.
8. Divide lysate into two portions, store one at 4°C and the other at -80°C. For the latter a cryoprotectant has to be added. Add sterile glycerol to a final concentration of 20% (4 mL phage lysate and 1 mL glycerol).

B: Confluent lysis (elution)

Steps

1. Plate between 10^4 and 10^5 phage on soft agar (even 10^6)
2. Incubate at 30°C for about 6 – 8 hours (or until confluent lysis occurs)
3. Add 5 mL of buffer to the plate and keep it in the cold room overnight (sealed the plate with parafilm to prevent accidental spilling
4. The following morning, collect the buffer using a pipette from the plate, and add 50 µL of chloroform and centrifuge at 5,000g for 5 minutes
5. Transfer the supernatant to a fresh and sterile tube and add about 25 µL of chloroform again. If things work well you should get a titre of more than 10^{10} pfu per mL
6. Store the lysate at 4°C for subsequent use.

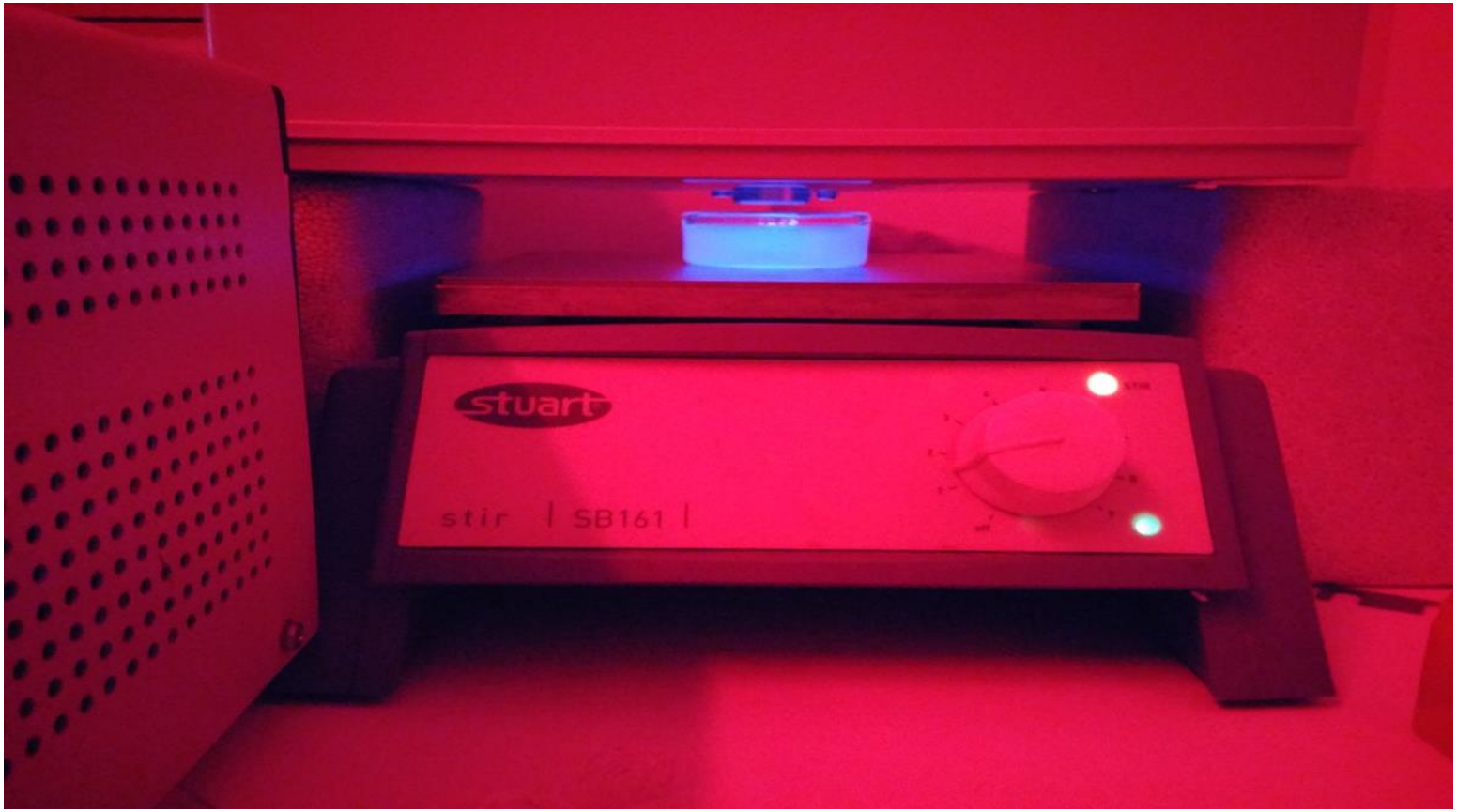


Figure B-1 UV-LED Experimental set up. The UV-LED system consist of a UVCLEAN lamp, which has a multi-chip arrangement of LEDs packed in a metal and glass casing. The wavelength of the UV light emitted by the LEDs was at 256 nm having a power output of 14 mW.

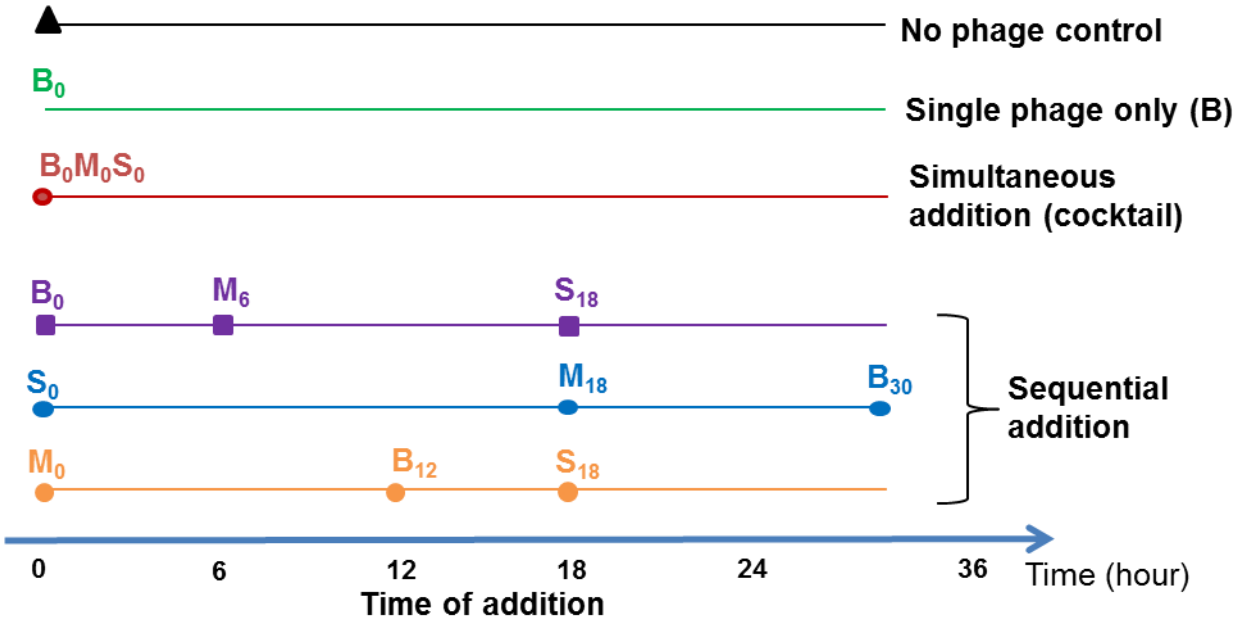


Figure B-2 Experimental strategy to add phages to an *E. coli* suspension. Subscript numbers indicate the timepoint in hours at which a particular phage was added. Time zero indicates the beginning of the experiment. In sequential additions, phages 2 and 3 (either B, M or S as the case may be) were always added at the time point of maximal lysis of the previous phage.