Characterisation and UV inactivation of bacteriophages infecting human-specific *Bacteroides* strain GB-124

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ABSTRACT

The pathogens contained in human faecal material can present a significant risk to human health, when discharged into surface waters. A recently isolated strain of *Bacteroides* spp., (GB-124) has shown good ability to detect viruses (phages) in waters exclusively contaminated by human faecal material, with widespread geographical applicability, proving a low-cost microbial source tracking tool (MST).

However, little is known about the morphology, ecology and inactivation of phages capable of infecting strain GB-124 during UV-B (representing the primary germicidal portion of sunlight) and UV-C radiation (a common tertiary disinfection stage in wastewater treatment). Therefore in order to address this knowledge gap, a total of twenty phages were isolated from municipal wastewater and characterised using transmission electron microscopy (TEM) and host range assessment. Ultraviolet radiation (UV) inactivation kinetics were determined using two phases of laboratory-based collimated beam experiments using UV-B and UV-C wavelengths.

TEM demonstrated that all phages were members of the *Siphoviridae* family, exhibiting little morphological variation. Results of Phase One collimated beam experiments were found to be more variable than those from Phase Two, with higher fluences required to achieve 4-$\log_{10}$ reductions in phage densities. 4-$\log_{10}$ phage reductions were achieved in Phase One experiments by mean fluences of 49 mJ/cm^2 (UV-C) and 540 mJ/cm^2 (UV-B), whereas Phase Two experiments required mean fluences of 36 mJ/cm^2 (UV-C) and 320 mJ/cm^2 (UV-B). All phages followed first-order inactivation kinetics. Comparison of Phase One and Phase Two experiments showed that the method used in Phase One may not be robust enough to quantify accurately inactivation fluences. This data supports the adoption of the standardised UV protocol used during Phase Two experiments.

This research demonstrates for the first time that B124 phages appear to be a homogenous group in both morphological and ecological terms. This observation supports their use as an indicator of human faecal material in surface waters. Comparisons with published data showed that B124 phages have greater resistance to UV radiation than many pathogenic viruses and bacteria and may therefore be used in QMRA as surrogates for enteroviruses.

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DECLARATION

I declare that the research contained in this thesis, unless otherwise formally indicated within the text, is the original work of the author. The thesis has not been previously submitted to this or any other university for a degree, and does not incorporate any material already submitted for a degree.

Signed.....

Dated.....

CHAPTER ONE

1. INTRODUCTION

1.1 Aim

The aim of the following study was to fill critical knowledge gaps regarding the use of bacterial viruses (bacteriophages or phages) as indicators of human faecal material within aquatic systems. A recently isolated *Bacteroides* strain (GB-124) has been shown to detect phages exclusively in human faecal material, and in waters subject to human faecal contamination, but no research has been undertaken into the ecology, or homogeneity of the phage group. If phages infecting GB-124 are to be used within Microbial Source Tracking (MST) studies, in Quantitative Microbial Risk Assessment (QMRA), or as surrogate organisms of selected pathogens, their behaviour within waterbodies and throughout wastewater treatment should be assessed. This study elucidates the morphology and ecology of phages infecting GB-124 and describes their inactivation characteristics during UV-B (representing the primary germicidal portion of sunlight) and UV-C radiation (a common tertiary disinfection stage in wastewater treatment).

This introductory chapter describes the risks associated with microbially-contaminated drinking and recreational waters, and some of the dominant pathogens responsible for the global water-related disease burden. This is followed by an overview of the concept of MST and the role that it can play in waterborne disease hazard management.

1.2 Waterborne disease burden

This study was undertaken in the context of global efforts to increase provision of improved drinking water supply, especially in less economically developed countries (LEDC). Provision of drinking water containing levels of pathogenic microorganisms low enough to avoid infectious disease, is an essential requirement for human wellbeing. Moreover, access to a secure, clean drinking water source is widely considered to be a basic human right (WHO, 2003a). Through bathing, drinking, and other domestic uses, water is a highly effective vehicle by which human populations can be exposed to disease causing pathogens. Approximately 1.8 million fatalities (90 % of which are among under 5 year olds in developing countries) per annum are caused by diarrhoeal diseases. 88 % of these fatalities are thought to be caused by inadequate sanitation, poor hygiene and unsafe water (Anon, 2008a).

Although harder to quantify, the economic impacts of poor water quality are also considerable; it has been estimated (Given *et al*., 2006) that US $21 to $51 million is lost annually in southern California as a result of swimming-related gastroenteritis resulting from exposure to (faecally) contaminated waters.

At the United Nations Millennium Summit in 2000, the UN set eight Millennium Development Goals (MDG) to be achieved by 2015. Two of these relate directly to health impacts of poor water quality, and others are considered to be related indirectly. Goal Four aims to reduce child mortality by two thirds (deaths are mainly attributed to water-related illness) and Goal Seven aims to reduce the number of people without access to safe drinking water by 50% from 2000 levels. Adoption of these goals reflects the emphasis placed on human illness and disease related to, or

associated with, water of poor microbial quality.

There is great disparity in the quality of drinking and recreational waters between LEDC and more economically development countries (MEDC). Illness resulting from ingestion of waterborne pathogens (primarily bacterial, protozoan and viral) in MEDC was dramatically reduced during the 20th Century, mainly as a result of chlorination of drinking water and advances in wastewater treatment (Perceval *et al*., 2004). However, in LEDC, drinking water disinfection is not as widespread, delivery systems are not as intensively maintained, and wastewater treatment is poor or absent (especially in rural areas). By 2050, it is estimated that the global population will be 8.9 billion, with population decreases in MEDC and population increases in LEDC (Anon, 2004a). Poorly funded, and less well-organised water infrastructures in LEDC are likely to come under greater pressure, putting many more lives at risk.

Outbreaks of disease from drinking water in MEDC have been reported (Short, 1988; Stanwell-Smith, 1994), as have outbreaks resulting from the use of recreational waters (Sellwood and Dadswell, 1991; Palmateer *et al*., 1991; Fleisher, *et al*., 1996), but the latter are more difficult to substantiate, with freshwater usage being more closely linked to viral outbreaks than seawater (Percival *et al*., 2004). Infections resulting from contaminated drinking water include *Shigella* spp., *Campylobacter* spp., and those resulting from recreational water use include *Pseudomonas* spp., and *Legionella* spp. (Moe 1997). Contaminated shellfish may also cause subsequent infection in humans (Scoging, 1991).

Estimates of waterborne disease outbreaks in MEDC are often contested: in the US, it has been estimated that 7,100,000 people (range 400, 000 to 27, 000, 000) annually contract mild infections, whilst 560,000 (520,000 to 690,000) contract moderate to severe infections with an annual mortality of 1200 (Morris and Levin, 1995). Other estimates include 627,800 to 1,479,200 people annually contracting gastroenteritis at beaches in Los Angeles (USA; Given *et al*., 2006)). The wide range shown for each of these two figures indicates the difficulty of accurate estimation. However, what is apparent is that water-borne diseases represent a significant burden on health and a barrier to economic growth and development.

1.3 Waterborne pathogens

In poorly treated wastewaters, and waters used for drinking and recreation, there may be present a wide variety of pathogenic organisms capable of causing infectious disease (Table 1.1) and it is estimated there may be more than 100 different types of pathogenic viruses in waterbodies polluted by human faecal material (Melnick and Gerba, 1980; Havelaar *et al*., 1993; Leclerc *et al*., 2000). Providing they are not inactivated, pathogens shed by infected persons during bathing, bowel movements or the washing of soiled bed sheets and clothing (Moe, 1997), may be transported through surface and sub-surface aquatic systems until they come into contact with a new host.

Waterborne infections are those caused by ingestion of faecally contaminated water, physical contact with faecally contaminated water (through bathing or other water-based activity) or consumption of food that has been exposed to faecally contaminated water (Hurst, 1997). In MEDC, secondary treatment of wastewater will not inactivate or completely remove viruses, but as shown in Table 1.1, chlorination is an effective means of disinfection. As a result of poor water treatment infrastructure and reduced disinfection of drinking water, pathogens may be present in significant numbers in both drinking and recreational waters in LEDC. Rural areas in LEDC may not be connected to the mains water network and access to safe, clean water may be limited. Such water scarcity may bring unsuitable water resources into use.

Table 1.1 Pathogens associated with waterborne disease and illness (adapted from Percival *et al*., 2004 and Moe 1997)

Organism type		Selected symptoms	Transmission	Risk mitigation
Viral	Adenovirus	Diarrhoea, respiratory illness, gastroenteritis, rash, cystitis and conjunctivitis dependent on serotype. Often asymptomatic.	Faecal-oral through recreational and possibly drinking water.	Chlorination and UV.
	Astrovirus	Mild or asymptomatic. May cause acute gastroenteritis, diarrhoea, mild fever, nausea and vomiting.	Faecal-oral.	Chlorination.
	Poliovirus, coxsackievirus and echovirus (together known as enterovirus)	Highly variable. Fever, malaise, headache, muscle ache, meningitis, neurological disease and conjunctivitis.	Faecal-oral and secretory. Found in all water types polluted by human sewage.	Chlorination, sodium hypoclorite, formaldehyde, gluteraldehyde and UV.
	Hepatitis A and E viruses	HAV - Mainly asymptomatic. Jaundice, malaise, fever, nausea and vomiting. HEV - Mainly asymptomatic. Jaundice, hepatomegaly, malaise, fever, nausea and vomiting. May cause fatality during pregnancy.	Faecal-oral through recreational and drinking water.	Vaccine (HAV only) and chlorination.
	Norovirus and sapovirus	Gastroenteritis, projectile vomiting, diarrhoea and fever.	Faecal-oral through recreational and drinking water. Aerosolization of vomit.	Chlorination.
	Rotavirus Group A and B	Severe diarrhoea (young children), vomiting and fever, acute gastroenteritis.	Faecal-oral.	Chlorination, ozone, and UV.
	Calicivirus	Acute gastroenteritis.	Human faeces.	
Bacterial	*Acinetobacter*	Septicaemia, uninary tract infections, skin infections, eye infections, meningitis, pneumonia, and endocarditis.	Found in the aquatic environment.	Chlorination (though pathogen can develop resistance).
	Aeromonas	Gastroenteritis and diarrhoea.	Found in the aquatic environment.	Chlorination decreases densities, but may not completely eliminate populations.
	Arcobacter	Diarrhoea and stomach cramps.	Found in the aquatic environment.	Chlorination.
	Campylobacter	Acute diarrhoea, bloody/mucoid faeces.	Faecal-oral through contaminated water.	Chlorination.

Table 1.1 (cont.) Pathogens associated with waterborne disease and illness (adapted from Percival *et al*., 2004 and Moe 1997)

Organism type		Selected symptoms	Transmission	Risk mitigation
Bacteria (cont.)	*Cyanobacteria*	Dermatitis, gastroenteritis, and toxins produced may cause death although this has not been reported.	Found in the aquatic environment, especially eutrophic lakes.	Prevention by reducing nutrients is best action.
	Escherichia coli	Diarrhoea, dysentery, meningitis, and in some cases acute kidney failure and death.	Contaminated water.	Chlorination and other disinfectants.
	Helicobacter pylori	Mostly asymptomatic, but may lead to peptic ulcer disease and gastric cancer.	Thought to be contaminated drinking water.	Not known.
	Legionella spp.	Pontiac fever and Legionnaires disease.	Found in the aquatic environment.	Heat and chlorine.
	The *Mycobacterium avium* complex	Pulmonary disease and cervical lymphadentis.	Found in the aquatic environment.	Chlorination.
	Salmonella spp.	Gastroenteritis, enteric fever and septicaemia.	Faecally contaminated water.	Chlorination.
	Shigella spp.	Dysentery, fever and malaise.	Faecal-Oral.	Chlorination.
	Vibrio cholerae	Asymptomatic to deadly. Vomiting and diarrhoea.	Found in surface and drinking water where disease is endemic.	Chlorination.
	Yersina spp.	Gastroenteritis, fever and diarrhoea.	Faecal-Oral.	Chlorination.
Protozoan	*Acanthamoeba* spp.	Fatal granulomatous and eye infections.	Present in the aquatic environment.	Not known.
	Balantidium coli	Usually asymptomatic, diarrhoea, nausea and dysentery.	Feacal-Oral.	Boiling.
	Cryptosporidium spp.	Diarrhoea, abdominal pain, fever, nausea and vomiting.	Present in the aquatic environment.	Ozone and UV.
	Cyclospora cayeyanensis	Explosive diarrhoea, nausea, vomiting, cramps and fatigue.	Present in wastewater.	Coagulation and filtration during water treatment.
	Entamoeba histolytica	May be asymptomatic. Fever, dysentery, diarrhoea.	Faecally contaminated water.	Hyperchlorination and iodination.
	Giardia duodenalis	May be asymptomatic. Nausea, malaise, diarrhoea and vomiting.	Faecal-oral through contaminated drinking/recreational waters.	Chemical disinfectants.

The number of organisms required to cause illness (termed the infectious dose) varies between pathogens. The infectious dose also varies from person to person, depending on the individual's immune response and general health. Viruses and protozoa generally have low infectious doses: often, fewer than fifty infectious units, oocysts, cysts or plaque forming units (Moe, 1997) are required to cause illness. For some enteric viruses, the infectious dose may be as low as one plaque forming unit (Percival *et al*., 2004). In terms of microbial water quality, this low infectious dose and environmental persistence makes them of great concern; as any waterborne virion is a potential hazard (Berg, 1967). For other enteric bacterial pathogens, infectious doses of 10^2 to 10^8 CFU/ml have been reported (Moe, 1997).

The consequences of infection by waterborne pathogens range from asymptomatic to fatal (Table 1.1). Mild to severe gastroenteritis is common, as are skin and other infections, diarrhoea (leading to dehydration), and conjunctivitis. Rotavirus infection (via faeco-oral route) is the most common cause of gastroenteritis worldwide (Parashar *et al*., 1998; Oh *et al*., 2003). Although the majority of water-related disease outbreaks are minor, some waterborne pathogens are deadly; enteric adenoviruses (serotypes 40 and 41) kill 50% of immuno-compromised individuals infected, whilst hepatitis E virus (HEV) has been reported as having a high mortality rate (around 30%) in pregnant women in LEDC (Hunter, 1997). Quantification of water-related illness is a problem in both MEDC and LEDC. Moreover, it is likely that the majority of waterborne disease outbreaks are not reported. As a result, it is often difficult to directly relate illness and water exposure, and targeted epidemiological studies are often needed in order to do so.

1.4 Pollution source and routes of transmission

The source and transmission routes of waterborne pathogens vary greatly. The main route of infection is faecal-oral (Percival *et al*., 2004), though as Figure 1.1 illustrates, the route from shedder to susceptible host may be highly complex. Pathogens may be transmitted by human-to-human contact (e.g., contact with faecally contaminated hands), ingesting food contaminated by either faeces or faecally contaminated water, or faecally contaminated fomites. However, the most important pathway with regards to this study is human reservoir → wastewater → surface water → domestic use/recreation → new human host. Amongst numerous other factors, the rate and success of faeco-oral transmission depends on the number of shedders in a catchment (determines density of pathogens in water), infectious dose required to initiate illness and exposure to the contaminated water source. These data help in the formulation of Quantitative Microbial Risk Assessments (discussed further in Section 2.4).

Faecal material within waterbodies close to human settlements is primarily derived from two sources, point source [PS; e.g., wastewater treatment works (WwTW) effluents – mostly human origin and usually attributable to a single outlet] or diffuse source (DS; e.g., field runoff – livestock origin, tending to be a composite of numerous smaller inputs dispersed over a greater area). Contamination of recreational and resource waters by human or animal faecal material may promote the transmission of bacterial, protozoan and viral pathogens, with human and animal faecal material presenting different health risks to water users (Jofre *et al.*, 1986; Araujo *et al.*, 1997a, 1997b).

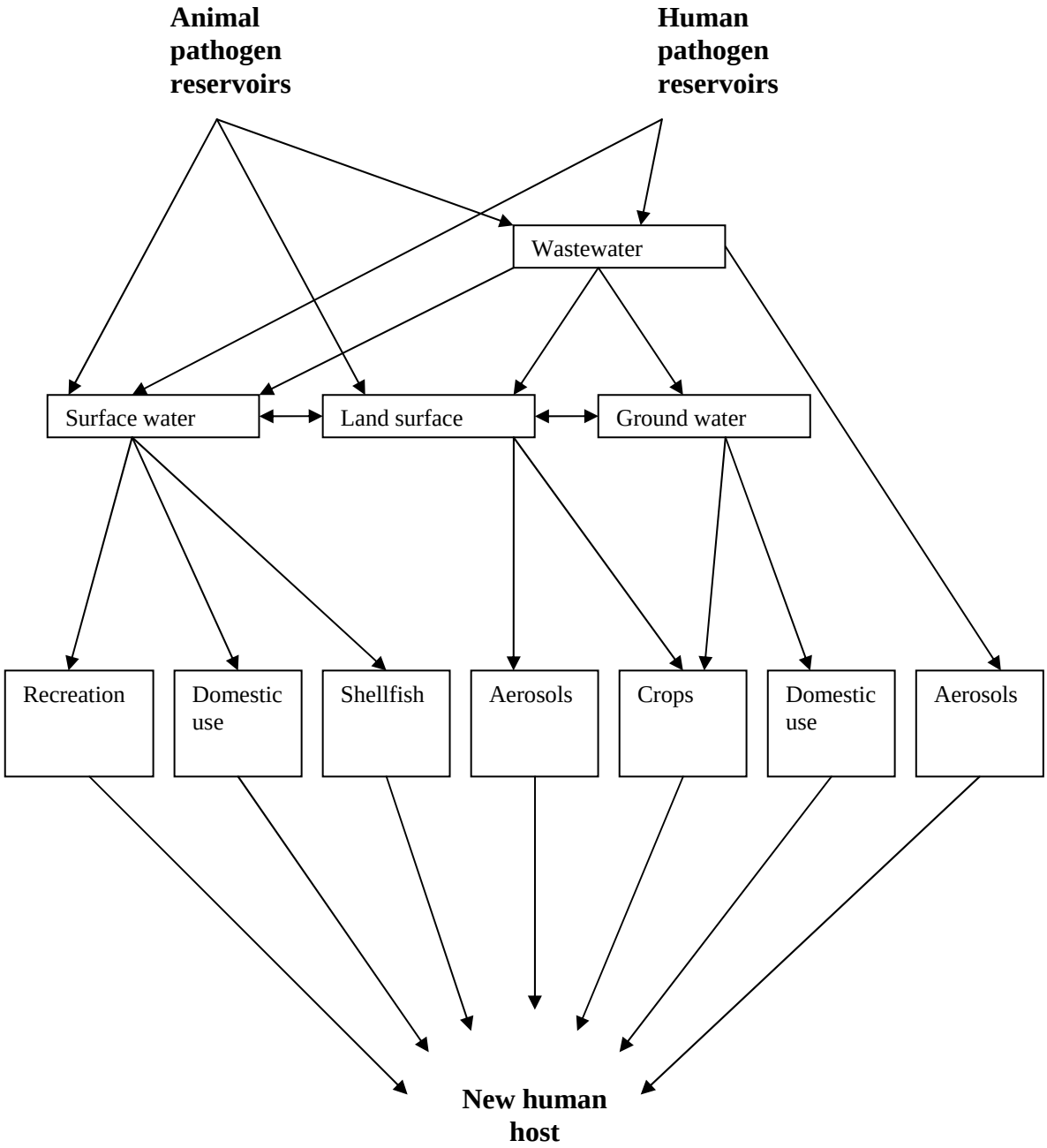


Figure 1.1 Water-related environmental routes by which infectious agents are transmitted to susceptible individuals (Hurst, 1997)

Human faecal pollution is associated with human-specific pathogens such as *Shigella* spp., *Salmonella*, hepatitis, poliovirus, adenovirus, reovirus, norovirus and rotaviruses (Knott, 1981; Scott *et al*., 2002; Field *et al*., 2003). Animal faecal material is associated with *Salmonella* spp., *Escherichia coli* (notably O157:H7), *Cryptosporidium parvum*, and *Giardia lamblia* (Field *et al*., 2003). Viruses are usually host-specific and the primary source of viruses capable of infecting humans is human faeces (IAWPRC, 1991; Field and Samadpour, 2007). The risk of pathogen transference from diffuse animal sources to human hosts (zoonotic infections) is thought to be low. Where insufficiently treated, or even untreated, WwTW effluents are discharged into water systems, the potential for infection of downstream users by pathogens is evident (typical pathogens present in untreated municipal wasters are presented in Table 1.2).

The ratio between PS and DS varies both spatially and temporally within, and between, river catchments. Factors such as topography, land-use, hydrology and population density all influence the PS:DS ratio. Pathogen load of both PS and DS microbial pollution varies geographically, annually and seasonally depending on number of infected individuals contributing to the sewage. Viruses are usually found in lower densities than bacteria (Percival *et al*., 2004), but as they have a lower infectious dose, fewer organisms are required to initiate infection. Aside from microbial health risks, both DS and PS pollution can promote eutrophication of receiving waterbodies, often leading to decreases in water quality and loss of floral and faunal biodiversity.

Table 1.2 Typical pathogen and index organism concentrations in untreated municipal wastewater (adapted from WHO, 2003b; Percival *et al*., 2004; da Silva *et al*., 2007; Villar *et* al., 2007)

Pathogen		**Disease/role**	**Numbers per 100 ml (PFU/cfu/genome copies)**
Bacteria	*Campylobacter* spp.	Gastroenteritis	$1 \times 10^4 – 1 \times 10^5$
	Clostridium perfringens spores	Index organism	6×10^4 to 8×10^4
	Escherichia coli	Index organism (except specific strains)	1×10^6 to 1×10^7
	Faecal streptococci/intestinal enterococci	Index organism	4.7×10^3 to 4×10^5
	Salmonella spp.	Gastroenteritis	0.2 to 8×10^3
	Shigella spp.	Bacillary dysentery	0.1 to 1×10^3
Viruses	HAV	Mainly asymptomatic. Jaundice, malaise, fever, nausea and vomiting.	Present in 92% wastewater samples (Real-time PCR; *n* = 50). No quantitative data available.
	Norovirus	Gastroenteritis, projectile vomiting, diarrhoea and fever.	Genotype 1 = 6×10^5 Genotype 2 = 3×10^5
	Polioviruses	Index organism (vaccine strains), poliomyelitis	1.80×10^2 to 5×10^5
	Rotaviruses	Diarrhoea, vomiting	4×10^2 to 8.5×10^4
Parasitic protozoa	*Cryptosporidium parvum* oocysts	Diarrhoea	0.1 to 3.9×10^1
	Entamoeba histolytica	Amoebic dysentery	0.4
	Giardia lamblia cysts	Diarrhoea	1.25×10^1 to 2×10^4
Helminths	*Ascaris* spp.	Ascariasis	0.5 to 1.1×10^1
	Ancylostoma spp. and *Necator* sp.	Anaemia	0.6 to 1.9×10^1
	Trichuris spp.	Diarrhoea	1 to 4

Numerous methods of reducing pollution from both PS and DS pollution are currently carried out in the UK. Methods to minimise impacts of diffuse pollution include run-off strips and field edge buffer (riparian) zones reducing the amount of faecal material deposited directly into waterbodies. Methods to minimise impact of PS pollution tend to be more stringent, and often result from EU Directives being transposed into UK law.

The EU Directives that are most relevant to this study are:

- *The Urban Wastewater Treatment Directive* (91/271/EEC; UWWTD)

The UWWTD requires the introduction of secondary biological treatment at WwTW greater than 10,000 population equivalent (p.e.) and also the introduction of tertiary nutrient removal at WwTW greater than 10,000 p.e. discharging into designated Sensitive Areas. Two hundred and fifty million pounds were allocated to the installation of such schemes during Asset Management Planning Stage 3 (AMP3), which ran from 2000-2005 (Water UK website, 2007). Although not all tertiary treatment methods are specifically aimed at reducing pathogen levels in wastewater (e.g., phosphate nutrient stripping), tertiary treatment (such as UV disinfection) may inactivate or remove pathogens rendering them ineffective; and

- *The Water Framework Directive* (2000/60/EC; WFD)

The adoption of the EU Water Framework Directive (WFD: 2000/60/EC) in October 2000, incorporates many existing directives, streamlining legislation and increasing management efficiency. Prescriptions outlined in the UWWTD are still applicable to UK waterbodies, whilst new objectives have been incorporated. The WFD is based around River Basin Management Plans (RBMP) aiming to protect all waterbodies in the basin from pollution and degradation. MST may prove a useful tool in identifying

sources of contamination, allowing effective remediation methods to be implemented whilst aiding ‘good ecological status’.

The UK water industry spends significant amounts of money on capital projects, such as the introduction of further WwTW process stages, to improve the quality of UK water. Spending during the last five year management period (2005 to 2010) was projected to be £3.6 billion (OFWAT, 2008).

Resulting from the transposition of EU Directives into national law, disinfection of final wastewater effluents using ultraviolet radiation is becoming increasingly common in Europe. This is also the case in the US. The efficiency and effectiveness of using UV as a disinfectant is discussed in further detail in Chapter Three. In LEDC, environmental legislation is not as well-developed, especially at river basin scale, and this may result in less, or inadequate, control over faecal pollution of waterbodies and increased risk to downstream users.

1.5 Risk, mitigation and traditional microbial source tracking methods

As pathogens are generally host-specific (Sinton *et al*., 1998; Percival *et al*., 2004), in order to assess and manage risk to human populations, it is necessary to distinguish accurately the source of faecal material (Jagals, 1995; Scott *et al*., 2002; Field *et al*., 2003; Gerba, 2006). Accurate identification of faecal pollution allows the implementation of targeted remediation strategies, benefitting both the water user and the taxpayer by an efficient allocation of resources.

Detection of the wide variety of viral and bacterial pathogens shown in Tables 1.1 and 1.2, is time-consuming, expensive, and in some cases unachievable in all but highly specialised laboratories, so indicator organisms of human or animal faecal material are often employed, highlighting the potential presence of viral and bacterial pathogens (Wyn-Jones and Sellwood, 2001). MST involves the use of chemical, phenotypic, genotypic or microbiological methods to define the origin of faecal material (Scott *et al*., 2002). Rather than attempt to monitor the levels of all waterborne pathogens, it is more cost-effective to monitor a number of selected non-pathogenic surrogate organisms, referred to as faecal indicator organisms (FIO). When FIO are detected in water samples, it may be assumed pathogenic organisms may also be present. FIO are shed in high numbers by the majority of individuals, and are continually present in pooled faecal material. Pathogens are only shed by infected individuals and will be present at lower levels in pooled sewage. No ‘perfect’ indicator organism has been found to indicate the presence of all pathogens in all environments, but a number have shown great promise. MST using indicator organisms may prove particularly useful in highlighting sources of long-term

pollution, but less useful when identifying isolated short-term sporadic waterborne disease events.

MST methods are based on the assumption that there is a characteristic within target faeces (e.g., phages, bacteria etc.) that indisputably identifies it as belonging to a particular host, which, after excretion, is detectable in water (Field and Samadpour, 2007). The ideal indicator of faecal material should have numerous qualities, including, but not limited to:

- presence in host faeces only;
- inability to replicate in natural waters;
- possession of a simple, reliable, rapid and inexpensive assay method, not requiring the culturing of isolates;
- not requiring a large reference strain library; and
- simple methods of sample collection and storage.

(Borrego *et al*., 1987; Havelaar, 1993; Field *et al*., 2003).

If the indicator employed is to be used as a surrogate of enteroviruses, rather than a general indicator of human or animal faecal pollution, it must possess additional qualities:

- have similar replication/inactivation kinetics to enteroviruses, particularly during wastewater treatment; and
- be present in greater numbers than enteroviruses in faecally polluted waters;

(Borrego *et al*., 1987; Havelaar, 1993)

In order to be an effective surrogate organism, consistent ecological behaviour and response to environmental pressures should be demonstrated. For bacteriophage based MST, this means host bacteria should detect a homogeneous phage group.

One of the first studies that may be interpreted as using MST was carried out in 1905 (Gordon, 1905). This study examined differential fermentation characteristics of streptococci isolated from human and animal (cow and horse) faeces. Streptococci isolated from human faeces fermented mannitol and not raffinose, whilst the inverse was observed for streptococci isolated from animal faeces. Since this pioneering study, numerous MST methods have been developed (Table 1.3).

Using faecal indicator bacteria (FIB) as diagnostic agents of faecal material source is a well established technique for assessing potential health risks of a waterbody. FIB used in MST studies include faecal coliforms, total coliforms, *Escherichia coli*, faecal streptococci, *Clostridium perfringens* and enterococci (Fujioka, 2002). However, FIB may not accurately reflect the environmental behaviour of enteric pathogens (Jofre *et al*., 1986; Gerba, 2006), especially human enteric viruses (Berg *et al*., 1973). For example, faecal coliforms are unsuitable indicators of faecal pollution because of low survival rates in challenging environments and interference by microbial flora such as *Proteus*, *Bacillus*, *Sarcina*, *Pseudomonas*, *Micrococcus*, *Flavobacterium*, *Actinomyces* and selected yeasts (Geldreich and Clarke, 1971).

Using streptococci in MST studies is also problematic; certain species such as *Streptococcus bovis*, *S. equinus*, and *S. avium* are unable to survive for long once expelled from animals (Borrego *et al*., 1990).

Table 1.3 Classification of MST methods (Cimenti *et al*., 2007)

Type	Examples
Pathogen monitoring	Human enteric viruses (enterovirus, adenovirus, Norovirus virus)
	Eggs of helminths (intestinal worms)
Culture	Faecal coliforms/faecal streptococci (FC-FS ratio)
	Faecal streptococci species identification
	FC-FS ratio shift
	Bifidobacteria spp. (sorbitol-fermenting *Bifidobacteria*)
	Rhodococcus coprophilus
	Bacteroides spp.
	Pseudomonas aeruginosa
	Phages of *Bacteroides fragilis*
	F-RNA phage subgroup
	Streptococcus bovis
Phenotypic	Antibiotic resistance analysis (ARA) or multiple antibiotics resistance analysis (MAR)
	Serogrouping
	Carbon utilisation profile
Genetic	Ribotyping
	Amplified fragment length polymorphism (AFLP)
	Pulse-field gel electrophoresis (PFGE)
	Repetitive PCR (rep-PCR)
	Denaturating gradient gel electrophoresis (DGGE)
	Host-specific molecular markers (LH-PCR; T-RFLP)
Chemical	Faecal stanols
	Fluorescent whitening agents
	Sodium tripolyphosphate
	Long-chain alkylbenzenes
	Caffeine
	Musk fragrances
	Estrogens
	Human pharmaceuticals
	Animal growth promoters

Berg *et al* (1973) showed that although more than 99.99 % of faecal coliforms, total coliforms and faecal streptococci were routinely destroyed during wastewater treatment (using chlorination), removal rates of unspecified enteroviruses was between 89 to 99 %. *Salmonella* has also been shown to have lower inactivation rates

than faecal coliforms (Borrego *et al.*, 1990). Such situations may lead to inaccurate assessments of microbial quality of waterbodies, resulting in an underestimation of risk to water users. Indeed, there have been recorded enteroviral epidemics in waterbodies judged safe by FIB enumeration (Geldreich, 1978; Borrego *et al*., 1987), indicating their failings as diagnostic health protection tools.

It has been demonstrated that enteroviruses are able to survive for long periods in natural waters (Akin *et al*., 1975), thus any substitute indicator must possess similar, or greater persistence.

Other drawbacks of FIB include water column multiplication (Solo-Gabriele *et al*., 2000), non-faecal sources (Scott *et al*., 2002) and lack of human/animal discriminatory power (Field *et al*., 2003); it is evident that FIB alone are unable to determine source of pollution (Jofre *et al*., 1986).

In conjunction with FIB, other methods have been proposed (Table 1.3). Antibiotic resistance patterns of faecal coliforms and streptococci have been used; however these methods require large numbers of isolates, tending to be both time consuming and labour intensive. There is also uncertainty regarding the accuracy of these techniques (Field *et al*., 2003; Field and Samadpour, 2007). Culture independent techniques are becoming more widespread in MST (Santo Domingo *et al*., 2007). These include using PCR to detect *Bacteroides* specific 16S marker sequences (Bernhard and Field, 2000), *Bifidobacterium* spp., or *Clostridium perfringens* (Savichtcheva and Okabe, 2007), and other DNA fingerprinting methods (Field and Samadpour, 2007).

Molecular methods of identifying faecal material are developing continuously (Field and Samadpour, 2007; Field *et al*., 2003; Meays *et al*., 2004; Santo Domingo *et al*., 2007), but the high level of expertise required and specialised laboratory equipment needed prohibit their use in many situations. Moreover, methods based on the detection of nucleic acids are of higher cost than most culture base indicators, and may produce false-negative/false positive results (Sobsey *et al*., 1998). As sensitivity of molecular techniques improves, these 'false' results may be reduced.

Using phages as indicators of human faecal material, and potentially surrogates for particular pathogens, is a rapid and inexpensive way of determining source of faecal material, applicable in all but the most basic laboratories, by operators with basic training. The introduction of international standard methods for three phage types (Anon, 2001a, b and c) has provided clear protocols allowing comparison of data obtained in different studies, and has improved the quality of phage based MST studies. The following chapter provides information on phage biology and the development of their use as indicators of human specific pollution, addressing some of the problems outlined above regarding 'traditional' FIO.

CHAPTER TWO

2. Bacteriophage characteristics and their use in MST and QMRA studies

2.1 General characteristics of phages

Phages are parasitic supramolecular viruses (Wurtz, 1992) capable of infecting prokaryotic organisms (Ackermann and DuBow, 1987a). Thought to be the most abundant organism on earth (possibly 10^{30} to 10^{32} in total), phages play a pivotal role in regulating both microbial and higher ecosystems (Kutter and Sulakvelidze, 2005). Phages were independently discovered twice, in quick succession, by Frederick W. Twort and Felix d'Hérelle working independently, in 1915 and 1917, respectively (Twort, 1915; d'Hérelle, 1917); Twort observed the glassy transformation of "*Micrococcus*" colonies, which he proposed was viral in nature (caused by T4 phage; Ackermann and DuBow, 1987a) whilst d'Hérelle described lysis (destruction of host cell) of *Shigella* cultures in broth. It was d'Hérelle who actively pursued phage research, naming them bacteriophages ('bacteria eater' in Latin). D'Hérelle also proposed that their method of multiplication was intracellular and briefly introduced the concept of phage therapy (Ackermann, 2003). An exhaustive history of the discovery and development of phage research would neither be possible nor desirable in this thesis, but a brief summary is given in Section 2.1.2. For more detailed summaries see Adams, 1959; Ackermann and DuBow, 1987a; or Summers, 2005.

Phages are found in the majority of bacterial families (Ackermann and DuBow, 1987a; Ackermann, 1999) with over 140 bacterial genera known to have associated phages. They are the largest known viral group (Ackermann, 2003) and are found in

diverse hosts: spirochetes, eubacteria, exo- and endospore formers, cyanobacteria, anaerobes, aerobes, mycoplasmas and chlamydias amongst others, and are delineated into 30 genera, 13 families and 1 order (Table 2.1 and Figure 2.1).

As of 2003, approximately 5100 phage 'species' have been viewed using electron microscopy, and it is thought that there are many more phages to be discovered (Ackermann, 2003). There are six basic types of phages: tailed, filamentous, and cubic (with either single or double stranded DNA or RNA as genetic material). Taxonomy and classification of bacteriophages below family level is contentious, and is an ongoing concern. Classification is carried out using the "polythetic species concept" (i.e., species are classified by property set (morphological, genetic etc.), of which, some may be absent in a particular member (Van Regenmortel, 2000).

Taxonomic names are derived from either Greek or Latin (orders = *virales*; families = *virivae*; genera = *virus*). At present, tailed phage genera have only vernacular names (i.e., T4 like, λ-like etc.).

Table 2.1 Classification and selected properties of phages (adapted from Ackermann, 2003 and Grabow, 2001)

Shape	Nucleic acid	Order (in bold) and families	Genera	Examples	Members	Morphological Characteristics
Tailed	dsDNA (L)	***Caudovirales***	15		4950	
		Myoviridae	6	T4, P1, Mu	1243	Contractile tail.
		Siphoviridae	6	λ, T5	3011	Long non-contractile tail.
		Podoviridae	3	T7, P22	696	Short tail.
Polyhedral	ssDNA (C)	*Microviridae*	4	ΦX174	40	Icosahedral capsid, no tail.
	dsDNA (C, T)	*Corticoviridae*	1	PM2	3?	Lipids, complex capsid, icosahedral
	dsDNA (L)	*Tectiviridae*	1	PRD1	18	Internal lipoprotein vesicle, double icosahedral
	ssRNA (L)	*Leviviridae*	2	MS2, Qß.	39	capsid
	dsRNA (L, S)	*Cystoviridae*	1	Φ6	1	Icosahedral capsid, no tail Envelope, lipids
Filamentous	ssDNA (C)	*Inoviridae*	2	fd, f1, M13	57	Rods or filaments
	dsDNA (L)	*Lipothrixviridae*	1	TTV1	6?	Envelope, lipids, rod shaped
	dsDNA (L)	*Rudiviridae*	1	SIRV1	2	TMV like, rod-shaped
Pleomorphic	dsDNA (C, T)	*Plasmaviridae*	1	L2	6	Envelope, lipids, without capsid
	dsDNA (C, T)	*Fuselloviridae*	1	SSV1	8?	Spindle/lemon shaped, without capsid

C = circular L = linear T = super-helical S =segmented ss = single-stranded ds = double-stranded

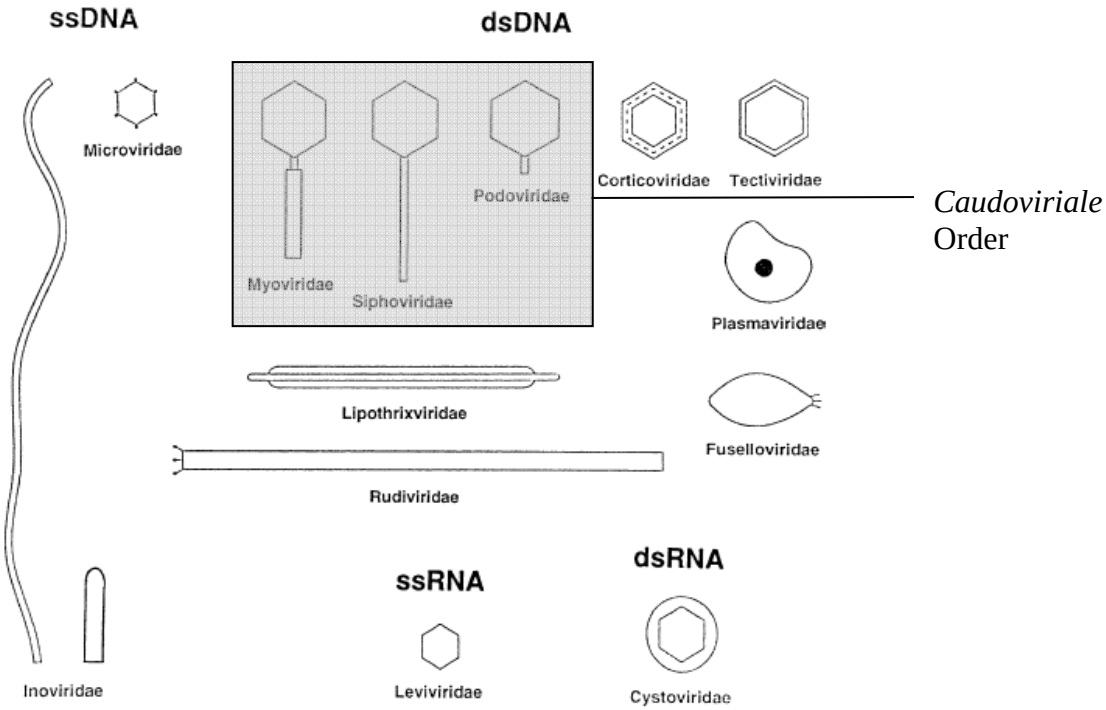


Figure 2.1 Major phage groups organised by nature of genetic material (adapted from Ackermann, 2003)

2.1.1 Phage structure and basic biology

As Figure 2.1 and Table 2.1 illustrate, phages constitute a diverse viral group, with tailed phages (delineated into the order *Caudovirales*) dominating. As of the year 2000, there were more than 4950 'species' members of the *Caudoviriale* order identified, representing 96 % of identified phages (Ackermann, 2001); this was an increase from 2700 members (95 % of identified phages) in 1987 (Ackermann and DuBow, 1987a). Tailed phages consist of a permanent cubic capsid (head) and a tubular tail structure. This combination is not seen in any other viral group (Ackermann, 2003) and *Caudovirales* are amongst the most complex viruses known (Lwoff *et al*., 1962). Phages have been found in 90 diverse host genera, encompassing a wide spectrum of bacterial types (Ackermann and DuBow, 1987a; Grabow, 2001). Phages not possessing binary morphology are in the minority; as of the year 2000, 186

(3.6%) polyhedral, pleomorphic and filamentous phages were identified (Ackermann, 2001).

The *Caudovirale* capsid is predominantly icosahedral (or derivatives thereof; 12 vertices, 20 faces and 30 edges and are composed of 2 pentagonal caps with one equatorial band of equilateral triangles) constructed from monomers (quasi-equivalent protein subunits) allowing for an economic genome. The monomers may be arranged by 2 (dimers), 3 (trimers), 5 (pentamers), or 6 (hexamers), the sum of which (the capsomer), may be viewed using transmission electron microscopy. Some capsids are icosadeltahedra, with each face being sub-divided into a number of smaller triangles (Ackermann and DuBow, 1987b).

Within the order *Caudovirales*, there are three phage families primarily defined by tail structure (see boxed area Figure 2.1):

- *Siphoviridae* family (61 % of *Caudovirales*; Ackermann, 2003) possess a simple long non-contractile tail, which can be either flexible or rigid. There is no neck between tail and capsid which is usually 55 to 65 nm in diameter. When stained with uranyl acetate and viewed using electron microscopy, flexible tails may appear rigid, whilst excess stain around the head may give the impression of a neck (Ackermann and DuBow, 1987b). λ (lambda) phage is one of the most studied siphoviruses;
- *Myoviridae* family (25 %; Ackermann, 2003) tails are long and contractile, consisting of a central core/tube and an outer sheath, which are always separated by a neck (free interval; Figure 2.2). They are constructed of protein sub-units arranged in rings/spirals and are usually rigid. Tails are contracted (made shorter and wider) when base plate makes contact with a

suitable receptor site (Ackermann and DuBow, 1987b). Myoviruses tend to have the largest capsid of the *Caudovirales*, and display a larger range in capsid size than other viral groups. Capsids range from 45 to 170 nm in diameter for non-elongated capsids and for elongated icosametric capsids, 46 x 33 to 230 x 50 nm (Ackermann and DuBow, 1987b). T4 is one of the most studied myoviruses; and

- *Podoviridae* family (14 %; Ackermann, 2003) possesses short tails that are similar in structure to long non-contractile tails with capsids around 55 to 65 nm in diameter (Ackermann and DuBow, 1987b). T7 is a notable member of the *Podoviridae* family.

Tails may contain numerous proteins of high molecular weight and can vary greatly in length; generally, contractile tails are shorter than non-contractile tails (Ackermann, 2003). Tails of phages belonging to the *Myoviridae* family range from around 60 to 455 nm in length whilst in the *Siphoviridae* family, tails range from 65 to 570 nm in length. Tail range observed within the *Podoviridae* family is very small, and the difference between podoviruses and siphoviruses is obvious, there being no intermediate tail length. Other dimensions of all tailed phages are remarkably similar (Table 2.2), which may be a legacy of *Caudovirale* shared evolution (Ackermann, 2001).

Table 2.2 Constant dimensions in *Caudovirales* (Ackermann and DuBow, 1987b)

Phage component and parameters	**Dimensions (nm)**
Capsid thickness	2 to 3
Neck	10 x 8
Collar	12 x 2
Extended sheath	
Diameter	18
Striations, repeat	4
Contracted sheath	
Diameter	24
Lumen (space within tail structure)	8 to 9
Wall, thickness	3
Tail tube	
Diameter	7 to 9
Lumen	2 to 2.5
Non-contractile tail	
Diameter	7 to 10
Striations, repeat	4
Wall, thickness	3 to 4
Lumen	2 to 2.5
Base plate	
Thickness	3 to 5

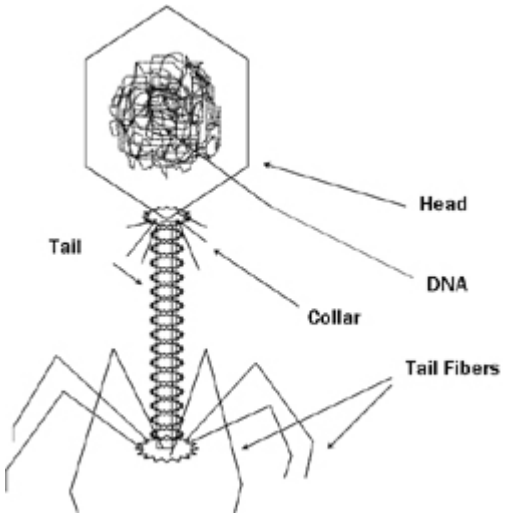


Figure 2.2 Components of phages belonging to the *Caudovirale* order (Anon, 2008b)

The degree of tail curvature can vary between virions within a pure culture of the same phage type. This is demonstrated in Figure 2.3 which shows showing a pure *B. fragilis* laboratory culture in which straight and kinked tails are shown on the same TEM grid, and Figure 2.4 which shows tail variation in somatic coliphages. Genomic sequencing of this sample in Figure 2.3, showed only one phage genotype was present, and all phages belonged to the same species (Hawking *et al*., 2008).

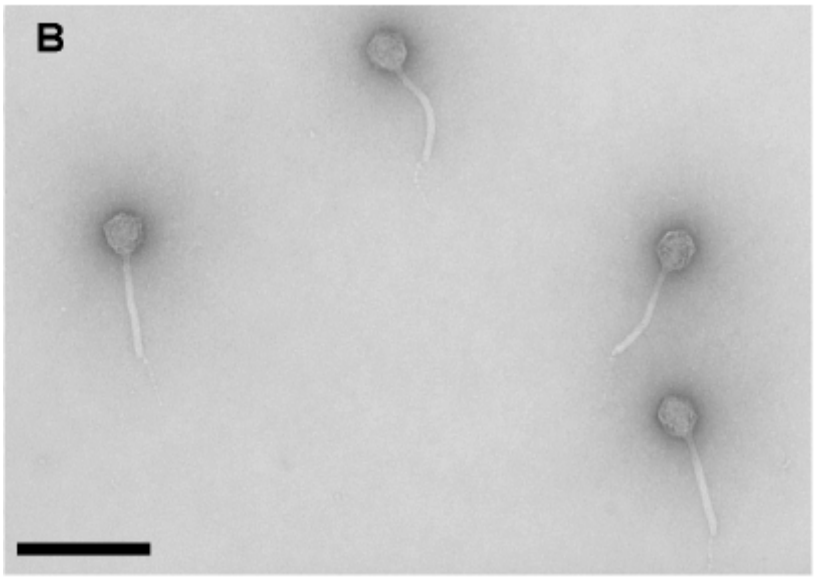


Figure 2.3 TEM of *B. fragilis* phage ATCC 51466 showing both kinked and straight tailed virions; bar is 200 nm (Hawkins *et al*., 2008)

Figure 2.4 shows somatic coliphages isolated from municipal final effluent with varying degrees of tail curl located on the same TEM grid (split for presentation reasons; phage on the left shows a 180 deg curl, the centre phage shows 540 deg curl and phage on the right shows a 360 deg curl; Dee and Fogleman, 1992).

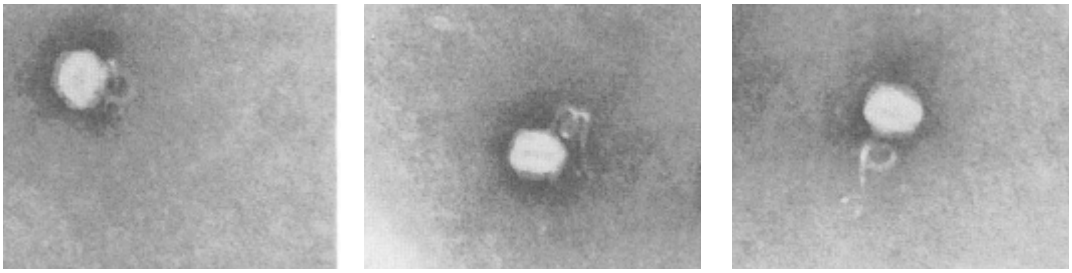


Figure 2.4 Tail variation of somatic coliphages isolated from municipal wastewater (Dee and Fogleman, 1992)

Delineation of phages into groups based upon tail curvature is therefore not advisable, as tail shape may vary between individual virions within a pure sample, and be altered by the electron microscopy staining process.

The connection between head and tail varies between phages, some phages have collars, whereas others have discs. Such structures are needed to connect the 5-fold symmetry of the capsid with the 6-, 5- fold symmetry of the tail. Tailed phages may also have a range of facultative organelles (fibres/knobs on phage head, collars/collar fibres, tail ornaments, and base plates/spikes) that have no apparent function, but are vital for taxonomy (Ackermann, 2001).

The majority of phages contain double stranded DNA (dsDNA), few contain double stranded RNA (dsRNA), single stranded RNA (ssRNA) or single stranded DNA (ssDNA; Ackermann, 2003). Tailed phages usually contain a single linear molecule of dsDNA usually between 11 and 490 Mdaltons in weight with length varying between 17 to 700+ kb (Ackermann, 2003). Protein and DNA composition of phages is roughly proportional (Schlesinger, 1934). The chemical composition of numerous phages has also been determined. *Caudovirale* G + C content (guanine-cytosine) appears to vary between 27 and 72 % with phages and host having similar G + C

contents (Ackermann and DuBow, 1987a). Unusual bases, such as 5-hydroxymethylcytosine (HMC), have been reported in many *Caudovirales*, the purposes of which may be to protect phage DNA against bacterial restriction endonucleases. Unusual bases may be indicated by abnormally high/low buoyancy in CsCl or melting temperatures.

Numerous members of the *Caudovirale* order have been genetically mapped; for example, phage T4 has around 290 genes, which are clustered together by function. The genome structure of 15 genera has been defined, and it is thought many more will be included in the future (Ackermann, 2003).

2.1.2 Early phage research

Although phages were discovered almost simultaneously by F.W. Twort and Felix d'Hérelle, in 1915 and 1917, respectively, it is possible that their effects were reported in the literature as early as the late 19th century. Hankin (1896) noted the antiseptic properties of Ganges (India) river water against *Vibrio cholerae*, assuming a volatile chemical agent was responsible, whilst Emmerich and Löw (1901) recorded lysis of bacterial cultures by an unknown agent. It is not certain whether observed phenomena recorded in the aforementioned studies were a result of phages, bacteriocins or other lytic enzymes.

Of the two co-discoverers of phage, it was d'Hérelle who actively pursued phage research, producing 97 books and papers in twenty years (Ackermann, 1987a). Using both solid and liquid media, he realised the particulate nature of phages, and developed the concept of plaque forming units (PFU) on agar as a way of phage

enumeration. The step-wise nature of phage replication was also highlighted in his experiments. The majority of early phage papers focused on phage therapy: d'Hérelle realised that as patient recovery was taking place, phage titers in patients' stools increased forming the conclusion that phages may be the causative agent of recovery. Notable early human phage therapy experiments included using staphylococcal phages to treat cutaneous boils (Bruynoghe and Maisin, 1921) and the bubonic plaque (d'Hérelle, 1925). The possibility of using phages medicinally within animal populations was also investigated by d'Hérelle (1921; 1926). By 1940, it is thought that approximately 560 phage papers had been published (Raettig 1958) and phage preparations were available both commercially and in field medical packs issued to German soldiers during the second world war (Summers, 2005). Research into phage therapy was made largely redundant by the introduction of antibiotics during the 1940s and research output fell considerably (Kellenberger, 1995).

As interest in phage therapy declined, research into the genetics, epidemiology and general characteristics of phages increased in the period 1940 to 1965 (Raettig 1967). The introduction of the electron microscope settled one of the early contentions regarding the state of phages; early electron microscope studies (e.g., Pfannkueh and Kauschc, 1940; Ruska, 1941; Luria and Anderson, 1942) showed that phages are particulate in character with specific morphologies.

Molecular studies of phages led to important discoveries (Grabow, 2001) such as establishing DNA as the genetic material (Hershey and Chase, 1952) and the nature of viral replication (Ellis and Delbrück, 1939). Phage research contributed to the Watson-Crick double helix DNA model (Watson and Crick, 1953; Kellenberger,

1995) and the development of the ‘central dogma’ of molecular biology. By examining phages it was shown that genetic material could be ssDNA (e.g., ΦX174 phage; Sinsheimer, 1959) or ssRNA (e.g., MS2; Fiers *et al*., 1976) and that genetic material could contain bases other than adenine, thymine, guanine or cytosine. Phages were important to geneticists as they could be rapidly propagated and compared to human, animal, insect and plant hosts for other viruses, allowing quicker experimentation, achievable in standard laboratories (Pelczar *et al*., 1988). Recently, post 1990, the number of papers publishing phage genomes has increased exponentially indicating renewed interest This renewed interest is because of the sheer abundance of phages being discovered in a range of terrestrial and aquatic environments, the discovery of phage importance in the host genome evolution (indicated by host genome sequencing), their use as indicators of specific pollution sources, and their use as therapeutic agents because of development of antibiotic resistance within humans (Kropinski and Clokie, 2009).

2.1.3 Phage infection of the host cell

The stages of phage life cycle vary in length, and therefore provide a useful taxonomic tool. The stages and kinetics of the infection process were demonstrated clearly by Ellis and Delbruck (1939) utilising one-step growth experiments. The main stages of the phage life cycle are:

1. Adsorption: the extracellular search by a virion for a host is diffusion driven, ending when adsorption structure (tail fibres, spikes) make contact with an adsorption site on the host bacterium (lipopolysaccharides, proteins, oligosaccharides). The specific site required by phages varies between species. Some phages require cofactors to bind successfully to the host; these include Ca^{2+}

and Mg^{2+} (and other divalent cations), tryptophan (for T4B phage) and maltose (for λ phage). Hosts often change binding structure to prohibit phage infection, but in most cases the effectiveness of such actions is limited as phages can adapt accordingly. Some phages may encode numerous versions of tail structures to cope with host mutations, whilst other may be able to utilize multiple adsorption sites;

2. Penetration: the transfer of genetic material from phage to host occurs after successful adsorption. Mechanisms vary from phage to phage, but generally the genetic material in *Caudovirales* is passed through the tail (which may be either contractile or non-contractile) into the host cell. The peptidoglycan and inner membranes of the host are penetrated by enzymes and phage genetic material begins to drive host mechanisms;

3. Direction of phage synthesis: transcription of phage immediate early genes is carried by host RNA, the function of which is to ensure successful phage replication. This may be achieved by disrupting host proteases, blocking restriction enzymes and destroying selected host proteins. Middle genes that direct phage DNA synthesis may then be produced, followed by late genes that encode phage particle;

4. Morphogenesis: DNA and phage heads are combined, producing procapsids, which are assembled using scaffolding proteins. Tails are assembled separately and combined when head is complete; and

5. The phage-host infection cycle may be concluded by lysis or lysogeny:
    - *lytic (virulent) phages*: once new phages have been synthesised, they are liberated by host cell destruction. Two components are utilized by tailed phages, a lysin (an enzyme that breaks the peptidoglycan layer of host) and a holin (a protein that creates a path in the inner membrane in which the lysin can pass through to reach the peptidoglycan matrix). The timing of cell lysis is a balance between maximum phage production within the host, and delay of phages finding a new host. Should the period between infection be too long, opportunities for instigation of new lytic cycle may be lost, and if too short, too few phages may be produced and the lytic cycle can break down.
    - *lysogenic (temperate) phages*: in contrast to the lytic cycle, lysogeny does not result in destruction of the host cell; phages are held in a non-infective state and are transferred to all host daughter cells. This research project utilizes virulent phage, thus temperate phages will not be discussed.

It is possible that in response to environmental stresses, typically lytic phages may enter a lysogenic state becoming integrated with the host bacterial genome.

2.2 Using phages as indicators of faecal pollution

To overcome the problems with 'traditional' FIO outlined in Section 1.5, several groups of bacteriophages have been proposed as indicators of the presence of human faecal material. Bacteriophages are persistent in the environment, are enumerated by a simple and economical assay, are non-pathogenic to humans, and tend to be host-specific (Ackermann and DuBow, 1987a; Grabow *et al.*, 1995; Van Cuyk and Siegrist, 2007). It has been hypothesised that, as phage structure, morphology, size and composition may be similar to enteric viruses, it is possible they can be used as surrogate organisms for enteric viruses (Jofre *et al.*, 1986; IAWPRC, 1991; Grabow *et al.*, 1995). Indeed, phages were first speculated to be acceptable models for enteric viruses as long ago as the 1940s (Guelin, 1948).

Phages are generally present in greater densities in faeces than are enteroviruses (Jofre, 2002), fulfilling a number of the requirements stated in Section 1.5. This research sets out to address some of the knowledge gaps (i.e., those stated by Borrego *et al*., 1987; Havelaar, 1993; Section 1.5) regarding the ecological similarity of *Bacteroides* phages and enteroviruses, thereby providing an evaluation of their potential to act as surrogates for enteroviruses in QMRA studies and water/wastewater treatment evaluations.

When evaluating the effectiveness of a phage group in MST and QMRA studies, differential inactivation within a phage group may present problems, and ideally a bacterial host capable of detecting a homogenous group of phages is recommended (Lasobras *et al*., 1997; Queralt *et al*., 2003). Therefore, identification of group homogeneity allows for a better understanding of how environmental conditions are

likely to influence the suitability and accuracy of GB-124 in MST studies. For example, if a host used in MST studies detects a heterogeneous group of phages, with the dominant phages being inactivated by high pH environments, the host bacterium may detect no phages in such environments, indicating an absence of faecal pollution (and associated enteric pathogens), resulting in mis-diagnosis of health risk (false negatives). However, if it was known that the dominant phage type was susceptible to high pH, other hosts could be used, achieving a more accurate assessment of risk to human health with the original host being successfully employed in other environments. By detecting a homogeneous group of phages, these false-negatives may be reduced, allowing the phages to be used more accurately in a diverse range of environments.

It is important to note that the composition of phage populations, in most situations, is not static. Indeed, it has been reported that the composition of phage populations may be altered by water and wastewater treatment processes. Dee and Fogleman (1992) studied the composition of somatic coliphage communities through a WwTW (Denver, USA). Six dsDNA phages, three from influent and three from effluent, were isolated and studied using electron microscopy. It was shown that the influent phage population was heterogeneous, containing two types of siphovirus (one group had tight curled tails and one group had non-contractile straight tails) and one type of microvirus (small cubic phage) whereas effluent phage populations were homogeneous (all siphoviruses with tightly curled tails). The study showed that the phage populations had differential inactivation kinetics, with siphoviruses being the most resistant to inactivation. However, it must be noted that the observations and

conclusions stated in this particular study were based on very small sample populations (three influent phage groups and three effluent phage groups).

Similar results were reported by Muniesa *et al*., (1999), who recorded a change in population ecology of somatic coliphages in sewage and sewage influenced waters: members of the *Siphoviridae* family dominated river waters downstream of WwTW, whilst members of the *Myoviridae* family were dominant in raw wastewater and at sites closer to the WwTW outfall. Lasobras *et al* (1997) recorded that the proportions of somatic coliphages belonging to the two families changed between two locations; proportions of phages belonging to the *Myoviridae* and *Siphoviridae* families were 91.0 % and 6.0 % in wastewater samples, respectively, and 67.6 % and 26.4 % in samples with persistent pollution. One conclusion from this study was that phage populations found in waters subject to persistent pollution (i.e., subject to either natural inactivation or by inactivation during treatment), are likely to be morphologically different than those in wastewater as a result of differential inactivation. The study concluded that phage morphology may significantly contribute to environmental persistence. Siphoviruses appeared to be more resistant to inactivation and this may explain why they are the most frequently recorded phage family so far (Francki *et al*., 1991). Phage homogeneity in wastewater final effluent is important, as this is the discharge of most concern to environmental managers.

Early research focused on phages as indicators of shigellae, cholera vibrios, and typhoid bacteria (Ackermann and DuBow, 1987a), from which three groups of phages capable of infecting enteric bacteria emerged as indicators of faecal pollution:

2.2.1 **Somatic coliphages** (SC; Hilton and Stotzky, 1973; IAWPRC, 1991)

Resulting from a decision in the 1940s to concentrate phage research on one particular type (Summers, 2005), SC are the most studied of all phages (Toranzos *et al*., 2002) and are capable of infecting *E. coli* and other closely related species (Gerba, 2006) from the family Enterobacteriacea (Grabow, 2001). Well known and extensively studied SC include T1-T7, PRD1 and ΦX174 (Gerba, 2006). Coliphages are a diverse group and no single host bacterium can propagate all phages (Ackermann and DuBow, 1987a), but strains such as WG-5 [*E. coli* mutant strain CN, American Type Culture Collection (ATCC) 700078] and *E. coli* strain C (ATCC 13706) have become standardized (Anon, 2001b) allowing comparison of inter-laboratory results. SC are found in the stools of many animal species, including humans, higher and lower primates, domestic animals and birds (Grabow *et al*., 1995). SC are lytic phages that utilise either protein receptors or lipopolysaccharides in the host cell wall as receptor sites (Anon, 2001b) and can belong to any of the *Caudoviriale* families identified in Section 2.1.1, or the *Microviridae* family (simple tailless structure, ssDNA, capsid may be up to 30 nm). The genetic material of SC is either ssDNA or dsDNA (Anon, 2001b).

Indications that SC may be used as indicators of faecal pollution/enteric pathogens were first given in Kott (1966). Although this study was primarily focused on the adaption of the most probable number enumeration technique to phages, this study presented data for SC active against *E. coli* B in WwTW (Austin, USA) and in the marine area surrounding the WwTW outfall. It was found that SC were present in both wastewater and sea water samples (mean = 85.6 MPN/100 ml and 23.2 MPN/100 ml respectively) and, although not stated in the paper, the data

demonstrated the potential for using SC as a tracer of WwTW effluent. A targeted study carried out by Wimpenny *et al* (1972) demonstrated (alongside *Serratia marcescens*, pigmented yeasts, and Bacillus) the ability of a λ-like phages active against *E. coli* K12 to detect faecal pollution in water. Numerous studies have employed SC as indicators of human faecal material since (e.g., Borrego *et al*., 1987; Borrego *et al*., 1990; Lucena *et al*., 1994; Paul *et al*., 1997).

There are numerous advantages of using SC as indicators of faecal pollution/enteric viruses, not least the simple and economical assay. SC display varying responses to environmental factors such as temperature and pH (Gerba, 2006), but generally tend to persist longer than FIB (Anon, 2007) and selected pathogenic viruses (Scarpino, 1978; Borrego *et al*., 1990). Somatic coliphages are often present in greater densities than pathogenic human enteric viruses, often outnumbering cytopathogenic viruses by a factor of around 500 (Grabow *et al*., 1993). A positive correlation with enteric viruses has been reported in a range of environments in varying geographical locations (Borrego *et al.*, 1987; Dutka *et al*., 1987; Borrego *et al*., 1990). Somatic coliphages were found to be lineally positively correlated with *Salmonella* bacteria, performing better than FC (Borrego *et al*., 1987) whilst Skraber *et al* (2004) found a correlation between SC and enteric viral genomes in French river waters.

SC tend to be more prevalent in wastewater (raw and treated) and wastewater polluted waters, than the two other phage types discussed below (F-specific RNA phages and *B. fragilis* phages; Grabow *et al*., 1993), and densities of SC in human stool samples have been found to be 60 to 100 times higher than those of *B. fragilis* phages in stool samples (Havelaar, 1993; Gantzer *et al*., 2002). Subsequently SC can be detected in

small sample volumes, 1 ml of faecally contaminated waters (Anon, 2007), and hence proving useful as a general indicator of faecal pollution (Gerba, 2006).

However, the disadvantages of using SC as an indicator of human faecal pollution are numerous:

- SC may infect bacteria related to *E. coli* that are capable of environmental replication, therefore facilitating SC replication (Grabow *et al*, 1995) presenting the potential for false-positive indications of faecal material. However, a recent review of the literature has assessed this potential as negligible (Jofre, 2009);
- SC are a heterogeneous group (Gerba, 2006), therefore differential inactivation kinetics may influence their use in MST studies in certain environments containing inactivation agents;
- There is limited information regarding the aquatic ecology of SC (particularly host range and their behaviour in different climatic regions; Anon, 2007) consequently it is not known how this effects their use in MST studies; and
- Although well correlated with wastewater effluent, SC are also associated with non-point pollution (Paul *et al*., 1997) and are found in areas not subject to faecal pollution (Seeley and Primrose, 1980) meaning they have little source identification ability (Gerba, 2006).

Although some positive correlation has been reported (Skraber *et al*., 2004), the use of SC as surrogate organisms of human enteric viruses in not recommended. This is primarily because of very different excretion patterns in terms of both timing and numbers (Jiang *et al*., 2001).

In summary, SC are a good indicator of faecal pollution (and of water quality in general) as they are found in high densities in faeces and faecally polluted waters, but they can not be used to distinguish between human and animal sources. In spite of all the aforementioned shortcomings, SC are still judged to provide a cheap, fast and effective method for detecting general faecal pollution.

2.2.2 **F-specific RNA phages** (FRNA phages; Havelaar *et al*., 1986; IAWPRC, 1991)

FRNA phages consist of ssRNA contained in a cubic capsid, usually between 21 nm and 30 nm in diameter (Anon, 2001a). Belonging to the *Leviviridae* family, FRNA phages are either leviviruses (e.g., MS2 phage) or allolevivirus (e.g., Qß phage; Anon, 2001a). FRNA phages are active against coliform bacteria and are genetically and morphologically similar to human enteroviruses (Fujioka, 2002). For example, FRNA phages have very similar morphology to the poliovirus (*Picornaviridae* family), in that both have icosahedral capsids of around 25 nm, and when viewed using electron microscopy, appear extremely similar (Grabow, 2001). FRNA phages have been recorded in stool samples from humans, higher primates, primates, domestic animals and birds (Grabow *et al*., 1995).

It is thought that FRNA phages are possibly the most suitable model organism for enteric viruses; Havelaar (1993) and Jiang *et al* (2001) reported good correlation in a range of water types between FRNA phages and enteric viruses, and absence of FRNA phages suggests absence of human enteric viruses (Havelaar and Nieuwstad, 1985; IAWPRC, 1991). Domestic wastewater has high densities of FRNA phages (albeit lower than somatic coliphage) and have been found to outnumber

cytopathogenic enteric viruses by up to 100 x (Gerba, 2006). FRNA phages have also been shown to accumulate in shellfish (Riou *et al*., 2007) and may provide an indicator of remote faecal pollution.

Two bacterial hosts are commonly used to detect FRNA coliphages:

- *Salmonella enterica* serovar typhimurium (WG-49; Havelaar and Pot-Hogeboom, 1988); and
- *E. coli* mutant HS(pFamp)R (Debartolomeis and Cabelli, 1991).

FRNA phages are only able to infect hosts possessing pili (sex fimbriae) coded by the F genetic factor, as these carry the required receptor sites. As the fimbriae are only produced at temperatures higher than 30 °C (Grabow *et al*., 1995), FRNA phages are unable to reproduce in most aquatic environments (Grabow, 2001).

The disadvantages of using FRNA phages as indicators of faecal pollution are that the phages are present in low numbers in human faeces, and the host bacterium must be kept in the mutated state (Fujioka, 2002). Since they are isolated from a range of human and animal faeces, they possess little ability to discriminate between human and animal faecal sources (Grabow, 2001). It has also been reported that they do not persist in the environment for as long as many enteric viruses (Jofre, 2002) and may possess higher decay rates than SC and *B. fragilis* phages (Lucena *et al*., 1994).

It is interesting to note that a number of FRNA coliphage serotypes have been identified (groups I – IV). These serogroups may prove useful for faecal source tracking studies, groups I and IV have only been detected in animal faeces, whereas

serogroups II and III have been almost exclusively detected in human faeces (the exception is 28% of porcine faeces; Furuse *et al*., 1983; Hsu *et al*., 1995; Grabow, 2001).

In summary, FRNA phages are unable to distinguish between human and animal faeces using the plaque assay method (Anon, 2001a) but analysis of serotypes may identify faecal source (hybridisation laboratory equipment would be required to perform these assays). There may be some validity in using them as models of selected human enteric viruses, especially as environmental replication is very unlikely, though environmental persistence may not correlate with enteric viruses. One advantage of using FRNA phages is the recent emergence of a new rapid (three hour) detection technique (Love and Sobsey, 2007) which could mean that incidents of general faecal contamination could be identified more quickly.

2.2.3 Phages infecting *Bacteroides* spp. (Tartera and Jofre, 1987; IAWPRC, 1991) Phages active against *B. fragilis* constitute the main focus of this project, and thus they are therefore discussed in detail in Section 2.3.

2.3 Using phages infecting *Bacteroides* spp., as indicators of human faecal material

Bacteroides bacteria are Gram negative, obligate anaerobes found in the human intestinal tract (Shah, 1992; Fujioka, 2002). Members of the *Bacteroides* genus are found at densities more than 10^9 to 10^{10} per gram of faeces (Geldreich, 1978; Salyers, 1984) and thus constitute the major component of the human faecal bacterial flora (Finegold *et al*., 1983). There are numerous *Bacteroides* species recognised: *B. fragilis* (the most commonly used in MST), *B. thetaiotaomicron, B. vulgatus, B. ovatus, B. distasonis, B. uniformis, B. stercoris, B. eggerthii, B. merdae, B. caccae* (Gherna and Woese, 1992), *B. oralis* and *B. melaninogenicus* (Keller and Traub, 1974).

B. fragilis phages are lytic, attaching to the host cell wall (Puig and Gironés, 1999), the majority of which belong to the *Siphoviridae* family. Before the practice of employing *B. fragilis* phages as an indicator of human faecal material was formalised (i.e., prior to the work of Jofre *et al*., 1986 and Tartera and Jofre, 1987), there were numerous references reported of *Bacteroides* phages being isolated from wastewater; Sabiston and Cohl (1969) briefly noted isolation of phages active against *B. distasonis*, Prévot *et al* (1970) isolated several phages active against *Ristella pseudoinsolita* (*B. fragilis*), and Nacescu *et al* (1972) isolated two phages active against *Bacteroides* spp.

Although belonging to the same taxonomic group, and probably being serologically related, no *Bacteroides* phage has an identical host range spectra and many phages are specific to only one *Bacteroides* strain (Kory and Booth, 1986). Booth *et al* (1979)

showed that *Bacteroides* phages were only able to infect other strains within the original species (i.e., *B. fragilis* phages could infect other *B. fragilis* strains, but were unable to infect *B. ovatus* or *B. thetaiotaomicron* etc.). Keller and Traub (1974) found that six *B. fragilis* phages were unable to infect any of the 36 *B. oralis* strains and six *B. melaninogenicus* they tested. Restricted host ranges of *Bacteroides* phages have also been reported by Tartera and Jofre (1987) and Bradley (1999).

Bacteroides phages tend to show little morphological variation, belonging mostly to the *Siphoviridae* family (Booth *et al*., 1979; Queralt *et al*., 2003).The dominance of siphoviruses does not appear to be geographically limited (Kory and Booth. 1986). Typical dimensions of *Bacteroides* phages are between 50 and 70 nm capsid diameter, with a straight, or slightly curved, tail of around 130 to 190 nm in length (Table 2.3). Although in the minority, descriptions of *Bacteroides* phages belonging to the *Myoviridae* and *Podoviridae* families do appear in the literature. Booth *et al*., (1979) isolated two *Myoviridae* (one on *B. ovatus* and one on *B. fragilis*), and one *Podoviridae* (on *B. fragilis*).

The validity of using phages infecting *Bacteroides* as indicators of human faecal material is reflected in the issue of an International Standard (Anon, 2001c) for their laboratory handling and assay. In the search for hosts that are able to detect species-specific phages, numerous *B. fragilis* strains have been studied, with varying degrees of phage recovery (Gerba, 2006).

Table 2.3 Selected *Bacteroides* phages found in the literature

Phage ID	Location isolated	Isolated from	Capsid diameter (nm)	Tail dimensions (nm)	Family	Host	Study
12	n/a	Animal Sera	68	180 (Straight/slightly curved)	*Siphoviridae*	*B. fragilis*	Keller and Traub, 1974
969	n/a	Animal Sera	68	180 (Straight/slightly curved)	*Siphoviridae*	*B. fragilis*	Keller and Traub, 1974
Bf-1	Virginia	Wastewater	70	190 x 16 (Straight/slightly curved)	*Siphoviridae*	*B. fragilis*	Booth *et al*., 1979
Bf-41	Virginia	Wastewater	106	50 x 16 (Short)	*Podoviridae*	*B. fragilis*	Booth *et al*., 1979
Bf-42	Virginia	Wastewater	90	167 x 24 (Straight)	*Myoviridae*	*B. ovatus/ B. fragilis*	Booth *et al*., 1979
Bf-71	Virginia	Wastewater	100	150 x 22 (Straight)	*Myoviridae*	*B. ovatus/ B. fragilis*	Booth *et al*., 1979
F1	Japan	Human Faeces	53	132 x 12 (straight)	*Siphoviridae*	*B. fragilis*	Kai *et al*., 1985
Baf-44, Baf-48, and Baf-64	Nebraska, USA	Wastewater	Dimensions given as between 51.1 x 49 and 55.9 x 52.3	Dimensions given as between 133 x 13.1 nm and 138 x 12.8	*Siphoviridae*	*B. fragilis*	Kory and Booth, 1986
B40-8	Barcelona region, Spain	Wastewater	62.8	156.8 x 9.3	*Siphoviridae*	*B. fragilis* HSP-40	Puig and Gironés, 1999
No ID stated (Type A/B in micrographs)	Barcelona region, Spain	Wastewater	53 to 55	180 nm long (Straight/slightly curved)	*Siphoviridae*	*B. fragilis* HSP-40 and RYC-2056	Queralt *et al*., 2003
No ID stated (Type C in micrographs)	Barcelona region	Wastewater	54 to 56	190 to 200 nm long (very flexible)	*Siphoviridae*	*B. fragilis* HSP-40 and RYC-2056	Queralt *et al*., 2003
No ID stated (Type D in micrographs)	Barcelona region	Wastewater	50 to 52	(Curly)	*Siphoviridae*	*B. fragilis* HSP-40 and RYC-2056	Queralt *et al*., 2003
ATCC 51477-B1	-	-	60	162 x 13.4 nm	*Siphoviridae*	*B. fragilis* RYC-2056	Hawkins *et al*., 2008

The isolation, identification and application of these strains are discussed below, and the advantages and disadvantages of each strain as a public health tool is described.

2.3.1 *Bacteroides fragilis* HSP-40

The first *B. fragilis* host strain proposed as an indicator of human faecal material was HSP-40 (ATCC51477), sourced from patient peritoneal exudates at the Hospital de Sant Pau Barcelona, Spain (Jofre *et al*., 1986). Phages active against HSP-40 are found in faeces from 4 % to 15 % of humans (Havelaar, 1993; Grabow *et al*., 1993), with excretion patterns varying over time (weeks and months). There appears to be no correlation between phage presence in human stools and age, ethnicity, intestinal illness or gender (Grabow *et al*., 1995).

Densities of HSP-40 phages in human faeces have been reported as being from 10^2 PFU/g (Kai *et al*., 1985) to 10^8 PFU/g (Tartera and Jofre, 1987). HSP-40 phages have been recorded as absent in the faeces of rabbits, pigs, cows, poultry, mice and quail (Tartera and Jofre, 1987), primates (chacma baboon and vervet monkey), higher primates (gorilla, chimpanzee, orang-utan), domestic animals (pig, cattle, sheep, horse, rabbit, dog and cat), and birds (goose and seabird; Grabow *et al*., 1993; Grabow *et al*., 1995). HSP-40 phages have been recorded in a single fowl sample, but it was speculated that this may have been the result of the fowl consuming human faecal material, or laboratory contamination (Grabow *et al*., 1993).

In order to be successful as an indicator of human faecal material, presence in waters polluted by human faeces, and absence in non-polluted waters must be demonstrated. Using the MPN technique (adapted for phage by Kott, 1966), in a study undertaken in

the Barcelona region, Jofre *et al* (1986) showed that phages active against HSP-40 were present in 100 % of water (n = 68) and sediment samples (n =6) taken from waterbodies near urban areas, whereas they were absent in water and sediment samples (n = 15 and 7, respectively) not subject to human faecal pollution. Presence of phages capable of infecting HSP-40 in waters contaminated with human faecal material, and absence in non-human faecally polluted waters was also demonstrated by Tartera and Jofre (1987). Phages infecting HSP-40 have been found in wastewater from slaughterhouses (Tartera *et al*., 1989), but at very low levels (less than three PFU per 100 ml), and it was speculated that there may have been some minor input of human faecal material.

The potential for using HSP-40 phages as an enteric virus surrogate organism is questionable. Phages infecting HSP-40 have been recovered in greater densities than enteric viruses (but lower than SC) from sediments receiving human faecal pollution (Jofre *et al*., 1989), but investigators have also reported absence of HSP-40 phages in water samples containing human enteric viruses (Grabow, 1993).

Alongside the confirmation of HSP-40 as a suitable indicator of human faecal material, other *Bacteroides* strains demonstrating similar or greater levels of phages recovery have been sought. Tartera and Jofre (1987) assessed eleven other *Bacteroides* strains, alongside HSP-40, with regard to their ability to detect phages in human faeces and WwTW effluent. HSP-40 was found to be the most effective strain, detecting more phages in thirteen wastewater samples (Table 2.4) than the others.

Table 2.4 Isolation of *Bacteroides* phages using twelve *Bacteroides* host strains (Tartera and Jofre, 1987)

WwTW Sample ID	*B. fragilis* HSP-40*	*B. fragilis* HSP-44	*B. fragilis* HSP-39	*B. fragilis* DSM-2151	*B. ovatus* DSM-1896	*B. vulgatus* DSM-1447	*B. hypermegas* DSM-1672	*B. microfusus* ATCC-29728	*B. eggerthii* ATCC-27254	*B. distasonis* ATCC-8503	*B. thetaiotaomicron* DSM-2079	*B. uniformis* ATCC-8492
1	230	0	0	-	-	-	-	-	-	-	-	-
2	230	20	0	-	-	-	-	-	-	-	-	-
3	230	0	0	-	-	-	-	-	-	-	-	-
4	230	-	-	0	-	0	40	-	-	-	-	-
5	230	-	-	20	-	0	130	-	-	-	-	-
6	230	-	-	0	-	0	0	-	-	-	-	-
7	1100	-	-	-	-	-	-	0	0	0	-	-
8	93	-	-	-	0	-	-	0	0	0	0	-
9	1100	-	-	-	0	-	-	0	0	0	0	-
10	1100	-	-	-	0	-	-	-	-	-	0	-
11	7	-	-	-	-	-	-	-	-	-	-	0
12	24	-	-	-	-	-	-	-	-	-	-	0
13	460	-	-	-	-	-	-		-	-	-	0

* all MPN per 100 ml; - not tested; 0 values less than 3 MPN/100 ml.

HSP-40 appears to be able to distinguish between human and animal faecal material in a range of fresh or saline water and sediment environments (Table 2.5). Seawater, marine sediments and groundwater samples contained phages active against HSP-40, albeit at diminished densities, which is likely to be a result of inactivation, increasing distance from pollution source, and dilution with unpolluted water.

Other studies have demonstrated the usefulness of phages infecting HSP-40 as indicators of human faecal material; Sun *et al* (1997) found that HSP-40 phages were present in 100 % of raw urban wastewater (n = 4), 100 % of river water downstream of WwTW (n = 4), and 50 % of river up-stream of WwTW (n = 2). It should be noted that these sample numbers are very small.

Table 2.5 HSP-40 phage densities in a range of environments, Spain (Tartera and Jofre, 1987)

Sample	No of samples	% positive for phage	Max (PFU/100 ml)	Minimum (PFU/100 ml)	Mean (PFU/100 ml)	Median (PFU/100 ml)
Wastewater	33	100	1.1×10^5	7	6.2×10^3	1.1×10^3
River water[a]	22	100	1.1×10^5	93	1.6×10^4	4.6×10^3
River sediment[a]	5	100	4.6×10^5	90	1.08×10^5	4.3×10^3
Seawater[a]	22	77.2	1.1×10^3	<3	1.2×10^2	4
Marine sediment[a]	12	91	43	<3	13.4	9
Groundwater[a]	19	21	Not known	0	-	-
Non-polluted[b]	50	0	-	-	-	-

[a] Areas exposed to wastewater
[b] Area not exposed to wastewater

Phages infecting HSP-40 have also been shown to bioaccumulate in mussels, in proportion to distance from pollution source, suggesting mussels containing HSP-40 phages are useful as indicators of remote faecal pollution (Lucena *et al*., 1994). They have also been found in oysters downstream of faecal pollution sources in North Carolina, USA (Chung *et al*., 1998). Other studies have successfully used phages active against HSP-40 as indicators of human faecal material in a range of environments (Lucena *et al*., 1996; Araujo 1997a, b; Puig *et al*., 1997).

Phages able to infect HSP-40 have been absent in faecal samples from the USA (Kator and Rhodes, 1992) and New Zealand (Sinton *et al*., 1998). Thus using HSP-40 to indicate presence of human faecal material in these, and possibly other, areas may not be valid (Puig *et al*., 1999).

Where found, recovery rates of HSP-40 phages from human faecal samples include 4 % in Holland (Havelaar, 1993), 5 % in Japan (Kai *et al*., 1985), 10 % in Spain (Tartera and Jofre, 1987), 11 % in France (Gantzer *et al*., 2002) and 15 % in South Africa (Grabow *et al*., 1993).

In order to be utilised as an accurate indicator of the presence of human faecal material and to avoid false-positive results, environmental replication of phages must not occur (satisfying the criteria for indicator organisms given in Section 1.5). For phages active against HSP-40, this issue has been addressed under both laboratory and field conditions (Jofre *et al*., 1986). Using siphovirus B40-8 (ATCC51477-B1) as a reference phage, replication was assessed in the presence of the host, in both fresh and seawater, sediment, under anaerobic and aerobic conditions and at two temperatures (22 °C and 30 °C). It was found that B40-8 was only capable of replication in the presence of the host bacteria and an external nutrient source (Modified Brucella-Broth; MBB). As such conditions are very unlikely to be present in natural environments (HSP-40 is anaerobic and unlikely to survive in the aquatic environment), it is assumed that *B. fragilis* phages are not capable of environmental replication.

HSP-40 phages are judged to be more resilient to environmental inactivation than SC and FRNA phages (Araujo *et al*., 1997a, b; Anon, 2001c; Gerba, 2006; Grabow, 2001; Fujioka, 2002; Sun *et al*., 1997) and FIB (Armon, 1993; Lucena *et al*., 1996; Jofre *et al*., 1986) possibly because of the prevalence of siphoviruses. Lasobras *et al* (1997) found that 100 % of HSP-40 phages isolated from both wastewater samples (n = 25) and waters subject to persistent pollution (n = 49) belonged to the *Siphoviridae* family.

Although HSP-40 shows good discriminatory powers, SC tend to outnumber HSP-40 phages in water (Tartera *et al*., 1989; Lucena *et al*., 1994) and sediments (Jofre *et al*., 1989; Lucena *et al*., 1996) contaminated with human faeces, although in groundwater,

densities of SC and HSP-40 phages were similar (2.5 and 2.7 PFU per 100 ml; Lucena *et al*., 1996). HSP-40 phages have been found in higher densities than enteroviruses in water and sediment samples (Lucena *et al*., 1994 and 1996) but it has also been reported that HSP-40 phages have been absent when enteroviruses have been found (Chung *et al*., 1998).

Araujo *et al* (1997a) compared levels of SC, HSP-40 phages and FRNA phages, reporting that in wastewater influent and samples from rivers subject to recent untreated domestic wastewater, SC were the most abundant (mean = 2.1×10^5 PFU/100 ml), followed by FRNA phages (mean = 7.2×10^4 PFU/100 ml), then HSP-40 phages (mean = 2.3×10^3 PFU/100 ml).

In summary, HSP-40 phages appear to be a structurally and phenotypically homogenous group that are found exclusively in human faeces and in waters and sediments contaminated with human faecal material. Numerous studies have demonstrated that they can be used successfully as an indicator of human faecal material, as they do not replicate in the environment, and have similar, if not greater persistence than commonly used FIB and enteroviruses. However low densities in human faeces and evidence of limited geographical applicability mean this indicator may not be ideal.

2.3.2 *Bacteroides fragilis* RYC-2056

HSP-40 was the *B. fragilis* host strain used in the majority of microbial source tracking studies for around ten years (Jofre *et al*., 1986 to Puig *et al*., 1997). In order to find a more effective *Bacteroides* host strain, overcoming the previously highlighted shortcomings of HSP-40, Puig *et al* (1997 and 1999) investigated 115 hospital sourced *B. fragilis* stains alongside HSP-40. Five of these strains RYC-2056, RYC-3234, RYC-3317, RYC-3318 and RYC-4023, were selected for further study as they detected high numbers of phages in urban wastewater.

These RYC strains and HSP-40 were examined in their ability to detect phages in animal faeces; only phages infecting HSP-40 and RYC-4023 were absent in animal faeces. HSP-40, RYC-2056 and RYC-4023 did detect phages in slaughterhouse wastewater, and it was speculated this was a result of minor human faecal contamination, as was described for HSP-40 in Tartera *et al* (1989). Phages infecting RYC-2056 were found in 100 % of slaughterhouse samples (mean = 3.7×10^1 PFU/ml) whereas HSP-40 phages were found in 39 % (mean = 1×10^0 PFU/ml) and RYC-4023 phages were found in 28 % of samples (mean = 1.4×10^0 PFU/ml).

The assessment of host strain ability to recover phages from human faecal material was limited. Of the six *B. fragilis* strains tested, detection rates within human faeces were only described for HSP-40 (10 to 13 %; Grabow *et al*., 1995 and Tartera and Jofre, 1987) and RYC-2056 (28 %). However, all host strains were assayed for their ability to recover phages from raw wastewater. All strains detected phages in WwTW samples, with RYC-2056 recovering more than HSP-40 (2.4×10^2 and 8.2×10^1 respectively). Moreover, HSP-40 detected the least phages of all strains in WwTW

samples. It is not made clear why strain RYC-4023 was not further assessed for suitability (it recovered phages exclusively from human faeces and was present in greater numbers than HSP-40).

In a large pan-European study, Blanch *et al* (2006) also found that RYC-2056 phages were detected in both human and animal faecal samples. Geographical spread of HSP-40 and RYC-2056 phages are shown in Table 2.6 (Puig *et al*., 1999) which shows that levels of HSP-40 phages were consistently lower than RYC-2056 phages (mean = 56.6 PFU/ml and 413.9 PFU/ml respectively).

Table 2.6 HSP-40 and RYC-2056 phage densities in urban wastewater samples in a variety of countries (Puig *et al*., 1999)

Country	**HSP-40 (PFU/ml)**	**RYC-2056 (PFU/ml)**
The Netherlands	1	4.6×10^2
The Netherlands	2.6	7.7×10^2
Ireland	1.4	3.0×10^2
Ireland	1.6	4.4×10^2
Austria	8.5	8.1×10^2
Austria	0.5	6.1×10^2
Portugal	0.4	1.8×10^2
Portugal	0.1	1.0×10^2
Portugal	0	1.0×10^2
Germany	2.2	7.8×10^2
Germany	1.3	6.0×10^2
Sweden	0.9	2.2×10^1
France	3.1×10^1	2.3×10^2
South Africa	1.1×10^2	1.8×10^2
South Africa	4.5×10^2	not done
South Africa	1.2×10^2	5.4×10^2
South Africa	2.3×10^2	5.0×10^2

Some geographical areas recorded low levels of HSP-40 phages, but moderate levels of RYC-2056 phages (e.g., Portugal; Puig *et al*., 1999) suggesting that RYC-2056 may be a more universally applicable indicator. The observed differences between densities of HSP-40 and RYC-2056 phages in urban wastewater may be explained by

differences in excretion rates. Around 30 % of humans excrete RYC-2056 phages (Puig *et al*., 1999), whereas less than 15 % excrete HSP-40 phages (Grabow *et al*., 1993).

Similar densities of RYC-2056 phages have been found in other areas: levels in Argentina, Columbia, France and Spain showed little difference, geometric mean over all sites in all countries being 4.8 $\log_{10}$ PFU/100 ml (n = 100; Lucena *et al*., 2003). Blanch *et al* (2004) showed that phages infecting RYC-2056 had limited ability to discriminate between faecal source (human and animal) in a range of geographical areas (UK, Spain, France, Sweden and Cyprus). Human (municipal and hospital) and animal (slaughterhouse) wastewaters were assayed and there was little difference between levels of phages infecting RYC-2056 (mean log PFU/100 ml were 3.82, n = 85, and 3.76, n = 85 respectively).

As with HSP-40 phages, RYC-2056 phages have been detected in shellfish (Spain, Greece, Sweden, UK), at lower densities than SC and FRNA phages (Formiga-Cruz, 2003). The validity of using RYC-2056 phages was assessed in a French river study by Skraber *et al* (2002). Levels of RYC-2056 phages were less than SC and FRNA in all water types tested (Table 2.7). Phages infecting RYC20-56 were influenced negatively by increased temperatures, but positively by increased flow and conductivity. It was reported that under favourable environmental conditions (high flows and low temperatures, i.e., winter) densities remained stable, whereas during unfavourable environmental conditions (high temperatures and low flows, i.e., summer) RYC-2056 phages were often undetectable (75 % of samples, n = 96).

Levels of phages infecting RYC-2056 have been shown to be lower than SC in sewage influent, septic tanks, and sewage-impacted rivers (Lucena *et al*., 2003). Also, an EU wide study reported that RYC-2056 phages were present at consistently lower densities than SC and FRNA phages in seawater sampling sites (n = 89) and freshwater sampling sites (n = 54; Contreras-Coll *et al*., 2002). Although lower than SC and FRNA phages, the widespread geographical prevalence of phages infecting RYC-2056 is an advantage over HSP-40 phages (as shown in Puig *et al*., 1999) as the latter's geographical spread is more limited.

Table 2.7 Mean levels of phages (log PFU/100 ml) in three types of French water (standard deviation in parenthesis; Skraber *et al*., 2002)

Phage type	**Raw wastewater (n = 9)**	**Treated wastewater (n = 9)**	**River water (n = 96)**
SC	6.27 (0.19)	3.96 (0.36)	3.25 (0.33)
FRNA	5.63 (0.77)	2.75 (0.54)	2.31 (0.45)
RYC-2056 phages	4.42 (0.41)	1.77 (0.44)	58% positive for phages (42% below detection limit (17 PFU/100 ml)

As with other *Bacteroides* phages, RYC-2056 detect phages with a narrow host range. (Puig *et al*., 1997; Puig *et al*., 1999)

Analysis of the RYC-2056, SC and FRNA phages in temperate river waters suggested that inactivation rates of all phages were similar, but greater than those of FIB (Lucena *et al*., 2003; Durán *et al*., 2002). Other studies have demonstrated that RYC-2056 phages are more persistent than SC and FRNA phages (Contreras-Coll *et al*., 2002).

In summary, RYC-2056 detects phages in both animal and human faeces showing less discriminatory ability than HSP-40. However, it detects a higher number of phages than HSP-40 in many geographical areas tested. The International Organisation for Standardization (Anon 2001c) recommends that RYC-2056 is the *Bacteroides fragilis* strain used when employing phages to indicate faecal pollution.

2.3.4 *Bacteroides thetaiotaomicron* strain GA-17

Bacteroides strain GA-17 was first isolated by Payan *et al* (2005), and proved successful in identifying human faecal pollution various European countries (Spain, France, Sweden, and Cyrus). However, it was found to produce turbid plaques when tested against municipal WwTW final effluent from southeast England. Costan-Longares *et al* (2008) found a good correlation between GA-17 phages and enteroviruses in water reclamation facilities (Spain). To date, a limited number of studies have been carried out using GA-17.

2.3.5 *Bacteroides* strain HB-73

Isolated from Hawaiian municipal WwTW effluent by Kannppan *et al* (2008), HB-73 has shown to detect phages within wastewater effluents from Hawaii, whereas GB-124 and GA-17 did not (Vijayavel *et al*., 2010). The geographical applicability of HB-73 is yet to be assessed as few studies have been undertaken.

2.3.6 *Bacteroides* strain GB-124

As has been shown, *Bacteroides* spp., host strains vary in their ability to distinguish between human and animal faecal material; strain RYC-2056 detects more phages than HSP-40 in a range of geographical areas, but does not discriminate between

human and animal faeces. Therefore more universally applicable, diagnostic host strains, able to detect phages in large numbers have been sought.

Bacteroides strain GB-124 isolated by Payan *et al* (2005), has been proposed as an indicator of human faecal material. GB-124 is most analogous to *B. ovatus* (Table 2.8) and was originally sourced from a raw municipal wastewater sample from south eastern England.

Table 2.8 Genetic characteristics of GB-124 (Payan *et al*., 2005)

Size of amplicon (bp)	1,488
Size sequenced (bp)	1,385
Most homologous strain	*B. ovatus* ATCC 8483T
Accession number	X83952
% Sequence identity	96.5

Ebdon *et al* (2007) built upon the work carried out by Payan *et al* (2005), assessing the discriminatory ability of GB-124 within waterbodies with different pollution regimes. Phages infecting *Bacteroides* strain GB-124 were recorded in all tested WwTW final effluent samples (n = 110; mean = 3.92 $\log_{10}$ PFU/100 ml; Table 2.9), and at monitoring locations thought to be contaminated with human faecal material (n = 47; mean = 3.39 $\log_{10}$ PFU/100 ml). GB-124 phages were present in samples taken from a WwTW with p.e. of 28, suggesting that GB-124 phages are excreted by a significant percentage of humans. They were recorded in lower densities in areas with both human and animal faecal contamination (n = 90; mean = 2.38 $\log_{10}$ PFU/100 ml) whilst they were absent in all water samples not subject to human faecal pollution (n = 4), and in animal faeces (n = 30).

Longitudinal analysis showed that phages infecting *Bacteroides* GB-124 decreased with increasing distance from WwTW, suggesting that WwTW effluent contributed the majority of human faecal material to the catchment. As GB-124 was isolated in 2005, the geographical applicability has not been widely assessed.

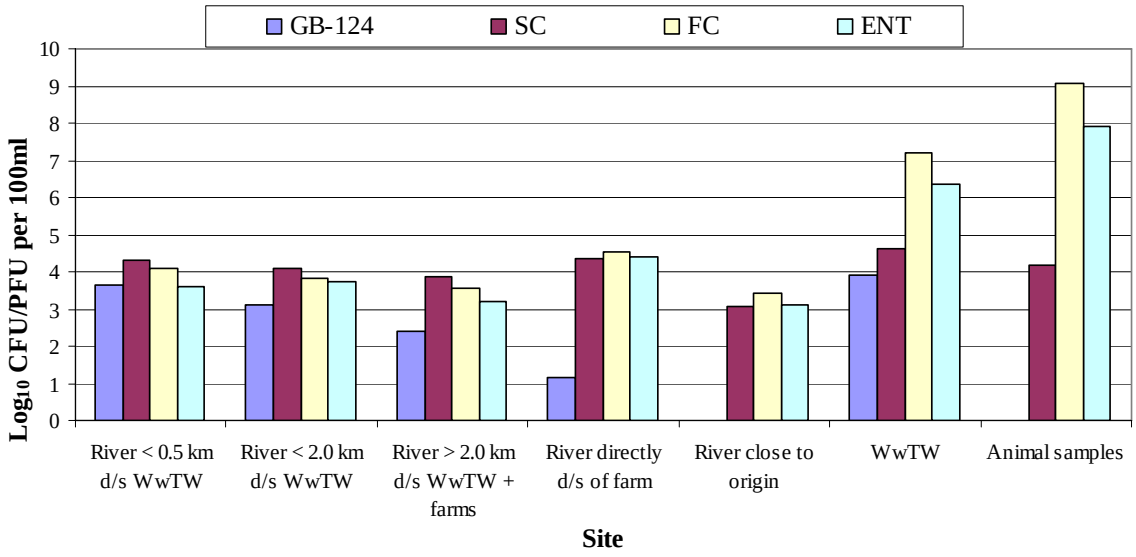
Table 2.9 Levels of phages infecting GB-124 in WwTW final effluent (Ebdon *et al*., 2007)

Site	**Population equivalent**	**Log_{10} PFU/100 ml**
Brighton Portobello	261,254	4.22–4.38
Eastbourne	119,336	3.76–4.70
Newhaven East	55,955	2.50–3.27
Goddards Green	49,824	3.98
Scaynes Hill	37,327	3.77–4.18
Hailsham South	27,448	2.30–4.26
Vines Cross	23,518	2.95
Uckfield	23,163	3.15–4.04
Crowborough Redgate	20,700	3.32
Hailsham North	14,725	2.00–3.00
Ringmer	4805	3.74
Forest Row	4456	2.30–3.63
Barcombe	3533	2.70
Cuckfield	3186	3.78
Wadhurst Whitegate	2675	2.00–3.00
Crowborough St Jon's	2377	2.00–2.30
Ditchling	1621	2.47–4.30
Maresfield	1542	3.47
Blackboys	1046	2.47
Hartfield	949	2.00–2.60
Kingston	946	2.30–3.32
Alfriston	769	3.07
East Hoathly	761	2.30–3.65
Rodmell	381	2.00–2.84
Poynings	371	2.30–2.84
Ansty	240	2.00
Streat	28	3.90
Esholt (N. England)	624,000	3.15
Aalborg (Denmark)	-	2.70

Phages active against GB-124 have been recorded in final effluents from south eastern England WwTW, north eastern England, Denmark (Ebdon *et al*., 2007), Spain, Sicily,

Hawaii (though not recorded by Vijayavel *et al*., 2010), Brazil and Uganda (unpublished data) suggesting that the usefulness of GB-124 is not limited to SE England. As has been demonstrated with other *Bacteroides* host strains, phages active against GB-124 were found at lower levels than SC, faecal coliforms (FC) and enterococci (ENT) in all samples (Figure 2.5).

Figure 2.5 Levels of GB-124 phages, SC, FC and enterococci in a range of samples (Ebdon *et al*., 2007)



In order to improve on existing techniques, GB-124 should detect phages in greater densities than other *Bacteroides* host strains. As GB-124 was isolated recently, extensive research has not been carried out, but the limited data suggest that the relationship between GB-124 and RYC-2056 phages may vary geographically. GB-124 detected fewer phages in UK wastewater than RYC-2056 (Table 2.10), whereas in Spanish samples, GB-124 outnumbered RYC-2056 phages.

Table 2.10 Levels of phages (PFU/ml) detected from various reference sources by RYC-2056 and GB-124 (Payan *et al*., 2005)

Strain	Human[1] (*n*)	Human[2] (*n*)	Cattle (*n*)	Pig (*n*)	Poultry (*n*)
RYC-2056	1.1×10^2 (12)	1.0×10^2 (9)	2.6×10^2 (7)	6.1×10^2 (8)	2.2×10^2 (8)
GB-124	5.0×10^2 (12)	2.5×10^1 (9)	0.17 (7)	0.33 (8)	0 (8)
No of sample GB-124>RYC-2056	11/12	3/9	0/7	0/8	0/8

[1] Spanish WwTW

[2] UK WwTW

Since the 'phage-lysis' method relies on traditional culture dependent methods rather than molecular techniques, the use of bacteriophages infecting *Bacteroides* strains for microbial source tracking, or simply as alternative FIO can be relatively low-cost, (Ebdon *et al*., 2007). This characteristic of the method would be useful in LEDC where access to laboratory equipment and media may be limited, and problems of unregulated PS discharges of human waste may be significant. The cost of the *Bacteroides* phage assay is estimated to be less than fifty pence per sample (author's own calculation including all lab consumables and media, but excluding operators time), whereas appropriate molecular methods may be more expensive.

Work is currently being carried out by researchers of EPHRU at the University of Brighton and the Instituto Superior Téchnico, Lisbon, into the relationship between human pathogenic viruses and densities of GB-124 phages in a range of environments. GB-124 has demonstrated an ability to detect phages of exclusively human faecal origin. At present, there is a lack of firm evidence supporting its use in different geographical areas, and nothing is known regarding the phages capable of infecting it and their ecology. This study aims to improve our understanding of these important knowledge gaps regarding the ecology of phages infecting GB-124.

2.4 Quantitative Microbial Risk Assessment (QMRA)

In order to assess more accurately the risk to users of pathogen contaminated waterbodies, QMRA may be used to assess the risk associated with a specified pathogen, in a particular water system. Risk assessments are ideally based on pathogen occurrence data, but as this is often missing, surrogates are often used (Haas *et al.*, 1999). Phages infecting *Bacteroides* strains may be used as surrogate microorganisms in QMRA.

QMRA produces an estimation of the disease burden from a water resource, based on the probable risks associated with the densities of surrogate organisms present. Health burden can be measured using the Disability Adjusted Life Years (DALYs) approach (Murray and Lopez, 1996) allowing comparison between health burdens of different pathogens. With respect to the present study, it appears that *Bacteroides* phages and enteroviruses may show similar ecological behaviour, and it is hypothesised that phages infecting GB-124 may be used in QMRA for assessments of the enteroviruses health burden. A relationship between surrogate microorganism and pathogen must be demonstrated before QMRA can be used.

When selecting a surrogate microorganism, assumptions relating to correlation of pathogen and surrogate ecology are made. This study aims to test the assumption that phages infecting GB-124 and enteroviruses share similar inactivation kinetics during exposure to UV-B and UV-C radiation. Assessment of phages infecting GB-124 inactivation kinetics will support the development of QMRA in a number of ways. The removal of enteroviruses during solar irradiation (UV-B driven) and during UV-C treatment within WwTW, may be indicated by the removal of phages infecting GB-124, thus providing better data for the formulation of QMRA.

Production of a QMRA is a complex process with many protocols available. As it is beyond the scope of the present research, production of QMRA will not be discussed in greater detail. However, further information is given by Haas *et al* (1999) or Medema and Ashbolt (2006).

CHAPTER THREE

3. Phage ecology and UV inactivation

3.1 Phage ecology

As stated in Section 2.2, differential inactivation/removal rates of enteric pathogens and their indicators may lead to inaccurate assessments of human health risk from faecally polluted waters. Many factors influence removal and inactivation of indicator organisms, some of which may be reversible. In order for GB-124 phages to be used as indicators of human faecal material in a range of geographical regions and physiochemical situations, a better understanding of their ecology is needed.

Major phage ecology studies have only been published during the past 25 years; the first monograph being *Phage Ecology* (Goyal, 1987). Studies detailing the ecology of phages in numerous environments have since been published; these include terrestrial (Ashelford *et al*., 2003), marine (Breitbart *et al*., 2002; Azam and Worden, 2004), freshwater (Paul and Kellogg, 2000), and dairy (Brüssow, 2001). Phage ecology has been dominated by two strands of enquiry, dairy processes and phage therapy in humans (Guttman and Kutter, 2005), but other 'real world' ecological situations are now being examined.

Numerous factors are thought to influence the survival of phages in natural environments (Grabow, 2001), including:

- the relationship between host and phage densities;
- organic compounds/matter (humic and fulvic acids);
- temperature and pH of surrounding environment;

- presence, and type, of solids;
- UV/visible light;
- other microorganisms; and
- presence of ions.

Phages are most vulnerable to inactivation when searching for a new host, or trapped in a host-free environment (Guttman and Kutter, 2005).

The majority of phages used in ecological experiments to date have been somatic coliphages. There is little information regarding the behaviour of *Bacteroides* phages in natural environments, though, as outlined in Chapter Two, available evidence suggests they are more persistent in the environment than traditional FIO.

3.2 UV inactivation technologies

3.2.1 Background

In order to meet mandatory consents for microbial indicators given in the EU Bathing Waters Directive (76/160/EEC) and the Shellfish Waters Directive (2006/113/EEC) disinfection of wastewaters is becoming more widespread and is also used in the food industry, notably with dairy, meat and vegetables (Ngadi *et al*., 2003). It is estimated that diarrhoea episodes could be reduced by up to 45% in less developed countries if drinking water disinfection was more widespread (WHO, 2004).

The first use of UV as a disinfecting agent in drinking water was recorded in 1910 (Henry *et al*., 1910), but high cost, introduction of chlorination and unreliability all restricted its use (Wolfe, 1990; Hoyer, 2004). The evidence of susceptibility of *Cryptosporidium* to UV (not readily inactivated by chlorination) encouraged the introduction of UV disinfection within drinking water plants (Clancy *et al*., 1998). The driver behind the introduction of UV treatment in the US, was the introduction of the Long Term 1 Surface Water Treatment Rule (USEPA, 2002) and subsequent water treatment rules requiring the 3-$\log_{10}$ removal/inactivation of *Giardia* cysts and *Cryptosporidium* and 4-$\log_{10}$ removal of viruses. In the US, UV disinfection has been widespread for over twenty years (Bolton and Smith, 2005) and in the UK, UV disinfection is becoming increasingly used during the tertiary stage at wastewater treatment works. Because of the reluctance of many European countries to introduce widespread chlorination of drinking water, UV is an attractive option, tending to be more economical than membrane or ozone treatment (Bolton and Smith, 2005). Moreover, UV disinfection does not produce harmful by-products (Shin *et al*., 2005). A number of countries have formulated regulation and guidance manuals regarding

the use of UV disinfection technologies (see ÖNORM, 2001 and 2003; USEPA, 2006).

UV disinfection systems are usually one of three types:

1. **low-pressure low intensity (LPLI)**: These systems generate monochromatic radiation usually around 254 nm, and are housed in quartz sleeves, preventing direct contact with water;
2. **low-pressure high intensity (LPHI):** As above, but with two to four times the UV-C output of LPLI systems; and
3. **medium-pressure high-intensity (MPHI):** These systems generate polychromatic radiation (more than one wavelength), of which 27 to 44 % of energy is UV-C and only 7 to 15 % in near 255 nm. However, total UV-C output is 50 to 100 times greater than LPLI systems.

Table 3.1 Typical operational characteristics for UV lamps used in WwTW (Metcalf and Eddy, 2003)

Item	**Unit**	**Type of lamp**		
		L pressure L intensity	**L pressure H intensity**	**M pressure H intensity**
Power	W	70 to100	200 to 500	Not stated
consumption	kW		1.2[a]	2 to 5
Lamp current	mA	350 to 550	Variable	Variable
Lamp voltage	V	220	Variable	Variable
Efficiency	%	30 to 40	25 to 35	10 to 12[b]
Lamp output at 254 nm	W	25 to 27	60 to 400	Not stated
Temperature	° C	35 to 45	90 to 150	600 to 800
Pressure	mmHg	0.007	0.001 to 0.01	Not stated
Lamp length	M	0.75 to 1.5	Variable	Variable
Lamp diameter	Mm	15 to 20	Variable	Variable

[a] Very high output lamp [b] Output in the germicidal range (~ 250 to 260 nm)

3.2.2 General UV inactivation characteristics of micro-organisms

UV radiation is the portion of the electromagnetic spectrum between 180 and 400 nm (UV-C: 180 to 280 nm, UV-B: 280 to 315 nm and UV-A: 315 to 400 nm; Hargreaves *et al*., 2007). The primary germicidal band is between 220 to 320 nm, located mainly in the UV-C range. However, UV-C produced by the sun is totally absorbed by the ozone layer, and does not reach the Earth's surface (Jagger, 1985). In the context of environmental microorganism inactivation, UV-C is not a factor. Solar UV-A and UV-B do reach the ground (95% and 5 % of solar UV respectively; Hargreaves *et al*., 2007), and have inactivating effects on organisms. Deleterious properties of sunlight on living cells are usually assigned to UV-B, although UV-A can also contribute significantly, having carcinogenic qualities (Stary and Sarasin, 2000).

The detrimental effects of UV-B have been widely documented. Studies have shown that UV-B is capable of inactivating viruses (Suttle and Chen, 1992; Noble and Fuhrman, 1997), bacteriophages (Suttle and Chen, 1992; Suttle and Chan, 1994), bacteria (Joux *et al*., 1999), oocysts (King *et al*., 2008), plants (Krizek *et al*., 1997), bacterioplankton communities (Winter *et al*., 2001) and more complex life forms (van der Horst *et al*., 2002; Nazari *et al*., 2010; Wang *et al*., 2010a).

UV is a physical rather than chemical disinfection method, primarily through UV photon absorption by the pyrimidine bases in DNA (thymine or cytosine), or within RNA (uracil or cytosine), preventing cell replication (Bolton and Linden, 2003). The level and manner of microorganism inactivation differ between UV wavelengths.

Examples of direct damage caused by DNA absorption of UV include cyclobutane pyrimidine dimers (most common type being T-T; Figure 3.1) and pyrimidine (4–6) pyrimidine photoproducts (most common type being T-C) which mostly create C → T transitions (Rünger and Kappes, 2008). Such pyrimidine dimers are the most common UV-B induced pre-mutagenic lesion (Jagger, 1985). In conjunction with direct DNA damage, capsid damage may also be caused by UV-C irradiation (Pecson *et al*., 2009) leading to microorganism inactivation.

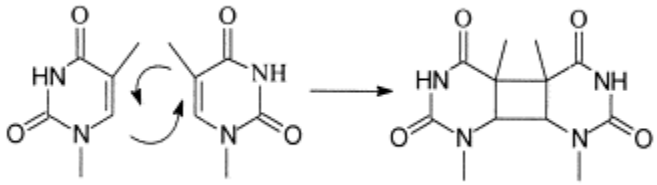


Figure 3.1 Formation of thymine cyclobutane dimers (Ravanat *et al*., 2001)

The dose of UV irradiation may alter the inactivation mechanism; Yang *et al* (1999) reported that sunlight exposure for a short period of time led to C to T mutations within M13mp2 phage, but at longer exposure the dominant mutation was G to C. The absorption of UV-B by DNA is less efficient than UV-C, whilst UV-A is thought not to be directly absorbed by DNA (Harm, 1980).

The effects of environmental UV-B and UV-C on microorganisms have been recorded by numerous authors, and the response of microorganisms to UV radiation has been shown to vary widely. Faecal indicator bacteria have been shown to be inactivated more quickly than enteric viruses (Sobsey, 1989). Bacteriophages may prove to have more similar inactivation characteristics to enteric viruses than enteric bacteria. They

may therefore prove to be more suitable surrogates for these pathogens (Shin *et al.*, 2005). This is a key knowledge gap addressed in this study.

Sunlight (in which the UV component is 95% UV-A, 5% UV-B) has been shown to decrease phage titres of surface waters by five percent per hour (Wommack *et al.*, 1996), and it is thought to be the dominant factor controlling loss of infectivity within surface waters (Garza and Suttle, 1998). The effects of sunlight have been observed up to 200 m below the surface of the water (Guttman and Kutter, 2005), and phages with smaller capsids (less than 60 nm) were found to be more susceptible to damage (Heldal and Bratbak, 1991; Mathias *et al.*, 1995). Phages capable of infecting the same host may have different decay rates in sunlight, with non-native phages decaying faster than native phages (Noble and Fuhrman, 1997) indicating that native phages adapt to ambient conditions.

3.3 Collimated Beam apparatus set-ups

In order to investigate the inactivation kinetics of phages, laboratory batch experiments using collimated beam (CB) apparatus are often carried out. CB experiments allow UV fluence rate and exposure times to be accurately controlled, giving narrow confidence intervals. UV inactivation experiments *in situ* (e.g., at wastewater treatment works/water treatment works) are more difficult to conduct because of complex water matrices (Scott *et al*., 2005), and difficulties in measuring average fluence rate and contact time (Sommer and Cabaj, 1993a, b; Hijnen *et al*., 2006). Modelling of predicted UV fluence levels and distribution within UV reactors based on water quality and reactor design has been carried out (Bolton *et al*., 2005; Cabaj *et al*., 2005; Ducoste and Linden, 2005), but collimated beam apparatus allow the close control of all parameters. CB experiments for microbial UV inactivation studies have become commonplace since the work of Qualls and Johnson (1983) who demonstrated the ease and accuracy of the method.

3.3.1 Hardware

UV CB inactivation experiments carried out by different authors often report conflicting results, despite using the same test organism. Differences in organism preparation and handling, UV irradiation apparatus, or the way fluences are calculated are likely to account for this. Numerous pieces of information are required in order for the reader to interpret results from a particular CB experiment. Sommer *et al*., (1995) states the following pieces of information are required:

- details of the vessel containing sample being irradiated;
- depth of the sample;
- initial density of the test organism suspension;

- the UV apparatus design;
- the method of fluence measurement; and
- the irradiation duration.

When all of the above information is known, inactivation data from different studies, or laboratories, may be compared. If some of the above information is missing, it should be noted that the results of the study may not be directly comparable to other studies. In circumstances where authors used a different irradiation method, or the method is not fully described, the data should be treated with caution. Conclusions drawn from such situations should be confirmed by running test organisms in parallel using the same apparatus and experimental design.

Although numerous configurations of CB apparatus are detailed in the literature, in order to conduct quality UV experiments, seven components are considered necessary (Bolton and Linden, 2003):

1. A UV impenetrable shutter: required to regulate exposure time;
2. a window/structure: UV lamp output is temperature sensitive and it is necessary to ensure enclosure is thermally stable;
3. a stable power supply;
4. a collimating tube: in order to provide a spatially homogeneous irradiation field, a collimating tube should be used. The inside of the tube must be ‘roughened’ and painted ‘flat black’ to prevent reflections within the tube. Alternatively, a series of apertures may be used;
5. an irradiation stage;

6. a stirring mechanism: suspension must be slowly stirred (avoiding vortex creation) during UV exposure to assume equal fluence; and
7. UV lamps: lamps should be vented to keep the temperature stable.

It is also important to recognise that although the term ‘collimated beam’ is commonly used, the UV rays are never truly parallel. It has been suggested that the term ‘quasi-parallel beam’ should be used instead (Sommer *et al*., 2001). The CB apparatus used in this study is described in Chapter Four: Materials and methods.

3.3.2 Fluence calculation and exposure methods

Methods of UV inactivation detailed in the literature are presented in Tables 3.2 and 3.3, and it is evident that many different arrangements of UV irradiation apparatus and fluence calculations have been used. In some studies microwells have been used to irradiate microorganisms, whereas some have used large Petri dishes. The arrangement and power of UV bulbs also varies widely between studies. Numerous authors have used no corrections to determine fluences (e.g., Kadavy *et al*., 2000), some have corrected for the attenuation of UV beam through sample depth (e.g., Hayes *et al*., 2006), and some have included absorption and a correction to account for unequal photon distribution across the irradiation vessel (e.g., Mofidi *et al*., 2002). Since the study of Bolton and Linden (2003), many authors have used the UVCalc spreadsheet which employs all necessary corrections (or calculating these corrections manually), giving a much more accurate estimation of the true fluence delivered to the sample being irradiated.

Table 3.2 Low pressure UV-C irradiation methods reported in the literature

Reference	Bolton and Linden 2003 corrections (or similar) used?	Other corrections made
Rauth, 1965	N	Absorbance corrections (Beer-Lambert law)
Antopol and Ellner, 1979	N	None
Rice and Hoff, 1981	N	None
Qualls and Johnson, 1983	N	Absorbance corrections (Beer-Lambert law)
Restifo et al., 1983	N	None
Chang *et al.*, 1985	N	Absorbance corrections (Beer-Lambert law)
Knudson, 1986	N	None
Butler, *et al*., 1987	N	UV absorbance of solutions; some divergence calculations; both corrections not used in all calculations
Harris *et al*., 1987	N	Absorbance corrections (Beer-Lambert law)
Havelaar *et al*., 1990	N	Fluence rate distribution (similar to Petri factor); Absorbance corrections (Beer-Lambert law)
Liltved and Landfald, 1996	N	Absorbance corrections (Beer-Lambert law)
Menga and Gerba, 1996	N	Absorbance corrections (Beer-Lambert law)
Lazarova *et al*., 1998	N	No detailed method stated
Shaffer *et al*., 1999	N	-
Kuluncsics *et al*., 1999	N	None
Tosa and Hirata, 1999	N	None
Kadavy *et al*., 2000	N	None
Martin *et al*., 2000	N	None
Shin *et al*., 2001	N	Fluence rate distribution (similar to Petri factor); Absorbance corrections (Beer-Lambert law)
Oguma *et al*., 2001	N	None
Mofidi *et al*., 2002	N	Fluence rate distribution (similar to Petri factor); Absorbance corrections (Beer-Lambert law)
Morita *et al*., 2002	N	None
Campbell and Wallis, 2002	N	Distribution factor (similar, but less comprehensive than Petri factor); UV absorbance
Kellogg and Paul, 2002	N	None
Linden *et al*., 2002b	N	Absorbance corrections (Beer-Lambert law); Petri factor (no details given)
Gerba *et al*., 2002	N	Absorbance corrections (Beer-Lambert law)
Oguma *et al*., 2002	N	Used biodosimeter (Qß phage)
Zimmer and Slawson, 2002	Y	
Hayes *et al*., 2003	N	None, though acknowledged attenuation may occur through Petri dish and solution
Marshall *et al*., 2003	Y	
Zimmer *et al*., 2003	Y	
Ngadi *et al*, 2003	N	None
Nicholson and Galeano, 2003	Y	-
Thurston-Enriquez *et al*., 2003	N	Absorbance corrections (Beer-Lambert law)

Table 3.2 (cont.) Low pressure UV-C irradiation methods reported in the literature

Reference	Bolton and Linden 2003 corrections (or similar) used?	Other corrections made
Caballero *et al*., 2004	N	None
Husman *et al*., 2004	N	Reflection and adsorption of sample
Lazarova and Savoye, 2004	Not stated	Method not given
Oguma *et al*., 2004	N	None
Duizer *et al*., 2004	N	None stated
Oguma *et al*., 2005	N	None
Scott *et al*., 2005	N	Absorbance corrections (Beer-Lambert law)
Shin *et al*., 2005	Y	-
Templeton *et al*., 2005	Y	-
Tree *et al*., 2005	N	Average depth through sample (Beer-Lambert law)
Wu *et al*., 2005	N	None
Nwachuku *et al*., 2005	N	Absorbance corrections (Beer-Lambert law)
Bohrerova *et al*., 2006	Y	-
Hayes *et al*., 2006	N	UV absorbance of solutions
Eischeid and Linden, 2007	Y	-
Hayes *et al*., 2008	Y	-
Sirikanchana *et al*., 2008	Y	-
Chun *et al*., 2009	Y	-
Eischeid *et al*., 2009	Y	-
McKinney *et a*l, 2009	N	Not stated
Wang *et al*., 2010b	Y	-

Table 3.3 Low pressure UV-B irradiation methods presented in the literature

Reference	Bolton and Linden 2003 corrections (or similar) used?	Other corrections made
Joux, 1999	N	None
Jacquet and Bratbak, 2003	N	None
Duizer *et al*., 2004	N	Not specified
Kumar *et al*., 2003	N	Not specified
Zenoff *et al*., 2006	N	None

3.4 Microorganism inactivation kinetics

3.4.1 UV-C

By comparing all the inactivation data from studies detailed in Appendix 1, general patterns of UV-C sensitivity may be observed. Within the literature there have been two major deviations from first-order kinetics reported:

1. The shoulder model – no inactivation at low fluences, with log-linear relationship at high fluences (Knudson, 1985; Hoyer, 1998; Sommer *et al*., 1998; Mamane-Gravetz *et al*., 2005); and
2. The tailing model – no subsequent increase in inactivation at higher fluences usually after 99% inactivation has occurred (Hijnen, *et al*., 2006). This may be caused by inadequate stirring (Budowsky *et al*., 1981a, b).

Bacteria are the most UV-C sensitive category of microorganisms, with a wide degree of fluences required to achieve 4-$\log_{10}$ reductions, from 1.1 mJ/cm^2 (*Escherichia coli* O157:H7; Sommer *et al*., 2000) to 50 mJ/cm^2 (*Salmonella typhimurium*; Maya *et al*., 2003). The mean fluence required for a 4-$\log_{10}$ reduction is 9.95 mJ/cm^2 (n = 54). Some bacteria display a "shoulder" (e.g., *Mycobacterium avium* W41; Hayes *et al*., 2008), whilst the majority do not. Protozoa are also easily inactivated requiring low fluences for 4-$\log_{10}$ reduction, the exception being excystation assays which show large fluence requirements (*Giardia muris* excystation assay requires 110 mJ/cm^2 to achieve a 2-$\log_{10}$ inactivation). Data in the literature suggest that inactivation of protozoan specimens is not $\log_{10}$ linear throughout the inactivation curve, with significant "tailing" observed (Craik *et al*., 2000; Campbell and Wallis, 2002; Zimmer *et al*., 2003).

Generally, the fluence requirements for the inactivation of viruses and phages are higher than those for bacteria and protozoa. Inactivation fluences for phages range from around 6.7 mJ/cm^2 to 119 mJ/cm^2 (n = 26) to achieve a 4-log$_{10}$ reduction (data presented in appendix I). These data are severely skewed by MS2, which has been consistently shown to be the most UV resistant phage, requiring fluences far higher than other phages tested. Inactivation data for viruses range from 7 mJ/cm^2 to 226 mJ/cm^2 (n = 37) to achieve a 4-log$_{10}$ decrease in titre. As with MS2 phage, adenovirus skews the data set as this requires far higher fluences than the other viruses tested: the various serotypes all require greater than 100 mJ/cm^2 to achieve a 4-log$_{10}$ inactivation.

Bacterial spores tend to be the most resistant organisms tested. Spores have been shown to display a "shoulder", and possible "tailing" (Knudson, 1986; Bohrerova *et al*., 2006). Spores tend not to be as susceptible to dimer formation (therefore UV-C inactivation) as the corresponding bacterium in its vegetative state.

Fluence requirements to achieve a 4-log$_{10}$ inactivation for the same microorganism varies between studies. Fluence required to achieve a 4-log$_{10}$ reduction of *E. coli* (ATCC11229) varied from 5 mJ/cm^2 (Harris *et al*., 1987) to 25 mJ/cm^2 (Zemke *et al*., 1990). This may be because of different methods of specimen isolation, processing, storage, purification, irradiation methods (Hoyer, 2000; Nicholson and Law, 1990; Nwachuku *et al*., 2005) or fluence calculation. For example, freeze-thawing many times during sample preparation may increase sensitivity to UV radiation; Meng and Gerba (1996) used 5x freeze-thaw for adenovirus whereas Thurston-Enriquez *et al* (2003) used 1x freeze-thaw and fluence requirement for 4-log$_{10}$ inactivation varied by 105 mJ/cm^2. The data for protozoa highlight the extent to which assay method can

influence the assessment of inactivation kinetics. The animal infectivity assay (e.g., Mofidi *et al*., 2002) and excitation assay (e.g., Karanis *et al*., 1992) for *Giardia lamblia* gave vastly different results. Morita *et al* (2002) studied infectivity and excystation assays of *Cryptosporidium parvum* in parallel and found the same thing, concluding that UV irradiated cysts may be able to excyst (i.e., exit the cyst), but not infect. The most credible way to compare required fluence of different organisms is to run both organisms using the same apparatus in parallel (Nichols and Galeano, 2003) using the same method of fluence calculation, irradiation and, where possible, sample preparation. Due to time constraints, in this study it was not possible to conduct both phages infecting GB-124 and pathogen inactivation experiments in parallel. Future work to confirm the results of this thesis should run both GB-124 phage inactivation experiments and pathogen inactivation experiments simultaneously using the same apparatus and methodology.

3.4.2 UV-B

Although *in situ* studies, identifying the role that UV-B plays in environmental microorganism inactivation, have been carried out, laboratory studies using collimated beam apparatus are rare (Tables 3.3 and 3.4), with the vast majority of UV studies using UV-C (Lytle and Sagripanti, 2005). Owing to the prevalence of UV-C as a tertiary treatment within both drinking water and wastewater treatment works, this is perhaps to be expected.

The six bacterial strains irradiated by Joux *et al* (1999) showed varying responses to UV-B, although all showed a log linear inactivation curve with increasing fluence and all strains were inactivated by a factor of 2-$\log_{10}$ using a fluence of 100 mJ/cm^2. As

was shown for UV-C, there appears to be little or no relationship between the bacterial genome size and UV-B fluence required to achieve a 4-$\log_{10}$ inactivation (Jacquet and Bratbak, 2003).

Table 3.4 Inactivation of microorganisms by low pressure UV-B radiation

Organism	**Fluence required per $\log_{10}$ reduction (mJ/cm^2)**					**Reference**
	1	**2**	**3**	**4**	**5**	
Canine calicivirus	~8	21	34	-	-	Duizer *et al*., 2004
Feline calicivirus	~15	22	34	-	-	Duizer *et al*., 2004
Deleya aquamarina	~95	150	225	-	-	Joux *et al*., 1999
Pseudomonas stutzeri	100	150	215	250	-	Joux *et al*., 1999
Pseudoalteromonas haloplanktis	~60	~90	~120	~140	-	Joux *et al*., 1999
Sphingomonas sp. RB2256	~185	>300	-	-	-	Joux *et al*., 1999
Salmonella typhimurium	50	100	175	210	250	Joux *et al*., 1999
Vibrio natriegens	37.5	75	100	130	150	Joux *et al*., 1999

Duizer *et al* (2004) present interesting results: to achieve a 3-$\log_{10}$ inactivation of feline and canine calicivirus by low-pressure UV-B radiation, 34 mJ/cm^2 is required, which is similar to fluences required for UV-C inactivation of caliciviruses (Thurston-Enriquez *et al*., 2003; Husman *et al*., 2004; Tree *et al*., 2005). This is a significant deviation from the 1:9/1:10 relationship for other microorganisms observed in other studies, indicating that mechanisms other than for formation of pyrimidine dimers may be functioning.

3.5 Applications of UV technologies

3.5.1 Drinking water plants

The USEPA recommends 21 mJ/cm^2 (for 2-log$_{10}$ reduction) and 36 mJ/cm^2 (for 3-log$_{10}$ reduction; Gerba *et al*., 2002) UV-C fluences for inactivation of enteric viruses in drinking water plants. These fluences may be less than is required to inactivate adenovirus type 2, though is sufficient for enteroviruses (Gerba *et al*., 2002). The Long Term 2 Enhanced Surface Water Treatment Rule (LT2ESWTR) was established to regulate UV-C treatment of drinking waters in the USA, and the fluences recommended are detailed in Table 3.5.

Table 3.5 The LT2ESWTR dose requirements in mJ/cm^2 (Cotton and Passantino, 2005)

	Log inactivation (mJ/cm^2)							
	0.5	**1**	**1.5**	**2**	**2.5**	**3**	**3.5**	**4**
Cryptosporidium	1.6	2.5	3.9	5.8	8.5	12	-	-
Giardia	1.5	2.1	3.0	5.2	7.7	11	-	-
Viruses	39	58	79	100	121	143	163	186

3.5.2 Wastewater treatment works

At fluences greater than 40 mJ/cm^2 virtually all viral and bacterial pathogens are inactivated (Bolton and Smith, 2005), and WwTW therefore aim to deliver fluences of this magnitude. However, in the UK fluences commonly used in WwTW vary. Southern Water Plc (the main water supply and sewerage company in south east England) have fluence consents (i.e., minimum requirements) of 32 and 35 mJ/cm^2, but it is common practice to deliver fluences of 50 mJ/cm^2 (I Mayor-Smith, pers comm). Data for New Romney WwTW (Southern Water, UK) show fluences delivered to be between 50.3 and 210 mJ/cm^2 with a mean of 79.8 mJ/cm^2

3.6 Environmental UV related microorganism inactivation

3.6.1 General considerations

Wavelengths shorter than 290 nm (UV-C) do not reach the Earth's surface as they are blocked by the atmosphere, (Harm, 1980; Coohill, 1996), with solar terrestrial UV composed of 5% UV-B and 95% UV-A (Kuluncsics *et al*., 1999). Therefore, when considering microorganism inactivation by environmental UV radiation, UV-C is not a factor. It has been calculated that around 90% of bacterial inactivation by solar UV is a function of UV-B wavelengths (Coohill and Sagripanti, 2009) and UV-B has been shown to have a greater effect than UV-A on virus inactivation (Noble and Fuhrman, 1997). The inactivation of phages and viruses in natural waters has been documented (Davies-Colley *et al*., 1997; Sinton *et al*., 2002; King and Monis, 2007; Sinton *et al*., 2007), with sunlight shown to be the dominant factor (Suttle and Chen, 1992; Suttle and Chan, 1994; Wommack *et al*., 1996; Davies *et al*., 2009). For example, it has been estimated that a 4.4 $\log_{10}$ reduction of poliovirus can be achieved using a 6h exposure (simulated conditions) using solar water disinfection (SODIS; Heaselgrave *et al*., 2006).

3.6.2 UV-B environmental inactivation

Because of localised ozone depletion during the latter part of the twentieth century, much of the literature focussing on UV-B radiation is based around the Antarctic (Cywinska *et al*., 2000; McKenzie *et al*., 2007; Hernández *et al*., 2009), but environmental studies detailing the effect of UV-B radiation in more temperate latitudes are also reported in the literature (Boelen *et al*., 2002; Jung *et al*., 2008). Environmental UV-B fluences vary depending on the latitude at which it is measured, altitude (it has been estimated UV-B fluence rate increases by 19% with every 1000m

increase in altitude; Blumthaler *et al*., 1993), time of day, season and overhead ozone concentration (Coohill and Sagripanti, 2009).

To give an indication of fluence rate found at the water surface in temperate latitudes, a figure of 0.022 mW/cm^2 has been used in previous studies (Jacquet and Bratbak, 2003) relating to a cloudy day with moderate sunlight intensity (Mostajir *et al*., 1999; Garde and Cailliau, 2000). Other studies have given UV-B peak fluence rates as approximately 0.20mW/cm^2 during a sunny April day, and a peak of around 0.10 mW/cm^2 during a sunny February day (Figure 3.2; Jung *et al*., 2008). Examples of total daily UV-B fluences have been measured as 55 kJ/m^2 in June and 10 kJ/m^2 in January (both Baltimore, USA; Heisler *et al*., 2004) and 570 to 611 kJ/m^2 (September, Red Sea, Boelen *et al*., 2002).

In the UK, UV-B data are difficult to obtain and are not widely measured (Sayers *et al*., 2009). For example, the UK Health Protection Agency, which supplies UV index data to the Meteorological Office, does not monitor UV-B levels directly (A Pearson, pers. comm.). UV-B levels recorded in Wales by UK amateur groups during March to June 2005 (http://www.uvguide.co.uk/uvinnature.htm) give maximum daily readings in the range of 0.04 mW/cm^2 (during cloudy conditions) to 0.375 mW/cm^2 (clear sky).

All data given by this group show a bell-shaped curve of UV-B recorded through the day, with a peak around solar noon, similar to those shown in Figure 3.2.

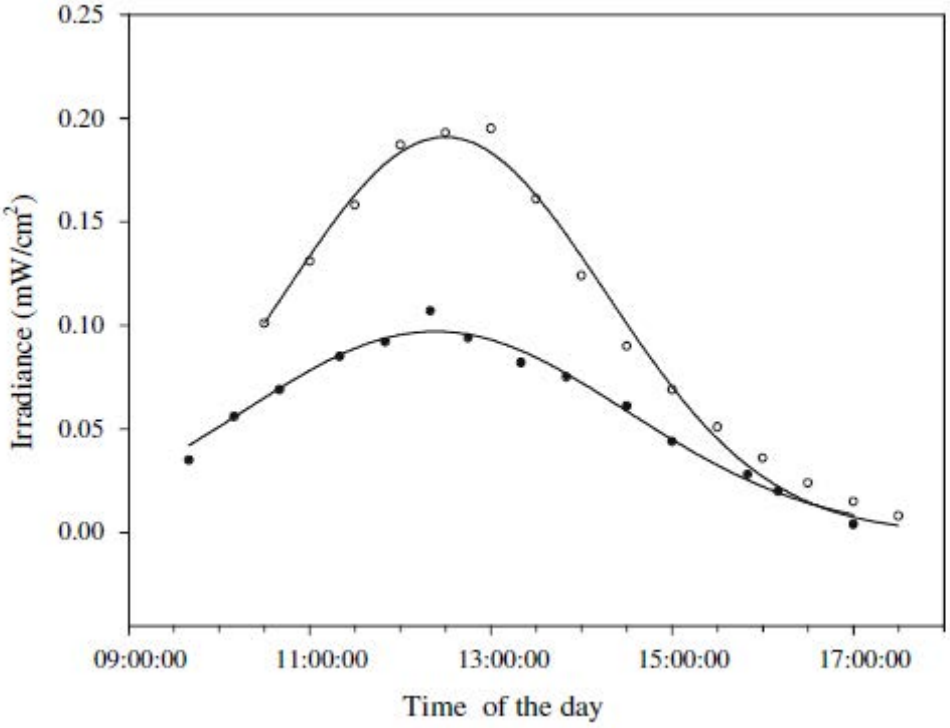


Figure 3.2 UV-B fluence rate recorded in Seoul, South Korea during a sunny February day (2005) represented by solid circles, and sunny April day (2005) represented by open circles (Jung *et al*., 2008)

Data from Québec (Canada) give peak environmental UV-B fluence rates of approximately 0.15 mW/cm^2 on a sunny day, and around 0.05 mW/cm^2 just below the water surface on a cloudy day during July 1996 (Mostajir *et al*., 1999). These figures are in accordance with those recorded in the UK (amateur group above).

It is clear from the literature that environmental UV-B fluences vary greatly, both geographically and diurnally. As UV-B fluences are not constant throughout the day, it is important to recognise that viral inactivation rates dependent on UV-B may also vary diurnally (Heldal and Bratbak, 1991). In order to prove useful, either direct measurements or estimates of UV-B fluence should be constructed when assessing how UV-B radiation will affect MST studies and QMRA in a particular location. For example, the UV-B Monitoring and Research Program (UVMRP, Colorado State

University, (http://uvb.nrel.colostate.edu/UVB/index.jsf) is a useful resource for further UV-B data within the United States. Elsewhere, national/regional databases may exist.

The potential for UV-B inactivation of phages has been shown by other authors, and may be the result of a rapid mechanism. Lytle and Sagripanti (2005) estimated that 63% of pathogenic virus would be inactivated by solar radiation (including UV-A and UV-B components) exposures between 8.4 minutes (phage T1) and 71 minutes (Tobacco Mosaic Virus) using data predicted from inactivation kinetics at 254nm. Changes in the genomic structure of solar-irradiated phages have also been observed. Research on the DNA phage M13mp2 (host *E. coli*) showed that when exposed to natural sunlight (therefore UV-A and UV-B), a change in bases occurred, with the G to C or G to T being the most prevalent changes (Negishi and Hao, 1992).

3.6.3 Microorganism protection from UV-B radiation

UV-B is attenuated as it passes through the water column, with the degree of attenuation determined by numerous physiochemical factors. In oligotrophic (low nutrient) water bodies, less UV-B attenuation occurs than in nutrient rich water bodies (Boelen *et al.*, 2000), with the degree of UV-B attenuation highly dependent on dissolved organic carbon and both organic and inorganic particulate matter (Häder *et al.*, 1998 and 2007; Bancroft *et al.*, 2007). Experiments carried out in oligotrophic (i.e., low nutrient) waters with low particulate matter content show average UV-B attenuation over three metres (based on 6 hour fluence data given in Table 3.6) is 73.4% (n = 5). At three metres, the UV-B attenuation is 98.9%. In waters with suspended organic and inorganic matter, and with higher nutrient status (i.e., river

systems), attenuation of UV-B is much greater. Other studies have shown UV-B to be detected at 6m depths in low dissolved organic carbon lakes (Williamson *et al*., 1996) and 15m in Gulf of Mexico (Wilhelm, *et al*., 2002). Attenuation of UV-B within marine water columns has been correlated with a decrease in UV induced DNA damage (Boelen *et al*., 2002).

Table 3.6 UV-B fluences measured at different depths through the water column at King George Island, Antarctica (Hernández *et al*., 2009)

Depth (m)	**2 hour UV-B fluence (mJ/cm^2)**	**6 hour UV-B fluence (mJ/cm^2)**
Repetition 1		
0	320	460
1	61	80
3	1.7	2.8
Rep. 2		
0	837	2011
1	209	502
3	0.8	0.2
Rep. 3		
0	120	433
1	33	120
3	2.5	9.3
Rep. 4		
0	-	950
1	-	190
3	-	4
Rep. 5		
0	-	480
1	-	121
3	-	7.6

There is also evidence to suggest that turbid waters with high levels of particulate matter may reduce the level of UV-B penetration, whilst shielding microorganisms from UV photons. Water spiked with montmorillonite has been shown to reduce the inactivation rates of phage T_7, and poliovirus 1 (Quignon *et al*., 1997). Kaolinite displays similar effects (Gerba and Schaiberger, 1975) as do organic humic acids

(which absorb UV strongly) (Bitton *et al*., 1972), possibly by coating the phage in a protective layer (Templeton *et al*., 2005).

Phages may also be protected from UV inactivation by deposition into bed-sediments. Skraber *et al* (2009) demonstrated that F-specific phage (MS2) and SC (ΦX174 and PRD-1) accumulated in natural clayey sediments sourced from canals, river and lakes. The binding ability of phages to clays has been reported (Schiffenbauer and Stotzky, 1982; Gantzer *et al*., 2001). These phages may become available when high flows re-suspend the bed sediments. The size of clay particle may also influence viral adsorption, with smaller particles capable of greater phage adsorption (Gerba and Schaiberger, 1975).

Viral adsorption rates to sediments may be temporally variable. Viruses can be adsorbed in large levels to suspended sediments, but after periods of rainfall, dilution of cations may retard adsorption (Schaub and Sagik, 1975). Natural sediments don't have the adsorptive capacity of kaolinite, with sand being particularly bad (Gerba and Schaiberger, 1975). Differential reaction to electrostatic charges by enteroviruses and phages may have implications for environmental studies and adsorption onto solid surfaces (Grabow, 2001).

Other protective features include phage coagulation, (reducing surface area subject to UV-B irradiation; Coohill and Sagripanti, 2008), and shading (by other microorganisms, surface and sub-surface plants, and other structures). Shading may be enhanced in eutrophic waters where primary production is greater than in nutrient poor waters.

The final protective feature that should be considered is the potential for phages such as those infecting GB-124 to undergo reactivation within the water column. There are two primary methods of reactivation: 1) photoreactivation (repair of pyrimidine dimers by UV-A exposure using the photolyase enzyme) and 2) dark repair (nucleotide excision repair; Oguma *et al*., 2001), which is more complex as it utilises at least twelve proteins (Freidberg *et al*., 1995). UV-A driven reactivation of UV-C irradiated organisms has been documented in bacteria (Kashimada *et al*., 1996; Tosa and Hirata, 1999; Sommer *et al*., 2000; Zimmer and Slawson, 2002; Oguma *et al*., 2004), protozoa (Oguma *et al*., 2001), spores (Knudson, 1986) and phages (Kadavy *et al*., 2000) and this can occur within a few hours (Oguma *et al*., 2002). The photolyase enzyme is crucial, and if absent from the microorganism, photoreactivation cannot take place (Oguma *et al*., 2001). It should be noted that although photorepair may be carried out, CPD repair is often done with errors leading to non-reactivation (Wang *et al*., 2010b).

During laboratory assays (i.e., the double agar assay for phages infecting GB-124), reactivation may occur: adenovirus serotype 40 has demonstrated a "shouldering" effect at low fluences (<30 mJ/cm; Thurston-Enriquez *et al*., 2003), which may be because of repair carried out by the host cell during assay. As the genetic material of adenoviruses is dsDNA, it is possible that only one strand of DNA is damaged during UV-C irradiation, which can then serve as a template for repair using the host cell (Harm, 1980). This may account for "shoulders" observed in some dsDNA virus inactivation curves. Rate of photorepair is limited by the amount of photolyase present. In *E. coli* there are around twenty photolyase enzymes in each cell, each of which can repair 5 dimers per minute (Friedberg *et al*., 1995).

The ability of microorganism to reactivate varies greatly from species to species, and specific target organisms should be assessed for their reactivation ability. As phages use the host cell to propagate, if the host has the photolyase enzyme the capacity for photoreactivation exists. It is not known if GB-124 has the photolyase enzyme, and therefore the ability to perform photoreactivation.

3.7 Rationale of study

As mentioned previously, there is no perfect indicator of human faecal pollution appropriate to all situations, but the indirect enumeration of bacteriophages infecting *Bacteroides* strains, by phage lysis of bacterial cultures, has shown promise, both for identifying faecal contamination, and for distinguishing faecal sources. However, since it is likely that there is no perfect indicator, a 'toolbox approach' to identifying faecal pollution, and ultimately to quantifying risk of human infection may be appropriate, using different indicators in different environments. In order to develop such a 'toolbox' the limitations of each indicator must be assessed systematically. If phages infecting *Bacteroides* spp., are to be used as indicators of human faecal contamination, further information regarding their ecology in natural aquatic environments is needed (Araujo *et al*., 1997; Weinbauer *et al*., 2007). The USEPA has identified research into phages infecting *Bacteroides* spp., as a high priority critical research need (Ashbolt *et al*., 2007).

Numerous physical, chemical and biological factors influence the survival of pathogens in waterbodies and effective indictors of faecal material and surrogate organisms must possess similar ecological characteristics to the pathogen or pathogens of concern. *Bacteroides* strain GB-124 has shown good potential as an indicator of human faecal material and as a possible surrogate for enteroviruses. However, no studies have been undertaken on the ecology of phages infecting GB-124. If phages of GB-124 are inactivated more rapidly than are common waterborne pathogens, then inaccurate water risk assessments may arise, resulting in human illness and mortality. The relationship between phages infecting GB-124, and waterborne viral pathogen levels needs to be demonstrated in order to allow accurate

assessment of risk through quantitative microbial risk assessment (QMRA) methodologies.

Differential environmental inactivation kinetics of *Bacteroides* phages and pathogens are likely to influence their application as an indicator of faecal contamination in aquatic environments (requirements for enterovirus surrogate organisms specified in Section 1.5). Bacterial hosts that detect a homogenous group of phages are recommended as homogeneity allows the comparison of different water types (as composition of phage population will not change) and ensure a consistent reaction to environmental pressures (Queralt *et al*., 2003).

As discussed above, one common method of pathogen/phage inactivation is UV radiation, delivered either naturally via sunlight (UV-B and UV-A) or artificially during effluent disinfection at WwTW (UV-C). UV disinfection of wastewater effluents using UV-C radiation is very effective in reducing the pathogen load of final effluents, and its use is becoming increasingly common (Cotton *et al*., 2001), primarily because of its effectiveness at inactivating *Cryptosporidium* (Cotton and Passantino, 2005). Sunlight-derived UV-B radiation has also been shown to inactivate microorganisms, and may influence the survival of phages (such as those infecting GB-124) in the environment (Jagger, 1985). These two important inactivation mechanisms may limit the use of phages infecting GB-124 in QMRA and MST studies if indicators and pathogens display different inactivation kinetics.

3.8 Aims and objectives of research project

To date, phages capable of infecting GB-124 have not been isolated and classified and hence assessment of their survival characteristics has not been investigated. What's more little is known of the ecology of such phages capable of infecting GB-124 and how this relates to types and densities of pathogens present in wastewater. In order to address these gaps in knowledge, this study contributes to the achievement of the following aim:

- To improve understanding of the environmental ecology of bacteriophages capable of infecting *Bacteroides* strain GB-124 and assess implications for phage-based MST studies and QMRA.

More specifically the research project set out to achieve two objectives:

- To isolate and characterise bacteriophages capable of infecting *Bacteroides* strain GB-124 assessing group homogeneity; and
- To ascertain UV inactivation kinetics of isolated phages using two UV wavelengths: UV-B (representing primary germicidal portion of sunlight) and UV-C (representing germicidal UV employed in WwTW) in controlled laboratory experiments.

This study will fill some of the critical knowledge gaps with regard to the homogeneity of phages able to infect GB-124, and how they are likely to behave and survive when exposed to tertiary disinfection stages during wastewater treatment, and in the environment. Results from this study may inform the decision as to whether phages infecting GB-124 can be used as indicators of human faecal contamination in areas downstream of WwTW with UV disinfection installed, and if they can be used

as surrogate organisms for enteroviruses after UV treatment and during solar UV-B exposure.

Through the better understanding of GB-124 phage ecology, results from this study may help to protect human health by informing the production of more accurate risk assessments.

CHAPTER FOUR

4. MATERIALS AND METHODS

This study used both international standard (e.g., ISO) methodologies and those detailed in scientific peer-reviewed literature by numerous authors. All glassware was acid-washed and sterilized (by autoclaving at 121 °C for 15 minutes) prior to use, and media and reagents were prepared according to the manufacturers' instructions. Methodologies are presented here chronologically with regard to the experiments undertaken, *vis a vis* phage isolation, characterisation, and finally irradiation using 254 nm (UV-C) and 302 nm (UV-B) wavelength ultraviolet radiation.

4.1 Isolation of bacteriophages infecting GB-124

4.1.1 Collection of samples and double-agar analysis

There are numerous phage isolation and concentration methods detailed in the literature, including ultrafiltration (Wommack *et al*., 1996; Breitbart *et al*., 2002), precipitation with polyethylene glycol (PEG; Yamamoto *et al*., 1970) and sequential adsorption and elution (Seeley and Primrose, 1982; Sobsey *et al*., 1990; Mendez *et al*., 2002). This study used the polyethylene glycol method developed by Yamamoto *et al*., (1970) as it has proved successful for bacteriophage concentration and purification in many previous studies (cited in over 1200 articles as of Jan 2010; Web of Science search). Extensive information regarding working with bacteriophages is detailed in Kutter and Sulakvelidze (2005).

On three occasions (1st October 2008, 13th October 2008 and 27th October 2008) one litre grab samples of wastewater final effluent from Scaynes Hill wastewater

treatment works (West Sussex, UK; 37, 327 p.e.) were collected in sterile polyethylene bottles (Fisher Scientific, UK), stored at 4°C in the dark and transported to the Environment and Public Health Research Unit (EPHRU) laboratory, University of Brighton. Effluent samples were filtered using sterile 10ml plastic syringes (Becton Dickinson, US) with a sterile 0.22 μm filter unit attached (Millipore, US) to retain bacteria and organic material, whilst allowing phages to pass through. Because of their low protein binding nature, these filters retain few phages (Tartera *et al*., 1992). Filtered effluent was stored in sterile glass tubes at 4°C in the dark until assayed. This was performed within four hours of collection.

A dilution series of filtered final effluent samples were assayed in triplicate (100 μl, 500 μl and 1 ml aliquots) using the double-agar protocol (ISO 10705-2) specifically for *B. fragilis* phages (Adams, 1959; Anon, 2001c). GB-124 working cultures were prepared by streaking a GB-124 culture on a BPRMA (*Bacteroides* phage recovery medium agar; Anon, 2001c) plate and incubating anaerobically [in anaerobic jars with an anaerobic sachet (Anaerogen, Oxoid, UK)] at 36 (± 2) $^{\circ}$C for 44 (± 4) hours. 12 ml of BPRMB (*Bacteroides* phage recovery medium broth; Anon, 2001c) in a Pyrex screw-topped glass test tube (SciLabware, UK), was then inoculated with 1/8th of the cells on BPRMA plate and incubated anaerobically at 36 (± 2) $^{\circ}$C for 21 (± 3) hours. 1 ml of this culture was then transferred to 11 ml of pre-warmed BPRMB in a Pyrex test tube and incubated anaerobically at 36 (± 2) $^{\circ}$C until the correct optical density was reached (0.33 OD at 620 nm). This optical density equates to 2 x 10^8 CFU/ml, representing the exponential bacterial growth phase (previously determined experimentally by GB-124 growth curves). Before optical density readings were taken, test tubes were wiped with a lint free cloth and with 70% ethanol to ensure

accurate readings. Pre-warmed (36 (± 2) °C) complete BPRMB was used to zero the spectrophotometer. The working culture was then mixed in a ratio of 1:1 (volume) with Bovine Albumin cryoprotector (Anon, 2001c), distributed into Eppendorf tubes (1.5 ml volume) and stored at -80 °C until required.

1 ml of GB-124 working culture was removed from storage at -80 °C and allowed to equilibrate to ambient room temperature (~20 °C). This was added to 11 ml of complete BPRMB in a 12 ml Pyrex screw-topped test tube excluding all air. The culture was incubated anaerobically overnight at 36 (± 2) °C. The following day, inoculum cultures were prepared by adding 1 ml of overnight GB-124 culture to 11 ml of pre-warmed BPRMB [36 (± 2) °C] in a 12 ml Pyrex test tube and incubated anaerobically at 36 (± 2) °C until the correct optical density was reached. Pre-warmed [36 (± 2) °C] complete BPRMB was used to zero the spectrophotometer and when the correct OD was reached, inoculum cultures were placed on ice and used within three hours.

For each assay, 1 ml of GB-124 inoculum culture was combined with 2.5 ml of molten (50 °C) semi-solid BPRM agar (0.8 % agar) and 1ml of sample (or dilution thereof) in a 13 ml plastic test tube, vortexed at low speed, poured over BPRM agar (1.6 % agar number 1) and left to set for approximately 10 minutes. Petri dishes were then inverted, placed in anaerobic jars with an anaerobic sachet (Anaerogen, Oxoid, UK) and incubated for 18 (± 2) hours at 36 (± 2) °C.

After incubation, zones of lysis were visible (plaques) where phages infected the confluent bacterial lawn, destroying the host *Bacteroides* cells. Each plaque represents one plaque forming unit (PFU) and PFU/ml of samples is given by:

$$n_{\text{pfp}} = \frac{N}{(n_1 V_1 F_1) + (n_2 V_2 F_2)}$$

Where:

n_{pfp} = the number of plaque-forming particles/units of bacteriophages infecting *Bacteroides fragilis* per millilitre (PFU/ml)

N = the total number of plaques counted on plates

n_1, n_2 = the number of replicates counted for dilution F_1, F_2 respectively

V_1, V_2 = the test volumes used with dilution F_1, F_2 respectively

F_1, F_2 = the dilution or concentration factors used for the test portions V_1, V_2 respectively (F = 1 for undiluted, F = 0.1 for ten-fold dilution etc.).

Anon (2001c)

4.1.2 Phage isolation, concentration and purification

Plates with well-spaced plaques were selected for isolation work. Plaques were selected at random in order to avoid selection based upon morphology (i.e., plaque appearance), and 20 plaques were picked during each round of isolation (this is considered the minimum number required to estimate population diversity; according to Bianchi and Bianchi, 1982). Agar plugs containing plaques were picked from plates using a sterile glass Pasteur pipette and suspended in 400 µl of buffer (19.5 mM Na_2HPO_4, 22 mM KH_2PO_4, 85.5 mM NaCl, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$) in a microcentrifuge tube (Fisherbrand). The suspension was then incubated for four hours

at 4°C (Puig and Girones, 1999) allowing phage diffusion from agar plug into the buffer. 100 µl of phage infected buffer (and dilutions thereof) were retested using the double-agar method (above) to confirm phage presence. Plaques were picked for a second time from plates with well spaced zones of lysis and suspended in 400µl of buffer for four hours at 4°C.

This suspension was added to 27 ml of GB-124 (at 0.33 OD) in a glass tube (filled to capacity to exclude air) and incubated anaerobically for 18 (± 2) hours at 36 (± 2) °C, to produce crude lysates. 1 ml of each crude lysate (and dilutions thereof) was assayed for phage viability using the double-agar method described above. Lysates that produced plaques were selected for further work. Remaining volumes of lysates that produced plaques were added to separate 620 ml cultures (500 ml Schott bottle filled to capacity to exclude air) of exponential GB-124 and incubated anaerobically for 18 (± 2) hours at 36 (± 2) °C. Crude lysates containing no lytic phages (absence of plaques) were discarded.

Phages were then concentrated and purified by precipitation with polyethylene glycol in accordance with Kutter and Sulakvelidze, (2005) and Yamamoto *et al* (1970). Following incubation, phage suspensions were kept at 4 °C and NaCl was added to a final concentration of 1M. After one to two hours, suspensions were centrifuged at 1,789 x *g* for 10 minutes to remove bacterial debris. Polyethylene glycol 8000 was added to a final concentration of 10 % (w/v), mixed for 30 minutes using a magnetic stirrer, and left overnight at 4 °C. The following morning, suspensions were centrifuged at 11,000 x *g* for 10 minutes at 4 °C in order to sediment precipitated phage. The supernatant was discarded and 30 ml of buffer (as above) were added.

Suspensions were stored at 4 °C overnight, allowing pellet to soften. The following morning, pellet and overlying buffer were mixed by gentle pipetting and further debris were removed by centrifugation at 1,500 x *g* for 10 minutes. The titre of resulting suspensions was between 10^8 and 10^{11} PFU/ml and they were stored in light tight glass tubes in the dark at 4°C.

Phages were named B124-1, B124-2 etc., following the system used in Tartera and Jofre (1987) for HSP-40 and related phage, where:

B = genus of host on which phages were isolated (in this example *Bacteroides*)

124 = strain of *Bacteroides* on which phages were isolated (GB-124)

-1 = the ID of the plaque picked (i.e., B124-1 was the first plaque picked)

4.2 Phage characterisation

4.2.1 Transmission Electron Microscopy

Electron microscopy employs electron dense solutions containing metal salts of high molecular weight and small molecular size, staining specimens and allowing visualisation under an electron beam. All twenty propagated phages were examined under the electron microscope. One drop (~ 10 µl) of phage preparation (10^{-1} dilution of original high titre lysate as detailed in 4.1.2 above) was deposited on 200 mesh Formvar/Carbon copper electron microscope grids (Agar Scientific, UK) and allowed to absorb for two minutes. The excess suspension was withdrawn using Whatman No. 1 filter paper (Whatman, UK) and one drop (~ 10 µl) of 0.22 µm filtered 1 % w/v uranyl acetate stain (pH 4 – 4.5) was added to the grid and left for 1 minute. The excess was withdrawn using filter paper and grids were left to air dry before viewing under the electron microscope (Hitachi-7100 at 100 kV). All phages were measured on micrograph printouts rather than on-screen (Ackermann, 2009).

4.2.2 Host range assessment

The ability of isolated B124 phages to infect other *Bacteroides* species/strains used in MST studies was assessed. The *B. fragilis* strains used were RYC-2056 (Puig *et al*., 1999) and HSP-40 (Jofre *et al*., 1986; Tartera and Jofre, 1987). 10-fold dilution series of phage suspensions were created and assayed in parallel with GB-124, using a control of sterile distilled water in place of phage dilution series. Samples were assayed using double-agar method and all dilutions were analysed in triplicate.

4.3 Construction of collimated beam UV apparatus

4.3.1 Collimated beam apparatus construction

Bacteriophage suspensions were irradiated in a custom-built CB UV box. It was decided (on grounds of cost and design flexibility) that the apparatus be constructed rather than purchased. The design of the box used in this study was based on the work of Bolton and Linden (2003) and Kuo *et al* (2003). The UV box built for this project is a combination of the examples shown in Figure 4.1 with a collimating tube, and opaque enclosure. The electrical diagram of the UV box is shown in Figure 4.2 and the UV box is shown in Figure 4.3.

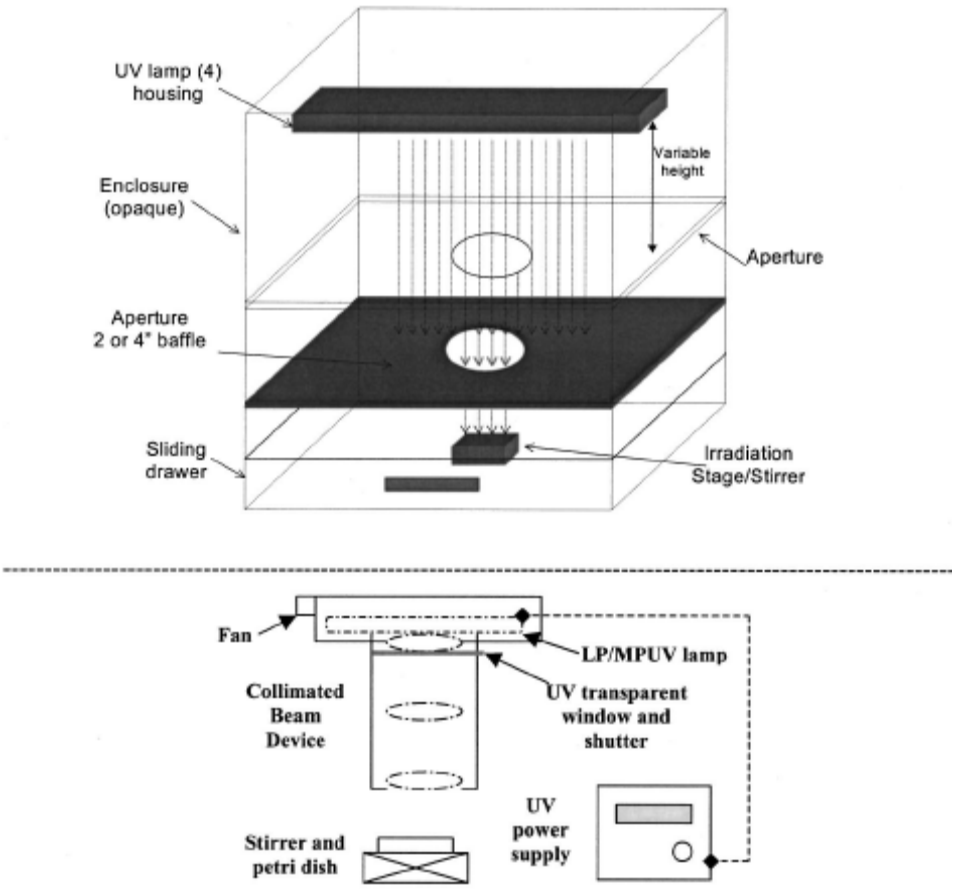


Figure 4.1 Examples of bench scale devices for conducting UV experiments (Bolton and Linden, 2003)

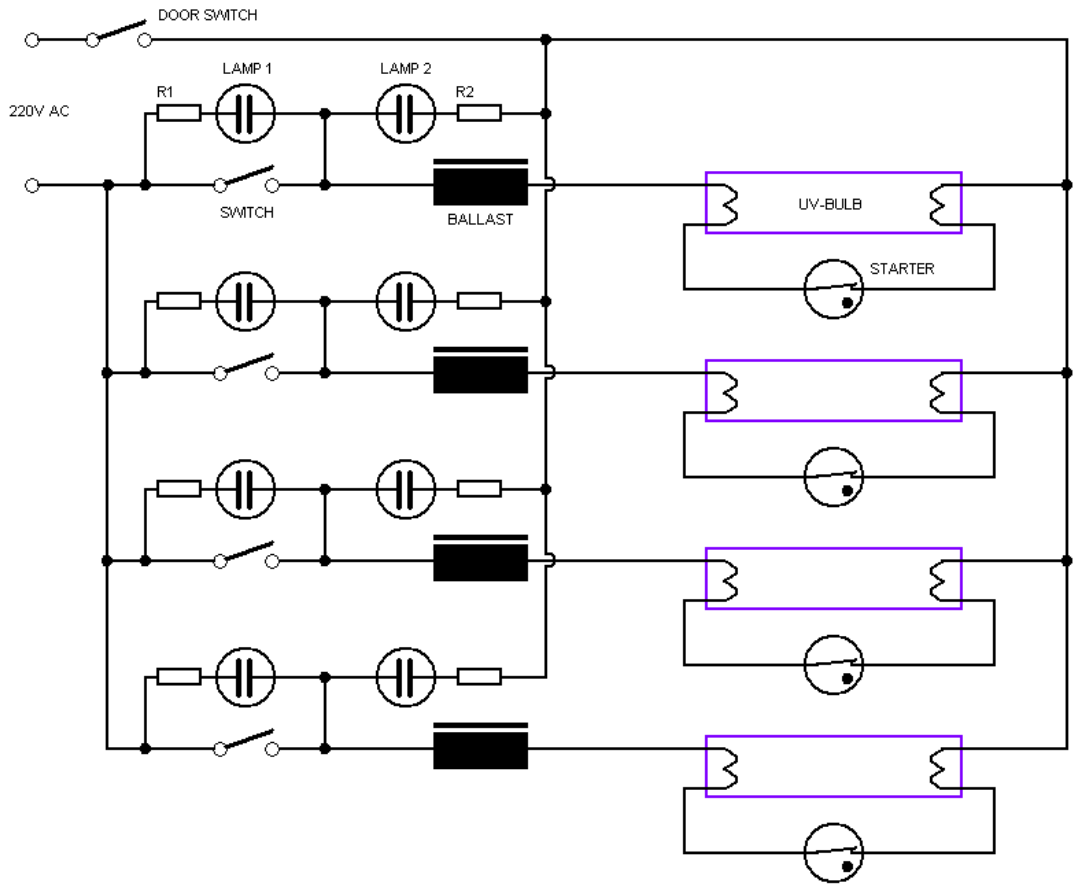


Figure 4.2 Electrical diagram of custom-built UV box (R = resistor)

The UV box enclosure was constructed using 90mm plywood, which was waterproofed using wood sealant and the interior painted flat black. The box exterior was covered with waterproof vinyl allowing easy cleaning. The bulb compartment housed four low-pressure low-intensity Hg 15W UV bulbs (UVP, UK), of which the central two were used to deliver light through a collimating tube, producing quasi-parallel rays (Figure 4.4). Bulbs were either of wavelength 254 or 302 nm, using a single wavelength at a time. 15W ballasts (UVP, UK) were used to regulate power supply to UV bulbs and starters (UVP, UK) were used to initialise UV bulbs.

A microswitch was fitted to the box door ensuring that the power supply to UV bulbs was cut when the door was opened, though because of changes in methodology, this

was bypassed for the tests. The box included two external LED lamps (with appropriate resistors) per UV bulb (Figure 4.5) for safety purposes: an amber lamp indicated that the UV bulb circuit was complete, and the bulb was switched off, a red lamp indicating that the UV bulb was on and emitting UV radiation. Any on/off configuration of the four bulbs was possible. Temperature sensors were located in the bulb compartment and in the experimental chamber.

Figure 4.3 UV box exterior (left) and interior (right) (the interior is shown with a shortened collimating tube)

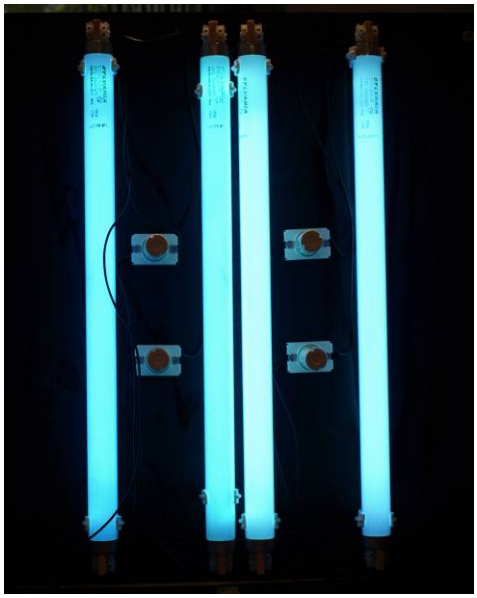


Figure 4.4 UV bulb arrangement

Figure 4.5 Lamps and switches on UV box

4.3.2 UV properties of CB apparatus

Two wavelengths were used in this study, and the spectral output of the UV-B and UV-C bulbs is shown in Figure 4.6. The UV-C bulbs had a monochromatic output at around 254 nm, whilst the UV-B bulb had a wider range of wavelengths with a peak at 300 to 325 nm (outputs above 400 nm were blocked by the filter mounted on the detector head). As this bulb had a wider output spectrum, its output is referred to as 'UV-B' rather than a specific wavelength (i.e., 302nm as described by manufacturers). No UV-C radiation is delivered by the UV-B bulb. Bulbs with similar properties to those used in this study have been used by several other authors: UV-C (Kellogg and Paul, 2002); UV-B (Kumar *et al*., 2003; Zenoff *et al*., 2006; Dib *et al*., 2008).

In the literature, the term 'UV dose' has been widely used. However this term is incorrect, as it implies that all UV output is absorbed by the test organism, whereas in reality little of the delivered UV energy is actually absorbed by the test organism. Fluence is the correct term. Fluence cannot be measured directly, but it can be inferred from two accurately monitored variables: fluence rate (power delivered from UV bulbs) and exposure time. Fluence rate was measured using an IL1400A

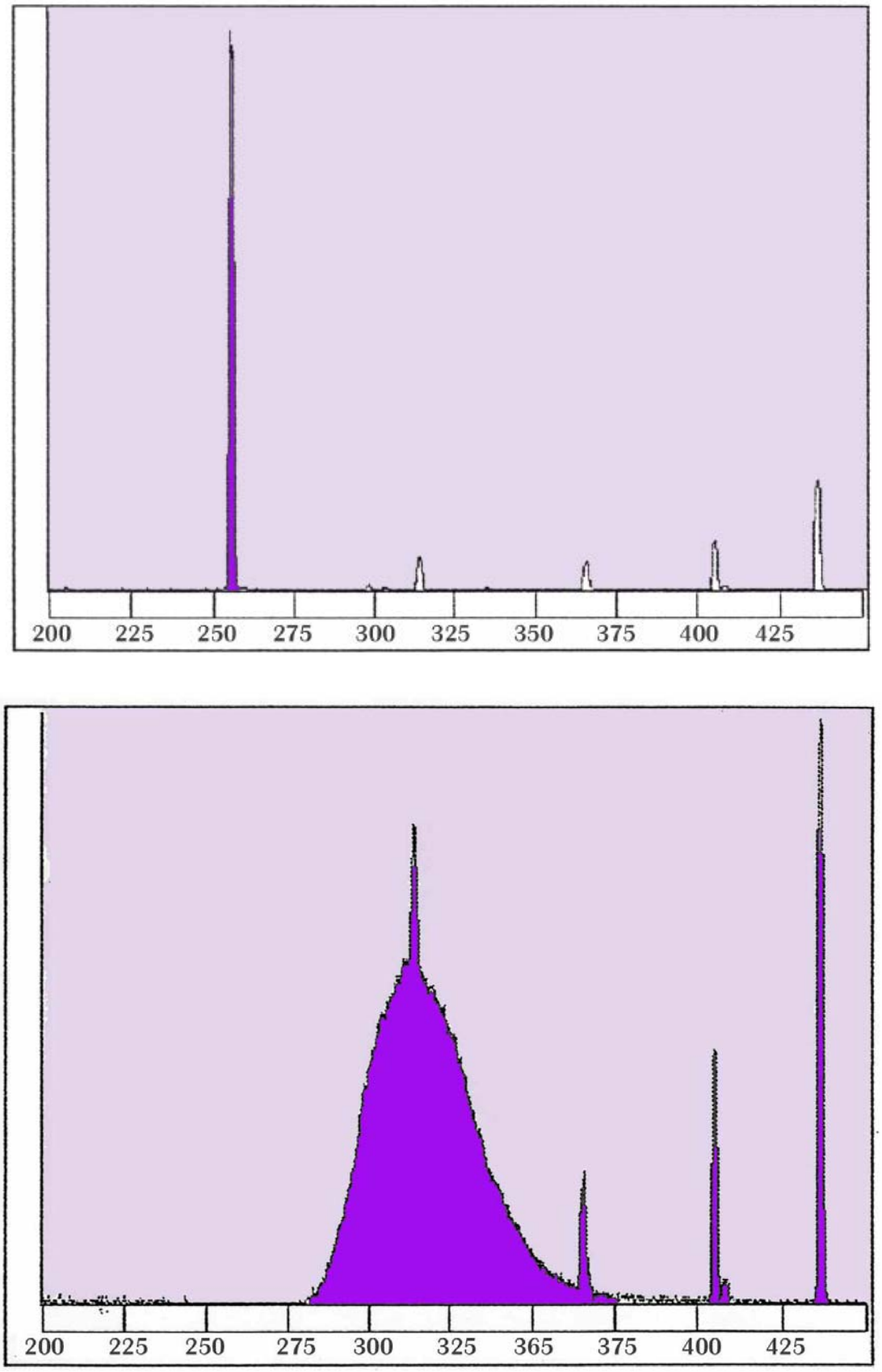


Figure 4.6 Spectral output of UV-C (top) and UV-B (bottom) bulbs (unspecified power unit on *y* axis, *x* axis shows wavelength in nm; UVP, 2010)

radiometer fitted with a SED005/U sensor and a WBS320 wide band UV-IR filter (both International Light; calibrated June 2009 by International Light). A UV-visible domed Teflon diffuser was also fitted and a spectral response curve of the sensor/filter unit (International Light) was used to determine the calibration factor to be used for different wavelengths.

Initial tests were carried out to characterise UV bulb output and to ascertain when stability was reached. The bulbs were labelled sequentially from one to four corresponding to their position in the box. To ensure continuity the same bulbs were used in the same positions for all experiments. These experiments were repeated on three consecutive days with the mean values being reported (Figures 4.7and 4.8).

UV bulb output (fluence rate) was stable after approximately 30 minutes. Temperatures in the bulb compartment and irradiation stage increased gradually, then stabilised. Bulb temperatures increased to ~40 °C whilst compartment temperature rose to ~23 °C. Efficacy of UV disinfection is not affected by pH or temperatures between 20 °C and 40 °C (Severin *et al*., 1983; Abu-Ghararah, 1997; USEPA, 2003), so the observed temperature increase in the sample compartment was not considered significant, in terms of its impact on the outcome of the experiment. During sample irradiation, bulbs were switched on one hour prior to commencement of inactivation to ensure consistency of UV output.

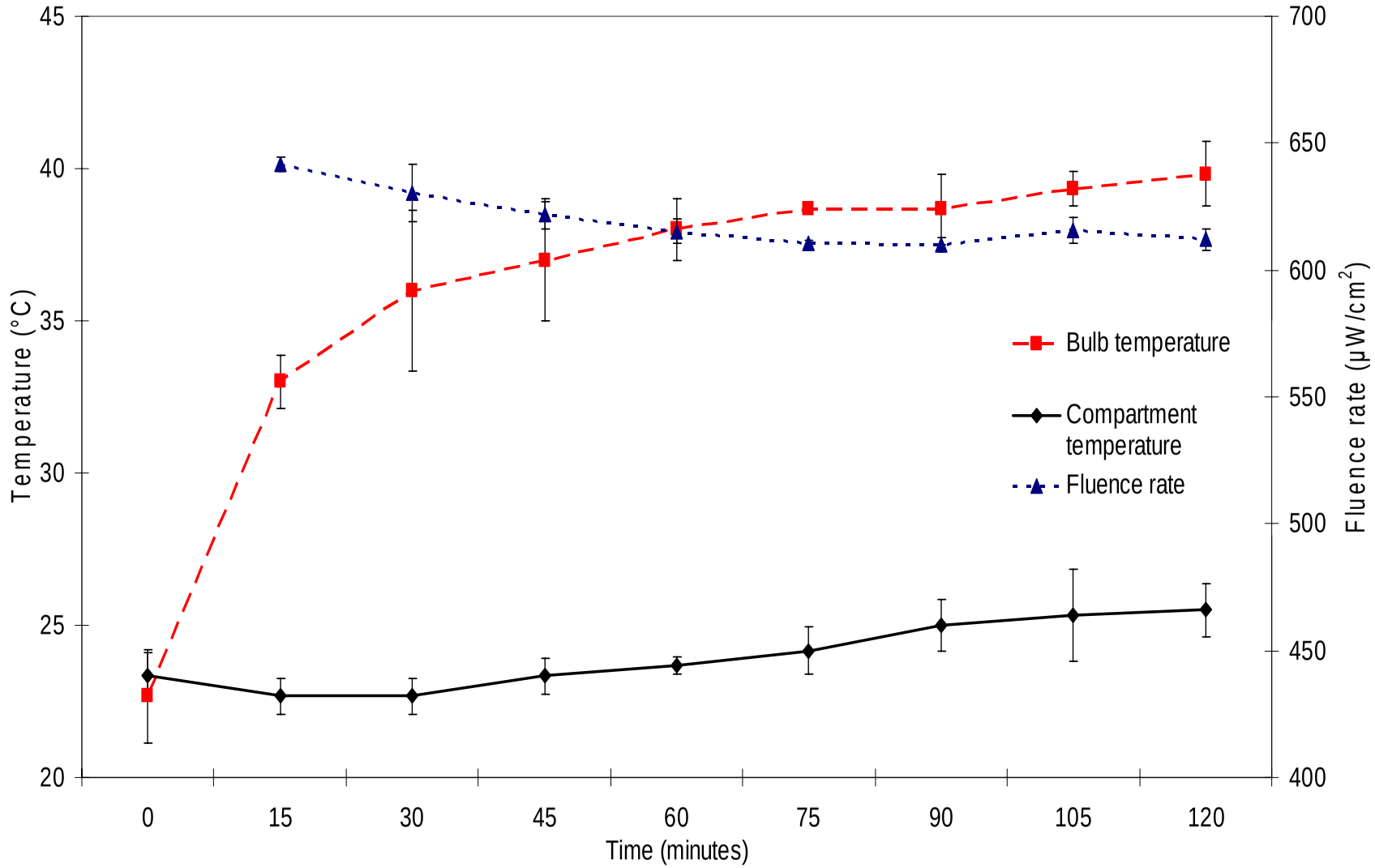


Figure 4.7 UV-B bulb fluence rate, compartment temperature and bulb compartment temperature during preliminary experiment. Each data point is the mean of three experiments and error bars represent 1 standard deviation above and below the mean.

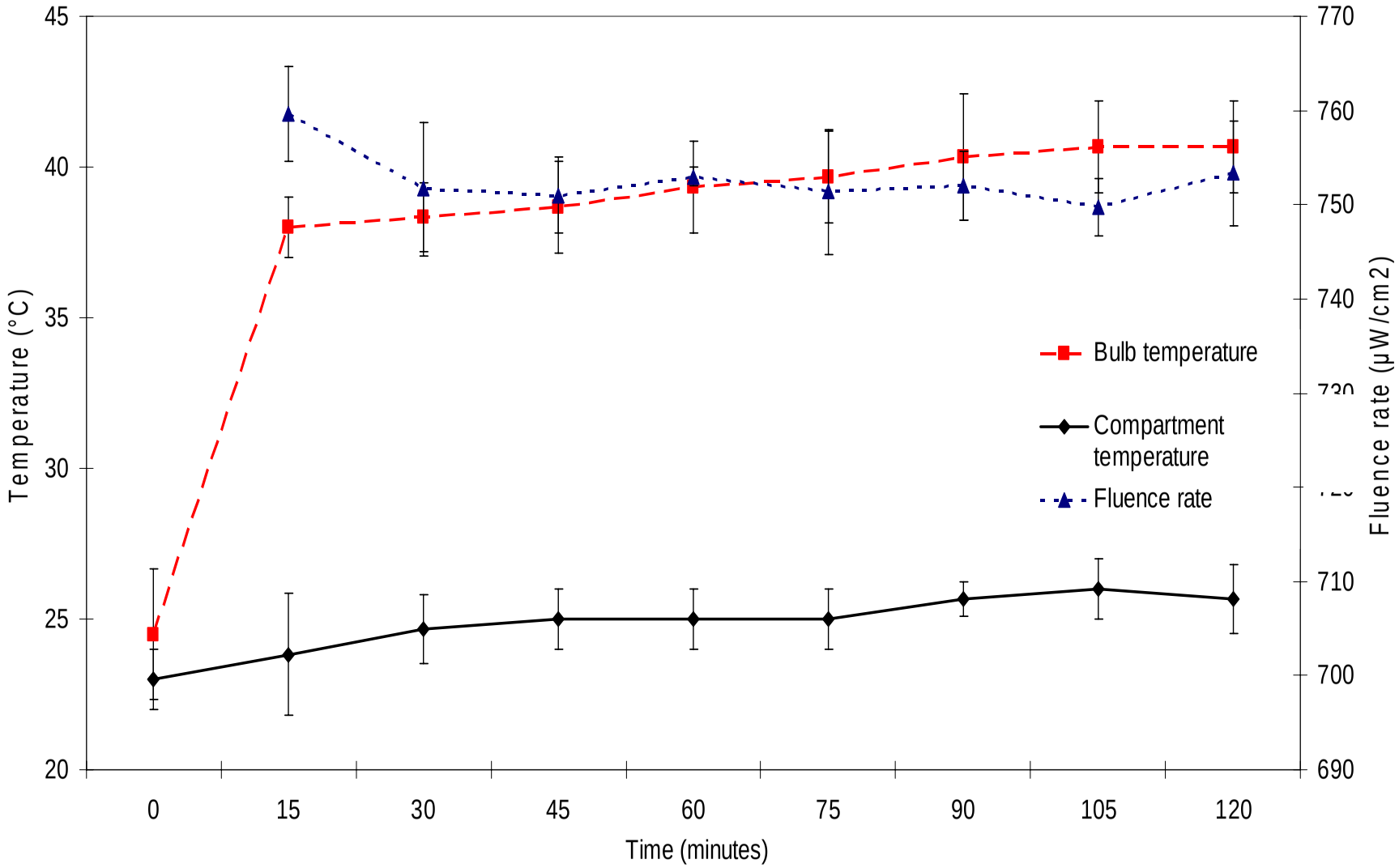


Figure 4.8 UV-C bulb fluence rate, compartment temperature and bulb compartment temperature during preliminary experiment. Each data point is the mean of three experiments and error bars represent 1 standard deviation above and below the mean.

4.4 UV Inactivation Methods

Irradiation of B124 phages was carried out in two phases. All phage suspensions were subjected to Phase One screening tests (to highlight differences between phage specimens), from which seven were selected for more thorough Phase Two investigation. To ensure an efficient use of time, media, and materials, the method used during Phase One screening experiments was simpler than the method used during the detailed experiments.

4.4.1 Phase One initial screening irradiation experiments

Initial screening irradiation experiments were conducted in accordance with previously described methods (Tartera *et al*., 1988a; Havelaar *et al*., 1990; Nicholson and Galeano, 2003; Shin *et al*., 2005).

For each phage specimen, 15 ml was warmed to room temperature, diluted to ~10^5 PFU/ml (using buffer given in Section 4.1.2) and pipetted into a 53mm diameter Petri dish. Immediately prior to phage exposure, the fluence rate directly below the collimating tube aperture was measured. This was the fluence rate used for the fluence calculation. Fluence rate was determined before each exposure. This petri dish containing the phage suspension was then placed directly below the collimating tube, with the shutter in place. The suspension surface was 20 cm from the base of the lamps, allowing higher fluence rate than would be used in the Phase Two experiments. Stirring was initialised ten seconds before UV exposure to ensure homogenous distribution of phages within samples. A preliminary experiment was carried out to determine if stirring decreased PFU/ml. Phage suspensions were stirred *in situ* for 4, 6, 8, and 10 minutes without UV and assayed in triplicate (along with a

control - no UV, no stirring) using the double-agar technique. No decrease in titre was observed, and it was assumed that slow stirring had no influence on phage inactivation. UV bulbs two and three were used during all screening experiments. A timer was started when the shutter was withdrawn and at predetermined intervals, the shutter was replaced, suspensions removed from the box, and a 400 µl aliquot taken for assay from which a dilution series was created. In order to avoid any shadowing of the solution by the meniscus (refracting UV light, excluding it from the edges of the Petri dish), aliquots were taken from the centre of the Petri dish (Havelaar *et al.*, 1990). All test aliquots were assayed in triplicate using the double-agar method detailed above. The suspension was then returned to the box, the shutter removed and the timer restarted. For UV-B experiments, aliquots were taken every minute, whereas for UV-C aliquots were taken every 10 seconds. These time intervals were determined in a series of pilot experiments to determine inactivation time frames. Temperature in both the bulb and sample compartments were consistent with the preliminary study (i.e., ~40 °C and ~23°C respectively, Figures 4.7 and 4.8). Each phage suspension was subject to three experimental runs for each wavelength.

The fluence received by the irradiated sample is given by:

$$D = It$$

where: D = fluence (mJ/cm^2)

I = fluence rate (mW/cm^2)

t = exposure time

(Metcalf and Eddy, 2003)

4.4.2 Phase Two inactivation experiments

Seven phage suspensions were selected for further experimentation (the rationale for selecting these phages is presented in Chapter Seven). For this set of experiments, the methodology was altered to ensure greater accuracy and precision, allowing data to be compared with other studies using CB apparatus. Fluences were calculated using the UVCalc spreadsheet (kindly provided by Professor James R. Bolton of Bolton Photosciences Ltd), which is based upon the work presented by Bolton and Linden, (2003) in which the collimated beam methodology was standardised (this paper has been cited by over 100 authors as of 24 March 2010, according to Web of Science). A broadly similar standardization protocol of collimated beam experimentation was presented by Kuo *et al* (2003).

The phage suspensions were diluted to a final concentration of 10^5 PFU/ml using the buffer described in Section 4.1.2. As with the screening experiments, the UV box was switched on one hour before the first experiment allowing the bulbs to reach a stable temperature. Phage suspensions were equilibrated to room temperature before irradiation. Samples were stirred *in situ*, protected from UV light by the shutter, for 10 seconds before UV exposure, ensuring a fully mixed sample. Prior to each exposure, the fluence rate at the centre of the beam (E_0) was measured and entered into the UVCalc spreadsheet. UV-C bulb output varied during the experimental run by ± 2 μW/cm^2 and UV-B bulb output tended to vary by ± 4 μW/cm^2. Exposure times were adjusted to correct for this. Data from the screening experiments identified estimated UV-C fluences required to inactivate samples to 99%, and seven fluences were chosen to capture this inactivation (0, 5, 10, 15, 20, 25, and 30 mJ/cm^2). For UV-B, fluences of 0, 50, 100, 150, 200, 250 and 300 mJ/cm^2 were used.

Rather than taking aliquots for assay from the Petri dish then returning suspension under UV beam until next target-time was reached (as in Phase One experiments), each fluence experiment was carried out separately using a new 10 ml volume of phage suspension.

Each exposure was repeated three times with samples being irradiated in a random order. Upon completion of exposure, the phage suspensions were removed from the UV box and stored in light-tight glass tubes, in the dark at 4 °C until all exposures had been carried out. UV-C test samples were assayed on the same day as inactivation experiments, whereas UV-B irradiated suspensions were assayed the next day; this was because longer inactivation periods were required by UV-B. Prior to inactivation experiments, stability of phages in light tight glass tubes stored at 4 °C was tested over three days and no decrease in the titre was observed.

A dilution series of each irradiated suspension was created immediately prior to analysis and assayed using the double-agar method described previously. Two dilutions were selected for assay, with each assayed in triplicate. Therefore 18 plaque assays were conducted for each fluence. Temperatures in both the bulb and sample compartments were consistent with the preliminary study (i.e., 40 °C and 23 °C respectively; Figures 4.7 and 4.8). Plates containing more than 300 PFU were not included in the analysis (as individual plaques were not clearly distinguishable), and no lower exclusion limit was used, as it was necessary to ascertain when total inactivation occurred (i.e., evidence of tailing). The lower limit of sensitivity of assay is < 1 PFU/100μl.

4.4.3 Statistical analysis of Phase Two experiments

In order to give accurate fluence measurements, a number of corrections to the equation given in Section 4.4.1 were considered necessary.

These were:

- *The Petri factor*

  Fluence rate varies over the surface of the Petri dish and it is necessary to correct for this. A 0.5 cm x 0.5 cm grid was produced, covering the Petri dish area and centred in the middle of the beam. UV fluence rate was measured every 0.5 cm along the *x* and *y* axis, and the ratio of each reading to the centre reading was calculated. The mean of these ratios gives the Petri factor. In order to improve accuracy, the sensor was partially blinded using UV impenetrable material, leaving a detection area of $2mm^2$. Using a partially blinded sensor does not give accurate readings of fluence rate, but this is of no consequence, as it is only the ratio of the readings that are important. The Petri factor was determined for each wavelength before commencement of exposures;

- *Reflection factor*

  UV light is reflected back towards the source when the beam passes between media (in this case air and water). For an air/water interface this is constant at 0.975 (Bolton and Linden, 2003);

- *Water factor*

  This accounts for the amount of UV adsorbed by liquid as the beam penetrates the phage suspension and is given by:

$$\text{Water Factor} = \frac{1 - 10^{-a\ell}}{a\ell \ln(10)}$$

where:

a = decadic absorption coefficient (cm^{-1})

l = vertical path length of water in Petri dish being irradiated

The above equation is only suitable for completely mixed samples, and is derived from integration of the Beer-Lambert law over the same depth (Morowitz, 1950; Bolton and Linden, 2003). The absorption coefficient for each phage suspension was measured using a UV visible spectrophotometer, which had been zeroed using distilled water. This was then replaced with phage suspension and the absorption coefficient (path length 1 cm) at the wavelength being investigated (254 nm and 302 nm); and

- *Divergence factor*

As the UV beam is not collimated perfectly (i.e., it displays divergence), a correction is necessary:

$$\text{Divergence Factor} = \frac{L}{(L + \ell)}$$

where:

L = distance from UV bulb to surface of suspension (cm)

l = vertical path length of liquid in Petri dish being irradiated (cm)

The distance from the UV bulb to the suspension surface, and the sample volume remained constant throughout the detailed experiments.

Determination of fluence rate

The average germicidal fluence rate (E'_{avg}) is therefore given by the following equation:

E'_{avg} = E_0 (fluence rate at the centre of the beam) X *Petri Factor* X *Reflection Factor* X *Water Factor* X *Divergence Factor*

(Bolton and Linden, 2003)

The fluence (mJ/cm^2) a suspension receives is given by E'_{avg} (mJ/cm^2) multiplied by exposure t (s).

The UVCalc spreadsheet was used to calculate exposure times required for chosen fluences.

The identification and observance of the factors outlined above helped ensure that the experimental design was sound, and that the results obtained during this investigation were robust, reproducible and reliable. What's more, it is possible to compare the results from this investigation with the findings from previous studies. As such, the results should further our understanding of the behaviour (persistence and survival) of such phages and consequently help determine their suitability as surrogates for human enteric viruses. The following chapter details the findings of the isolation and characterisation experiments, whilst the next chapters present the results of the inactivation experiments.

CHAPTER FIVE

5. RESULTS OF ISOLATION AND CHARACTERISATION OF B124 PHAGES

In order to act as an effective phage-lysis MST tool, it is desirable that any bacterial host strain detects a homogenous group of phage, as diverse groups of phage, such as somatic coliphages may demonstrate differential inactivation kinetics in different water matrices, making inter-site comparisons problematic. This chapter assesses the similarity of isolated B124 phages using morphological and host range characteristics. These data, and those detailed in the next chapter that present the results of Phase One inactivation experiments, informed the decision as to which phages would be selected for Phase Two inactivation experiments.

5.1 Phage isolation

During each round of phage isolation, 20 or 21 plaques were picked from final effluent double-agar plates; 61 plaques being picked in total. The proportion of picked plaques successfully propagated and stored in high titre (~10^{10} PFU/ml) culture was 33%. The remaining 67% of plaques unsuccessfully propagated may have contained lysogenic phage strains (unlikely because of presence of initial plaque), bacteriocins able to destroy bacterial lawn, thus giving the appearance of plaques (as reported by Booth *et al*., 1979), or may have been damaged in some way by the culturing technique. As detailed in Chapter Four, to achieve 27 mls of high titre phage suspension, two rounds of host infection were necessary. Firstly, an inoculation of a 27 ml GB-124 culture in exponential growth phase with a picked plaque, and secondly, this 27 ml lysate was used to inoculate a 620 ml GB-124 culture in

exponential growth phase. After each stage of infection, the titre of lysate was tested in triplicate by double-agar assay (Table 5.1).

Table 5.1 Titres and plaque size of B124 phages during isolation process

Phage ID (B124-)	Stage 1 lysate (PFU/ml)	Stage 2 lysate (PFU/ml)	Plaque size range (mm)
1	1.00×10^{2}	1.47×10^{10}	0.2 to 1.5
2	1.42×10^{4}	4.00×10^{8}	0.2 to 1.1
3	1.21×10^{3}	1.02×10^{10}	0.5
4	9.20×10^{3}	1.00×10^{10}	0.3 to 1.5
10	4.50×10^{2}	1.60×10^{9}	1 to 3.6
12	3.00×10^{2}	2.22×10^{10}	0.3 to 2.5
14	3.56×10^{2}	1.30×10^{10}	0.2 to 1
17	1.43×10^{4}	7.50×10^{9}	1 to 3.8
20	2.00×10^{1}	4.40×10^{8}	0.2 to 1.5
21	1.23×10^{4}	1.80×10^{10}	0.2 to 1
22	9.00×10^{2}	5.75×10^{10}	0.9 to 3.8
25	1.00×10^{4}	5.00×10^{10}	0.2 to 3.2
26	5.00×10^{3}	5.60×10^{10}	0.2 to 1
29	4.00×10^{3}	3.63×10^{10}	1 to 3
30	1.10×10^{4}	2.44×10^{10}	1 to 4.9
31	2.10×10^{1}	2.10×10^{8}	0.2 to 4
35	3.00×10^{2}	1.80×10^{11}	1.2 to 3.1
45	1.00×10^{3}	7.00×10^{10}	2.1 to 3.1
54	2.36×10^{3}	1.10×10^{11}	1 to 3
57	4.80×10^{2}	1.00×10^{11}	3 to 4.2

Single plaques produced ‘stage one’ non chloroform treated lysates of between 2.00×10^{1} PFU/ml (B124-20) and 1.43×10^{4} PFU/ml (B124-17). The range of stage one lysates was 1.42×10^{4} PFU/ml with a mean value of 4.37×10^{3} PFU/ml ($n = 20$), and PFU/ml of stage two lysates was between 2.10×10^{8} PFU/ml and 1.80×10^{11} PFU/ml (range = 1.80×10^{11} PFU/ml, mean = 3.91×10^{10} PFU/ml, $n = 20$). Correlation analysis showed that the titre of the second stage lysate was not significantly related to the titre of the first stage lysate ($R^2 = 0.076$ %, $n = 20$).

Plaque sizes during stage one and stage two isolation varied between phage specimens, and also within plates of the same phage specimen (see Table 5.1). Figure 5.1 shows typical plaques caused by lytic phages infecting GB-124.

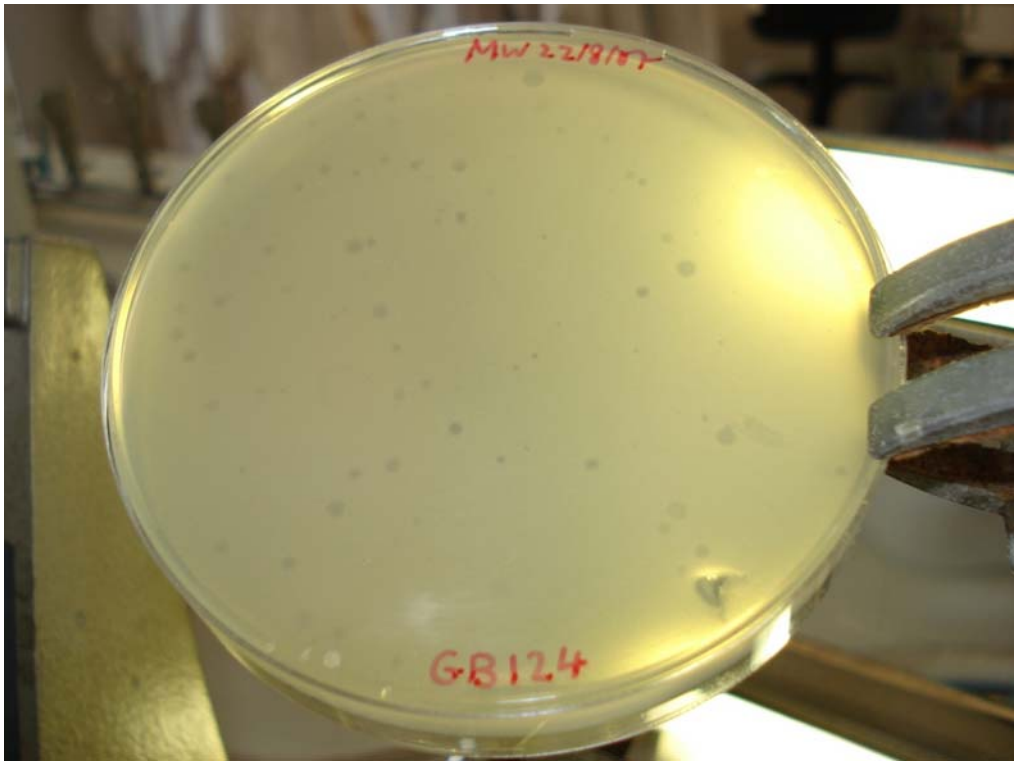


Figure 5.1 Example of plaques caused by phage infecting GB-124 present in municipal wastewater

The range of plaque sizes recorded ranged from approximately 0.2 mm to 4.9 mm in diameter. It was interesting to note that the plaque size range was constant between stage one and stage two lysates. There are numerous reasons why plaque size might be expected to vary on the same plate (e.g., the gel-strength of the semi-solid agar). Phage isolates from the environment, rather than from pure laboratory strains, may have different replication and adsorption rates when re-plated. Delayed adsorption to new host cell by certain replicated phages once on the plate, may create greater variation in plaque size (Adams, 1959). This can be minimised by allowing phage and host to mix prior to plating, though this was shown to have no effect on plaque size

variation during the research. To facilitate faster phage diffusion through agar, reducing differential plaque sizes, agar density of both ssBPRMA and BPRMA was lowered to 0.65% and 1% respectively (original values were 0.8% and 1.6%). Resulting plaques were the same size as those recorded on original strength plates and PFU/ml of samples were consistent. For all subsequent analyses original agar strengths were used.

5.2 Transmission Electron Microscopy

In order to assess the morphology of the twenty isolated B124 phages, they were examined using transmission electron microscopy (TEM). Phage micrographs are shown in Figures 5.2 to 5.7, and the dimensions are summarised in Table 5.2. For reasons of space, not all phages are presented in the figures below. Both phosphotungstic acid and uranyl acetate stains were used during TEM, with uranyl acetate producing clearer images. However, staining with uranyl acetate produced both positively (black coloured) and negatively (white coloured) stained virions, often on the same grid (as has been described elsewhere; Ackermann, 2009). Examples of both a positively and negatively stained B124-17 phages are shown in Figure 5.5. Each dimension given in table 5.2 is the mean value of five virion measurements.

All isolated B124 phages had binary morphology with an icosahedral capsid and a non-contractile helical tail, placing them in the *Caudoviriale* order, *Siphoviridae* family. As described in the literature review, the *Caudovirale* order is divided into three families, *Siphoviridae, Myoviridae* and *Podoviridae*. Members of these families are distinguishable by tail length and structure (long non-contractile, long contractile, and short non-contractile respectively). The presence of both an icosahedral capsid and helical tail identifies the genetic material of all isolated B124 phages as dsDNA.

All phages had capsids that were non-elongated, with diameters ranging from 50 nm to 70 nm, and it is apparent from the micrographs that both positively and negatively stained capsids of the same phage specimen appear to be different sizes (Figure 5.5). As is consistent with the literature, positively stained capsids appear smaller than negatively stained capsid, and as the majority of phages were only positively stained,

the capsid measurements based on positive stains only (given in Table 5.2) should be treated with caution. However, as some phage specimen micrographs show both types of staining of the same phage specimen, it is possible to estimate the negatively stained capsid size from positively stained virions A shrink of positively stained specimens between 10 nm – 26 nm was observed.

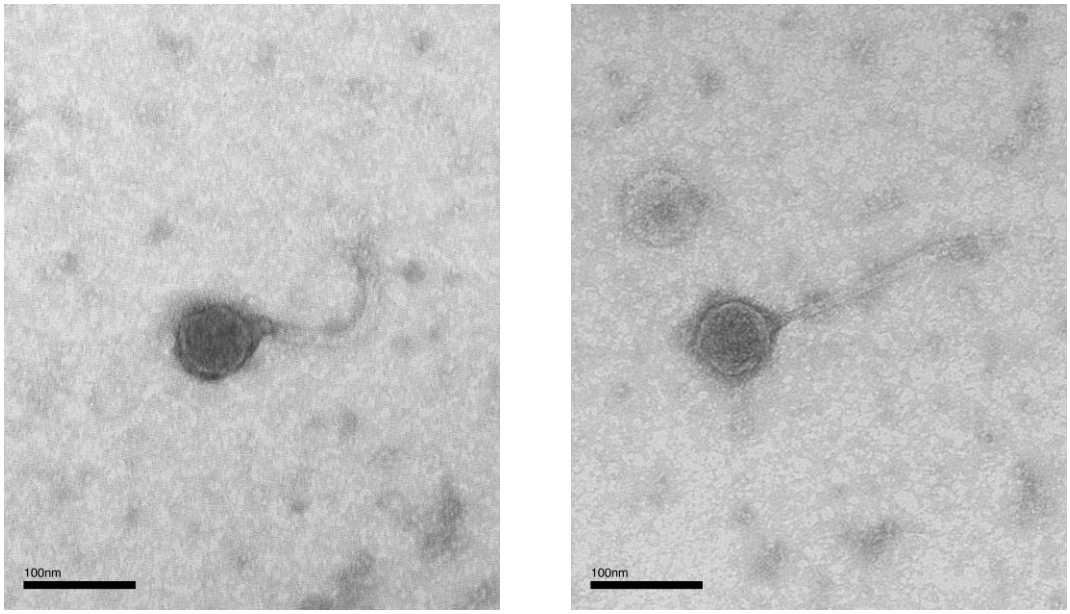


Figure 5.2 Positively stained B124-1 siphovirus showing ‘curly’ tail (L) and straight tail (R)

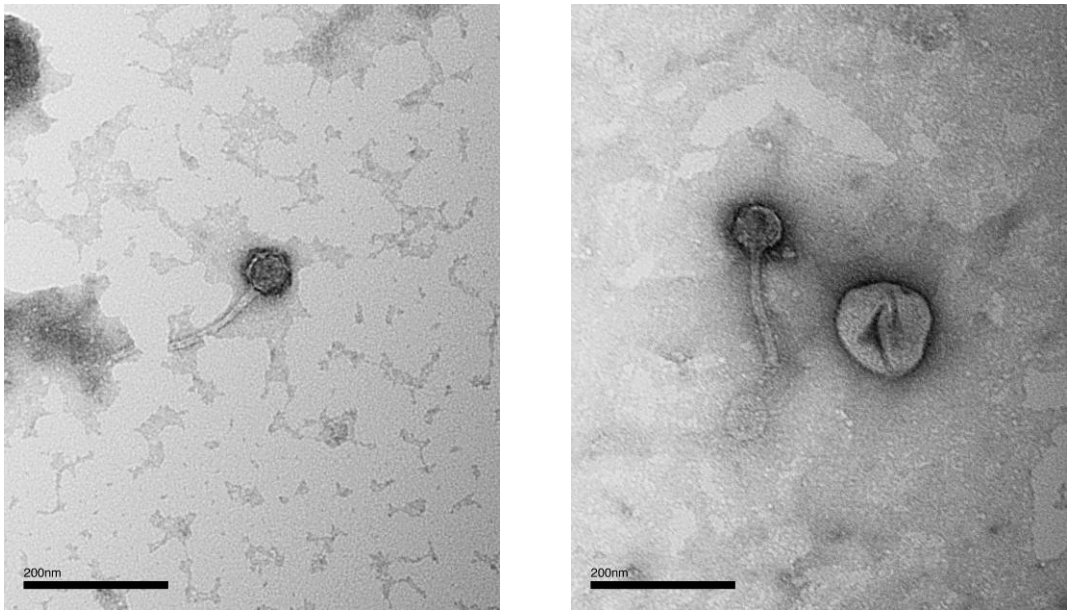


Figure 5.3 Positively stained B124-3 siphovirus showing ‘curved’ tail (L) and ‘kinked’ tail (R)

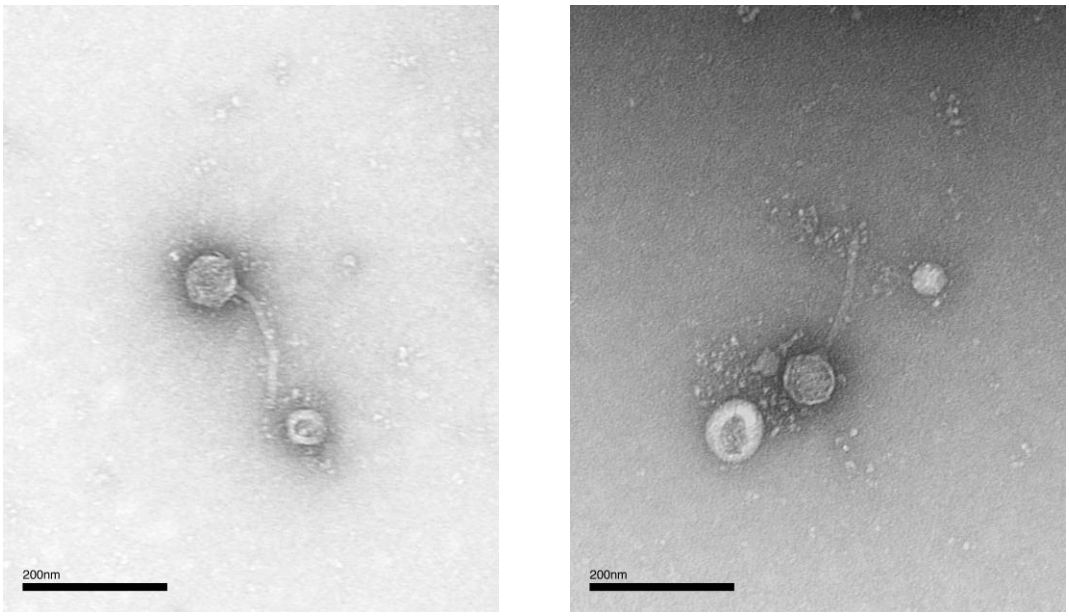


Figure 5.4 Negatively stained B124-12 siphovirus showing 'curved' tail (L) and straight tail (R)

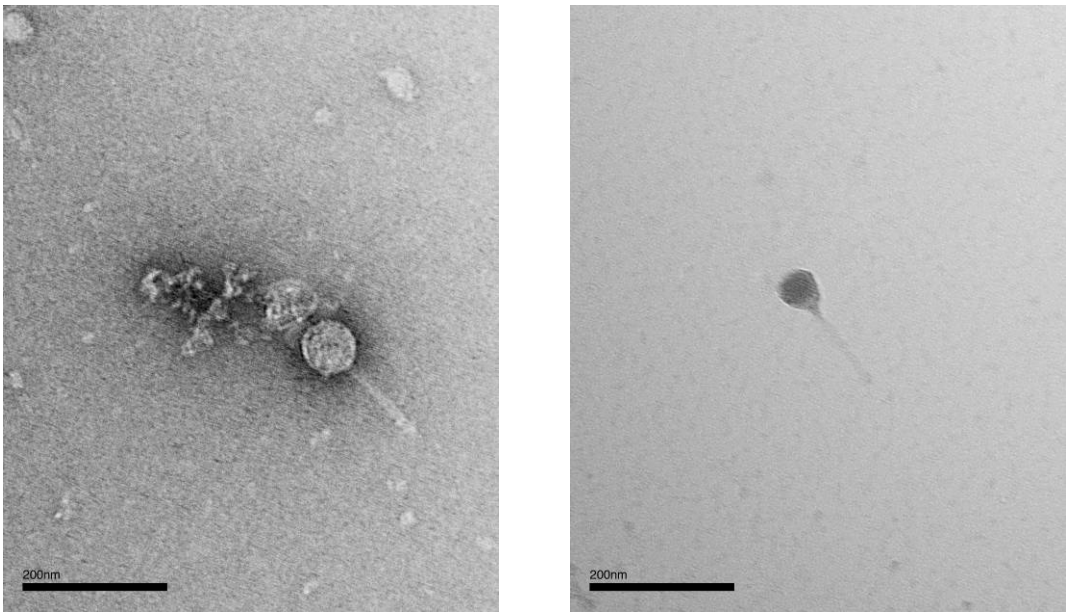


Figure 5.5 B124-17 straight-tailed siphovirus showing negative staining (L) and positive staining (R)

Table 5.2 Morphological characteristics of B124 bacteriophages

Phage ID (B124-)	Capsid shape	Mean capsid diameter (nm)	Tail shape	Mean tail width (nm)	Mean tail length (nm)	Family
1	Icosahedral	(P)† 64 nm (SD = 4.16) (*n* = 5)	Curled/straight	13 nm (SD = 1.67) (*n* = 5)	195 nm (SD = 9.09) (*n* = 5)	*Siphoviridae*
2	Icosahedral	(P) 59 nm (SD = 4.98) (*n* = 5)	Straight	12 nm (SD = 1.58) (*n* = 5)	*	*Siphoviridae*
3	Icosahedral	(N)‡ 72 nm (SD = n/a) (*n* = 1)	Slightly curved/straight	-	-	*Siphoviridae*
		(P) 62 nm (SD = 5.90) (*n* = 5)		11 nm (SD = 1.82) (*n* = 5)	166 nm (SD = 5.86) (*n* = 5)	
4	Icosahedral	(P) 60 nm (SD = 7.30) (*n* = 5)	Slightly curved/straight	13 nm (SD = 1.10) (*n* = 5)	210 nm (SD = 9.35) (*n* = 5)	*Siphoviridae*
10	Icosahedral	(P) 60 nm (SD = 5.37) (*n* = 5)	Straight	13 nm (SD = 0.89) (*n* = 5)	*	*Siphoviridae*
12	Icosahedral	(N) 70 nm (SD = 3.70) (*n* = 5)	Slightly curved/straight	12 nm (SD = 1.48) (*n* = 5)	167 nm (SD = 5.26) (*n* = 5)	*Siphoviridae*
14	Icosahedral	(P) 65 nm (SD = 5.54) (*n* = 5)	Straight	12 nm (SD = 1.79) (*n* = 5)	165 nm (SD = 6.08) (*n* = 5)	*Siphoviridae*
17	Icosahedral	(N) 71 nm (SD = 5.32) (*n* = 5)	Straight	12 nm (SD = 1.58) (*n* = 5)	120 nm (SD = 6.12) (*n* = 5)	*Siphoviridae*
		(P) 45 nm (SD = n/a) (*n* = 1)		-	-	
20	Icosahedral	(P) 60 nm (SD = 2.68) (*n* = 5)	Slightly curved	12 nm (SD = 0.84) (*n* = 5)	120 nm (SD = 4.82) (*n* = 5)	*Siphoviridae*
21	Icosahedral	(P) 65 nm (SD = 5.90) (*n* = 5)	Long wavy tail	13 nm (SD = 1.10) (*n* = 5)	245 nm (SD = 7.13) (*n* = 5)	*Siphoviridae*
22	Icosahedral	(N) 70 nm (SD = 4.38) (*n* = 5)	Straight and curved	13 nm (SD = 0.84) (*n* = 5)	163 nm (SD = 23.93) (*n* = 5)	*Siphoviridae*
		(P) 50 nm (SD = n/a) (*n* = 1)		-	-	
25	Icosahedral	(P) 50 nm (SD = 4.12) (*n* = 5)	Straight and curved	12 nm (SD = 0.89) (*n* = 5)	135 nm (SD = 5.64) (*n* = 5)	*Siphoviridae*
26	Icosahedral	(P) 50 nm (SD = 4.53) (*n* = 5)	Straight and curved	12 nm (SD = 0.71) (*n* = 5)	135 nm (SD = 7.33) (*n* = 5)	*Siphoviridae*
29	Icosahedral	(P) 59 nm (SD = 4.32) (*n* = 5)	Straight and curved	13 nm (SD = 1.22) (*n* = 5)	160 nm (SD = 9.50) (*n* = 5)	*Siphoviridae*
30	Icosahedral	(P) 65 nm (SD = 5.22) (*n* = 5)	Slightly curved	12 nm (SD = 2.28) (*n* = 5)	181 nm (SD = 8.89) (*n* = 5)	*Siphoviridae*
31	Icosahedral	(P) 58 nm (SD = 6.06) (*n* = 5)	Straight	12 nm (SD = 0.84) (*n* = 5)	164 nm (SD = 4.92) (*n* = 5)	*Siphoviridae*
35	Icosahedral	(P) 59 nm (SD = 5.76) (*n* = 5)	Straight	12 nm (SD = 1.64) (*n* = 5)	145 nm (SD = 13.56) (*n* = 5)	*Siphoviridae*
45	Icosahedral	(P) 60 nm (SD = 5.41) (*n* = 5)	Straight	12 nm (SD = 1.79) (*n* = 5)	148 nm (SD = 13.67) (*n* = 5)	*Siphoviridae*
54	Icosahedral	(P) 59 nm (SD = 5.45) (*n* = 5)	Straight	12 nm (SD = 1.22) (*n* = 5)	200 nm (SD = 8.79) (*n* = 5)	*Siphoviridae*
57	Icosahedral	(P) 58 nm (SD = 4.56) (*n* = 5)	Slightly curved	13 nm (SD = 1.67) (*n* = 5)	160 nm (SD = 10.16) (*n* = 5)	*Siphoviridae*

† P = positive stain
‡ N= negative stain
* It was not possible to determine tail lengths of B124-2 and B124-10 on micrographs

Tail shape appeared to be homogenous, showing little variation and the majority of phages having straight (Figure 5.2 R) or slightly curved tails (Figure 5.3 L and 5.4 L). However, tail length varied from 120 nm (B124-17, Figure 5.5) to 245 nm (B124-21, Figure 5.6). Tail width was consistent throughout, being around 12.5 nm, and tail fibres or other appendages were not observed. Small kinks or curves may be caused by the staining process, and may not be indicative of true morphological variation (see Section 2.2.1). Of all 20 samples, two phages had distinct tails: B124-21 had a long (245 nm) 'wavy' tail and some B124-1 virions had a curled tail [though this may be a result of virion damage indicated by the offset head-neck joint; Figure 5.2 (L)]. Capsid damage was also evident on positively stained phage, notably B124-2 and other phage micrographs show head and tail disassociation (B124-22, Figure 5.7 L). Susceptibility of phages to damage may indicate why only 33% of picked plaques were successfully propagated.

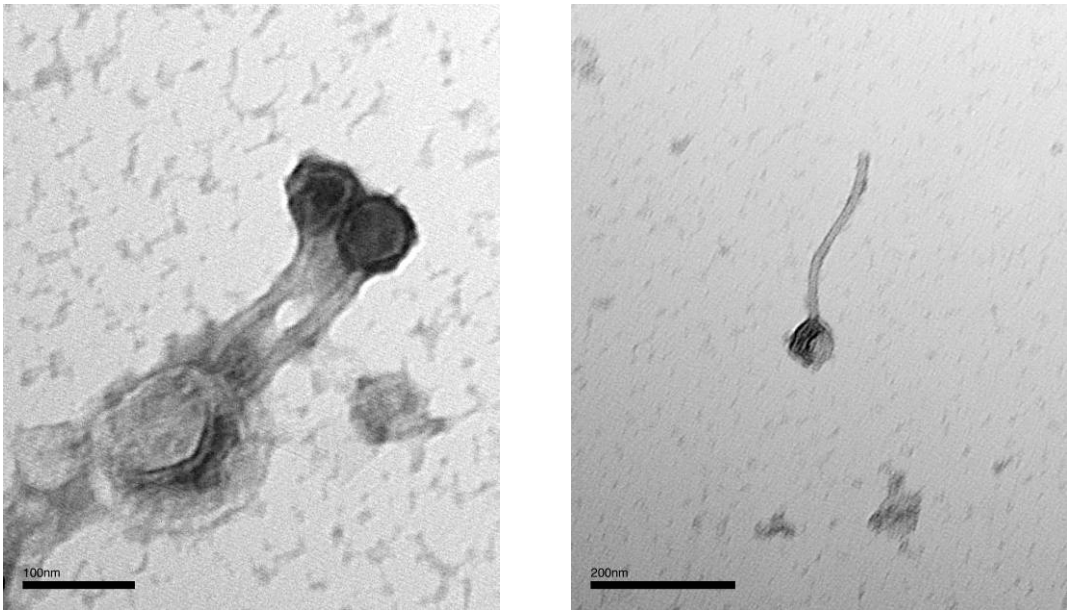


Figure 5.6 Phage B124-21 with head damage/shrinkage (L), and showing long wavy tail (R)

The damage to some virions suggested at first glance that *Podoviridae* were present in some samples (see Figure 5.7 R). However the presence of *Podoviridae* in the isolated phage suspensions is disputable. When examined under the electron microscope, phage suspensions containing presumed *Podoviridae* (B124-3, B124-31, B124-45 and B124-57) also contained disassociated tails. Tail-less phages observed may be siphoviruses with tail damage, and as these damaged virions were found in isolation (i.e., one or two per grid), it suggests virion damage rather than genuine *Podoviridae*. Micrographs of bf-1 phage given in Booth *et* al (1979) also show disassociated tails with kinked and straight tailed varieties of the same phage.

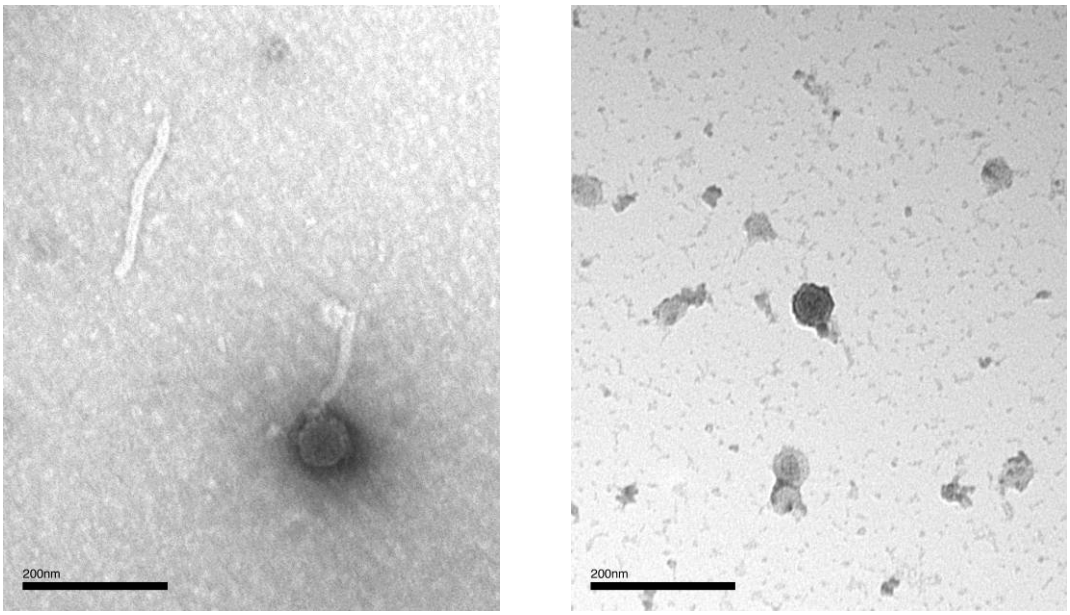


Figure 5.7 Phage B124-22 with head damage and disassociated tails (L) and false *Podoviridae* B124-57 (R)

5.3 B124 phage host range

The host range of B124 phages was assessed using two *B. fragilis* strains previously proposed as detecting phage exclusively present in human faecal material: HSP-40 (Tartera and Jofre, 1987) and RYC-2056 (Puig *et al*., 1997, 1999). The results are shown in Table 5.3. All B124 phage, excluding B124-35, were able to infect RYC-2056, tending to have marginally lower titres (though not statistically significant; paired t test; P = 0.06) when plated on RYC-2056 [mean GB-124 = 3.17 x 10^{10} PFU/ml (n = 20), mean RYC-2056 = 3.67 x 10^{9} PFU/ml (n = 20)]. Morphology of B124-35 has no distinctive characteristics, suggesting morphology may not influence phage host range. There was no correlation between PFU/ml when plated on GB-124 and RYC-2056 (R^2 = 0.11 %) and no clear relationship was evident between B124 phage plaque size when plated on RYC-2056 and GB-124. Some phages produced larger maximum plaques on GB-124 than RYC-2056 (B124-1, -10, -12, -14, 17, -22, -25, -29, -30, -31, -45, -54 and -57), whilst some produced smaller plaques (B124-2, -3, -4, -20, -21 and -26). Overall, plaques tended to be more homogenous in size when plated on RYC-2056 than when plated on GB-124 with less 'in-plate' variation (see standard deviation data in table 5.3.) The mean standard deviation of all RYC-2056 plaques was 0.30 (n = 200) and GB -124 plaques was 0.66 (n = 200). None of the isolated B124 phages were able to cause bacterial lysis when plated on HSP-40.

The high homogeneity of isolated B124 phages is evident not only from virion morphology but also in host range. Moreover, intra-Family variation appears to be limited, with the majority of isolated phages having a capsid of 50 nm to 70 nm in diameter, and a straight/slightly curved tail between 120 nm and 200 nm (the exception being B124-21 which had a relatively long wavy tail). This high

homogeneity is a positive characteristic in relation to MST. The following chapters report the UV inactivation of B124 phage, assessing if morphological and host range homogeneity corresponds to congruent behaviour during UV irradiation.

Table 5.3 Host range of B124 phages using HSP-40 and RYC-2056

Phage ID (B124-)	PFU/ml GB-124	Mean GB-124 Plaque size (mm)	Able to infect RYC-2056	PFU/ml RYC-2056	Mean RYC-2056 Plaque size (mm)	Able to infect HSP-40
1	1.47×10^{10}	1.1 mm (SD = 0.51) (*n* = 10)	**Y**	5.60×10^{9}	1.0 mm (SD = 0.28) (*n* = 10)	**N**
2	4.00×10^{8}	0.9 mm (SD = 0.44) (*n* = 10)	**Y**	7.60×10^{8}	1.3 mm (SD = 0.26) (*n* = 10)	**N**
3	1.02×10^{10}	0.6 mm (SD = 0.21) (*n* = 10)	**Y**	1.69×10^{9}	1.0 mm (SD = 0.28) (*n* = 10)	**N**
4	1.00×10^{10}	0.9 mm (SD = 0.40) (*n* = 10)	**Y**	3.70×10^{9}	1.7 mm (SD = 0.53) (*n* = 10)	**N**
10	1.60×10^{9}	2.2 mm (SD = 0.80) (*n* = 10)	**Y**	2.06×10^{8}	0.6 mm (SD = 0.21) (*n* = 10)	**N**
12	2.22×10^{10}	1.5 mm (SD = 0.76) (*n* = 10)	**Y**	4.70×10^{8}	1.2 mm (SD = 0.26) (*n* = 10)	**N**
14	1.30×10^{10}	0.7 mm (SD = 0.30) (*n* = 10)	**Y**	6.40×10^{9}	0.6 mm (SD = 0.21) (*n* = 10)	**N**
17	7.50×10^{9}	2.4 mm (SD = 0.77) (*n* = 10)	**Y**	5.40×10^{9}	1.4 mm (SD = 0.58) (*n* = 10)	**N**
20	4.40×10^{8}	1.4 mm (SD = 0.81) (*n* = 10)	**Y**	7.70×10^{8}	1.6 mm (SD = 0.44) (*n* = 10)	**N**
21	1.80×10^{10}	0.6 mm (SD = 0.28) (*n* = 10)	**Y**	1.04×10^{9}	1.2 mm (SD = 0.35) (*n* = 10)	**N**
22	5.75×10^{10}	2.1 mm (SD = 1.31) (*n* = 10)	**Y**	1.50×10^{10}	1.3 mm (SD = 0.48) (*n* = 10)	**N**
25	5.00×10^{10}	2.0 mm (SD = 0.92) (*n* = 10)	**Y**	1.30×10^{9}	1.7 mm (SD = 0.26) (*n* = 10)	**N**
26	5.60×10^{10}	0.7 mm (SD = 0.31) (*n* = 10)	**Y**	1.68×10^{10}	2.1 mm (SD = 0.21) (*n* = 10)	**N**
29	3.63×10^{10}	1.8 mm (SD = 0.67) (*n* = 10)	**Y**	7.00×10^{8}	1.0 mm (SD = 0.16) (*n* = 10)	**N**
30	2.44×10^{10}	2.9 mm (SD = 1.26) (*n* = 10)	**Y**	2.70×10^{8}	1.1 mm (SD = 0.21) (*n* = 10)	**N**
31	2.10×10^{8}	2.1 mm (SD = 1.25) (*n* = 10)	**Y**	4.50×10^{7}	0.9 mm (SD = 0.24) (*n* = 10)*	**N**
35	1.80×10^{11}	2.1 mm (SD = 0.62) (*n* = 10)	**N**	-	-	**N**
45	7.00×10^{10}	2.6 mm (SD = 0.37) (*n* = 10)	**Y**	6.20×10^{8}	0.8 mm (SD = 0.26) (*n* = 10)*	**N**
54	1.10×10^{11}	1.8 mm (SD = 0.86) (*n* = 10)	**Y**	5.20×10^{9}	2.1 mm (SD = 0.21) (*n* = 10)	**N**
57	1.00×10^{11}	3.6 mm (SD = 0.46) (*n* = 10)	**Y**	3.75×10^{9}	1.2 mm (SD = 0.26) (*n* = 10)	**N**

***** At low dilutions there were many large plaques (3mm) and an almost confluent lysis of small plaques (~1mm). At higher dilutions only small plaques were evident and this figure used to ascertain PFU/ml

CHAPTER SIX

6. RESULTS OF PHASE ONE UV INACTIVATION EXPERIMENTS

Numerous different methods of phage inactivation using collimated beam apparatus have been presented in the literature. This chapter presents the results of Phase One screening experiments of all 20 isolated phage specimens, carried out in order to determine general patterns of UV inactivation. Data from Phase One experiments, in conjunction with morphological and host range data given in Chapter Five, were used to select seven phages to undergo Phase Two inactivation experiments. Note, all *P* values given are based on 95% confidence interval.

6.1 Phase One UV-C inactivation kinetics of B124 phages

6.1.1 Correlation between fluence and phage inactivation during Phase One experiments

All 20 phage specimens showed a significant ($P = 0.000$) negative linear relationship between $\log_{10}$ PFU/100 µl and fluence (mJ/cm^2), ranging from -0.97 % (B124-12 and B124-29) to -1.00 % (B124-2 and B124-30), with a mean of -0.98 % and 0.008 SD (Table 6.1). Variance of correlation between phages was very limited, with all phages showing a statistically significant relationship, meaning that the null hypothesis of the relationship between fluence and $\log_{10}$ PFU/100 µl occurring as a result of chance could be rejected.

Table 6.1 Pearson product-moment correlation values for $\log_{10}$ PFU/100µl and UV-C fluence during Phase One experiments

Phage specimen ID (B124-)	Pearson product-moment correlation	*P*-value
1	-0.99	0.000
2	-1.00	0.000
3	-0.99	0.000
4	-0.99	0.000
10	-0.98	0.000
12	-0.97	0.000
14	-0.98	0.000
17	-0.98	0.000
20	-0.99	0.000
21	-0.99	0.000
22	-0.99	0.000
25	-0.99	0.000
26	-0.98	0.000
29	-0.97	0.000
30	-1.00	0.000
31	-0.98	0.000
35	-0.99	0.000
45	-0.98	0.000
54	-0.98	0.000
57	-0.98	0.000

6.1.2 UV-C inactivation rate coefficient and fluence required for 1-, 2-, 3- and 4-$\log_{10}$ inactivation of B124 phages during Phase One experiments

To determine the inactivation rate coefficient (*k*), i.e., how readily the microorganism is inactivated, the first-order disinfection model (Chick, 1908; Watson, 1908) can be applied. The linear relationship between fluence and $\log_{10}$ inactivation given by:

$$^{10}\log\left(\frac{N_t}{N}\right) = -k^* \text{ Fluence},$$

where N_t is the organism concentration after time *t* (seconds), and *k* is the inactivation rate coefficient (Hijnen *et al.*, 2006). Inactivation rate coefficients allow the

comparison of inactivation kinetics of different organisms from different studies. Higher *k* values indicate that the test organism is more easily inactivated, hence requiring low fluences. Organisms with lower *k* values require more fluence for inactivation.

The dose-response curves for all 20 phage specimens irradiated by UV-C are shown in Figures 6.1 to 6.4, and it is evident that the phages do not display a significant shoulder (i.e., a fluence threshold needed to initiate inactivation; shoulders are mainly found in bacteria and bacterial spores).

All linear regression analysis (used in production of *k* values and fluence required for $\log_{10}$ reduction in titre) was calculated with an intercept of 0 (Havelaar *et al.*, 1990; Hijnen *et al.*, 2006; Hijnen, pers. comm.) allowing the data to be compared with those of other UV inactivation studies.

The presence of a minimal shoulder in B124-17 and B124-57 was unexpected and it is likely that this could be explained by the methodology employed during Phase One experiments, rather than by a real dose-response relationship. This issue will be discussed further in Chapter Eight.

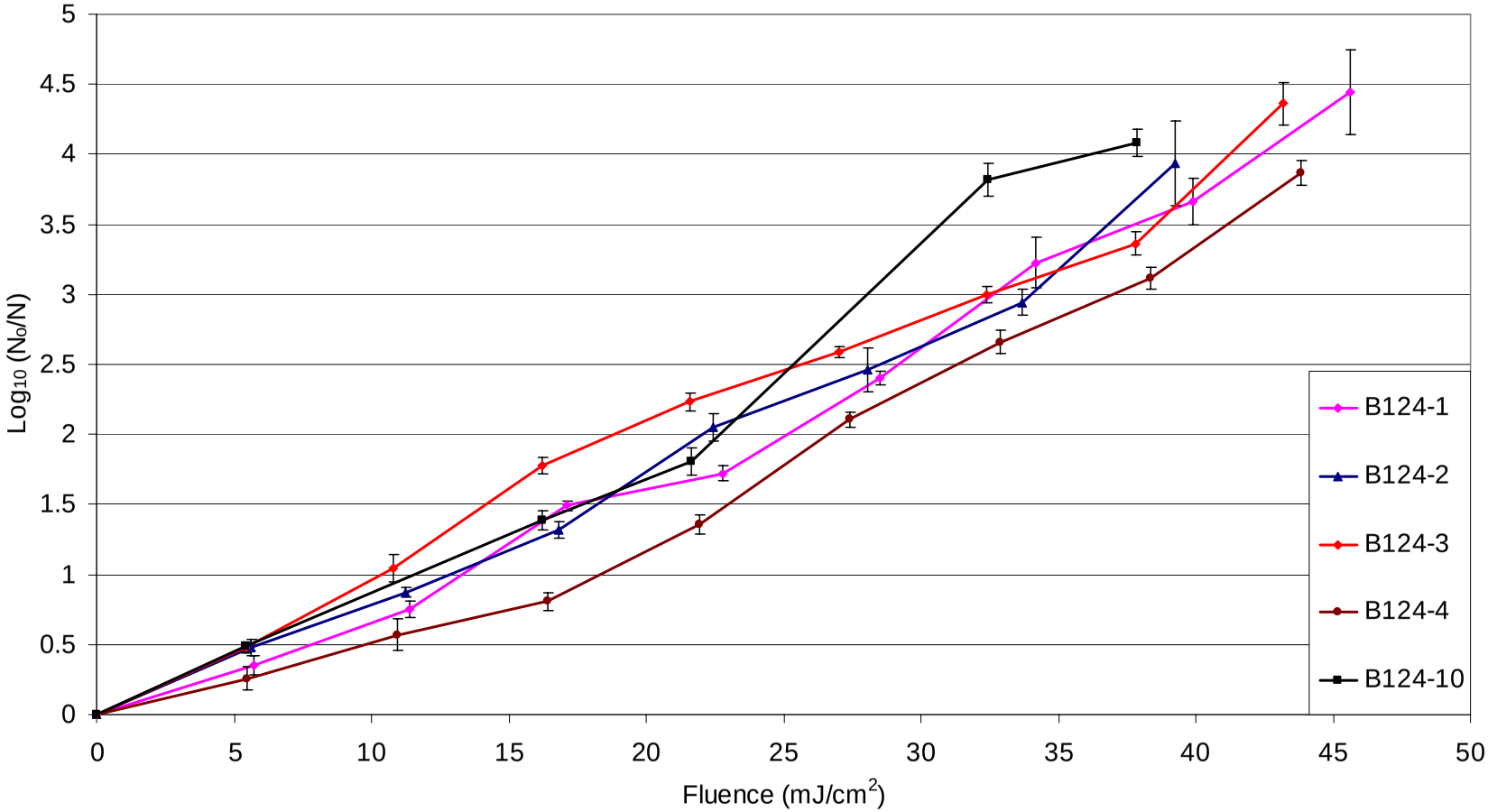


Figure 6.1 Dose-response curves of B124-1, B124-2, B124-3, B124-4 and B124-10 phages during Phase One UV-C inactivation experiments (data points show the mean of 9 values. Error bars represent one standard deviation above and below the mean)

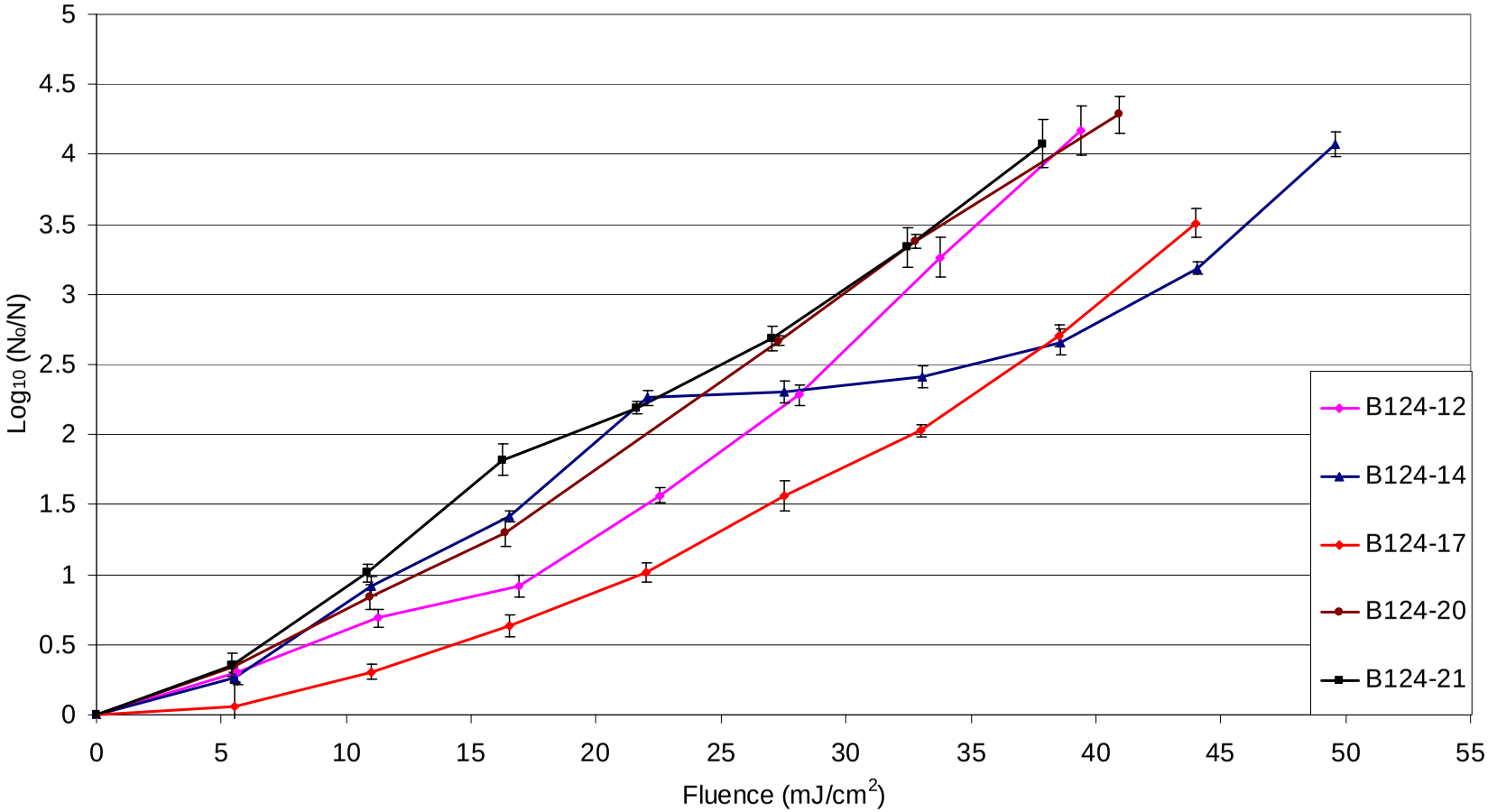


Figure 6.2 Dose-response curves of B124-12, B124-14, B124-17, B124-20 and B124-21 phages during Phase One UV-C inactivation experiments (data points show the mean of 9 values. Error bars represent one standard deviation above and below the mean)

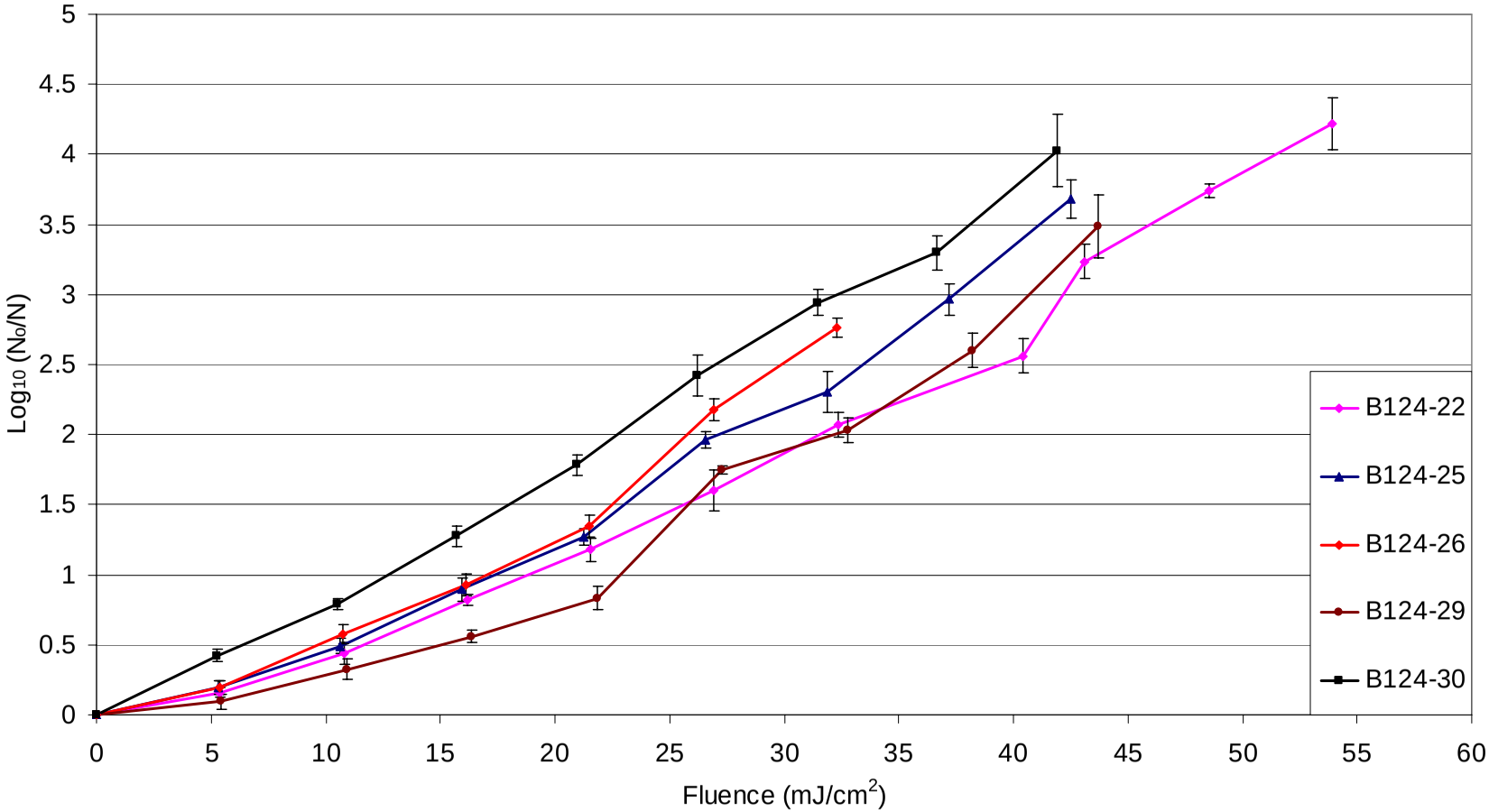


Figure 6.3 Dose-response curves of B124-22, B124-25, B124-26, B124-29 and B124-30 phages during Phase One UV-C inactivation experiments (data points show the mean of 9 values. Error bars represent one standard deviation above and below the mean)

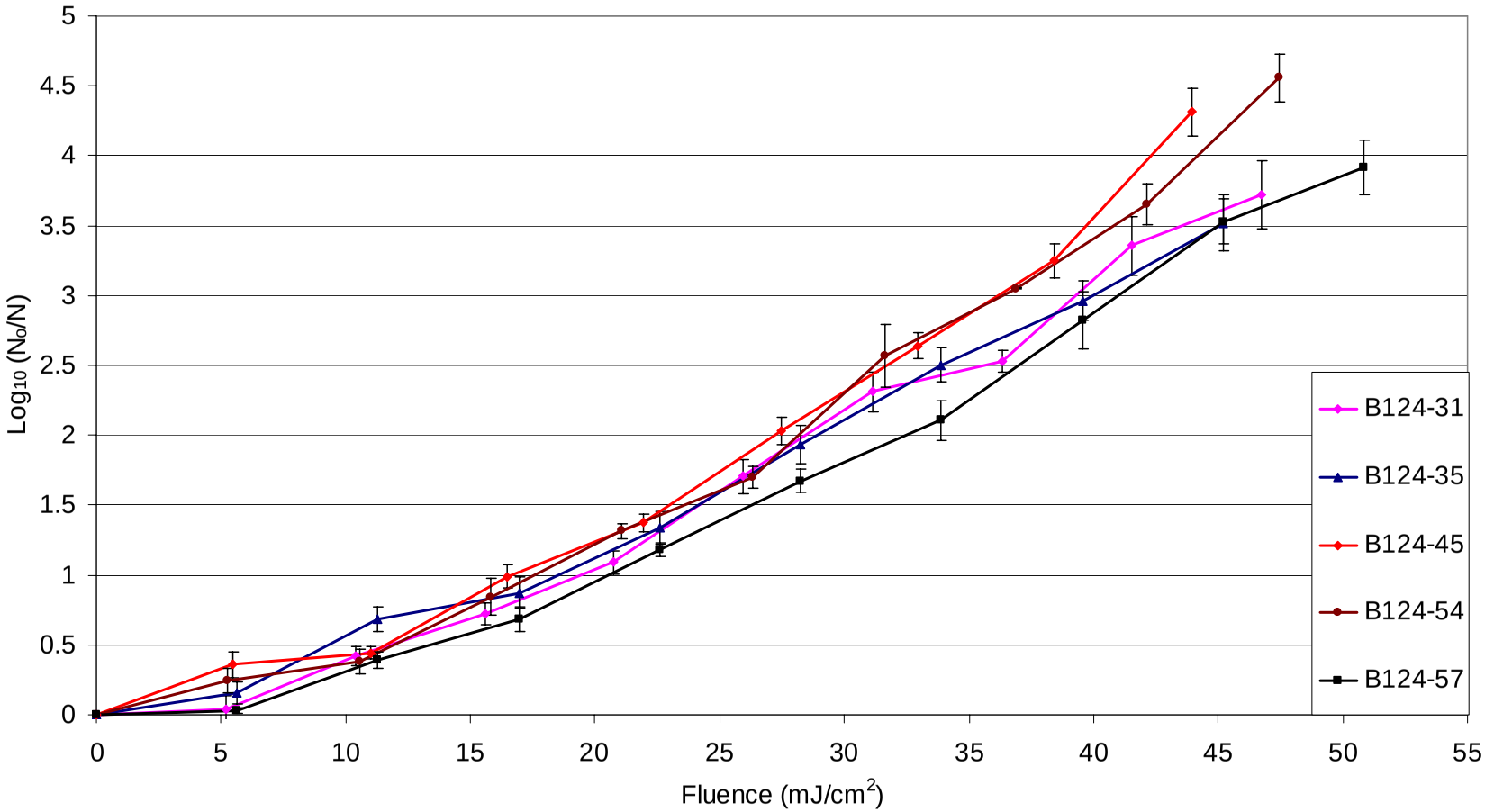


Figure 6.4 Dose-response curves of B124-31, B124-35, B124-45, B124-54 and B124-57 phages during Phase One UV-C inactivation experiments (data points show the mean of 9 values. Error bars represent one standard deviation above and below the mean)

Data are presented as $\log_{10}$ phage inactivation (N_o/N) against fluence (mJ/cm^2). As was shown statistically in Table 6.1, the relationship between fluence and PFU/100µl is linear negative, and when expressed in terms of $\log_{10}$ inactivation and fluence, the inverse is apparent (i.e., as fluence increases, $\log_{10}$ inactivation also increases). The dose-response curves are not entirely linear, with the majority showing a slight curve. Phage B124-14 inactivation appears to reach a plateau between 20 mJ/cm^2 and 32 mJ/cm^2 (Figure 6.2). All phage specimens presented a similar relationship to increasing fluence, and the inactivation rate coefficient can be expressed statistically, derived from linear regression analysis (Table 6.2). *k* values vary from 0.065 mJ/cm^2 (B124-29) to 0.106 mJ/cm^2 (B124-10) with a mean value of 0.084 mJ/cm^2 (SD = 0.041). All phage *k* values lie within ±2 SD from the mean and 4 groups based upon SD from mean *k* values can be delineated (Table 6.3). The inactivation curves are statistically different from each other (t test; $P = 0.018$).

Phages B124-10, -20 and -21 showed higher inactivation rate constants (0.106 mJ/cm^2, 0.100 mJ/cm^2 and 0.104 mJ/cm^2) lying within +2 SD of the mean *k* value. The largest group of phages (50%) is 1 SD below the mean (between 0.072 mJ/cm^2 and 0.083 mJ/cm^2). Phages with the lowest inactivation rate constants are B124-17 and -29 (0.066 mJ/cm^2 and 0.065 mJ/cm^2).

Determination of fluence required for $\log_{10}$ reductions in viral titre is a useful statistic when assessing efficacy of UV disinfection units in water and wastewater treatment plants. This is particularly important where effluents have pathogen removal consents outlined in environmental legislation (e.g., when UV tertiary treatment of effluent is mandatory, prior to discharge to shellfish waters).

Table 6.2 Fluence required using UV-C for each log_{10} reduction of phage specimens during Phase One experiments

Phage specimen ID (B124-)	*k* (mJ/cm^2)	*P*-value	Initial log_{10} PFU/100µl	-1 log_{10} reduction (mJ/cm^2)	-2 log_{10} reduction (mJ/cm^2)	-3 log_{10} reduction (mJ/cm^2)	-4 log_{10} reduction (mJ/cm^2)
1 (72)*	0.091	0.000	4.74	11	22	33	44
2 (63)	0.092	0.000	4.84	11	22	33	44
3 (72)	0.096	0.000	5.06	10	21	31	41
4 (72)	0.082	0.000	5.01	12	25	37	49
10 (54)	0.106	0.003	4.93	9	19	28	38
12 (63)	0.090	0.000	4.47	11	22	33	44
14 (81)	0.080	0.000	6.06	12	25	37	50
17 (81)	0.066	0.000	3.99	15	30	46	61
20 (63)	0.100	0.000	5.24	10	20	30	40
21 (72)	0.104	0.000	4.38	10	19	29	38
22 (90)	0.073	0.000	5.67	14	27	41	55
25 (81)	0.082	0.000	4.64	12	24	36	49
26 (63)	0.076	0.000	5.31	13	26	40	53
29 (81)	0.065	0.000	4.39	15	31	46	61
30 (81)	0.092	0.000	4.93	11	22	33	44
31 (90)	0.073	0.000	4.83	14	27	41	55
35 (81)	0.072	0.000	4.36	14	28	42	55
45 (81)	0.083	0.000	4.61	12	24	36	48
54 (90)	0.083	0.000	4.86	12	24	36	48
57 (90)	0.074	0.000	4.87	14	27	41	54

* number of data points included in regression analysis for each phage specimen (3 exposures for each fluence, each assayed in triplicate)

Table 6.3 Identification of phage groups based on SD of *k* (UV-C) during Phase One experiments

±SD	*k* value (mJ/cm^2)	Phage specimen ID (B124-)
Within +2 SD	0.097 – 0.108	10, 20, 21
Within +1 SD	0.085 – 0.096	1, 2, 3, 12, 30
Mean	0.084	-
Within -1 SD	0.072 – 0.083	4, 14, 22, 25, 26, 31, 35, 45, 54, 57
Within -2 SD	0.060 – 0.071	17, 29

Rearrangement of the standard linear regression equation allows the calculation of fluence values for the required $\log_{10}$ inactivation. The rearranged equation used was:

$$N \log_{10} \text{ inactivation} = N/k$$

where

N = required $\log_{10}$ inactivation

k = slope of line (inactivation rate constant)

Fluences required for 1-$\log_{10}$, 2-$\log_{10}$, 3-$\log_{10}$ and 4-$\log_{10}$ inactivation of phage specimens are also given in Table 6.2. As is often reported by other authors, fluences required to inactivate organisms are presented with no decimal places.

As the inactivation rate coefficient is constant, it is not necessary to discuss comparative 1-$\log_{10}$, 2-$\log_{10}$ etc. values for each phage specimen and, as they are often reported in the literature, only 4-$\log_{10}$ reduction values will be discussed. Fluence required to reach 4-$\log_{10}$ inactivation varied between phage specimens from 38 mJ/cm^2 (B124-10) to 61 mJ/cm^2 (B124-29) with a mean of 49 mJ/cm^2 (SD = 6.96 a range of 23 mJ/cm^2). As expected, when distributed into groups based on SD from the mean, the pattern is broadly inverted to that found for k values (Table 6.4) with only a few phages changing groups (this is a function of the data spread and resulting SD calculation). This is as expected, as higher k equates to a higher rate of inactivation, and therefore lower fluence required for 4-$\log_{10}$ reductions in titre. As 4-$\log_{10}$ reduction values are a function of k, the k values and 4-$\log_{10}$ reduction values are highly correlated ($R^2 = 0.98$)

Table 6.4 Identification of phage groups based on SD of 4-$\log_{10}$ reduction value (UV-C) during Phase One experiments

±SD	4-$\log_{10}$ reduction value (mJ/cm^2)	Phage specimen ID (B124-)
Within +2 SD	55.55 – 62.52	17, 29
Within +1 SD	48.60 – 55.54	4, 14, 22, 25, 26, 31, 35, 57
Mean	48.59	-
Within -1 SD	41.63 - 48.58	1, 2, 10, 12, 30, 45, 54
Within -2 SD	34.67 – 41.62	3, 20, 21

Neither the inactivation rate constant or the fluence required to reach 4-$\log_{10}$ inactivation were correlated with the initial PFU/100µl of the phage specimen (R^2 = 0.011% and R^2 = 0.028 % respectively), indicating that initial titre of phage suspension being irradiated has little effect on either the rate of inactivation, or the fluence required to reach 4-$\log_{10}$ inactivation. An indirect way in which phage titre may influence fluence required to achieve a 4-$\log_{10}$ reduction is because of UV absorbance of phage suspension (i.e., higher titre phage suspensions are likely to have higher absorbance). In Phase Two experiments this will be corrected for using the method given in Bolton and Linden (2003) and the UV-Calc spreadsheet.

6.2 Phase One UV-B inactivation kinetics of B124 phages

6.2.1 Correlation between fluence and phage inactivation during Phase One experiments

As was shown with UV-C radiation, all 20 phage specimens demonstrated a significant (P = 0.000) negative linear relationship between $\log_{10}$ PFU/100 µl and fluence, in this instance ranging from -0.95 % (B124-10) to -1.00 % (B124-2, B124-20 and B124-31), with a mean value of -0.98 % and 0.012 SD (Table 6.5).

Table 6.5 Pearson product-moment correlation values for $\log_{10}$ PFU/100µl and UV-B fluence during Phase One experiments

Phage specimen ID (B124-)	Pearson product-moment correlation	*P*-value
1	-0.96	0.000
2	-1.00	0.000
3	-0.99	0.000
4	-0.99	0.000
10	-0.95	0.000
12	-0.99	0.000
14	-0.99	0.000
17	-0.99	0.000
20	-1.00	0.000
21	-0.98	0.000
22	-0.98	0.000
25	-0.98	0.000
26	-0.98	0.000
29	-0.98	0.000
30	-0.99	0.000
31	-1.00	0.000
35	-0.98	0.000
45	-0.99	0.000
54	-0.98	0.000
57	-0.98	0.000

The variance in correlation values is extremely small, though slightly larger than that recorded for UV-C, with all phages showing a statistically significant relationship.

The null hypothesis of the relationship between fluence and $\log_{10}$ PFU/100 µl occurring because of chance can therefore be rejected.

6.2.2 UV-B inactivation rate coefficient and fluence required for 1-, 2-, 3- and 4-$\log_{10}$ inactivation of B124 phages during Phase One experiments

The UV-B dose-response curves for all 20 phage specimens are shown in Figures 6.5 to 6.8. Phage inactivation displays a positive relationship with fluence, but a number of anomalies are presented in the dose-response curves. B124-1 appears to display a sudden increase in the rate of inactivation between 115 mJ/cm^2 to 170 mJ/cm^2, which then appears to plateau until 260 mJ/cm^2 before rising exponentially again (Figure 6.5). A plateau is also displayed in B124-35 between 120 mJ/cm^2 and 190 mJ/cm^2 (Figure 6.8) and B124-54 between 50 and 100 mJ/cm^2 . Phage B124-22 appears to display a decrease in $\log_{10}$ inactivation between 50 mJ/cm^2 and 100 mJ/cm^2 (Figure 6.7). The reason for these anomalies is unclear, though it's likely to be related to methodology used during Phase One experiments. This issue will be discussed further in Chapter Eight.

UV-B inactivation rate constants are generally one order of magnitude lower than those given for UV-C radiation and are significantly related (paired t-test; $P = 0.000$; Table 6.6), varying from 0.0056 mJ/cm^2 (B124-31) to 0.0107 mJ/cm^2 (B124-1), with a mean value of 0.0076 mJ/cm^2 (SD = 0.0014). All phage *k* values lie within ±3 SD from the mean and 5 groups based upon SD from mean *k* values can be delineated (Table 6.7). As with UV-C data, the largest group of phages (50%) is located 1 SD below the mean. The inactivation curves are statistically different from each other (t test; $P = 0.000$).

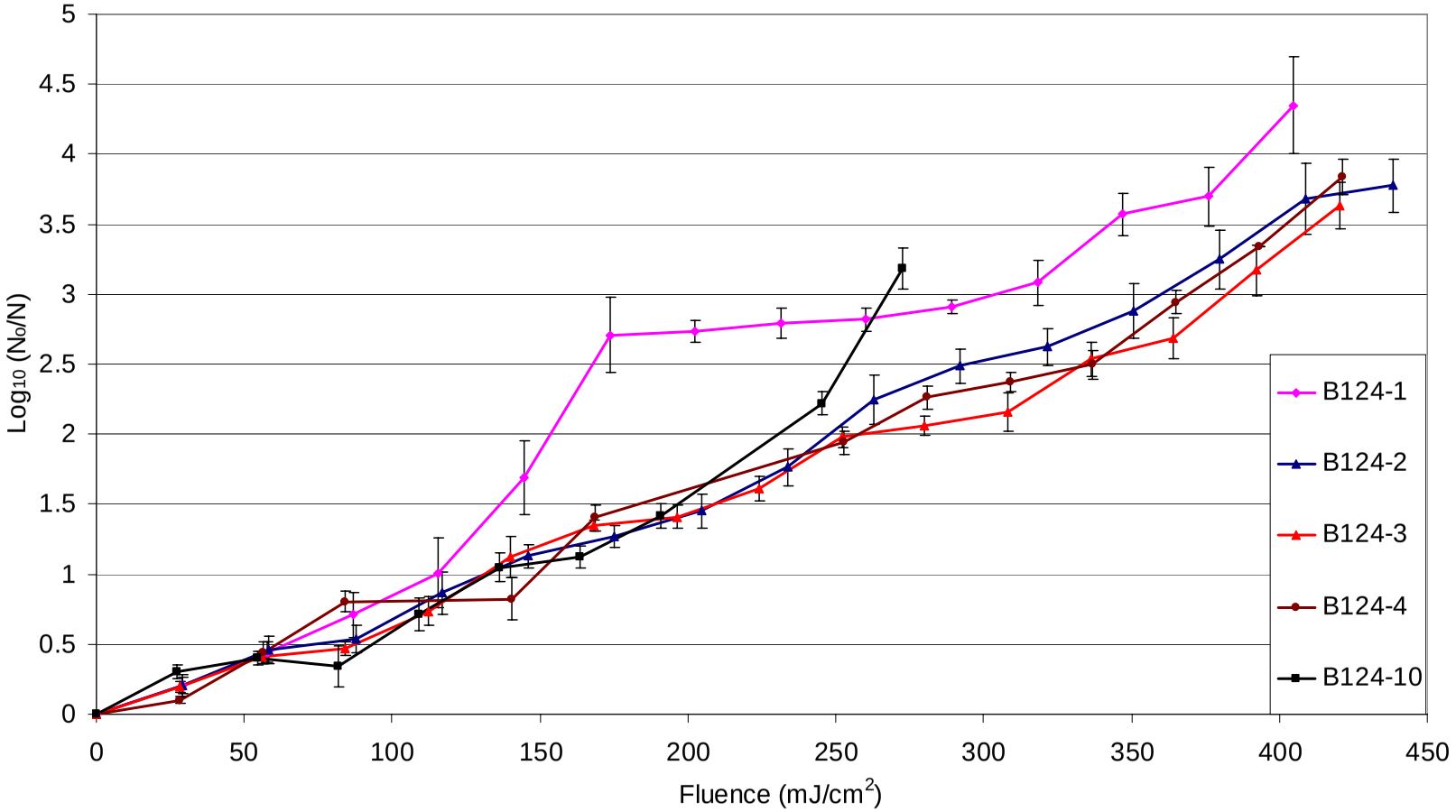


Figure 6.5 Dose-response curves of B124-1, B124-2, B124-3, B124-4 and B124-10 phages during Phase One UV-B inactivation experiments (data points show the mean of 9 values. Error bars represent one standard deviation above and below the mean)

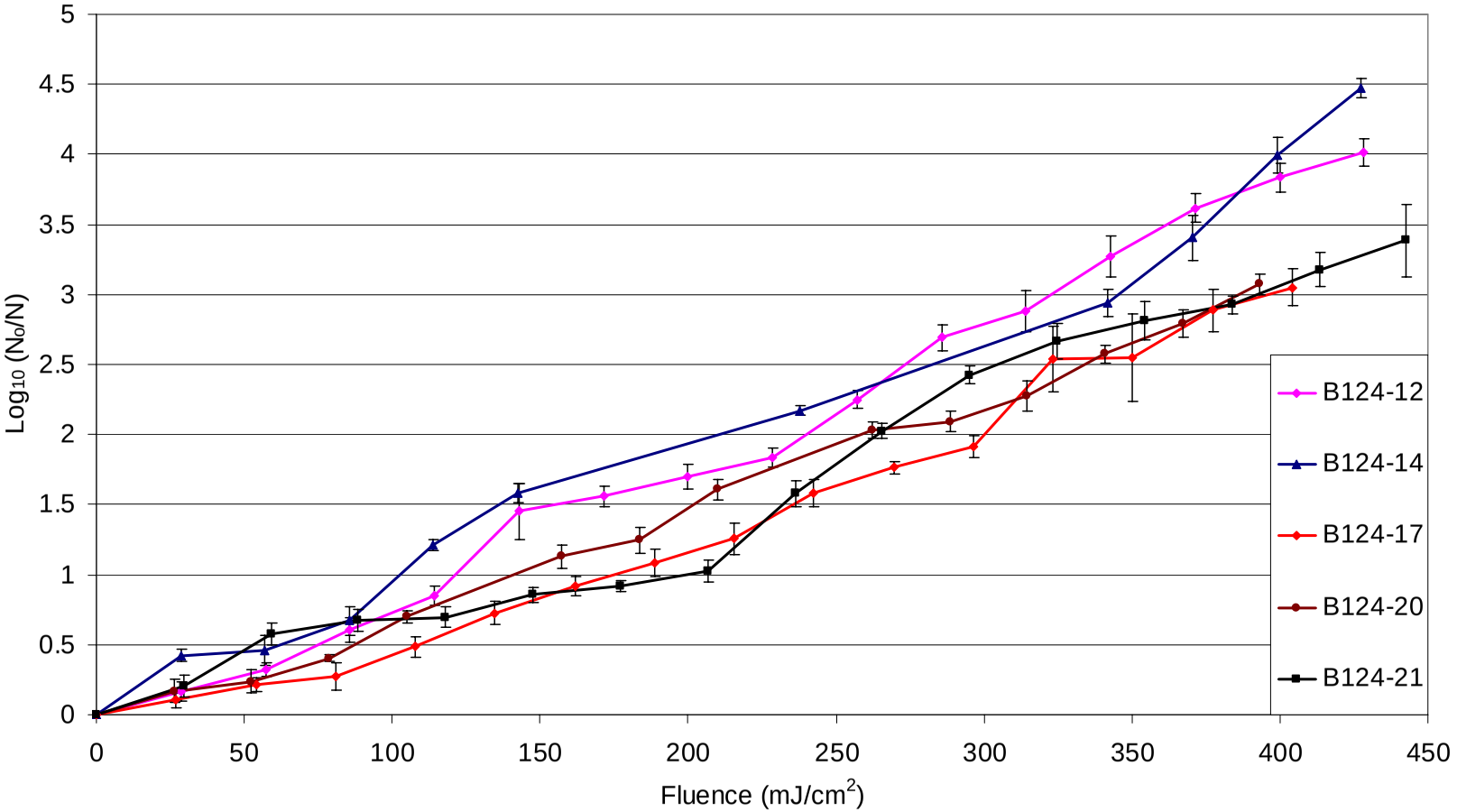


Figure 6.6 Dose-response curves of B124-12, B124-14, B124-17, B124-20 and B124-21 phages during Phase One UV-B inactivation experiments (data points show the mean of 9 values. Error bars represent one standard deviation above and below the mean)

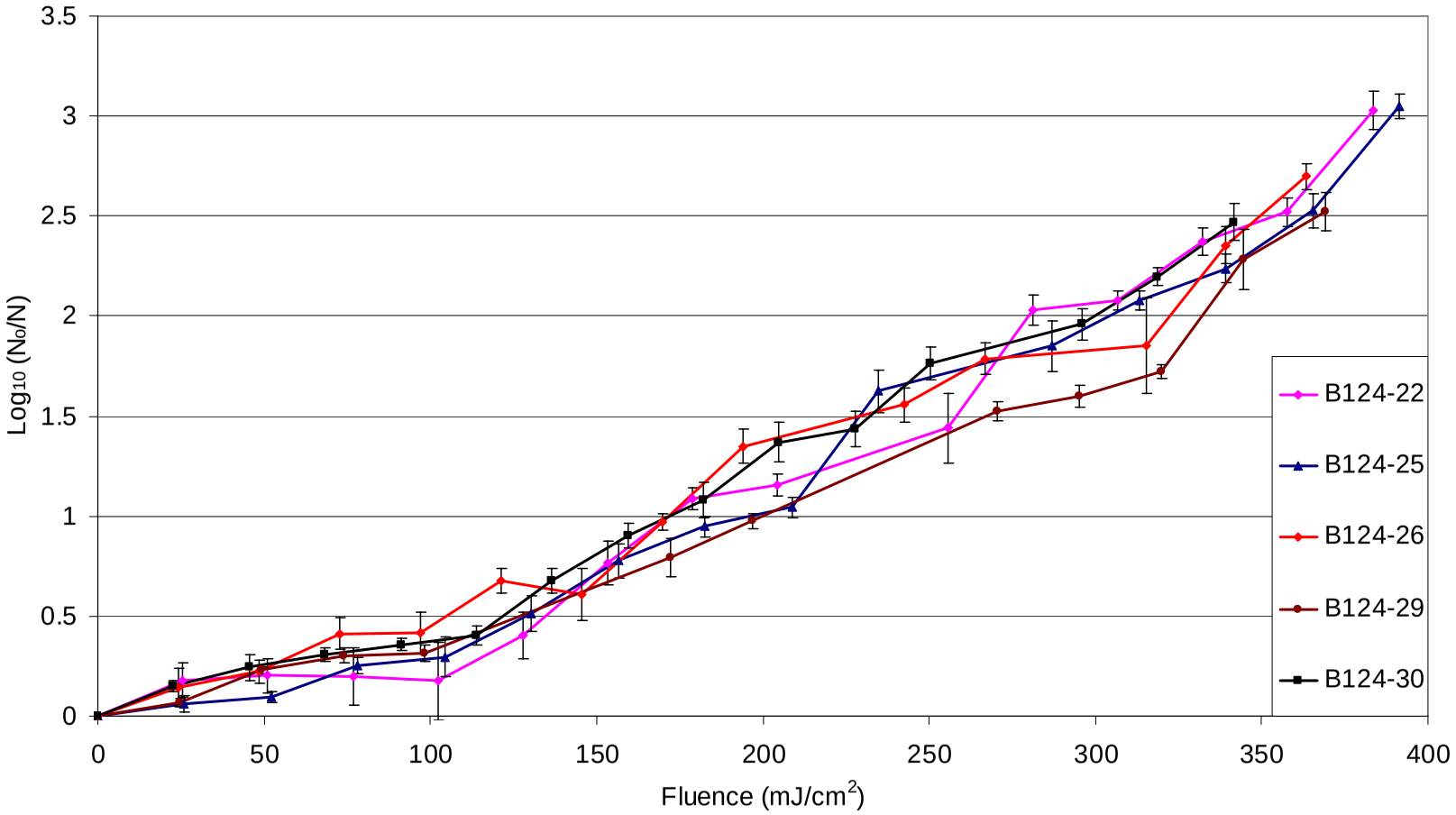


Figure 6.7 Dose-response curves of B124-22, B124-25, B124-26, B124-29 and B124-30 phages during Phase One UV-B inactivation experiments (data points show the mean of 9 values. Error bars represent one standard deviation above and below the mean)

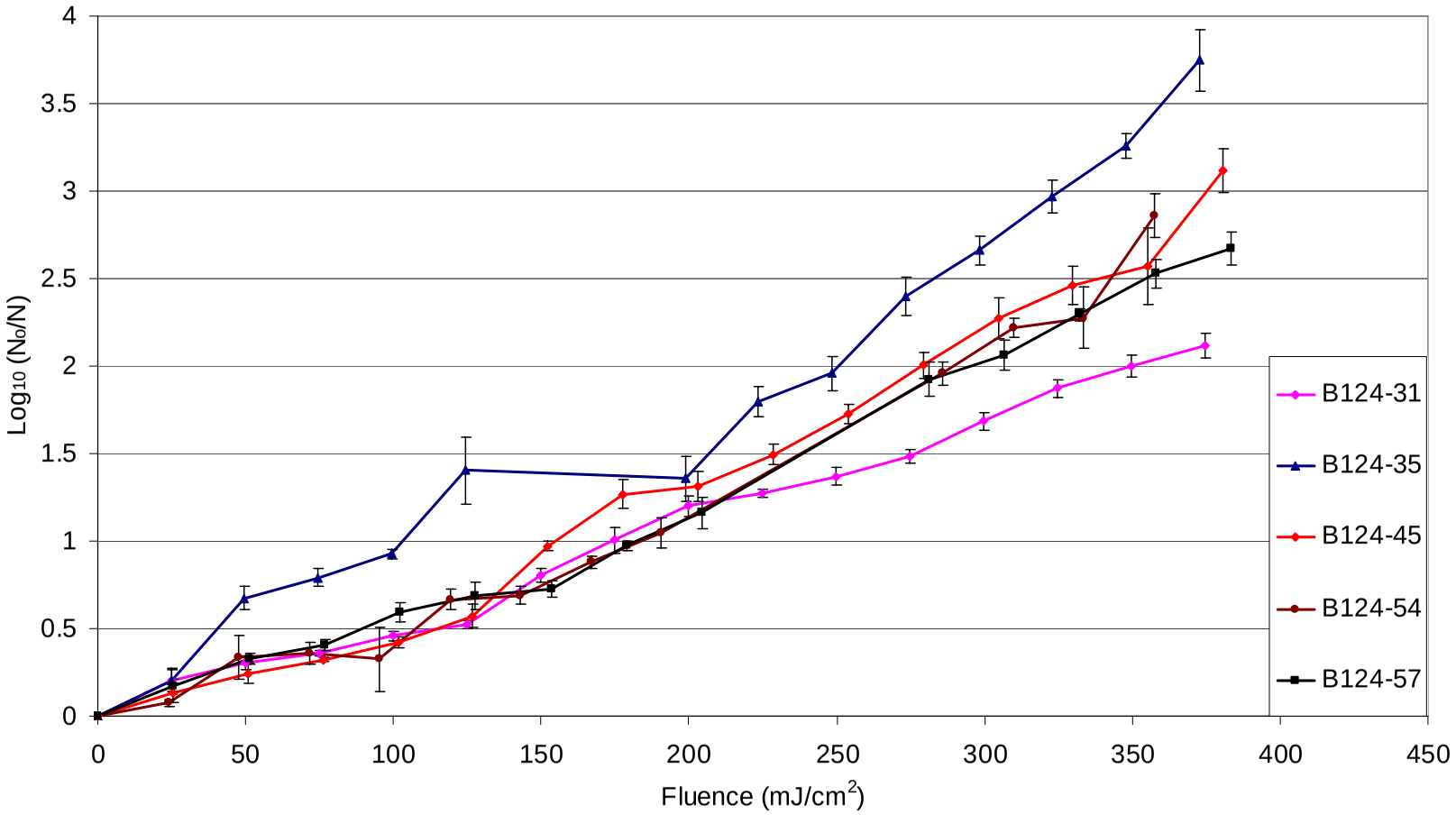


Figure 6.8 Dose-response curves of B124-31, B124-35, B124-45, B124-54 and B124-57 phages during Phase One UV-B inactivation experiments (data points show the mean of 9 values. Error bars represent one standard deviation above and below the mean)

Table 6.6 Fluence required using UV-B for each log_{10} reduction of phage specimens during Phase One experiments

Phage specimen ID (B124-)	*k* (mJ/cm^2)	*P*-value	Initial log_{10} PFU/100µl	-1 log_{10} reduction (mJ/cm^2)	-2 log_{10} reduction (mJ/cm^2)	-3 log_{10} reduction (mJ/cm^2)	-4 log_{10} reduction (mJ/cm^2)
1 (117)*	0.0107	0.000	4.65	93	187	280	374
2 (126)	0.0084	0.000	4.68	120	240	359	479
3 (135)	0.0077	0.000	5.13	130	259	389	518
4 (108)	0.0082	0.000	4.95	123	245	368	490
10 (81)	0.0090	0.000	4.82	111	222	333	444
12 (135)	0.0093	0.000	4.31	108	216	324	432
14 (90)	0.0097	0.000	6.05	103	206	309	412
17 (135)	0.0070	0.000	4.60	143	287	430	574
20 (117)	0.0075	0.000	5.34	134	267	401	535
21 (135)	0.0075	0.000	4.29	133	267	400	533
22 (126)	0.0067	0.000	4.79	149	298	447	596
25 (126)	0.0065	0.000	4.72	153	306	459	612
26 (117)	0.0065	0.000	4.45	153	306	459	612
29 (99)	0.0059	0.000	4.77	170	341	511	681
30 (126)	0.0066	0.000	4.39	153	305	458	611
31 (135)	0.0056	0.000	5.32	178	357	535	713
35 (117)	0.0091	0.000	4.75	110	221	331	442
45 (135)	0.0072	0.000	4.48	139	279	418	557
54 (108)	0.0068	0.000	5.03	147	293	440	587
57 (117)	0.0066	0.000	4.90	151	302	452	603

* number of data points included in regression analysis for each phage specimen (3 exposures for each fluence, each assayed in triplicate)

Table 6.7 Identification of phage groups based on SD of *k* (UV-B) during Phase One experiments

±SD	*k* value (mJ/cm^2)	Phage specimen ID (B124-)
Within +3 SD	0.0105 – 0.0117	1
Within +2 SD	0.0091 – 0.0104	12, 14, 35
Within +1 SD	0.0077 – 0.0090	2, 3, 4, 10
Mean	0.0076	-
Within -1 SD	0.0063 - 0.0075	17, 20, 21, 22, 25, 26, 30, 45, 54, 57
Within -2 SD	0.0049 – 0.0062	29, 31

Based upon Phase One UV-B *k* values, B124-1 is the only phage located in the group +3 SD above the mean. 4-log_{10} inactivation fluences ranged from 374 mJ/cm^2 (B124-1) to 713 mJ/cm^2 (B124-31) with a mean value of 540 mJ/cm^2 (range = 339 mJcm^2; SD = 91.05; Table 6.6). Fluence required to achieve 4-log_{10} inactivation of all 20

phages were within ±2SD of the mean, with the largest group being within +1 SD (45% of phage; Table 6.8).

Table 6.8 Identification of phage groups based on SD of 4-$\log_{10}$ reduction value (UV-B) during Phase One experiments

±SD	**4-$\log_{10}$ reduction value (mJ/cm^2)**	**Phage specimen ID (B124-)**
Within +2 SD	631.22 - 722.26	29, 31
Within +1 SD	540.16 - 631.21	17, 22, 25, 26, 30, 45, 54, 57
Mean	540.15	-
Within -1 SD	449.10 – 540.14	1, 2, 3, 4, 20, 21
Within -2 SD	358.05 – 449.09	10, 12, 14, 35

6.3 Relationship between UV-B and UV-C inactivation rates of B124 phages during Phase One experiments

Fluences required for a 4-$\log_{10}$ inactivation during U V-B and UV-C exposures were only slightly correlated (R^2 = 0.48; Figure 6.9), as were *k* values (R^2 = 0.41), suggesting that behaviour during UV-B and UV-C radiation (based solely on Phase One results) is reasonably consistent for phage. However, this lower than expected correlation may be a consequence of the methodology used during the Phase One screening experiments. This issue will be discussed further in Chapter Eight.

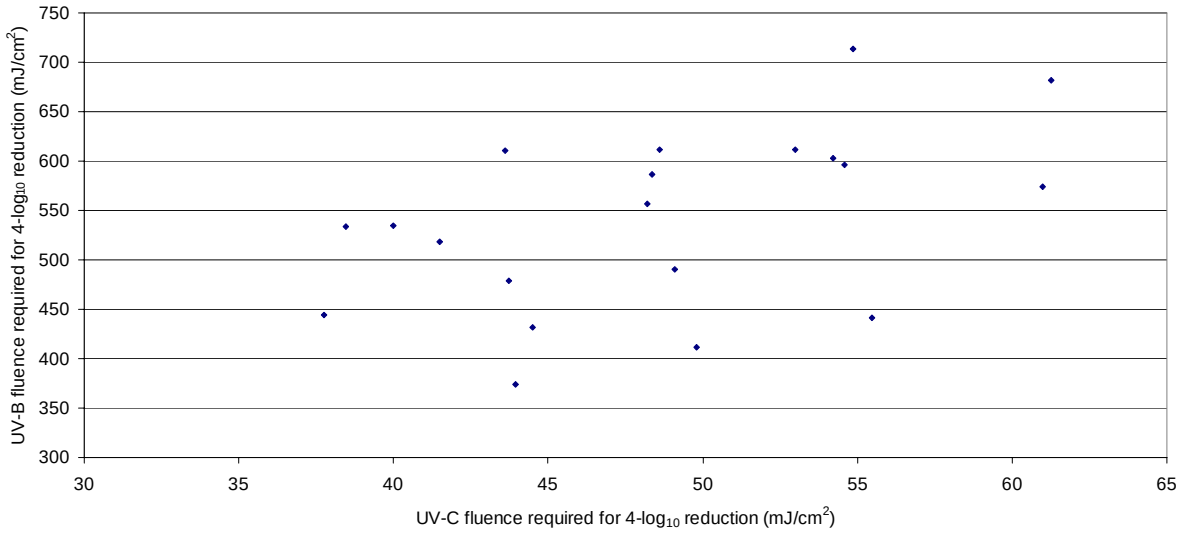


Figure 6.9 Relationship between 4-$\log_{10}$ reduction B-124 phage fluence requirements for UV-B and UV-C radiation during Phase One experiments

Phase One experiments revealed some interesting results that were consequently used to inform the design of Phase Two experiments. These results gave general indications of the behaviour of B124 phages during UV-B and UV-C radiation, and Phase Two experiments presented in the next chapter will attempt to quantify inactivation relationships using a more detailed and in-depth methodology.

CHAPTER SEVEN

7. RESULTS OF PHASE TWO UV INACTIVATION EXPERIMENTS

The results of the Phase One screening experiments presented in Chapters Five and Six reporting the morphology and host range of different phage, identified a sub-set of seven phages with distinct characteristics, suitable for further investigation (Phase Two). These were:

- B124-1 (selected because of its low *k* value and non-characteristic dose-response curve during UV-B irradiation);
- B124-10 (because of its contrasting response to different UV wavelengths i.e., highest group of *k* values during Phase One UV-C experiments and just above mean *k* value during Phase One UV-B experiments);
- B124-12 (because it demonstrated the second highest *k* group in both UV-B and UV-C Phase One experiments);
- B124-21 (because it demonstrated one of the highest *k* values found during Phase One UV-C experiments but medium *k* during UV-B experiments, and it was the only phage with a distinct wavy tail);
- B124-29 (because of its very low *k* value during both UV-B and UV-C Phase One experiments);
- B124-35 (because it was the only phage unable to infect *Bacteroides* strain RYC-2056); and
- B124-54 (because it was one of the most consistent 'typical' phage with near mean *k* values for both UV-B and UV-C during Phase One experiments and had

an inactivation plateau between 50 mJ/cm^2 and 100 mJ/cm^2 during UV-B irradiation).

A total of eighteen plates (two dilutions) were used for each fluence exposure (three replicates), of which there were six (giving a total of 108 plates for each phage inactivation curve) and each data point in the dose-response curves is a mean value from at least nine plates. A total of 1512 plates were analysed during Phase Two experiments. During these experiments, plaque size was recorded, and it was found that fluence had no discernable impact on plaque dimensions or appearance (for both UV-B and UV-C wavelengths), with plaque morphology being consistent with that when isolated (Table 5.1).

7.1 Phase Two UV-C inactivation kinetics of B124 phages

7.1.1 Correlation between fluence and phage inactivation during Phase Two experiments

All seven phage specimens showed a significant (P = 0.000) negative linear relationship between $\log_{10}$ PFU/100 µl and fluence, displaying minimal range: from -0.98 % (B124-10, -12, -29 and -35) to -0.99 % (B124-1, -21 and -54), with a mean value of -0.98 % and 0.006 SD (Table 7.1). Therefore, the null hypothesis that the relationship between fluence and $\log_{10}$ PFU/100 µl resulted from chance alone can be rejected.

Table 7.1 Pearson product-moment correlation values for $\log_{10}$ PFU/100µl and UV-C during Phase Two experiments

Phage specimen ID (B124-)	Pearson product-moment correlation	*P*-value
1	-0.99	0.000
10	-0.98	0.000
12	-0.98	0.000
21	-0.99	0.000
29	-0.98	0.000
35	-0.98	0.000
54	-0.99	0.000

7.1.2 UV-C inactivation rate coefficient and fluence required for 1-, 2-, 3- and 4-$\log_{10}$ inactivation of B124 phages during Phase Two experiments

The dose-response curves for UV-C irradiation of all seven phage specimens are shown in Figure 7.1. As in Chapter Six, data are presented as $\log_{10}$ phage inactivation (N_o/N) against fluence (mJ/cm^2).

The relationship between $\log_{10}$ inactivation and fluence is linear positive (i.e., as fluence increases, $\log_{10}$ inactivation increases accordingly) and no shoulder or tail were present in the data, indicating that all phages followed first order kinetics (i.e., the rate of inactivation was proportional to the fluence delivered). All phage specimens displayed a similar relationship to increasing fluence, and the slope of the inactivation curves derived from linear regression analysis is shown in Table 7.2. *k* values varied from 0.099 mJ/cm^2 (B124-54) to 0.139 mJ/cm^2 (B124-10) with a mean value of 0.114 mJ/cm^2 (SD = 0.014, range = 0.04 mJ/cm^2). All phage *k* values lay within ±2 SD from the mean, and four groups were delineated (Table 7.3).

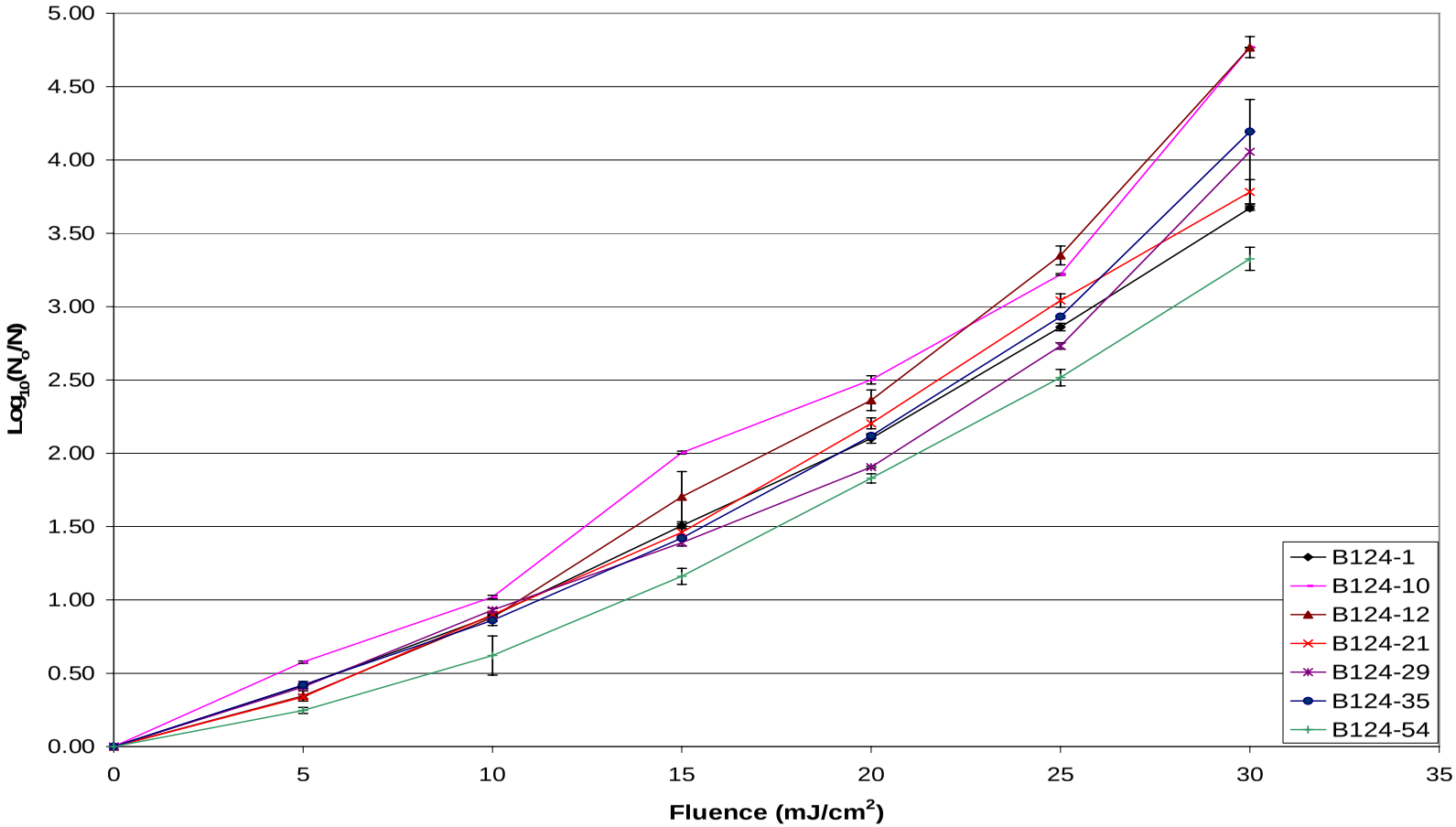


Figure 7.1 Dose-response curves of phages B124-1, B124-10, B124-12, B124-21, B124-29 B124- 35 and B124-54 during Phase Two UV-C inactivation experiments (data points show the mean of 9 values. Error bars represent one standard deviation above and below the mean)

Phage B124-10 had the largest inactivation rate constant, laying within +2 SD of the mean *k* value, and B124-54 the lowest *k* value, located -2 SD below the mean. The largest group of phages (42%) was located one SD below the mean with 28% lying one SD above the mean. Although groups based on SD can be identified, statistical analysis shows that the inactivation curves are not significantly different (t test; $P = 0.874$).

Table 7.2 Fluence required for each $\log_{10}$ reduction of phage specimens during Phase Two UV-C irradiation

Phage specimen ID (B124-)	*k* (mJ/cm^2)	*P*-value	Initial $\log_{10}$ PFU/100µl	-1 $\log_{10}$ reduction (mJ/cm^2)	-2 $\log_{10}$ reduction (mJ/cm^2)	-3 $\log_{10}$ reduction (mJ/cm^2)	-4 $\log_{10}$ reduction (mJ/cm^2)
1 (54)	0.113	0.000	5.00	9	18	27	35
10 (54)	0.139	0.000	4.84	7	14	22	29
12 (54)	0.121	0.000	4.77	8	17	25	33
21 (54)	0.117	0.000	4.96	9	17	26	34
29 (54)	0.101	0.000	4.24	10	20	30	40
35 (54)	0.107	0.001	4.19	9	19	28	37
54 (54)	0.099	0.000	4.10	10	20	30	41

* number of data points included in regression analysis for each phage specimen (3 exposures for each fluence, each assayed in triplicate)

Fluences required to achieve a 4-$\log_{10}$ phage inactivation ranged from 29 mJ/cm^2 (B124-10) to 41 mJ/cm^2 (B124-54). The mean was 36 mJ/cm^2, the range was 12 mJ/cm^2 and the SD was 4.07 mJ/cm^2.

Table 7.3 Identification of phage groups based on SD of *k* (UV-C) during Phase Two experiments

±SD	*k* value (mJ/cm^2)	Phage specimen ID (B124-)
Within +2 SD	0.129 – 0.141	10
Within +1 SD	0.114 – 0.128	12, 21
Mean	0.114	-
Within -1 SD	0.100 – 0.113	1, 29, 35
Within -2 SD	0.086 – 0.099	54

Log_{10} reduction values were highly correlated with phage inactivation coefficients (R^2 = -0.99%), with the phage specimens delineated into groups based on SD from the mean (Table 7.4). The groups were almost exactly the inverse of groups shown in Table 7.3: the exception being B124-1. As *k* and 4-log_{10} fluence values are a function of the same regression equation, this was expected.

Table 7.4 Identification of phage groups based on SD of 4-log_{10} reduction value (UV-C) during Phase Two experiments

±SD	**Fluence for 4-log_{10} inactivation (mJ/cm^2)**	**Phage specimen ID (B124-)**
Within +2 SD	39.65 - 43.71	54
Within +1 SD	35-58 - 39.64	29, 35
Mean	35.57	
Within -1 SD	31.50 – 35.56	1, 12, 21
Within -2 SD	27.44 – 31.49	10

An interesting feature of the dose-response curves shown in Figure 7.1, was the clustering of points during low fluences, and the data showing greater spread at higher fluences. This is explained by the relatively small differences in the inactivation rate coefficient outlined above.

7.2 Phase Two UV-B inactivation kinetics of B124 phages

7.2.1 Correlation between fluence and phage inactivation during Phase Two experiments

As with all previous experiments conducted for both UV-B and UV-C wavelengths during Phase One and Phase Two, a negative linear relationship between $\log_{10}$ PFU/100 µl and fluence was evident for UV-B irradiation during Phase Two experiments, displaying minimal range: from -0.98 % (B124-1, -10 and -54) to -0.99 % (B124-12, -21, -29 and -35), with a mean value of -0.99 % and 0.007 SD (Table 7.5). Therefore, the null hypothesis that the relationship between fluence and $\log_{10}$ PFU/100 µl occurred as a result of change could be rejected.

Table 7.5 Pearson product-moment correlation values for $\log_{10}$ PFU/100µl and UV-B during Phase Two experiments

Phage specimen ID (B124-)	Pearson product-moment correlation	*P*-value
1	-0.98	0.000
10	-0.98	0.000
12	-0.99	0.000
21	-0.99	0.000
29	-0.99	0.000
35	-0.99	0.000
54	-0.98	0.000

7.2.3 UV-B inactivation rate coefficient and fluence required for 1-, 2-, 3- and 4-$\log_{10}$ inactivation of B124 phages during Phase Two experiments

The dose-response curves for UV-B irradiation of all seven phage specimens are shown in Figure 7.2 and in Table 7.6.

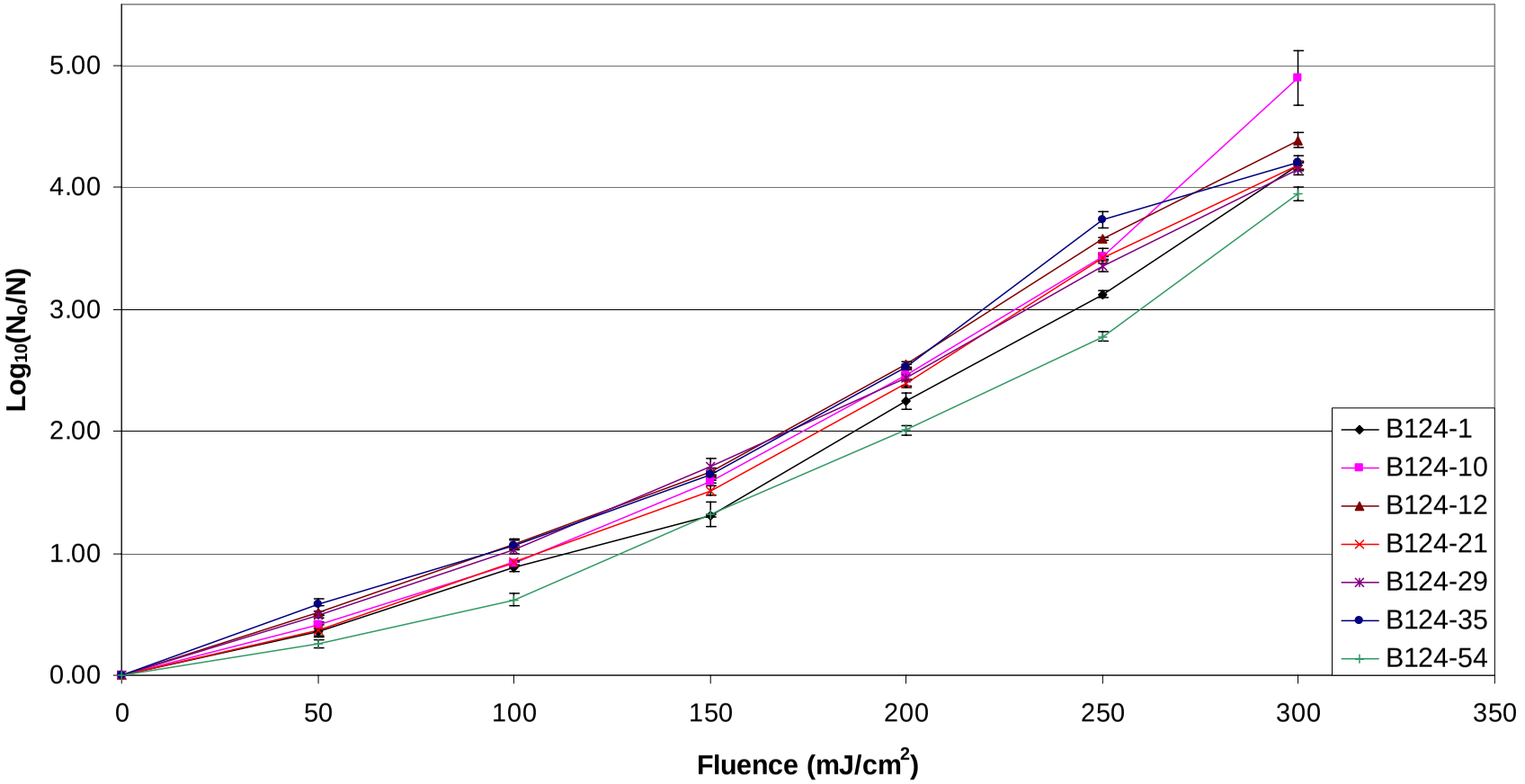


Figure 7.2 Dose-response curves of phages B124-1, B124-10, B124-12, B124-21, B124-29 B124- 35 and B124-54 during Phase Two UV-B inactivation experiments. Data points show the mean of 9 values. Error bars represent one standard deviation above and below the mean.

Table 7.6 Fluence required for each log_{10} reduction of phage specimens during Phase Two UV-B irradiation

Phage specimen ID (B124-)	*k* (mJ/cm^2)	*P*-value	Initial log_{10} PFU/100µl	-1 log_{10} reduction (mJ/cm^2)	-2 log_{10} reduction (mJ/cm^2)	-3 log_{10} reduction (mJ/cm^2)	-4 log_{10} reduction (mJ/cm^2)
1 (54) *	0.0123	0.000	5.00	81	163	244	325
10 (54)	0.0139	0.000	5.28	72	144	216	288
12 (54)	0.0136	0.000	5.14	74	147	221	294
21 (54)	0.0129	0.000	4.99	78	155	233	310
29 (54)	0.0124	0.000	4.14	81	161	242	323
35 (54)	0.0133	0.000	4.21	75	150	226	301
54 (54)	0.0100	0.000	4.07	101	201	302	402

* number of data points included in regression analysis for each phage specimen (3 exposures for each fluence, each assayed in triplicate)

The data showed no shouldering or tailing and the relationship between log_{10} inactivation and fluence was linear positive (i.e., as fluence increased, log_{10} inactivation increased) and no deviation from first order kinetics was observed. All phage specimens displayed a similar relationship to increasing fluence. The slope of the inactivation curves, derived from linear regression analysis, are shown in Table 7.6. *k* values varied from 0.01 mJ/cm^2 (B124-54) to 0.0139 mJ/cm^2 (B124-10) with a mean value of 0.0126 mJ/cm^2 (range = 0.013, SD = 0.004). All phage *k* values lay within ±2 SD from the mean and 3 groups based upon SD from mean *k* values could be delineated (Table 7.7). The majority group (57%) was within + 1 SD (B124-10, -12, 21 and -35). The inactivation curves are not statistically different from each other (t test; $P = 0.529$).

Table 7.7 Identification of phage groups based on SD of *k* (UV-C) during Phase Two experiments

±SD	*k* value (mJ/cm^2)	Phage specimen ID (B124-)
Within +1 SD	0.0127 - 0.0139	10, 12, 21, 35,
Mean	0.0126	-
Within -1 SD	0.0113 – 0.0125	1, 29,
Within -2 SD	0.0100 – 0.0112	54

Fluences required to inactivate phages by 4-$\log_{10}$ ranged from 288 mJ/cm^2 (B124-10) to 402 mJ/cm^2 (B124-54). The mean was 320 mJ/cm^2, the range was 114 mJ/cm^2 and the SD was 38.59 mJ/cm^2. Log_{10} reduction values were highly correlated with phage inactivation coefficients (R^2 = -0.99%), and phage specimens were delineated into groups based upon SD from the mean (Table 7.8). The groups were almost exactly the inverse of the groups shown in Table 7.7 (with the exception of B124-54) which, because of its lowest *k* value, is located +3 SD of the mean. No 4-$\log_{10}$ fluence requirement was located below 1 SD of the mean.

Table 7.8 Identification of phage groups based on SD of 4-$\log_{10}$ reduction value (UV-B) during Phase Two experiments

±SD	**Fluence for 4-$\log_{10}$ inactivation (mJ/cm^2)**	**Phage specimen ID (B124-)**
Within +3 SD	397.55 – 436.13	54
Within +2 SD	358.96 - 397.54	-
Within +1 SD	320.37 - 358.95	1, 29,
Mean	320.36	-
Within -1 SD	281.77 – 320.35	10, 12, 21, 35,

7.3 Relationship between UV-B and UV-C inactivation kinetics of B124 phages during Phase Two experiments

The inactivation kinetics of each phage during UV-B and UV-C irradiation were similar and statistically significant. Correlation of inactivation rate coefficients required during UV-B and UV-C radiation show a positive linear relationship (R^2 = 0.73 %, P = 0.063). The relationship between k for UV-C and UV-B was approximately 1:0.11 (i.e., phage inactivation occurs 1-$\log_{10}$ slower during UV-B than UV-C; Table 7.9). This relationship was relatively stable between phage specimens, varying from 1:0.10 (B124-10 and -54) to 1:0.12 (B124-29 and -35).

Table 7.9 Relationship between k for phage specimens during UV-B and UV-C irradiation during Phase Two experiments

Phage specimen ID (B124-)	UV-C k values (mJ/cm^2)	UV-B k values (mJ/cm^2)	Ratio (UV-C:UV-B)
1	0.113	0.0123	0.11
10	0.139	0.0139	0.10
12	0.121	0.0136	0.11
21	0.117	0.0129	0.11
29	0.101	0.0124	0.12
35	0.107	0.0133	0.12
54	0.0985	0.0100	0.10

Fluences required for 4-$\log_{10}$ inactivation of each phage during UV-B and UV-C radiation were also positively correlated (R^2 = 0.73 %, P = 0.062; Table 7.10 and Figure 7.3) with an average ratio of 1:9.03 (UV-C requirement:UV-B requirement). The ratio varied from 1:8 (B124-35) to 1:10 (B124-10), indicating that nearly ten times the amount of UV-B radiation than UV-C radiation was required for a 4-$\log_{10}$ reduction in phage titre.

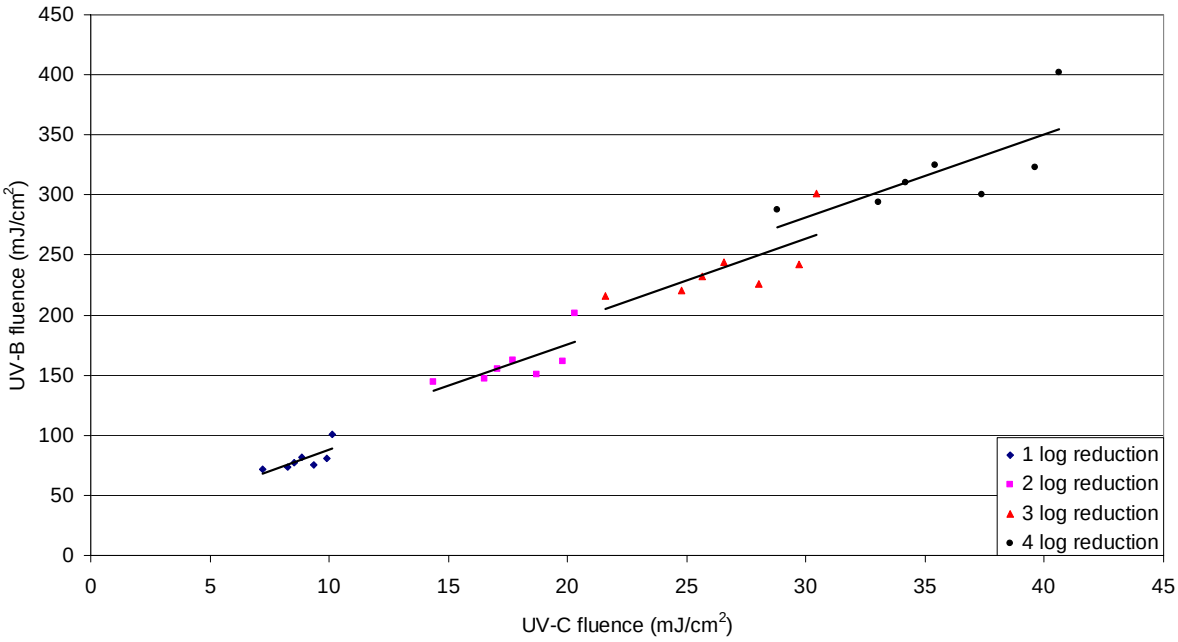


Figure 7.3 Relationship between UV-C and UV-B fluences required for log_{10} reduction in B124 phage titres.

Table 7.10 Relationship between 4-log_{10} reduction fluence for phage specimens during UV-B and UV-C irradiation during Phase Two experiments

Phage specimen ID (B124-)	UV-C 4-log_{10} inactivation fluence (mJ/cm^2)	UV-B 4-log_{10} inactivation fluence (mJ/cm^2)	UV-C:UV-B ratio
1	35	325	1:9.19
10	29	288	1:10.00
12	33	294	1:8.90
21	34	310	1:9.07
29	40	323	1:8.15
35	37	301	1:8.05
54	41	402	1:9.90

7.4. Comparison of Phase One and Phase Two experiments

Comparing the results from Phase One and Phase Two experiments (which can be done directly for seven phage specimens), it is evident that a disparity exists between the two methods. 4-$\log_{10}$ reduction values for the seven phage during UV-B and UV-C irradiation are shown in Table 7.11. The differences for UV-B range from 15% (B124-1) to 111 % (B124-29). For UV-C the differences tend to be lower (because of shorter exposure times), ranging from 12% (B124-21) to 55 % (B124-29).

Table 7.11 Comparison of Phase One (P1) and Phase Two (P2) 4-$\log_{10}$ reduction fluences

Phage ID (B124-)	UV-B			UV-C		
	P1 (mJ/cm^2)	P2 (mJ/cm^2)	Difference (mJ/cm^2)	P1 (mJ/cm^2)	P2 (mJ/cm^2)	Difference (mJ/cm^2)
1	374	325	49	44	35	9
10	444	288	156	38	29	9
12	432	294	138	44	33	11
21	533	310	223	38	34	4
29	681	323	358	61	40	21
35	442	301	141	55	37	18
54	587	402	185	48	41	7

The previous two chapters have demonstrated how phages capable of infecting *Bacteroides* strain GB-124 present in partially treated municipal wastewater, are inactivated under conditions similar to those found in both wastewater treatment works (UV-C) and also in the natural environment (UV-B). As phages infecting GB-124 have been proposed as indicators of faecal contamination, it is important to establish how these results may impact upon MST studies, and if there is the possibility of using GB-124 phages as surrogate organisms for enteroviruses in both WwTW and river catchments. In Chapter Eight these findings will be discussed within the context of QMRA, and the potential use of B124 phages as surrogates of pathogens within waterbodies will be assessed, with conclusions and suggestions for further work presented.

CHAPTER EIGHT

8. DISCUSSION, CONCLUSIONS AND FURTHER WORK

This work was designed to contribute to the aim of improving our understanding of the ecology of phages capable of infecting *Bacteroides* strain GB-124, in order to better assess their suitability for use in MST studies and as surrogate organisms of enteric viruses in QMRA. This study was the first to isolate and assess the homogeneity of B124 phage, and to determine their inactivation kinetics during exposure to UV-B and UV-C radiation (representing germicidal UV wavelengths associated with sunlight and wastewater treatment processes, respectively). This chapter discusses how the results reported in the three preceding chapters, influence the potential use of B124 phages within MST and QMRA studies, and evaluate if they may be able to indicate risk of enteric virus infection to water-users.

8.1 Phage homogeneity

8.1.1 Isolation

Bacterial hosts used in bacteriophage-based MST and QMRA, should detect a homogenous phage group. In this study, although 61 plaques were selected from final effluent double agar plates, only 20 plaques caused bacterial lysis when re-plated on GB-124. As these plaques were able to cause lysis on the original agar plate, it was unexpected that subsequent lysis was not observed. It is possible that the plaques were unable to cause lysis after being selected because they had entered a dormant lysogenic state (see Section 2.1.3). Host infection by phages may have taken place, but phage DNA was integrated in the host cell rather than causing lysis. Moreover, when the selected plaques were introduced to GB-124 broth cultures, no bacterial

clearing was observed (i.e., reduction of culture turbidity or production of 'stringy' cultures), suggesting that the phages had entered a pseudolysogenic, or phage-carrier state which has been observed for other phages including those infecting other strains of *Bacteroides* (Lwoff, 1953; Keller and Traub, 1974; Booth *et al*., 1979; Lythgoe and Chao, 2003). Pseudolysogeny is where a mixed bacterial population develops in broth culture, including a significant portion of bacterial phenotypes resistant to phage infection, causing retardation of phage replication. It is possible that two phage phenotypes are present in WwTW final effluent, one group that can lyse GB-124 after re-plating, and one group that cannot, either entering a lysogenic state or having been sufficiently damaged to inhibit host infection.

8.1.2 Morphology and host range

All B124 phages selected during this study belonged to the *Siphoviridae* family (binary morphology with icosahedral capsid and non-contractile tail), showing little variation between specimens. Minor tail variations were evident (i.e., straight, slightly curved, kinked etc.). However, this tail shape variation may have been because of the effects of the uranyl acetate staining, rather than being true morphological characteristics (see Section 2.1.1). The only phage to possess different tail morphology was B124-21, which had a distinct long (~245 nm) wavy tail. Compared with tail variation reported by other authors, the variation in tail shape observed in B124 phages was minimal. As shown in Figures 2.3 and 2.4, it is evident that the degree of tail curvature can vary between virions in the same sample, on the same TEM grid. The delineation of B124 phages into distinct groups, based on the degree of tail curvature alone, is therefore not possible.

The dominance of phages belonging to the *Siphoviridae* family within the B124 phage population is consistent with reports of *Bacteroides* phages given in the literature (Prévot *et al*., 1970; Nacescu *et al*., 1972; Keller and Traub, 1974; Burt and Woods, 1977; Booth *et al*., 1979; Masanori *et al*., 1985; Kory and Booth, 1986; Tartera and Jofre, 1987; Lasobras *et al*., 1997; Queralt *et al*., 2003) showing high within-family homogeneity (i.e., *siphoviruses* with similar dimensions and morphology). It is however possible that the culturing technique used in the present study favoured the survival of members of the *Siphoviridae* family. Only 33% of selected plaques resulted in propagation, and there is the possibility that the unsuccessfully propagated phages may have belonged to other families (some of which are more fragile and easily damaged: e.g., *Myoviridae* or *Podoviridae* families).

B124 phages appear to be a more homogeneous group than those reported to infect *Bacteroides* strains HSP-40 or RYC-2056 (e.g., variations recorded by Queralt *et al*., 2003), and therefore may be better indicators of human faecal pollution. During initial data analysis for this study, it was thought that members of the *Podoviridae* family had been isolated (icosahedral head with short tail). However, the presence of podoviruses was rejected, as disassociated tails were observed in micrographs, suggesting virion damage rather than genuine podoviruses. Disassociated heads and tails have been observed by other authors, and such virion damage may account for the low recovery of lytic phages from the selected plaques. It is also possible that the TEM staining procedure damaged virions.

All B124 phages were isolated from municipal wastewater final effluent, and it is possible that the high homogeneity of B124 phages in such wastewater effluent may

not be an accurate reflection of the phage population in raw and partially treated wastewater at other stages of wastewater treatment. As was discussed in Section 2.2, the composition of phage populations through WwTW is not constant, and when using phages infecting GB-124 in MST studies, it is important to be aware of water sample origin. For example, septic tanks may have a higher percentage of myoviruses and podoviruses, whereas treated sewage may have a greater percentage of siphoviruses (Dee and Fogleman, 1992). If siphoviruses are more persistent in both the environment and through treatment plants, WwTW effluent would be detectable longer in the receiving waterbody than septic tank effluent because of the higher proportion of resistant siphoviruses in the final effluent. This may become an issue if quantitative estimations of faecal pollution (and risk to health) are attempted using B124 phages as an indicator of viral loads.

The majority of capsid dimensions for phages infecting *Bacteroides* given in the literature are within the range displayed by B124 phages isolated in this study (Tables 2.3 and 5.2), and appear to be representative of siphoviruses isolated from wastewater. The only significant deviation from the dimensions given in Table 2.3 was B124-21, which as previously mentioned, had a long (~245 nm) wavy tail.

The host range patterns exhibited by B124 phages were found to be homogenous. B124 phages were found to infect *Bacteroides* strain GB-124 and RYC-2056 (with the exception of B124-35) but were unable to infect and lyse *Bacteroides* strain HSP-40. Host range analysis during this study was not extensive enough to draw exhaustive conclusions. However, the homogeneity of the host range when tested on three more widely researched *Bacteroides* host strains has been demonstrated.

All the morphological and infectious characteristics described above suggest that B124 phages are a highly homogenous group consisting of predominantly siphoviruses, with either a straight or slightly curved tail, capable of infecting *Bacteroides* strain RYC-2056, but not HSP-40.

8.2 Issues arising from different UV methodologies

8.2.1 Fluence calculations

When discussing microorganism inactivation kinetics it is useful to determine both the inactivation rate coefficient (*k*) and the 4-$\log_{10}$ reduction value. However, in the majority of studies, $\log_{10}$ reduction values are given, rather than *k* values. As the two items are derived from the same regression equation and the *k* value (slope of line) is used in calculation of the 4-$\log_{10}$ reduction value, the two are essentially different measurements of inactivation kinetics. In the discussion below, 4-$\log_{10}$ inactivation values are discussed, allowing easier comparison with previous studies reported in the literature.

As shown in Chapter Seven, the degree of correlation between Phase One and Phase Two UV experiments differs according to wavelength. Correlation between fluences required for a 4-$\log_{10}$ inactivation using UV-B during Phase One and Phase Two is weaker than that for UV-C (R^2 = 0.20 and 0.58 respectively). The mean 4- $\log_{10}$ reduction value for the seven phages that were used during both experimental phases was 47 mJ/cm^2 for Phase One and 36 mJ/cm^2 for Phase Two (UV-C). For UV-B, the Figures were 499 mJ/cm^2 and 320 mJ/cm^2 (Phase One and Phase Two respectively). However, it is evident that the methodology employed during Phase One experimentation may over-estimate the fluences required to achieve 4-$\log_{10}$ inactivation.

Fluence over-estimation can result from using an overly simplistic method of fluence calculation. The method used in Phase One was based on the equation used by numerous authors (e.g., Oguma *et al*., 2001; Kellogg and Paul, 2002; Wu *et al*., 2005),

where fluence (mJ/cm^2) is the product of fluence rate (i.e., the fluence rate measurement at the water surface of irradiation vessel, mW/cm^2) and exposure time (seconds). This calculation does not take into account any other factors, such as light reflection from the water surface, UV absorbance of the phage suspension, and unequal distribution of UV photons across the Petri dish. This has important implications, not just in this study, but for research conducted by other authors where correction factors have not been used (Tables 3.2 and 3.3).

If fluence rate correction factors are not used, fluence is invariably over-estimated. For example, during Phase Two irradiation of B124-1 (using 254nm UV), the radiometer reading at the centre of the Petri dish located below the aperture of the collimating tube was 0.129 mW/cm^2. When applying the Bolton and Linden (2003) corrections (Petri factor, reflection factor, water factor and divergence factor as detailed in Materials and methods) the true fluence rate across the Petri dish was calculated as 0.103 mW/cm^2 (a difference of 0.026 mW/cm^2). To deliver a fluence of 10 mJ/cm to the sample using the corrected fluence rate values, the exposure time would be 1 minute and 43 seconds. If using uncorrected fluence rate readings, the calculated fluence delivered in 1 minute 43 seconds would have been 13.32 mJ/cm^2 a difference of 3.32 mJ/cm^2). During longer exposure, the disparity between uncorrected and corrected fluences is exaggerated. For example, an exposure time of 5 minutes, 1 second would deliver a fluence of 30 mJ/cm^2 when calculated using the corrected values (fluence rate as previous example). Using uncorrected values this fluence would have been 39.96 mJ/cm^2 (a difference of 9.96 mJ/cm^2).

When evaluating UV-B fluences, the generally longer exposure times used (up to one hour) compound the problem yet further. For example, when irradiating B124-1 using UV-B during Phase Two experiments, the radiometer reading at the centre of the Petri dish was 0. 0117 mW/cm^2, and the corrected value for true fluence rate across the Petri dish was 0.094 mW/cm^2. An exposure time of 56 minutes and 1 second delivered a fluence of 300 mJ/cm^2 using the corrected fluence rate, whereas the uncorrected figure would have delivered a fluence of 393.22 mJ/cm^2 (a difference of 93.22 mJ/cm^2). This would partially explain the disparity between Phase One and Phase Two experiments. Fluences delivered were the same during both experimental phases, it was the method of fluence calculation that was different.

Data from studies using the Bolton and Linden (2003) method can be directly compared with data recorded during this research, whereas studies using no fluence corrections cannot. Without knowing the precise details of the experimental set-up of studies not using the fluence corrections, it is impossible to alter these results to reflect true fluence rates.

8.2.2 Anomalies produced during Phase One experiments

Another interesting feature of the data that was present in Phase One experiments only, was the apparent “shouldering” (i.e., little inactivation during initial exposures) observed in B124-17 and B124-57 (during UV-C irradiation) and the inactivation curve fluctuations observed during Phase One UV-B irradiation (e.g., inactivation plateaus shown by B124-1, B124-4, B124-21, B124- 22, B124-35 and B124-54). One reason why data produced during Phase One may be more variable is that aliquots were withdrawn from the Petri dish for assay rather than using a fresh sample for each

fluence exposure. By withdrawing an aliquot for assay, the sample volume within the irradiation vessel decreased, reducing UV attenuation and leading to possible miscalculation of fluence. Aliquots were withdrawn from the centre of the Petri dish, and as this is where the stir bar was suspended during stirring, a degree of phage shadowing may have occurred. Therefore, it is possible that aliquots contained phages that had been shielded from UV light by the stir bar. Also, phage aggregation may have caused apparent increased inactivation shown in the Phase One results (e.g., B124-1 during UV-B exposure between 100 and 200 mJ/cm^2). Phase One exposure times were shorter, and any phage clusters may not have had sufficient time to break up during irradiation. As these "shoulders" were not observed during Phase Two experiments, they are likely to be a result of the methodology employed, rather than true biological characteristics. These data support the adoption of the standardised UV protocol used during Phase Two experiments. The results from Phase Two experiments are discussed in detail below.

8.3 Comparison of B124 phages UV-C inactivation kinetics to those of other microorganisms

Although the method of UV exposure and fluence calculation varies between studies reported in the literature, it is important to compare the inactivation kinetics of B124 phages with those of specific pathogenic microorganisms. This will inform the decision as to whether B124 phages can be used as a suitable indicator organisms of human faecal pollution, or surrogates for particular pathogens. For the sake of clarity, not all the data identified during the course of the literature review are presented here. Only data for selected organisms that have similar fluence requirements to those shown by B124 phages, or which are of particular interest in terms of water quality monitoring are presented here. Full data concerning fluence rates for microorganisms are given in Appendix 1 (inactivation data for over 60 bacterial, 40 phage, 40 viral, 20 protozoan and 10 spore experiments).

8.3.1 Bacteria, Protozoa and Spores

It is evident from data provided in Appendix 1, that B124 phages require different fluences than bacteria, protozoa and spores in order to achieve 4-$\log_{10}$ inactivation. Protozoa and bacteria both require lower fluences, whereas spores require higher fluences to achieve the same reduction as B124 phages. This indicates that B124 phages may not be suitable as surrogate organisms of spore-forming pathogens (such as *Bacillus anthracis*) as B124 phages will be inactivated more rapidly. In comparison with bacteria and protozoa, B124 phages appear to be more persistent, and may therefore be used to indicate their potential presence, but there exists a potential for false-positive indication (over-estimation) of bacterial and protozoan pathogens.

8.3.2 Viruses and bacteriophages

Importantly, the fluence requirements of B124 phages were found to be much more closely aligned with those of other viruses and bacteriophages, than the other groups of microorganisms reported above (Table 8.1). The similarity of fluence requirement for B124 phages and previously reported B40-8 phage was not unexpected, as B40-8 is a siphovirus isolated using *Bacteroides* strain HSP-40, also from municipal wastewater. Data for the other phages are variable. Although inactivation data for PRD-1 given by Meng and Gerba (1996) are similar to those for B124 phage, data for PRD-1 given by Shin *et al* (2005) are less so. These authors state that a fluence of 110 mJ/cm^2 was required for a 4-log_{-10} inactivation. *Staphylococcus aureus* phage A994 is a siphovirus with dsDNA (44kbp in length) and a non-elongated head (Kwan *et al*., 2005); this the same morphology as B124 phages.

The viruses that require similar fluences to B124 phages are calicivirus canine, calicivirus feline, coxsackievirus (B3 and B5), echovirus (I and II), hepatitis A HM175 - though other data, namely those of Battigelli *et al* (1993) do not agree - poliovirus type 1 and rotavirus SA 11. Also, although no exact figures for 1-, 2-log_{10} etc., are given, greater than 3.6 log inactivation of poliovirus type 1 (LSc 1, BGMK cell line) was achieved by Shin *et al* (2005) using 30 mJ/cm^2, and greater than 5.2 log^{10} inactivation of coxsackievirus 4 (BGMK cell line) was achieved using 30 mJ/cm^2.

Table 8.1 Phages and viruses with similar UV-C inactivation kinetics to B124 phages (FRNA phages are also shown for comparison)

Organism	Type/Species	Genetic material	Fluence required for log inactivation				Reference
			-1	-2	-3	-4	
Phage	**B124-1**	dsDNA	**9**	**18**	**27**	**35**	This study
	B124-10	dsDNA	**7**	**14**	**22**	**29**	This study
	B124-12	dsDNA	**8**	**17**	**25**	**33**	This study
	B124-21	dsDNA	**9**	**17**	**26**	**34**	This study
	B124-29	dsDNA	**10**	**20**	**30**	**40**	This study
	B124-35	dsDNA	**9**	**19**	**28**	**37**	This study
	B124-54	dsDNA	**10**	**20**	**30**	**41**	This study
	B40-8 (HSP-40)	dsDNA	12	18	23	28	Sommer *et al*., 1998
	B40-8 (HSP-40)	dsDNA	11	17	23	29	Sommer *et al*., 2001
	B40-8 (HSP-40)	dsDNA	7.1	14.3	21.4	28.6	Clancy *et al*., 2004
	PRD-1 (*S. typhimurium* Lt2)	dsDNA	9.9	17.2	23.5	30.1	Meng and Gerba, 1996
	Staphylococcus aureus phage A994 (*Staphylococcus aureus* A994)	dsDNA	8	17	25	36	Sommer *et al*., 1989
	FRNA (MS2)	ssRNA	23	55	87	119	Thurston-Enriquez *et al*., 2003
Virus	Calicivirus canine (MDCK cell line)	ssRNA	7	15	22	30	Husman *et al*., 2004
	Calicivirus canine (CRFK cell line)	ssRNA	7	16	25	-	Husman *et al*., 2004
	Calicivirus feline (CRFK cell line)	ssRNA	6	16	26	36	Thurston-Enriquez *et al*., 2003
	Coxsackievirus B3 (BGM cell line)	ssRNA	8	16	24.5	32.5	Gerba *et al*., 2002
	Coxsackievirus B5 (BGM cell line)	ssRNA	9.5	18	27	36	Gerba *et al*., 2002
	Echovirus I (BGM cell line)	ssRNA	8	16.5	25	33	Gerba *et al*., 2002
	Echovirus II (BGM cell line)	ssRNA	7	14	20.5	28	Gerba *et al*., 2002
	Enterovirus	ssRNA	7.5	15	24	34.5	Lazarova and Savoye, 2004
	Hepatitis A HM175 (FRhK-4 cell)	ssRNA	5.1	13.7	22	29.6	Wilson *et al*., 1992
	Poliovirus	ssRNA	6	14	22	32.5	Lazarova and Savoye, 2004
	Poliovirus Type 1 (Mahoney)	ssRNA	3	7	14	40	Sommer *et al*., 1989
	Poliovirus 1 (BGM cell line)	ssRNA	8	15.5	23	31	Gerba *et al*., 2002
	Poliovirus 1 [CaCo2 cell-line (ATCC HTB37)]	ssRNA	7	17	28	37	Thompson *et al*., 2003
	Poliovirus Type 1 LSc-1	ssRNA	-	-	-	23 - 29	Snicer *et al*., 1998
	Rotavirus SA 11 (MA 104)	dsRNA	8	15	27	38	Sommer *et al*., 1989
	Rotavirus SA 11 (MA 104)	dsRNA	9.1	19	26	36	Wilson *et al*., 1992

The data indicate that B124 phages may be used as indicator and surrogate organisms for enterovirus (polio-, coxsackie- and echovirus) and there may be potential for B124 phages to act as a surrogate organism of norovirus (for which feline calicivirus are often used as surrogates; Doultree *et al*., 1999). The data for enterovirus inactivation derive from studies where not all the Bolton and Linden (2003) corrections were applied, and it is possible therefore, that slightly lower fluences may be required to achieve 4-$\log_{10}$ inactivation than those given.

The data show that there appears to be no clear relationship between inactivation kinetics and phage morphology. Phages that require similar fluences to B124 to achieve a 4-$\log_{10}$ inactivation are all dsDNA, but have different morphologies. B40-8 and *S. aureus* phage A994 are members of the *Siphoviridae* family (therefore binary morphology with a long non-contractile tail), PRD-1 is a tail-less virion belonging to the *Tectiviridae* family. Other members of the *Siphoviridae* family, for which inactivation data are available suggest that 3-$\log_{10}$ inactivation occurs at around 11.6 mJ/cm^2 and 18.1 mJ/cm^2 (siphoviruses D3C3, F116 and G101 in Kadavy *et al*., 2000), whereas B124 phages require between 22 and 30 mJ/cm^2 to inactivate by 3-$\log_{10}$. The morphology of the viruses requiring the same fluences as B124 phages are also different: unlike B124 phage, the enteroviruses and caliciviruses both have icosahedral capsids without tails, indicating that inactivation kinetics are independent of the presence/absence of tail structures.

Persistence of phages belonging to different families was also reported by Shin *et al* (2005). One siphovirus, one podovirus and one myovirus were irradiated (host *E. coli* WG5) and were inactivated very quickly (10 mJ/cm^2 inactivated phages by greater

than 5.5-, greater than 3.48- and greater than 5.99-$\log_{10}$, respectively). Interestingly, and in contrast to other studies, the difference between *Myoviridae* and *Siphoviridae* was minimal.

As dsDNA, ssRNA and dsRNA phages and viruses are inactivated at similar fluences to B124 phages (data presented in Appendix 1), no clear relationship is evident between the nature of genetic material and inactivation kinetics. If the nature of genetic material were to be the factor determining inactivation kinetics, then B124 phages would be inactivated at similar fluences as siphoviruses as reported by Shin *et al* (2005) and adenovirus (this is dsDNA with a genome of between 30 to 40 kbp, of a similar size to *Bacteroides* phage). This is not the case: MS2 phage, commonly used as a biodosimeter, requires the highest fluences to achieve 4-log inactivation. The genetic material of MS2 phage is ssRNA, with a similar structure and size to enteroviruses. However, MS2 phage requires a greater fluence dose to achieve the same level of inactivation as enteroviruses. In Section 2.2.2, it was noted that FRNA phages (of which MS2 is the most studied) may be used as surrogates for enteroviruses. The difference in UV-C inactivation requirements may make this inadvisable (Table 8.1).

Although no clear relationship is evident, it has been suggested that double stranded viruses may be more UV tolerant, and in the case of adenovirus, it is hypothesised that this is because they can utilise host enzymes to repair damage (Gerba *et al*., 2002). There is also no evident relationship between genome size and UV-C inactivation kinetics. For example, although B40-8 has a genome of 51.7 kbp, and echovirus has a genome of around 7.5 kbp, they require similar fluences for 4-$\log_{10}$ inactivation.

8.4 Comparison of B124 phages UV-B inactivation kinetics with those of other microorganisms

From B124 phage inactivation data derived from this study, and enterovirus data reported in the literature, the UV-C inactivation characteristics of B124 phages and enterovirus appear well correlated. However, as solar UV-C does not reach the surface of the earth, in order to be useful in QMST and QMRA studies, the response to UV-B irradiation should also be correlated. As detailed in the Chapter Three, solar UV-B is one of the primary environmental inactivation methods of microorganisms.

8.4.1 Bacteria and viruses

Few data are available from studies in which organisms have been irradiated in CB apparatus with UV-B. As was demonstrated with UV-C inactivation kinetics, B124 phages are inactivated at a slower rate than selected bacteria, but insufficient data are available to be able to assess how B124 phage inactivation kinetics relate with respect other phage, viruses, spores and protozoa.

In Section 8.3.2, it was hypothesised that B124 phages may have similar UV-C inactivation kinetics to feline/canine caliciviruses (which are commonly used as surrogates for noroviruses). When irradiated using UV-B, 34 mJ/cm^2 were required to reduce both feline and canine caliciviruses by 3-log$_{10}$ (Duizer *et al.*, 2004). These data indicate that B124 phages may not be used as indicators of feline/canine caliciviruses in waterbodies where UV-B is the dominant inactivating factor, as feline/canine caliciviruses may be inactivated more rapidly than B124 phages. However, as these studies were not carried out in parallel, using the same apparatus (e.g., Duizer *et al.*, 2004 irradiated samples in microwells, whereas the present study used Petri dishes), a

degree of experimental error may be present. No UV-B/enterovirus data were found in the literature, and therefore it is not currently possible to examine the applicability of B124 phages as an indicator index for enteroviruses during solar irradiation.

The results of inactivation experiments using UV-B undertaken during this research, show B124 phages to be inactivated by 4-$\log_{10}$ at fluences between 288 mJ/cm^2 and 402 mJ/cm^2, whereas 4-$\log_{10}$ inactivation for UV-C required from 29 to 41 mJ/cm^2. The data show that ten times the amount of UV-B fluence is required than UV-C fluence in order to achieve the same level of inactivation. The difference in fluence requirement is most probably because of the greater efficiency of UV photon absorption by DNA at wavelengths close to 250 nm than at 302 nm, and therefore the greater efficiency of dimer formation (i.e., disruption in base pairing). It has been demonstrated that dimer formation during UV-B is around 90% less efficient than dimer formation during UV-C (Ravanat *et al*., 2001; Giacomoni, 1995) and as this is similar to the observed ratio for B124 phage, it can be postulated that the main mechanism of UV-B inactivation of B124 phages is via the production of dimers.

8.5 The use of Collimated Beam experiments to inform QMST and QMRA studies

8.5.1 Using B124 phages as index/indicator organisms during and after UV-C treatment

An objective of this research was to assess the suitability of B124 phages as surrogate organisms for particular pathogens, both in the natural environment and during wastewater treatment processes. This work also informs QMRA studies, as the levels of B124 phages in wastewater may suggest the degree of treatment needed to reduce illness and morbidity, caused by selected pathogens, among consumers of shellfish and recreational users of the receiving waters.

A key knowledge gap is the comparative environmental behaviour of GB-124 phages and enteroviruses. As presented in Section 1.5, in order to be a useful indicator for enteroviruses in wastewater and wastewater contaminated waterbodies, GB-124 phages should be present in greater numbers and have similar inactivation characteristics (Borrego *et al*., 1987; Havelaar, 1993).

From the data presented in Table 8.1, it appears that B124 phages and enteroviruses require similar UV-C fluences for a 4-$\log_{10}$ inactivation, and it is therefore proposed that B124 phages may be able to act as surrogate organisms for enteroviruses during WwTW UV tertiary treatment, and in QMRA studies. As echovirus and coxsackievirus have been implicated in waterborne disease outbreaks (e.g., Kapadia *et al*., 1984; Kukkula *et al*., 1997; Amvrosieva *et al*., 2001) and poliovirus is prevalent in LEDC, the use of a safe, easily assayed surrogate phage is a very useful tool for QMRA and health protection.

Moreover, as phages infecting GB-124 assays are relatively low-cost, its use would prove beneficial in areas where poliovirus is endemic (India, Nigeria and areas surrounding the Afghanistan/Pakistan border). By using B124 phages as surrogates of enteroviruses in WwTW final effluent samples and in faecally-impacted surface waters, a cheaper and rapid estimation of risk to users of waterbodies could be achieved. This would benefit both the individuals using the water resource, and by reducing the number of days lost to illness, the cost of medical care for infected individuals, and the overall enteric disease burden. More expensive techniques to detect poliovirus (and other enteric viruses) may be provided by WHO poliovirus eradication programme, providing equipment and expertise to affected areas.

Given the data from the Phase Two experiments, the fluences delivered by UK WwTW (up to 50 mJ/cm^2; Mayor-Smith, pers. comm.) would be enough for a 4-log_{10} inactivation of B124 phages. An extensive record of the densities of B124 phages in final effluents from the southeast of England are presented by Ebdon *et al* (2007; Table 2.9), and it is clear that phages infecting GB-124 would be inactivated by 'typical' WwTW fluences, and would therefore be absent from UV irradiated final effluents.

Enterovirus densities of final effluent detailed in the literature vary. Dahling *et al* (1989) recorded between 1 and 24,000 PFU/litre in Puerto Rico, and the data vary depending on the efficacy, and process stages, of WwTW. Generally, the ratio of enterovirus to HSP-40 phages in wastewater has been given as approximately 1:5 (South Africa; Grabow *et al*., 1993) and if this ratio were to be consistent in all scenarios, as B124 phages appear to be a conservative indicator of enteroviruses, it

can be assumed that enteroviruses would also be absent from UV treated final effluent (Jacangelo, *et al*., 2003). As the fluence requirements necessary to achieve a 4-$\log_{10}$ inactivation of enteroviruses are similar to those required for a 4-$\log_{10}$ reduction of B124 phages, it is expected that no enterovirus virions would be present in the final effluent of tertiary treated UV WwTW (based on 100 ml sample), providing that a fluence greater than 40 mJ/cm^2 is delivered. Also, as the majority of bacterial and viral pathogens (excluding adenovirus) require lower fluences than B124 phages to achieve a 4-$\log_{10}$ reduction, B124 phages can act as a conservative indicator for these organisms, aiding the development of QMRA protocols.

Another group of viruses for which B124 phages may be an index indicator organism are noroviruses. At present, norovirus cannot be cultivated in the laboratory and feline/canine caliciviruses are often used as a model system during disinfection studies (Slomka and Appleton, 1998; Doultree *et al*., 1999). As B124 phages and canine/feline calicivirus appear to require similar fluences in order to achieve the same level of inactivation using UV-C, B124 phages may prove to be a useful indicator/index organism for noroviruses. However, unlike *Bacteroides* GB-124 phages, noroviruses are not necessarily human specific and because of this, B124 phages may be an unsuitable indicator of animal-derived noroviruses in the environment.

Finally, recent work (unpublished data) suggests that there may be a positive correlation between adenovirus and B124 phages in surface waters in the UK and Portugal (James Ebdon, Pers comm.). However, this relationship may break down in waterbodies where effluents are subject to UV sterilisation as adenovirus requires far

higher fluences (greater than 100 mJ/cm^2), than B124 phages. This means that adenovirus would still be present after UV treatment, whereas B124 phages are likely to be absent. If B124 phages were to be used as an indicator of adenoviruses, false negative results may be obtained and QMRA models based on B124 phages as a surrogate would be inappropriate.

8.5.2 Using B124 phages as index/indicator organisms during UV-B irradiation

The correlation of enteroviruses and B124 phages when studied using CB apparatus under UV-C radiation is useful in determining the efficiency of UV inactivation treatment methods, and the risk to human populations coming into contact with final effluent. However, in MEDC it is unlikely that people will be exposed to minimally diluted, recently discharged effluent, with the majority of contact taking place downstream of the discharge point in recreational waters (i.e., after significant mixing and dilution has occurred). In LEDC, contact with minimally treated wastewater is more likely and therefore the risk to human health is greater. In between the final effluent release point and the point of contact with human populations, numerous physiochemical and biological factors influence phage and enterovirus survival. As described in Chapter Three, sunlight inactivation by UV-B is an important factor influencing the survival of microorganisms in river systems, and for B124 phages to be used as indicator/index organisms of enteroviruses through the receiving drainage system, B124 phages should ideally have higher UV-B resistance than the target pathogen.

Using the CB apparatus detailed in this study (Chapter Four), exposure times necessary to deliver required fluences can be calculated (Table 8.2). These

calculations use example fluence rates of 0.022 mW/cm^2 (Jacquet and Bratbak, 2003) and 0.20 mW/cm^2 (Jung *et al*., 2008) and assume that microorganisms will be exposed to a constant fluence with no virus reactivation occurring. In river systems, constant fluence is unlikely, because microorganisms move within the water column. Moreover, as most UV-B is delivered around a four hour period (two hours either side of the solar noon) a constant fluence cannot be assumed (Coohill and Sagripanti, 2009).

Table 8.2 Exposure times for target fluences using UV-B fluence rate values detailed in the literature (Jacquet and Bratbak, 2003)

Target fluence (mJ/cm^2)	**Time required (Jacquet and Bratbak, 2003)**	**Time required (Jung *et al*., 2008)**
50	37m 53s	4m 10s
100	1h 15m 45s	8m 20
150	1h 53m 38s	12m 30s
200	2h 31m 31s	16m 40s
250	3h 9m 24s	23m 59s
300	3h 47m 16s	33m 30s

Data from this study indicate that a UV-B fluence of between 288 mJ/cm^2 and 402 mJ/cm^2 is required to achieve a 4-log$_{10}$ inactivation of B124 phage. Given the fluence rate of 0.022 mW/cm^2, this would equate to environmental exposure times of between 3h 38m and 5h 4m 33s. As this is the peak exposure, it is unlikely that 4-log$_{10}$ inactivation could be achieved during a single diurnal cycle. However, if a higher fluence rate were used in the calculation (i.e., 0.20 mW/cm^2 as recorded by Jung *et al*., 2008, and similar to those recorded in the UK) 4-log$_{10}$ inactivation would occur within 35 minutes. Based on a fluence rate of 0.20 mW/cm^2, the potential for environmental inactivation of B124 phages within a diurnal UV-B exposure cycle is evident.

In determining the possibility of B124 phage inactivation in a waterbody by UV-B, it is useful to determine daily fluence loads. Examples given in the literature include 10 kJ/m^2 in January and 55 kJ/m^2 in June in Baltimore, USA (Heisler *et al*., 2004), and 570 to 611 kJ/m^2 in September at the Red Sea (Boelen *et al*., 2002). A 4-log_{10} reduction of B124 phages could be achieved during a single day based upon these total fluence loads, as this requires from 2.8 kJ/m^2 to 4 kJ/m^2. This calculation is highly dependent on local solar conditions, as the UV-B load delivered in sunnier climates will be far higher. This may have important ramifications for the use of GB-124 phages as a tool for QMST and QMRA in latitudes where solar UV-B levels are higher. Therefore, local climatic conditions should be assessed when using B124 phages in river systems, and factors such as cloud cover must be taken into account. This is pivotal in controlling the amount of UV-B radiation reaching the water surface.

To date, no *in situ* ecological studies of B124 phages have been reported, but data collected by researchers at the Environment & Public Health Research Unit at the University of Brighton, show the reduction/decline of phages infecting GB-124 along a longitudinal river transect. Phages infecting GB-124 at monitoring sites less than 0.5km downstream of a WwTW in a chalkland river catchment in southern England, were demonstrated to range from 3.11 log_{10} PFU/100ml during base-flow, to 3.76 log_{10} PFU/100ml during high-flow events. At sites less than 2km downstream the mean values decreased to 2.49 log_{10} PFU/100ml during base-flow, to 3.14 log_{10} PFU/100ml during high-flow events. The furthest sites, greater than 2km from WwTW, phage densities fell to 1.21 log_{10} PFU/100ml during base-flow, to 1.88 log_{10} PFU/100ml during high-flow events (Ebdon, pers. comm.). These figures concisely

demonstrate the decline of GB-124 phages over a longitudinal river profile downstream of a point source of human faecal material. One of the explanations for the decline could be the influence of UV-B radiation, as the fluences likely to be delivered during a sunny day are capable of inactivating the phage. Another explanation could be the dilution of wastewater effluent through the river catchment (though conversely, other point sources could be contributing phage load to water system mitigating the dilution effect).

In pilot tests for this study, river water samples were taken downstream of a WwTW in an area subject to long water retention times (the Pevensey Levels, East Sussex, UK). Phages infecting GB-124 were found in non-UV disinfected WwTW final effluent (approximately 3.70 $\log_{10}$ PFU/100ml), but were not discovered at any points in the receiving drainage system (closest monitoring point is approximately 1km downstream of WwTW outlet). Retention times in this area may be greater than five days, and it is likely that sufficient UV-B would be delivered to inactivate B124 phage.

Moreover, as mean densities of phages infecting GB-124 found in the River Ouse (south east England) have been recorded as less than 10^4 PFU per 100 ml throughout the river transect (Ebdon *et al*., 2007), a fluence of less than that required for 4-$\log_{10}$ inactivation will be required to remove B124 phages completely. For a 3-$\log_{10}$ inactivation of B124-phage, between 216 mJ/cm^2 and 302 mJ/cm^2 would be required. This corresponds to exposures times between 17 minutes and 59 seconds to 25 minutes and 8 seconds (0.2 mW/cm^2 fluence rate).

As phages infecting GB-124 have been enumerated in river water samples in many countries (Ebdon, pers. comm.), it is likely that virions are not exposed to the full amount of solar UV-B reaching the water surface. So far it has been assumed that all phage virions are exposed to the full UV-B fluence throughout the water column. However, it has been shown that UV-B radiation is attenuated through the water column (Table 3.6) and this may extend phage survival. As WwTW effluents are typically nutrient rich and more turbid than surrounding waters, UV-B penetration into the receiving water column will not be as great as in waters with a lower nutrient status/turbidity, thus protecting the phages from inactivation. However, in areas subject to faecal pollution other than from WwTW (i.e., rural septic tanks and soakways, usually in isolated rural locations), the receiving water may be nutrient poor, and UV-B irradiation of phages may be greater. As septic tanks may have greater proportions of myoviruses and podoviruses (which may be inactivated more quickly than siphoviruses), when using GB-124 to detect phages downstream of septic tanks, this problem will be compounded, and low levels of phages may be present.

The protective influence of sediments may also extend B124 survival in river systems. After rainfall events, phage protection will be enhanced because of increased deposition of terrestrial sediments into the river. Increased phage densities have been observed after heavy rainfall (Ebdon *et al.*, 2007), but it is difficult to separate the influence of WwTW overflows, re-suspension of phage-rich river bed sediments, and decreased UV-B water column penetration, as no detailed studies have been conducted to date. The sediment absorption characteristics of B124 phages have yet to be assessed.

As well as physiochemical factors reducing phage exposure to UV-B radiation, the method and time of water sampling may influence the density of phages (infecting GB-124) present. Samples taken after the period of greatest inactivation (i.e., two hours post solar-noon) may contain more inactivated B124 phages than those taken two hours before solar noon. Also, in winter, less solar UV-B is delivered, therefore less UV induced phage-inactivation will take place. The location of sample collection point may also influence density of phages recorded. For example, if a slow moving river is being sampled, with limited mixing between bank and centre water columns, the difference between UV-B exposure in the centre (i.e., exposed section) of the river will be greater than the UV-B exposure on the banks (which may be shaded for part of the day).

It is evident that when using phages infecting GB-124 in environmental aquatic systems, particular attention must be paid to the local environmental conditions within the system. Characteristics such as type of point source (septic tank or WwTW), cloud cover, sediment transport characteristics, river flows, and water chemistry may all influence the densities of B124 phages recorded. If such environmental factors are underestimated, then there is a chance that there may be a mis-diagnosis of risk, resulting in negative health impacts for resource users.

8.6 Conclusions

The principal findings from this study are:

1. Phages infecting GB-124 are a morphologically homogenous group belonging to the *Siphoviridae* family with capsid diameters between 50 and 70nm and tails of 12.5 x 130 to 200 nm. B124-21 appears to be the only phage with a distinct tail, longer (245nm) and with a wave pattern. As staining procedures may produce tail deformation, delineation of B124 phages into group based on tail morphology alone is not advisable. Low recovery rate of picked plaques may indicate experimental bias, favouring propagation of siphoviruses;

2. This study has shown that UV inactivation kinetics of phages infecting GB-124 are highly homogenous and when exposed to UV-C and UV-B radiation, all B124 phages show first order inactivation kinetics with a positive log linear relationship between fluence and inactivation. B124 phages are inactivated by both UV-B and UV-C radiation and fluence required to inactivate phages between UV-B and UV-C are highly correlated. Fluences required to inactivate phages by 4-$\log_{10}$ when using UV-C are approximately an order of magnitude lower than UV-B and it is possible to estimate environmental inactivation by UV-B from laboratory based UV-C experiments. This has important implications for MST studies and the application of using phages infecting GB-124 in river systems and downstream of WwTW using tertiary UV disinfection;

3. B124 phages are likely to be inactivated by the fluences delivered both in WwTW (UV-C) and in the natural environment (UV-B). Also, because of UV-B inactivation data, faecally polluted river systems with long water retention times

may not contain any phages able to infect GB-124, therefore leading to false negatives of human faecal material. However, as the majority of pathogens would also be removed, there would be no risk associated with diagnosis;

4. Results from Phase Two experiments show that B124 phages may be used as an index, or indicator organism for enteric viruses, and possibly norovirus during UV-C wastewater disinfection. The potential for using B124 phages as index/indicator organisms within in the environment is unknown, as data for UV-B inactivation of enteroviruses and norovirus (feline/caninecalicivirus) have not been produced.; and

5. Rapid methods, as used in Phase One experiments, may lead to fluctuation of inactivation rates over experimental period, and produce microorganism inactivation ‘shoulders’. The detailed methodology used in Phase Two gives more rational inactivation curves. Phase One and Phase Two experimental results were only weakly correlated and studies employing methods similar to those used in Phase One should be treated with caution.

8.7 Recommendations for future work

1. When discussing the inactivation kinetics of B124 phages and how this may influence their use as surrogates of enteroviruses and indicators of human faecal pollution, it is important to recognise the limitations of applying results from collimated beam studies to 'real world' situations (i.e., wastewater treatment works with complex biochemical water matrices or river systems). This is because CB tests are carried out using Petri dishes where sample depth (path length) etc. is constant, CB test usually use organisms in suspension with low turbidity/high UV transmission and flow rate in WwTW may be variable, where in CB tests there is no flow. Assessment of B124 phage inactivation in wastewater treatment would prove useful for their application in QMRA;

2. During initial phage isolation from final effluent agar plates, only 33% of picked plaques were successfully propagated and able to re-infect GB-124. It would be useful to investigate if the method of phage isolation damaged particular phage types, favouring the survival of others;

3. The community ecology of WwTW influent and effluent phage populations may have an influence on MST and should be investigated. Septic tanks may have higher proportion of myoviruses and podoviruses (more easily inactivated than siphoviruses), whereas treated sewage may have greater percentage of siphoviruses. This would mean that WwTW effluent would be detectable longer than septic tank effluent because of the presence of more resistant phage;

4. Reactivation of UV-C irradiated adenovirus during assay has been observed, and it may also be useful to investigate if repair of damaged B124 phage occurs in host cells during assay. It would also be useful to investigate if GB-124 can cause photoreactivation of B124 phages when in river systems. If it were able to so, there would be implications for both MST and QMRA;

5. As it is possible that B124 phages may be suitable surrogate organisms of enteroviruses, more work needs to be carried out on the relationship between the two organisms *in situ* (i.e., in river systems and WwTW). It would be useful to determine if a predictive relationship between the two microorganisms could be determined, thus giving a better estimation of risk to users of receiving waterbodies, enhancing QMRA;

6. It would be useful to assess UV-A inactivation fluences and B124 phage inactivation, to further investigate the role of solar UV in phage decay. This would be of particular interest in river systems. This was planned in this study, but the exposure times involved did not permit it;

7. This study only investigated low-pressure UV radiation, and there is some evidence to suggest microorganisms are inactivated more quickly using medium-pressure (MP) polychromatic UV (Malley *et al*., 2004; Mamane-Gravetz *et al*., 2005). If polychromatic UV is to be installed in more WwTW, this may influence the use of B124 phages as indicators of human faecal material and surrogates for enteroviruses; and

8. An investigation into B124 phages accumulation in sediments would also help to determine how much sediment re-suspension (associated with high flow events) contributes to phage levels in river systems. This would aid in the formulation of QMRA by better distinguishing phage sources.

9. It would be useful to investigate the development of a B124 phage detection assay based on molecular methods. This may facilitate quicker determination of human faecal pollution in affected waterbodies. Further UV inactivation studies using a new B124 molecular technique should be carried out parallel with pathogen inactivation studies, also using appropriate molecular techniques.

This study has identified and contributed to key knowledge gaps regarding the group composition and ecological behaviour of phages infecting GB-124. Within the wider context of public health protection, this study has shown that non-pathogenic bacteriophages infecting GB-124 may prove useful indicator organisms for pathogenic enteroviruses, assisting in the development of risk assessments and reducing health burden from waterborne disease. Moreover, as the GB-124 phage-lysis method is low-cost and implementable in basic laboratories, use of the technique may aid in the achievement of the Millennium Development Goals relating to water quality (four and seven).

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APPENDIX I – Inactivation data reported in the literature

Table A1 UV-C fluences required for bacterial inactivation presented in the literature

Species	Fluence required per log_{10} reduction (mJ/cm^2)					Reference
	1	2	3	4	5	
Aeromonas hydrophila ATCC 7966	1.1	2.6	3.9	5	6.7	Wilson *et al*., 1992
Aeromonas salmonicida	1.5	2.7	3.1	5.9	-	Liltved and Landfald, 1996
Campylobacter jejuni biotype 1 strain 709/84	-	-	1.8		-	Butler *et al*., 1987
Campylobacter jejuni ATCC 43429	1.6	3.4	4	4.6	5.9	Wilson *et al*., 1992
Citrobacter freundii ATCC 8090	4.2	9.3	15	22	-	Zemke *et al*., 1990
Enterobacter cloacae ATCC 13047	7	12	22	34	-	Zemke *et al*., 1990
E. coli (wastewater isolate)	-	-	5	-	-	Butler *et al*., 1987
Escherichia coli ATCC 11229	2.5	3	3.5	5	10	Harris *et al*., 1987
Escherichia coli ATCC 11229	3	4.8	6.7	8.4	10.5	Chang *et al*., 1985
Escherichia coli ATCC 11229	5.1	8.4	15	25	34	Zemke *et al*., 1990
Escherichia coli ATCC 11229	<5	5.5	6.6	7.7	10	Zimmer and Slawson, 2002
Escherichia coli ATCC 11229	7	8	9	11	12	Hoyer, 1998
Escherichia coli ATCC 11229	3.95	5.3	6.4	7.3	8.4	Sommer *et al*., 1998
Escherichia coli ATCC 11303	4	6	9	10	13	Wu *et al*., 2005
Escherichia coli ATCC 29222	6	6.5	7	8	9	Sommer *et al*., 1998
Escherichia coli ATCC 25922	6	6.5	7	8	9	Sommer *et al*., 2000
Escherichia coli C	2	3	4	5.6	6.5	Otaki *et al*., 2003
Escherichia coli C300	2.5	4.8	6	7.5	10	Eischeid and Linden, 2007
Escherichia coli IFO3301	2	4	6	-	-	Oguma *et al*., 2001

Table A1 (cont.) UV-C fluences required for bacterial inactivation presented in the literature

Species	1	2	3	4	5	Reference
Escherichia coli K-12 IFO3301	1.5	2	3.5	4.2	5.5	Otaki *et al*., 2003
Escherichia coli (NCTC 10418)	1.33	-	-	5.32	-	Tree *et al*., 2005
Escherichia coli O157:H7	1.5	3	4.5	6	-	Tosa and Hirata, 1999
Escherichia coli O157:H7	<2	<2	2.5	4	8	Yaun *et al*., 2003
Escherichia coli O157:H7 ATCC 43894	1.5	2.8	4.1	5.6	6.8	Wilson *et al*., 1992
Escherichia coli O157:H7 CCUG 29193	3.5	4.7	5.5	7	-	Sommer *et al*., 2000
Escherichia coli O157:H7 CCUG 29197	2.5	3	4.6	5	5.5	Sommer *et al*., 2000
Escherichia coli O157:H7 CCUG 29199	0.4	0.7	1	1.1	1.3	Sommer *et al*., 2000
Escherichia coli O25:K98:NM	5	7.5	9	10	11.5	Sommer *et al*., 2000
Escherichia coli O26	5.4	8	10.5	12.8	-	Tosa and Hirata, 1999
Escherichia coli O50:H7	2.5	3	3.5	4.5	5	Sommer *et al*., 2000
Escherichia coli O78:H11	4	5	5.5	6	7	Sommer *et al*., 2000
Escherichia coli wild type	4.4	6.2	7.3	8.1	9.2	Sommer *et al*., 1998
Halobacterium elongata ATCC33173	0.4	0.7	1	-	-	Martin *et al*., 2000
Halobacterium salinarum ATCC43214	12	15	17.5	20	-	Martin *et al*., 2000
Helicobacter pylori (clinical isolate, Texas)	<2.3	<3.9	<5.4	<5.4	-	Hayes *et al*., 2006
Helicobacter pylori ATCC 43504	<6.2	<6.2	<6.3	<6.2	-	Hayes *et al*., 2006

Table A1 (cont.) UV-C fluences required for bacterial inactivation presented in the literature

Species	1	2	3	4	5	Reference
Helicobacter pylori ATCC 49503	<2.4	<4.3	<6	<6	-	Hayes *et al*., 2006
Klebsiella pneumoniae ATCC 13047	4.2	8.8	15	23	-	Zemke *et al*., 1990
Klebsiella terrigena ATCC 33257	4.6	6.7	8.9	11	-	Wilson *et al*., 1992
Legionella pneumohila Philadelphia 2 strain	0.92	1.8	2.8	-	-	Antopol and Ellner, 1979
Legionella pneumohila ATCC 33152	1.6	3.2	4.8	6.4	8.0	Oguma *et al*., 2004
Legionella pneumohila ATCC 43660	3.1	5	6.9	9.4	-	Wilson *et al*., 1992
Listeria monocytogenes (in fresh brine)	-	-	-	-	16.8	McKinney *et al*., 2009
Mycobacterium avium 33B	5.76	8.10	10.4	12.8	-	Hayes *et al*., 2008
Mycobacterium avium W41	5.74	7.93	10.1	12.3	-	Hayes *et al*., 2008
M. avium D55A01	6.39	9.37	12.4	15.3	-	Hayes *et al*., 2008
M. intracellulare B12CC2	7.83	10.6	13.4	16.1	-	Hayes *et al*., 2008
M. intracellulare ATCC13950	7.42	11.2	15	18.7	-	Hayes *et al*., 2008
Salmonella spp.	<2	2	3.5	7	14	Yaun *et al*., 2003
Salmonella anatum (human faeces)	7.5	12	15	-	-	Tosa and Hirata, 1998
Salmonella derby (human faeces)	3.5	7.5	-	-	-	Tosa and Hirata, 1998
Salmonella enteritidis (human faeces)	5	7	9	10	-	Tosa and Hirata, 1998
Salmonella infantis (human faeces)	2	4	6	-	-	Tosa and Hirata, 1998
Salmonella typhi ATCC 6539	2.7	4.1	5.5	7.1	8.5	Chang *et al*., 1985
Salmonella typhi ATCC 19430	1.8	4.8	6.4	8.2	-	Wilson *et al*., 1992

Table A1 (cont.) UV-C fluences required for bacterial inactivation presented in the literature

Species	1	2	3	4	5	Reference
Salmonella typhimurium (human faeces)	2	3.5	5	9	-	Tosa and Hirata, 1998
Salmonella typhimurium (activated sludge)	3	11.5	22	50	-	Maya *et al*., 2003
Shigella dysenteriae ATCC 29027	0.5	1.2	2	3	4	Wilson *et al*., 1992
Shigella sonnei ATCC 9290	3.2	4.9	6.5	8.2	-	Chang *et al*., 1985
Staphylococcus aureus ATCC 25923	3.9	5.4	6.5	10.4	-	Chang *et al*., 1985
Streptococcus faecalis	6.6	8.8	9.9	1.2	-	Chang *et al*., 1985
Streptococcus faecalis (secondary effluent)	5.5	6.5	8	9	12	Harris *et al*., 1987
Vibrio anguillarum	0.5	1.2	1.5	2	-	Liltved and Landfald, 1996
Vibrio cholerae ATCC 25872	0.8	1.4	2.2	2.9	3.6	Wilson *et al*., 1992
Yersina enterocolitica serogroup 0:3 biotype 4 strain 304/84	-	-	2.7	-	-	Butler *et al*., 1987
Yersina enterocolitica ATCC 27729	1.7	2.8	3.7	4.6	-	Wilson *et al*., 1992
Yersinia ruckeri	1	2	3	5	-	Liltved and Landfald, 1996

Table A2 UV-C fluences required for bacteriophage inactivation presented in the literature

Type (host)	Fluence required per log_{10} reduction (mJ/cm^2)				Reference
	1	2	3	4	
B40-8 (HSP-40)	12	18	23	28	Sommer *et al*., 1998
B40-8 (HSP-40)	11	17	23	29	Sommer *et al*., 2001
B40-8 (HSP-40)	7.1	14.3	21.4	28.6	Clancy *et al*., 2004b
MS2	3.9	25.3	46.7	68	Snicer *et al*., 1998
MS2	15.3	39.3	63.3	87.4	Snicer *et al*., 1998
MS2	-	-	-	64 - 93	Snicer *et al*., 1998
MS2	28.9	57.9	86.9	115.9	Roessler and Severin, 1996
MS2 ATCC 15977-B1 (*E. coli* ATCC 15977–B1)	15.9	34	52	71	Wilson *et al*., 1992
MS2 (*E. coli* ATCC 15597)	19	40	61	-	Oppenheimer *et al*., 1993
MS2 (*E. coli* ATCC 15977)	13.4	28.6	44.8	61.9	Meng and Gerba, 1996
MS2 (*E. coli* ATCC 15597)	23	55	87	119	Thurston-Enriquez *et al*., 2003
MS2 (*E. coli* ATCC 15597)	20	42	70	98	Lazarova and Savoye, 2004
MS2 (*E. coli* ATCC 15597)	~11	~28 to 30	~45 to ~48	-	Bohrerova *et al*., 2006
MS2 (*E. coli* CR63)	16.9	33.8	-	-	Rauth, 1965
MS2 (*E. coli* C3000)	35	-	-	-	Battigelli *et al*., 1993
MS2 (*E. coli* C3000)	~13	30	-	-	Shin *et al*., 2001
MS2 (*E. coli* C3000)	20	42	68	90	Linden *et al*., 2002a
MS2 (*E. coli* C3000)	20	42	69	92	Batch *et al*., 2004
MS2 (*E. coli* HS(pFamp)R)	-	45	75	100	Thompson *et al*., 2003
MS2 (*E. coli* K-12 Hfr)	21	36	-	-	Sommer *et al*., 1998
MS2 (*E. coli* K-12)	18.5	36	55	-	Sommer *et al*., 2001
MS2 DSM 5694 (*E. coli* NCIB 9481)	4	16	38	68	Wiedenmann *et al*., 1993
MS2 (*E. coli* NCIMB 9481)	14	-	-	62.5	Tree *et al*., 2005
MS2 (*Salmonella typhimurium* WG49)	16.3	35	57	83	Nieuwstad and Havelaar, 1994

Table A2 (cont.) UV-C fluences required for bacteriophage inactivation presented in the literature

Type	1	2	3	4	Reference
MS2 NCIMB 10108 (*Salmonella typhimurium* WG49)	12.1	30.1	-	-	Tree *et al*., 1997
Pseudomonas aeruginosa phage D3C3 (PAO303)	~6.2	~10.6	~17.5	-	Kadavy *et al*., 2000
Pseudomonas aeruginosa phage F116 (PAO303)	~8.6	~14.6	~18.1	-	Kadavy *et al*., 2000
Pseudomonas aeruginosa phage G101 (PAO303)	~4.3	~8.6	~11.6	-	Kadavy *et al*., 2000
Pseudomonas aeruginosa phage UNL-1 (PAO303)	~1.3	~2.5	~4.3	~6.7	Kadavy *et al*., 2000
PHI X 174 (E. coli ATCC 15597)	4	8	12	-	Oppenheimer *et al*., 1993
PHI X 174 (E. coli WG5)	2.2	5.3	7.3	10.5	Sommer *et al*., 1998
PHI X 174 (E. coli WG5)					Lazarova *et al*., 1998
PHI X 174 (E. coli WG 5)	3	5	7.5	10	Sommer *et al*., 2001
PHI X 174 (E. coli C3000)	2.1	4.2	6.4	8.5	Battigelli *et al*., 1993
PRD-1 (S. typhimurium Lt2)	9.9	17.2	23.5	30.1	Meng and Gerba, 1996
PRD-1 (S. typhimurium Lt2)	-	50	-	110	Shin *et al*., 2005
Staphylococcus aureus phage A994 (*Staphylococcus aureus* A994)	8	17	25	36	Sommer *et al*., 1989
T7	4.3	8.6	12.9	17.2	Clancy *et al*., 2004
Qβ	11.9	23.8	35.7	47.6	Clancy *et al*., 2004

Table A3 UV–C fluences required for viral inactivation presented in the literature

Species/type	Fluence required per log_{10} reduction (mJ/cm^2)				Reference
	1	2	3	4	
Adenovirus type 1 (CCL-2; ATTC)	34.4	68.9	103.4	137.9	Nwachuku *et al*., 2005
Adenovirus type 2 (A549 cell line)	20	45	80	110	Shin *et al*., 2005
Adenovirus type 2 (Human lung cell line)	35	55	75	100	Ballester and Malley, 2004
Adenovirus type 2 (PLC / PRF / 5 cell line)	40	78	119	160	Gerba *et al*., 2002
Adenovirus type 2 (A549 cell line)	45	85	125	165	Sirikanchana *et al*., 2008
Adenovirus type 2 (A549 cell line)	-	30	50	80	Eischeid *et al*., 2009
Adenovirus type 6 (CCL-2; ATTC)	38.5	76.9	115.4	153.8	Nwachuku *et al*., 2005
Adenovirus type 15 (A549 cell line)	40	80	122	165	Thompson *et al*., 2003
Adenovirus 40 (PLC / PRF / 5 cell line)	50	109	167	226	Thurston-Enriquez *et al*., 2003
Adenovirus 40 (ATCC – Dugan)	29.5	59.4	89.8	120.9	Meng and Gerba, 1996
Adenovirus 41 (ATCC – TAK)	22.4	49.5	80.2	-	Meng and Gerba, 1996
Calicivirus canine (MDCK cell line)	7	15	22	30	Husman *et al*., 2004
Calicivirus canine (CRFK cell line)	7	16	25	-	Husman *et al*., 2004
Calicivirus feline (CRFK cell line)	4	9	14	19	Tree *et al*., 2005
Calicivirus feline (CRFK cell line)	6	16	26	36	Thurston-Enriquez *et al*., 2003

Table A3 (cont.) UV-C fluences required for viral inactivation presented in the literature

Species/type (host)	1	2	3	4	Reference
Coxsackievirus B3 (BGM cell line)	8	16	24.5	32.5	Gerba *et al*., 2002
Coxsackievirus B5 (Buffalo Green Monkey cell line)	6.9	13.7	20.6	-	Battigelli *et al*., 1993
Coxsackievirus B5 (BGM cell line)	9.5	18	27	36	Gerba *et al*., 2002
Echovirus I (BGM cell line)	8	16.5	25	33	Gerba *et al*., 2002
Echovirus II (BGM cell line)	7	14	20.5	28	Gerba *et al*., 2002
Enterovirus	7.5	15	24	34.5	Lazarova and Savoye, 2004
Hepatitis A (HAV/HFS/GBM)	5.5	9.8	15	21	Wiedenmann *et al*., 1993
Hepatitis A HM175 (FRhK-4 cell)	5.1	13.7	22	29.6	Wilson *et al*., 1992
Hepatitis A HM175 (FRhK-4 cell)	-	-	-	7	Snicer *et al*., 1998
Hepatitis A HM175 (FRhK-4 cell)	4.1	8.2	12.3	16.4	Battigelli *et al*., 1993
Poliovirus	6	14	22	32.5	Lazarova and Savoye, 2004
Poliovirus Type 1 (Mahoney)	3	7	14	40	Sommer *et al*., 1989
Poliovirus Type 1 (ATCC Mahoney)	6	14	23	30	Harris *et al*., 1987
Poliovirus 1 (BGM cell line)	5	11	18	27	Tree *et al*., 2005
Poliovirus 1 (BGM cell line)	8	15.5	23	31	Gerba *et al*., 2002
Poliovirus 1 [CaCo2 cell-line (ATCC HTB37)]	7	17	28	37	Thompson *et al*., 2003
Poliovirus Type 1 LSc-1	4	8.7	14.2	20.6	Meng and Gerba, 1996
Poliovirus Type 1 LSc-1 (MA104 cell)	5.6	11	16.5	21.5	Chang *et al*., 1985

Table A3 (cont.) UV-C fluences required for viral inactivation presented in the literature

Species/type	1	2	3	4	Reference
Poliovirus Type 1 LSc-1	5.7	11	17.6	23.3	Wilson *et al*., 1992
Poliovirus Type 1 LSc-1	-	-	-	23 – 29	Snicer *et al*., 1998
Reovirus Type 1 Lang strain	16	36	-	-	Harris *et al*., 1987
Reovirus -3 (Mouse L-60)	11.2	22.4	-	-	Rauth, 1965
Rotavirus (MA 104) in freshwater	20	80	140	200	Caballero *et al*., 2004
Rotavirus (MA 104) in seawater	50	110	160	220	Caballero *et al*., 2004
Rotavirus SA 11 (MA 104)	8	15	27	38	Sommer *et al*., 1989
Rotavirus SA 11 (MA 104)	7.6	15.3	23	-	Battigelli *et al*., 1993
Rotavirus SA 11 (MA 104)	7.1	14.8	25	-	Chang *et al*., 1985
Rotavirus SA 11 (MA 104)	9.1	19	26	36	Wilson *et al*., 1992
Rotavirus WA 11 (MA 104)	-	-	-	50	Snicer *et al*., 1998

Table A4 UV-C fluences required for protozoan inactivation presented in the literature

Species (assay method)	Fluence required per log_{10} reduction (mJ/cm^2)				Reference
	1	2	3	4	
Cryptosporidium parvum (cell culture infectivity)	1	2	<5	-	Shin *et al*., 2001
Cryptosporidium parvum (animal?)	<2	<2	<2	<4	Clancy *et al*., 2004a
Cryptosporidium parvum (animal?)	<3	<3	3 - 6	<16	Clancy *et a*l., 2000
Cryptosporidium parvum (animal infectivity)	0.5	1	1.4	2.2	Morita *et al*., 2002
Cryptosporidium parvum	2	<3	<3	-	Zimmer *et al*., 2003
Cryptosporidium parum	-	-	-	<19	Bukhari *et al*., 1999
Cryptosporidium parum	-	-	<10	-	Shin *et al*., 1999
Cryptosporidium parvum, oocysts, tissue culture assay	1.3	2.3	3.2	-	Shin *et al*., 2000
Encephalitozoon cuniculi, microsporidia	4	9	13	-	Marshall *et al*., 2003
Encephalitozoon hellem, microsporidia	8	12	18	-	Marshall *et al*., 2003
Encephalitozoon intestinalis, Microsporidia	3	5	6	-	Marshall *et al*., 2003
Giardia lamblia	<1			<2	Shin *et al*., 2000
Giardia lamblia	-	-	-	-	Malley *et al*., 2000
Giardia lamblia	<10	~10	<20	-	Campbell *et al*., 2002
Giardia lamblia	<2	<2	<4	-	Mofidi *et al*., 2002
Giardia lamblia excystation assay	>63	-	-	-	Rice and Hoff, 1981

Table A4 (cont.) UV-C fluences required for protozoan inactivation presented in the literature

Species (assay method)	1	2	3	4	Reference
Giardia lamblia excystation assay	40	180	-	-	Karanis *et al*., 1992
Giardia lamblia, gerbil infectivity assay	<0.5	<0.5	<0.5	<1	Linden *et al*., 2002b
Giardia muris	<1.9	<1.9	~2	~2.3	Hayes *et al*., 2003
Giardia muris	<2	<2	<4		Mofidi *et al*., 2002
Giardia muris excystation assay	77	110	-	-	Carlson *et al*., 1985

Table A5 UV-C fluences required for sporal inactivation presented in the literature

Spore	Fluence required per log_{10} reduction (mJ/cm^2)				Reference
	1	2	3	4	
Bacillus anthracis Sterne	81	-	-	-	Knudson, 1986
B. anthracis Sterne	27.5	-	-	-	Nicholson and Galeano, 2003
B. subtilis	~35	40.4	~55	~70	Wang *et al*., 2010
B. subtilis (ATCC 6633)	36	48.6	61	78	Chang *et al*., 1985
B. subtilis (ATCC 6633)	29	40	51	-	Sommer *et al*., 1998
B. subtilis (ATCC 6633)	20	39	60	81	Sommer *et al*., 1999
B. subtilis (ATCC 6633)	26	-	-	-	Hoyer, 2000
B. subtilis (ATCC 6633)	24.5	-	-	-	Nicholson and Galeano, 2003
B. subtilis (ATCC 6633)	24	35	47	79	Mamane-Gravetz and Linden, 2004
B. subtilis (ATCC 6633)	24.5	-	-	-	Nicholson and Galeano, 2003
B. subtilis (ATCC 6633)	-	22.8	-	-	Bohrerova *et al*., 2006
B. subtilis (ATCC WN626)	24.5	-	-	-	Nicholson and Galeano, 2003
B. subtilis (ATCC WN626)	0.4	0.9	1.3	2	Marshall *et al*., 2003