THE CONTRIBUTION OF TEMPERATE PHAGES TO COLIPHAGE NUMBERS
DETECTED BY PLAQUE ASSAY

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A thesis submitted to the faculty at the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Master of Science in the Department of Environmental Sciences and Engineering in the Gillings School of Global Public Health.

Chapel Hill
2017

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ABSTRACT

Sharon Jiang: The contribution of temperate phages to coliphage numbers detected by plaque assay
(Under the direction of Jill Stewart)

Coliphages are potential indicators of human fecal contamination and enteric viruses in ambient water, but current detection methods may underestimate counts by overlooking effects of temperate phages that may reside within the cells of native bacteria. This study evaluated the contribution of temperate phages to male-specific and somatic coliphages numbers from diluted sewage detected by EPA Method 1602, the single agar layer plaque assay. Twenty-two parallel assays were run with and without induction using mitomycin C or ultraviolet light as inducing agents. Ultraviolet light appeared to have toxic effects but coliphage counts of samples exposed to mitomycin C were significantly higher (Wilcoxon signed rank, $p < 0.05$) than non-induced counts for both somatic and male-specific coliphages. The difference between induced and non-induced counts tended to be small but could affect regulatory decisions for counts close to threshold values.

ACKNOWLEDGEMENTS

I would like to thank my thesis advisor, Dr. Jill Stewart for her support and guidance throughout the course of my undergraduate and graduate work. I would also like to thank my committee members Dr. Louise Ball and Dr. Mark Sobsey for their valuable contributions to this thesis. This work was also supported by the advice and assistance of Sarah Rhodes, Claire Tipton, Emily Bailey, Collin Coleman, Yvonne Yuen, and the rest of the members of the Stewart and Sobsey lab groups. I am also grateful to KS Anderson for his aid with ultraviolet dose calculations and assumptions. This accomplishment would not have been possible without the help and support from all mentioned.

Thank you.

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LIST OF ABBREVIATIONS

CI   Confidence interval

DAL   Double agar layer

DNA   Deoxyribonucleic acid

EPA   Environmental Protection Agency

FIB   Fecal indicator bacteria

MMC   Mitomycin C

NRC   National Research Council

OD   Optical density

PBS   Phosphate-buffered saline

PFU   Plaque-forming units

PSI   Pounds per square inch

RNA   Ribonucleic acid

SAL   Single agar layer

UWRRC   Urban Water Resources Research Council

UV   Ultraviolet

CHAPTER 1: INTRODUCTION

Fecal indicator organisms, such as thermotolerant coliforms and *Escherichia coli* (*E. coli*), are easy-to-measure microorganisms that indicate the presence of fecal contamination in environmental media. When these organisms are detected, they imply the presence of pathogens and are considered the best available practical alternative to direct monitoring for fecal pathogens (Ashbolt et al. 2001; UWRRC 2014). The validity of an effective fecal indicator organism depends on its similarity in characteristics to fecal pathogens such as having similar survival and multiplication rates in the environment and similar physical, chemical and morphological properties. Because fecal indicator bacteria, a widely-used indicator group, are poor surrogates in representing and detecting human enteric viruses in water, coliphages, bacteriophages of *E. coli* bacteria, have been proposed as better indicators of viruses in fecally-contaminated water (EPA Office of Water 2015; Jofre et al. 2016).

Currently, somatic and male-specific coliphages are approved indicators for source water and shellfish monitoring in the United States (EPA 2009; NSSP 2011) and for Type 2 reclaimed water in North Carolina (NCAC 2011). Somatic coliphages are DNA viruses that infect by attaching to receptor sites located on the cell wall of *E. coli*. Male-specific coliphages are single-stranded RNA (*Leviviridae* group) and DNA (*Inoviridae* group) viruses that infect by attaching to receptor sites located on the fertility fimbriae of male strains of *E. coli* (NRC 2004). Recently, coliphages have begun to be reviewed as ambient water quality indicators due to the need for a viral indicator of recreational water quality (EPA Office of Water 2015). A limitation to their usefulness as indicators is their potential to replicate within *E. coli* cells outside the intestinal

microflora of warm-blooded animals, through lytic infection and lysogenic induction. Wiggins and Alexander found that different coliphages require different *E. coli* densities to replicate, usually between 10^3 and 10^4 colony-forming units per milliliter (CFU mL^{-1}) (1985). Similarly, Woody and Cliver suggested that F-specific RNA coliphage cannot replicate in nutrient-poor environments when host cell density is below 10^4 CFU mL^{-1} (1997). However they found that the addition of *Enterococcus* to the host cell culture resulted in increased phage yields, suggesting that the addition of *Enterococcus* supplies a factor that enhances phage replication (Woody and Cliver 1997). These two studies suggest that coliphages are not likely to replicate in environmental waters of interest such as groundwater because the host cell density is not high enough to support replication. Otherwise information is sparse on whether there is an underestimation of coliphages from environmental samples by current detection methods because they may neglect the impact of temperate phages, bacteriophages that have the ability to enter the lysogenic life cycle. Rather than immediately lysing the host bacteria they infect, temperate phages can integrate into the genome of their hosts as a prophage or occur as an extrachromosomal plasmid within the host bacterium. The host bacterium continues to function and reproduce normally until the lytic cycle is triggered by an induction event.

This study aims to evaluate the significance of the contribution of temperate phages to male-specific and somatic coliphages counts from diluted raw sewage detected by EPA Method 1602. Prophage induction on native bacteria from diluted sewage was done by mitomycin C and ultraviolet radiation. If the contribution of temperate phages is significant enough to surpass coliphage criteria levels set by government regulations, then it would suggest that more research is needed to approve coliphages as fecal indicators for ambient water quality monitoring.

CHAPTER 2: OBJECTIVES

1. To compare plaque counts of somatic coliphages from diluted sewage samples with and without induction of lysogenic bacteriophages.

2. To compare plaque counts of male-specific coliphages from diluted sewage samples with and without induction of lysogenic bacteriophages.

CHAPTER 3: REVIEW OF LITERATURE

Historically, fecal indicator bacteria (FIB) have been used to detect and determine the

level of fecal contamination in environmental waters in order to protect the general population

from water-related pathogens associated with human excreta (EPA 2006; EPA Office of Water

2015). However, FIB are poor indicators of waterborne pathogens that are not bacteria such as

enteric viruses which have been suggested as significant causative agents of recreational

waterborne illnesses (Sinclair et al. 2009; Soller et al. 2010). Instead of FIB, bacteriophages

(phages), or viruses which infect bacteria, have been proposed as potentially valuable indicators

for enteric viruses from fecal contamination because they share fundamental characteristics with

viral pathogens such as structure, composition, morphology, size, and site of replication (Grabow

2001; Ashbolt et al. 2001; EPA Office of Water 2015). Another advantage of using phages as

fecal indicators is that they are relatively easy, quick, and inexpensive to cultivate in laboratories.

Coliphages, in particular male-specific and somatic coliphages, have been given the most

attention in the United States as an indicator of human viral pathogens. Currently, coliphages are

one of the approved fecal indicator microorganisms that can be used to monitor microbial water

quality in public groundwater systems (EPA 2006).

Coliphages as fecal indicators

An ideal fecal indicator should be correlated to health risk, have similar or greater

survival to fecal pathogens, have similar or greater transport to fecal pathogens, be present in

greater numbers than fecal pathogens, and be specific to a fecal source or an identifiable source

of origin (NRC 2004). While no ideal indicator exists, coliphages exhibit many desirable

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attributes because they are specific to and numerous in the gastrointestinal tract of humans and other warm-blooded animals (Grabow 2001). In one gram of human feces, there can be up to 10^4 coliphages (Fortier and Sekulovic 2013). Coliphages also survive in similar ways as other enteric viruses because they are non-enveloped viruses, making them more resistant to degradation and inactivation in the environment (Grabow 2001).

Currently, somatic and male-specific coliphages are considered approved indicators for monitoring shellfish and source water quality (EPA 2009; NSSP 2011). Somatic coliphages are DNA viruses that infect by attaching to receptor sites located on the cell wall of *E. coli*. Male-specific coliphages are single-stranded RNA (*Leviviridae* group) and DNA (*Inoviridae* group) viruses that infect by attaching to receptor sites located on the fertility fimbriae of male strains of *E. coli* (NRC 2004). These fertility fimbriae are produced only when the temperature is above 30°C. Because of this temperature requirement, male-specific coliphages are unlikely to replicate in the environment, further supporting their ability as surrogates for many enteric viruses which also fail to multiply in the environment (Grabow 2001). A subgroup of male-specific coliphages is the male-specific RNA coliphage group. Male-specific RNA coliphages in particular have physical structures, composition, and morphology that closely resemble those of many human enteric viruses, making them attractive viral indicator candidates (Grabow 2001). Male-specific RNA coliphages can be further divided into four groups, through serotyping or genotyping, by whether they are of human or animal origin, although there is some overlap of the groups (NRC 2004).

Some studies indicate that somatic coliphages numbers may exceed those of male-specific coliphages in fecally-contaminated water, and may be more persistent in warmer water than male-specific coliphages (Grabow 2001; Lee and Sobsey 2011; Jofre et al. 2016). But

overall, coliphages can be considered equally persistent as or more persistent than enteric viruses (EPA Office of Water 2015). Although there have been many disparate results, there exists epidemiological evidence suggesting a relationship between coliphages and gastrointestinal illness in humans from recreational exposure (Colford et al. 2007; Abdelzaher et al. 2011; EPA Office of Water 2015; Griffith et al. 2016; Jofre et al. 2016). Even without a clear correlation between coliphage densities and human viruses in water, there is evidence that somatic and male-specific coliphages are more strongly associated with pathogenic viruses than traditional FIB (EPA Office of Water 2015; Jofre et al. 2016). A summary of how coliphages compare to *Enterococci* and *E. coli*, traditional fecal indicator bacteria, in terms of ideal indicator attributes can be found in Table 1.

According to Table 1, coliphages meet or exceed *Enterococci* and *E. coli* in many indicator attributes, supporting coliphages as fecal indicator microorganisms. However, a potential objection to the use of coliphages as indicators of ambient water quality is their potential replication within *E. coli* cells that may occur in aquatic systems (EPA Office of Water 2015; Jofre et al. 2016). Coliphages can replicate in the environment by the lytic and lysogenic life cycles, the details of which are described in the subsequent subsections.

Table 1. Attribute comparison of *Enterococci*, *E. coli*, and coliphages as indicators of fecal contamination in water

Indicator Attribute	*Enterococci*	*E. coli*	Coliphages
Exist in the intestinal microflora of humans and other warm-blooded animals	Yes	Yes	Yes
Present when pathogens are present	Yes, but not indicative of viruses in wastewater treatment plant effluent	Yes, but not indicative of viruses in wastewater treatment plant effluent	Yes, and a better surrogate for viruses in wastewater treatment plant effluent
Absent in uncontaminated samples	May be present in nonfecally-contaminated ambient water	May be present in nonfecally-contaminated ambient water	Likely but not always absent in nonfecally-contaminated ambient water
Present in greater numbers than human viruses	In raw sewage, but not in treated effluent	In raw sewage, but not in treated effluent	In most cases
Equally resistant as viruses to environmental factors	No	No	Under most conditions
Equally resistant as viruses to disinfection in water and wastewater treatment plants	No, except for ozone	No, except for ozone	Under most conditions, but not as resistant as adenovirus
Do not multiply in the environment	Can in some environments in association with vegetative matter	Can under certain conditions	No, but not likely enough to affect criteria levels
Quick, easy, and inexpensive to detect	By EPA Method 1600 and 1611	By EPA Method 1603, but not considered rapid	By EPA Method 1601 and 1602, but quantification validation is needed
Nonpathogenic	Can be in some settings like hospitals, but generally not in ambient water	Generally nonpathogenic, but certain pathogenic strains are known.	Yes
Demonstrated association with illness from epidemiological studies	Yes	Yes	Yes
Specific to a fecal source or identifiable as to source of origin	Not by EPA Method 1600, but methods are being developed	Not by EPA Method 1603, but methods are being developed	Not by EPA Method 1601 and 1602, but methods are being developed

Table adapted from EPA Office of Water 2015.

The lytic and lysogenic life cycles

The two main lifecycle categories of phages are virulent and temperate phages. While virulent phages can only replicate through a lytic cycle, temperate phages can replicate through a lytic or lysogenic cycle. After the phage attaches to the host cell at a receptor site, injection of the

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phage genome into the host occurs. If the phage follows the lytic life cycle, then the genome of the virulent phage is immediately replicated and packaged into virions using the replication mechanism of the host cell. The host cell bursts, or lyses, and the newly-created virions are released into the environment. The alternative to the lytic cycle is the lysogenic cycle, where lytic multiplication is repressed and the temperate phage genome is incorporated into the genome of the host. The repressed phage DNA, or prophage, is passed on to daughter cells every time the host cell undergoes cell division (Golais et al 2013; Bondy-Denomy and Davidson 2014). A lysogen, or lysogenic bacterium, is a bacterial cell that contains prophage, which is usually integrated into the host bacteria's genome. Although different individuals of a specific temperate phage always have the same integration site on the phage chromosome, the integration of phage DNA doesn't necessarily occur at the same site in a bacterial chromosome. For example in *E. coli*, phage λ DNA usually integrates at one site while phage Mu DNA integrates randomly into host DNA (Casjens 2003). Sometimes rather than integrating, the prophage can be present as or in an extrachromosomal plasmid within the host bacterium.

Prophages are one of the main sources of genetic diversity and strain variation of *E. coli* (Fortier and Sekulovic 2013). Ubiquitous and numerous in bacterial communities, prophages can constitute up to 20% of a bacterial genome. However, some of these prophages can be defective or undergoing mutational decay (Paepe et al. 2016). Such prophages that may still contain functional genes but are unable to cause the full phage replication cycle are referred to as defective or cryptic prophages (Casjens 2003). If the prophage remains fully functional, lysogeny is typically maintained through a phage repressor binding to phage operator sequences to repress early promoters of the lytic cycle (Fortier and Sekulovic 2013). The lysogenic cycle can lead to the lytic cycle following an induction event which can occur spontaneously at random or after an

environmental trigger such as exposure of the host bacterium to a chemical or physical stressor. An induction event usually requires proteolytic cleavage and displacement of the phage repressor, causing prophage genes required for lytic growth to be turned on (Fortier and Sekulovic 2013). In a laboratory setting, proper prophage induction depends on factors such as the concentration of the inducing agent and temperature of incubation to protect against mortality of bacteria that are not lysogens (Raya and Hébert 2009). In the environment, induction rates are usually too low to be considered a cost to host bacteria communities. Instead, prophages and other prophage-related entities can provide a competitive advantage for the remaining lysogenic population (Casjens 2003).

Prophage and prophage-related entities

Besides fully functional and defective prophages, there are three other types of prophage-related entities: satellite prophages, bacteriocins, and gene transfer agents. Rather than carrying their own virion structural genes, satellite prophages have evolved to rely on being encapsidated by the virion proteins of other phages. Bacteriocins are bacterially-produced proteins that kill or inhibit the growth of other bacteria. Gene transfer agents are bacterially-produced phage-like particles that mediate horizontal gene transfer. The universal presence and the evolutionary stability of prophage-related bacteriocins and gene transfer agents suggests that there may be a positively-selected function associated with such entities (Casjens 2003). Sometimes fully functional prophage gene products expressed by the lysogen are also advantageous to the bacterial host. In the λ genome, the products of the *bor* gene expressed during lysogeny become outer membrane lipoproteins of *E. coli* lysogens. Similar to previously studied virulence gene products, Bor aids the lysogen in resisting serum complement killing perhaps by altering serum

sensitivity (Barondess and Beckwith 1995). Such genes that are expressed from the prophage in a lysogen and alter the properties of the lysogen are called lysogenic conversion genes.

Inducing agents

In bacteriophage λ, lysis and lysogeny depend on the relative expression of regulatory proteins CI (promotes lysogeny) and Cro (promotes lysis). The CII protein, which represses transcription from the lytic promoters and positively regulates its own synthesis, depends on factors that measure the cellular energy level of the host. In low nutrient conditions, the signaling molecule cyclic AMP (cAMP) is high, promoting CII stabilization and lysogeny. Under sufficient nutrient conditions, cAMP levels are reduced within the host which also reduces the level of lysogenization. The lysogenic induction, also known as prophage induction, occurs when the SOS system of the host bacterium is activated in response to DNA damage. The SOS response activates a protein that degrades the CI repressor (the lysogeny promoter), allowing for the excision of the prophage and the recommencement of the lytic cycle (Raya and Hébert 2009). As DNA-damaging agents, mitomycin C (hereafter referred to as MMC, although MC is another common abbreviation) and ultraviolet (UV) radiation are often used for activating the SOS system of the host bacterium, resulting in prophage induction (Raya and Hébert 2009; Fortier and Sekulovic 2013).

Mitomycin C. After observing that the impaired DNA synthesis of *E.coli B* cells treated with MMC could be restored by infection with phage *T2r²*, Otsuji et al. found that MMC could also induce the development of active phages in the lysogenic strain of *E. coli* K-12 (1959). Since then MMC has been used to induce prophages from a variety of bacteria, including *Yersinia* strains and *Streptococcus* strains (Casjens 2003). MMC is an antibiotic that damages bacterial DNA which activates the lytic life cycle of temperate phages (Raya and Hébert 2009).

It can form crosslinks between complementary DNA strands, which can lead to mutagenesis, apoptosis, the inhibition of DNA synthesis, and the initiation of DNA repair events. Rather than MMC reacting directly with DNA, it is only after a reductive activation cascade of MMC, culminating in the opening of an aziridine ring, that the activated MMC can covalently react to DNA and create cross links, see Figure 1 (Tomasz 1995).

The suggested concentration of MMC that should be used for prophage induction ranges from 0.1 to 2 µg mL^{-1}, as higher concentrations can be toxic for bacterial cells (Raya and Hébert 2009). The use of MMC as an inducing agent for prophages from water samples can be found in many studies on lysogenic bacteria, strengthening its status as a commonly used inducing agent along with ultraviolet radiation (Jiang and Paul 1996; Jiang and Paul 1997; Muniesa and Jofre 2007).

Figure 1. The reductive activation cascade of mitomycin C resulting in interstrand crosslinking of DNA
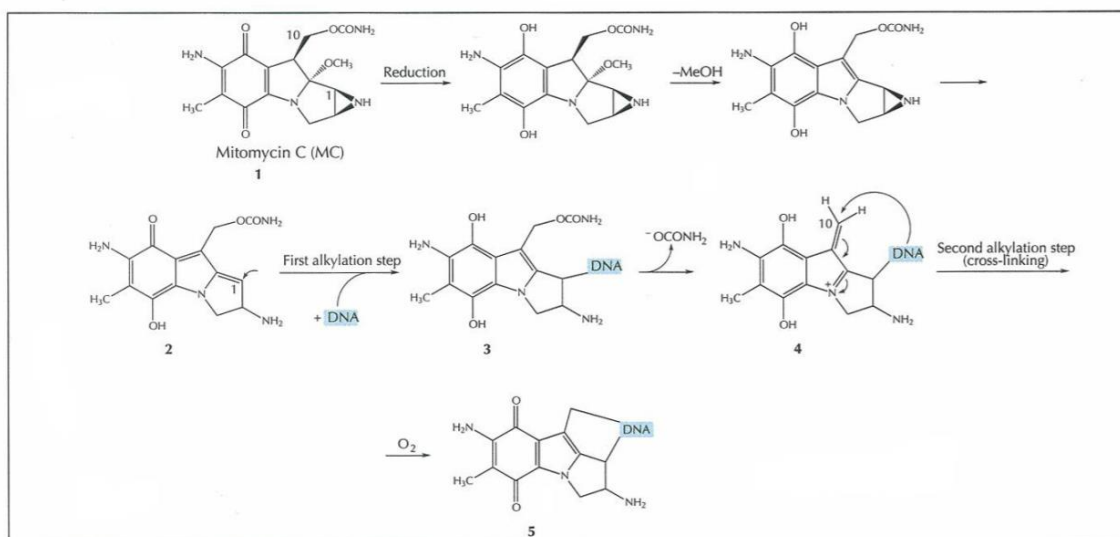
Figure originally from Tomasz 1995.

Ultraviolet radiation. UV radiation is form of electromagnetic radiation involving the wavelength region between 100 and 480 nm. The most energetic UV wavelengths, wavelengths between 100 and 200 nm, are referred to as vacuum UV or Schumann UV. UV-C radiation involves wavelengths between 200 and 280 nm, while UV-B and UV-A radiation involves wavelengths from 280 to 315 nm and 315 to 380 nm, respectively. UV radiation, particularly in the UV-B and UV-C regions, is absorbed by DNA, which leads to photoproducts such as pyrimidine dimers. Photoproducts directly related to exposure to UV-C radiation include DNA-protein cross-links and DNA single-strand breaks. (Kiefer 2007). DNA-protein cross-links caused by UV radiation activates cellular repair systems, including the RecA protein degradation of a repressor that suppresses lysis which allows a prophage to excise and begin the lytic cycle (Weinbauer and Suttle 1996). Many studies use UV-C to induce prophages, though the final UV dose can vary substantially, see Table 2 (Jiang and Paul 1996; Weinbauer and Suttle 1996; Jiang and Paul 1997; Muniesa and Jofre 2007). The variation in UV doses likely depends on the volume of sample that was subjected to UV radiation and the length of time the induced sample was incubated afterward.

Table 2. Ultraviolet (254 nm) doses used in selected prophage induction studies

Induction study (Authors, Year Published)	Ultraviolet dose for prophage induction ($J\ m^{-2}$)
Jiang and Paul 1996	1.476×10^2
Jiang and Paul 1997	$6.96 \times 10^4 - 1.62 \times 10^5$
Muniesa and Jofre 2007	45
Weinbauer and Suttle 1996	$20 - 3 \times 10^3$

See Appendix A for calculation steps used in determining these doses.

Currently, there are relatively few studies on the potential contribution of temperate phages to coliphage numbers in fecally-contaminated water. Several past studies on lysogeny and coliphages have focused on the contribution with regards to marine samples and native bacteria, not specifically coliphages. The following subsections will focus on the currently available literature on lysogenic contribution to bacterial numbers.

Current knowledge of lysogenic contribution in environmental samples

It is theorized that lysogeny is the preferred lifecycle when the host bacteria population is too low for the maintenance of continued lytic infections. This theory has found support by observations that demonstrate an increased prevalence of lysogeny in oligotrophic environments where there is a low density of slow-growing bacteria, but also contradiction from studies finding no significant difference between the percentage of lysogenic cells in oligotrophic and eutrophic environments (Weinbauer and Suttle 1996; Jiang and Paul 1997; Chibani-Chennoufi et al. 2004).

Marine environments. Study results on marine environments are highly varied concerning the incidence of lysogenic bacteria in marine environments and whether they are an important source of phage production. Weinbauer and Suttle (1996) reported that lysogenic bacteria induced by MMC ranged from 0.07 to 4.4% of the total bacterial community while Jiang and Paul found that 43% of their studied bacterial community was lysogenized (1996). Although these percentages vary significantly, both studies concluded that under normal conditions, lysogenic viral production does not contribute significantly to the total population (Weinbauer and Suttle 1996; Jiang and Paul 1997). Weinbauer and Suttle also noted that induction with MMC seemed most efficient although no statistical significance could be established (1996). In summary, prophages can be induced from marine samples, though the percentage of the total

bacterial community that lysogenic bacteria constitutes is highly variable, but overall are not considered to contribute significantly to viral counts in the environment.

Fecally-contaminated waters. Muniesa and Jofre found that prophages did not significantly contribute to the numbers of free somatic coliphages in a variety of environmental samples including raw sewage, river water, and sea water (2007). Although they found that induced samples were significantly different from non-induced ones (Students' *t*-test, $p < 0.05$), in most samples the ratio between the numbers of induced phages and non-induced phages approached 1, meaning the difference was small. Using the Most Probable Number method, the authors estimated that in a sample containing more than 10^5 *E. coli* cells there were 6.5 [3.5, 12] MMC-inducible cells per mL, 4.2 [1.7, 10.2] ciprofloxacin-inducible cells per mL and 1.4 [0.5, 4.0] UV-inducible cells per mL (Muniesa and Jofre 2007). Similar to Weinbauer and Suttle (1996), the authors noted that induction with MMC seemed most efficient but could not establish a significant difference due to the overlap of the lower and upper limit ranges (Muniesa and Jofre 2007). Muniesa and Jofre concluded that the induction of prophages, whether from contamination or from native bacteria, does not significantly contribute to the numbers of free somatic coliphages in environmental waters (2007).

Study design concerns. It is possible that studies searching for the effect of prophage induction may result in a minimum number of functional prophages because they depend on successful induction and use of permissive indicator strains (Casjens 2003). On the other hand, it is also possible that laboratory-induced prophage numbers could be higher than the numbers that are induced in the environment due to the controlled and ideal conditions set during the specific search of prophages. Additionally, commonly used inducing agents such as MMC and UV-C are unlikely to be found in the environment. Jiang and Paul noted that without inducing agents,

lysogenic bacteria are likely a small source of phage numbers (1997). In short, there is likely a difference between the number of prophages that exist in the environment, the number that is naturally induced in the environment, and the number that is detected and induced in a laboratory setting. Jiang and Paul noted that lysogenic production of viruses may have a delayed amplification effect, where progenies of the originally infected bacterium after an induction event may produce in turn a larger number of viruses (1997). Finally, the variety of modeling methods and statistical tests were used to understand prophage induction data may also affect the reported results. For example, in a study done by Muniesa and Jofre, Student's *t*-test was applied to understand the difference between assays of non-induced and induced coliphages (2007). However, an assumption of Student's *t*-test is that there is an underlying Normal distribution, which may not be the case for coliphages in the environment.

    Coliphages and water quality guidelines. Because many regulatory water quality guidelines are based on threshold levels, even small differences in indicator concentrations may affect whether or not waters are in compliance. For example, according to the National Shellfish Sanitation Program, emergency-closed shellfish growing areas cannot be reopened for harvest if male-specific coliphage counts exceed background levels or a level of 50 coliphages per 100 grams of shellfish sample (NSSP 2011). It may be possible for a growing area to be falsely opened to harvest because temperate phages are not being detected with current plaque assay methods. Another example of a regulation that involves coliphage threshold levels is the regulation of Type 2 reclaimed water in North Carolina. In North Carolina, Type 2 reclaimed water is highly treated wastewater reclaimed water that can be used for indirect contact irrigation of food crops that do not need to be peeled, skinned, cooked, or thermally processed before consumption (NCAC 2011). Type 2 reclaimed water requires a log 5 or greater reduction in

coliphages as well as a monthly geometric mean of less than or equal to 5 plaque-forming units per 100 milliliters and a daily maximum of less than or equal to 25 plaque-forming units per 100 milliliters (NCAC 2011). While lysogenic contribution may be small in native bacterial communities, the contribution may be significant in compliance situations based on such threshold levels where the addition of a few coliphages may result in a significantly different compliance status. Thus, more research is required to better understand the contribution of temperate phages to counts from plaque assays that may be used for determining water quality compliance status.

CHAPTER 4: METHODS

Sample collection and processing

The raw sewage samples used in these experiments came from the Mason Farm Wastewater Treatment Plant run by the Orange County Water and Sewer Authority, a public, non-profit agency that services the southern Orange County area of North Carolina. The grab samples were collected in autoclaved bottles in the morning, usually between 7:00 and 9:00AM, and stored on ice until processing which occurred within eight hours of collection. Samples were collected from October 19, 2016 to February 16, 2017. The samples for somatic coliphage detection were diluted 100-fold for processing while the samples for male-specific coliphage detection were diluted 10-, 50-, or 100-fold in order to achieve countable dilutions.

Enumeration of coliphages by the single agar layer procedure

The standard single agar layer (SAL) plaque assay method (EPA 2001) was used to quantify male-specific and somatic coliphages from the diluted raw sewage samples. For practical reasons, 60 mL of sample water was processed using EPA Method 1602. The sewage samples were diluted with sterile 0.01M phosphate-buffered saline (PBS), up to a hundred-fold, for readable plate counts. Each experiment consisted of a standard SAL assay on non-induced coliphages, an assay of MMC-induced coliphages, and an assay of ultraviolet-induced coliphages. Duplicates were run for each assay. Overall, the SAL assay required three days to complete, not including experimental preparations. Preparation of stock solutions, coliphage stock, and host bacteria stock cultures were prepared in advance when possible. Preparation of the overnight *E. coli* host occurred on the first day. Sample collection and processing occurred

on the second day, and assay results were read on the third and final day. These processes are detailed in the subsequent sections.

Preparation of stock solutions. The antibiotic stock solutions, stock magnesium chloride, and MMC stock solution were prepared in advance whenever possible. Stock nalidixic acid for the growth of *E. coli* CN-13 was prepared by dissolving 1 g of nalidixic acid sodium salt in 100 mL of autoclaved deionized water and filtering the solution through a sterile, 0.22-µm-pore-size membrane filter assembly. Stock ampicillin/streptomycin for the growth of *E. coli* F_{amp} was prepared by dissolving 0.15 g of ampicillin sodium salt and 0.15 g streptomycin sulfate in 100 mL of autoclaved deionized water and filtering the solution through a sterile, 0.22-µm-pore-size membrane filter assembly. For long-term storage, 10 mL aliquots of the antibiotic stocks were labeled, dated, and stored at -20°C. Prior to use, the antibiotic stocks were thawed at room temperature and vortexed well.

Stock magnesium chloride (80×, 4M) was prepared by adding 300 mL deionized water to 814 g of magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$). After all of the $MgCl_2 \cdot 6H_2O$ was dissolved, the stock solution was brought to a final volume of 1 L with additional deionized water, mixed thoroughly, and autoclaved for 15 minutes at 121°C and 15 psi. The stock magnesium chloride was stored at 4°C. Stock MMC ($C_{15}H_{18}N_4O_5$) was prepared by dissolving 2 mg of solid MMC in 5 mL of autoclaved deionized water (0.4 mg mL^{-1}). The stock MMC was stored in the dark at 4°C. Prior to use, the stock magnesium chloride and MMC were mixed or vortexed well.

Preparation of coliphage stock and host bacteria stock cultures. Preparation of coliphage stock requires three consecutive days. The frozen coliphage stocks that were prepared were MS2 (ATCC® 15597-B1™) and phi-X 174 (ATCC® 13706-B1™). Overnight *E. coli* host culture

must be prepared on the first day: *E. coli* F_{amp} (ATCC® 700891^{TM}) for MS2 coliphage and *E. coli* CN-13 (ATCC® 700609^{TM}) for phi-X 174 coliphage. On the second day, log-phase host bacteria culture must be prepared using the overnight culture. Detailed steps on making the overnight and log-phase host culture can be found in the subsequent sections. In an autoclaved shaker flask, 1 mL of the log-phase host bacteria was added to 50 mL half-strength tryptic soy broth along with 500 µL antibiotic (ampicillin/streptomycin for MS2, nalidixic acid for phi-X 174), 625 µL 4M $MgCl_2$, and 10 µL frozen virus stock. The flask was then incubated overnight (18 to 20 hours) at 36°C ± 1.0°C with the shaker set to 100 rpm. After incubation, the overnight coliphage stock was poured into a sterile 50 mL conical centrifuge tube along with 5 mL of chloroform ($CHCl_3$), vortexed for two minutes, and then centrifuged at 4°C and 3000 rpm for 30 minutes. After centrifugation, the supernatant was extracted from the conical centrifuge tube and pipetted into a 100 mm sterile petri plate, all the while being careful not to disturb the chloroform layer or the pellet at the bottom of the tube. The plate with the supernatant was then left open inside a laminar flow hood for about thirty minutes to allow any remaining chloroform to off-gas. The coliphage stock was then pipetted into a new sterile 50 mL conical centrifuge tube along with a 40% glycerol solution in a 1:1 ratio. The coliphage stock was divided into 1 mL aliquots, labelled, dated, and stored in cryogenic storage vials at -80°C. Prior to use, the coliphage stock was thawed at room temperature and vortexed well. The double agar layer plaque assay procedure was used to enumerate stock suspensions of MS2 and phi-X 174 for use in spiking the positive controls for ongoing precision and recovery.

To create frozen host bacteria stock cultures of *E. coli* F_{amp} and *E. coli* CN-13, a reference host bacterial culture of each strain was streaked onto a 1.5% tryptic soy agar plate with the appropriate antibiotic (ampicillin/streptomycin for *E. coli* F_{amp}, nalidixic acid for *E. coli* CN-13)

and incubated overnight at 36°C ± 1.0°C. After incubation, an individual colony was picked, inoculated into tryptic soy broth with the appropriate antibiotic, and allowed to grow to log phase. The log-phase host bacteria broth was harvested by mixing sterile glycerol and broth in a ratio of 1:4 in 2 mL cryogenic freezer vials which were labeled, dated, and stored at -80°C until use.

Day one: Preparation of overnight E. coli host cultures. An overnight *E. coli* host culture was used to create the log-phase culture because an inoculum from the overnight culture reaches log phase faster than an inoculum from frozen stock (EPA 2001). In a sterile shaker flask, a loopful of *E. coli* frozen stock culture was added to 25 mL of tryptic soy broth and 250 µL of appropriate antibiotic (ampicillin/streptomycin for *E. coli* F_{amp}, nalidixic acid for *E. coli* CN-13). The flask was then capped, labeled, and secured in a shaker and incubated at 36°C ± 1.0°C and 100 rpm overnight (18 to 20 hours). After incubation, the flasks containing overnight *E. coli* F_{amp} and *E. coli* CN-13 cultures were chilled on ice until ready for use.

Day two: Sample collection and processing. After returning from sample collection, the raw sewage samples were kept at 4°C until use. The overnight *E. coli* F_{amp} and *E. coli* CN-13 cultures were used to inoculate log-phase *E. coli* F_{amp} and *E. coli* CN-13 cultures. In a sterile shaker flask, 0.7 mL of overnight *E. coli* F_{amp} was added to 50 mL tryptic soy broth and 500 µL ampicillin/streptomycin stock solution. In a separate sterile shaker, 0.9 mL of overnight *E. coli* CN-13 was added to 50 mL tryptic soy broth and 500 µL nalidixic acid stock solution. More *E. coli* CN-13 was inoculated into its log-phase flask relative to the *E. coli* F_{amp} log-phase flask because it was observed that *E. coli* CN-13 took slightly longer time compared to *E. coli* F_{amp} to reach a similar absorbance reading as an estimate of concentration. The flasks were capped and incubated at 36°C ± 1.0°C and 100 rpm for about an hour. After an hour, 1 mL of culture from

each flask was dispensed into a cuvette for absorbance reading at 520 nm. An absorbance reading between 0.1 and 0.5 optical density (OD) is an indication of log-phase growth (EPA 2001). After an hour, the absorbance reading at 520 nm for both flasks was between 0.2 and 0.3 OD. While the log-phase host bacteria cultures were growing, 850 mL of double-strength agar was prepared in two bottles by dissolving and heating 25.5 g tryptic soy broth and 7.65 g agar in deionized water. The double-strength agar was then autoclaved for 15 minutes at 121° and 15 psi. After autoclaving, the bottles containing double-strength agar were placed on stir plates to cool.

Bottles containing 0.01 M PBS as diluent were prepared for the raw sewage dilutions. Because duplicates of each assay were done, a total of twelve 250 mL bottles were used for sample analysis. The somatic bottles contained 59.4 mL of diluent for a one-hundred-fold dilution while the male-specific bottles contained 58.8 mL diluent for a one-fiftieth-fold dilution. After the log-phase cultures were confirmed ready for use by the absorbance readings, the diluted sewage sample (600 µL for somatic bottles, 1200 µL for male-specific bottles) was added to the bottle of diluent along with 750 µL magnesium chloride and 1200 µL appropriate antibiotic (final concentration of 100 µg mL^{-1} of nalidixic acid for somatic coliphages; and 15 µg mL^{-1} of streptomycin and 15 µg mL^{-1} of ampicillin for male-specific coliphages). To the MMC bottles, 75 µL of stock MMC was added so that the concentration in the sample, before the addition of double-strength tryptic soy agar, was 0.05 µg mL^{-1}, a concentration known to induce prophages (Raya and Hébert 2009). Before being combined with double-strength tryptic soy agar, the raw sewage of UV sample bottles was first subjected to a UV dose equivalent to 45 J m^{-2}. This was achieved by pipetting raw sewage into a 90 mm sterile petri dish 10 in beneath a short wave (UV-C) 254nm bench lamp (Spectroline XX-15F) for 2.9 ± 0.3 s. This approach has

been previously used by Muniesa and Jofre (2007) to induce coliphages in a similar study and the UV dose has been previously evaluated by O'Brien et al. (1984) to induce lysogenic bacteriophage 933W at similar rates to a final concentration of 0.5 ug mL^{-1} MMC. To see how the UV dose was calculated, refer to Appendix A.

Once all sample bottles contained diluted sewage, antibiotics, and magnesium chloride, they were placed into a water bath of 45° to 48°C for 5 minutes. The double-strength agar bottles were also kept in the same water bath to keep the agar molten. Next, 6 mL of the appropriate log-phase *E. coli* host (*E. coli* F$_{amp}$ for male-specific and *E. coli* CN-13 for somatic) and 60 mL double-strength agar was added to the sample bottle and gently mixed before being poured into three labelled, sterile 150×15 mm petri dishes, about 40 mL per dish. During this procedure, the sample and host bacteria remained in contact for at least three but no more than ten minutes before plating to prevent replication of phages (EPA 2001). Laboratory prototype bacteriophage strains MS2 and phi-X 174 were spiked into sterile 0.01M PBS as positive controls for male-specific and somatic coliphages, respectively. Sterile 0.01M PBS was used as the negative control. The controls were processed and plated in the same way as the samples, with magnesium chloride, double-strength agar, and the appropriate log-phase host bacterial culture and antibiotic. After allowing the agar to harden, the plates were covered, inverted, and incubated for 16 to 24 hours at 36°C ± 1.0°C.

Day three: Interpretation of single agar layer results. On the third day of analysis, circular zones of clearing, typically 1 to 10 mm in diameter, in the lawn of host bacteria are considered to be plaques (EPA 2001). All plaques were assumed to be caused by lytic coliphages and not by other agents or sources of lysis such as from bacteriocins. All plaques per plate series were counted and recorded in order to determine plaque-forming units (PFU) per 100 mL.

Because the samples analyzed were in 60 mL rather than 100 mL volumes, PFU per 100 mL were calculated from the recorded results.

Statistical methods

The distributions of the raw data and the log_{10}-transformed data were investigated to determine whether a parametric or non-parametric method should be used. Once it was determined that a non-parametric method should be chosen, the Kruskal-Wallis test was used for testing whether the samples originate from the same distribution. *Post hoc* analysis was done using Dunn's multiple comparisons test, a non-parametric pairwise multiple comparisons procedure, and Wilcoxon signed rank test, a non-parametric equivalent to the one-sample paired *t*-test, to determine whether the phage numbers from the assays of induced coliphages were different from those of the assays of non-induced coliphages at $p < 0.05$. Four pairs of Wilcoxon signed rank tests were done, comparing the assays of non-induced and MMC-induced somatic coliphages, of non-induced and UV-induced somatic coliphages, of non-induced and MMC-induced male-specific coliphages, and of non-induced and UV-induced male-specific coliphages. Additionally, the probability of superiority was calculated for each inducing agent.

The average difference between the induced and non-induced counts and the average of the non-induced counts were used to calculate the average percent increase between non-induced and induced counts. Linear regression on counts from induced assays versus counts from non-induced assays was also done to investigate this percent increase. The results from the two methods was compared. The probability of superiority, PS_{dep} is the probability that in a randomly sampled pair of scores (one matched pair of scores) the score from Condition B, the condition which most frequently has the higher score, will be greater than the score from Condition A, the condition which most frequently has the lower score (Grissom and Kim 2012). For this study,

Condition B refers to induction and Condition A refers to no induction. These calculations were done using Statistical Analysis System (SAS® 9.4) software (Cary, NC) and GraphPad InStat 3 software (La Jolla, CA).

CHAPTER 5: RESULTS

Descriptive statistics: Assays of somatic coliphages

A total of eleven experiments were completed, resulting in twenty-two assays per induction group. However, the assays of UV-induced coliphages did not begin until the second experiment, resulting in twenty, rather than twenty-two, UV-induced coliphage counts for the assays of somatic and male-specific coliphages ($n = 20$). Because it was more than five times greater than the average and not observed in duplicate, it was considered an extreme outlier, one MMC-induced somatic coliphage count was dropped, resulting in a total of twenty-one MMC-induced somatic coliphage counts ($n = 21$). If the outlier had been included in the analysis, it likely would have resulted in misleadingly inflated results regarding the distribution of MMC-induced somatic coliphages. The skewness, kurtosis, and Shapiro-Wilk test p-values of each distribution was examined for evidence of non-Normality to support the use of non-parametric statistical tests. The descriptive statistics of each induction group for the assays of somatic coliphages are presented in Table 3.

Of the three groups, the MMC-induced counts have the greatest mean (3.16×10^4 PFU/100 mL $\pm$ 3.75×10^3 PFU/100 mL) coliphage counts while the UV-induced counts have the lowest mean (1.40×10^4 PFU/100 mL $\pm$ 2.13×10^3 PFU/100 mL) coliphage counts. The higher skewness and kurtosis values of all groups and the low *p*-values of the Shapiro-Wilk tests ($p < 0.05$) support the necessity of a non-parametric statistical test when comparing the assays of induced coliphages to those of non-induced coliphages.

Table 3. Statistical measures of coliphage counts from assays of somatic coliphages

	Non-induced (PFU/100 mL)	Mitomycin C-induced (PFU/100 mL)	UV-induced (PFU/100mL)
N	22	21	20
Mean	2.75×10^4	3.16×10^4	1.40×10^4
Standard error	3.75×10^3	3.99×10^3	2.13×10^3
Median	2.17×10^4	2.32×10^4	1.37×10^4
Skewness	1.45	1.39	1.19
Kurtosis	2.12	1.92	1.58
Shapiro-Wilk test p-value	0.0067	0.0114	0.0108

The positive skewness of the distribution of coliphage counts of non-induced somatic coliphages (Figure 2) visually supports the premise that the data have a non-Normal distribution. The distribution of the non-induced counts seems to peak between 1×10^4 and 2×10^4 PFU/100 mL. Like the distribution of the somatic non-induced coliphage counts, the distribution of the MMC-induced counts appears to be non-Normal with observable positive skewness. The upper coliphage counts of the UV-induced somatic coliphages is a little more than half that of the upper coliphage counts of the non-induced and MMC-induced counts. The distribution of the UV-induced somatic coliphage counts also appears non-Normal. The positive kurtosis values for all induction groups mean that all three distributions have heavier tails and sharper peaks compared to a Normal distribution.

Figure 2. Sample distributions of coliphage counts from assays of somatic coliphages
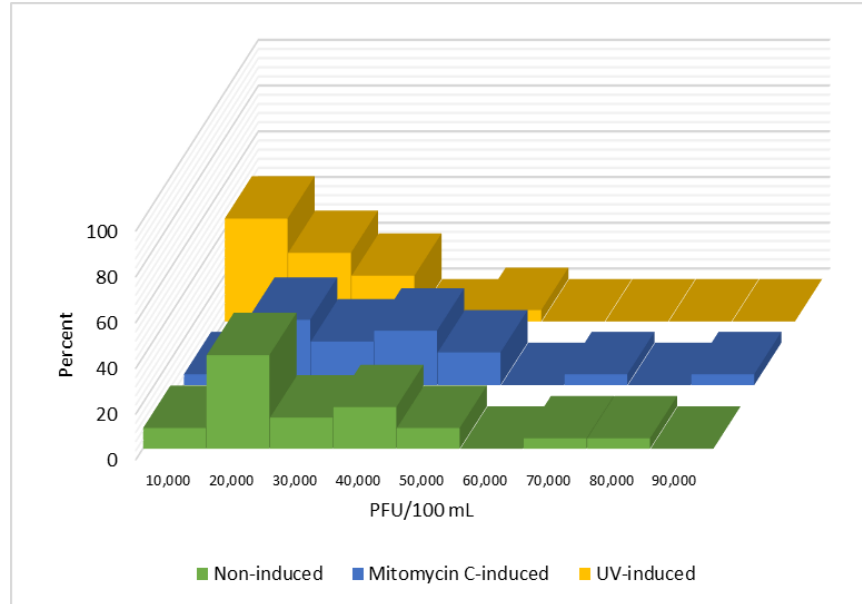
Overall, the non-Normality of the distributions supports the use of non-parametric statistical tests. However, it is often the case that non-Normal microbial concentrations require $\log_{10}$-transformation to result in Normal distributions. Although $\log_{10}$-transforming the data improved the skewness, kurtosis, and Shapiro-Wilk test *p*-values of some groups, the success was inconsistent between group types. For example, the $\log_{10}$-transformed UV-induced somatic coliphages still had a high kurtosis value (Kurtosis = -1.32) and significant statistical evidence that the distribution was non-Normal (Shapiro-Wilk test *p*-value = 0.099). The inconsistency was even more pronounced for the $\log_{10}$-transformed male-specific data, with the transformed MMC-induced and UV-induced data still violating Normality (detailed in the subsequent section on descriptive statistics of assays of male-specific coliphages).

Descriptive statistics: Assays of male-specific coliphages

Again, a total of eleven experiments were completed, resulting in twenty-two assays of non-induced and MMC-induced coliphages and twenty assays of UV-induced coliphages. The

descriptive statistics of each induction group for the assays of male-specific coliphages are

presented in Table 4.

Table 4. Statistical measures of coliphage counts from assays of male-specific coliphages

	Non-induced (PFU/100 mL)	Mitomycin C-induced (PFU/100 mL)	UV-induced (PFU/100 mL)
n	22	22	20
Mean	6.56×10^3	8.74×10^3	2.55×10^3
Standard error	1.61×10^3	2.10×10^3	7.50×10^2
Median	1.93×10^3	5.15×10^3	1.14×10^3
Skewness	1.03	1.33	2.75
Kurtosis	-0.316	0.719	8.97
Shapiro-Wilk test p-value	0.0005	0.0007	<0.0001

Similar to the somatic counts, the MMC-induced male-specific counts have the greatest

mean ($8.74E \times 10^3$ PFU/100 mL $\pm 2.10 \times 10^3$ PFU/100 mL) coliphage counts while the UV-

induced assays have the lowest mean (2.55×10^3 PFU/100 mL $\pm 7.50 \times 10^2$ PFU/100 mL)

coliphage counts. While the somatic induction groups had similar skewness and kurtosis values,

the values of the assays of male-specific coliphages are more variable, with the UV-induced

distribution being the most extreme. The high positive skewness and kurtosis values of the UV-

induced male-specific coliphage counts points to a non-Normal distribution. Further support for

suggesting a non-Normal distribution of coliphage counts can be seen in Figure 3.

Figure 3. Sample distributions of coliphage counts from assays of male-specific coliphages
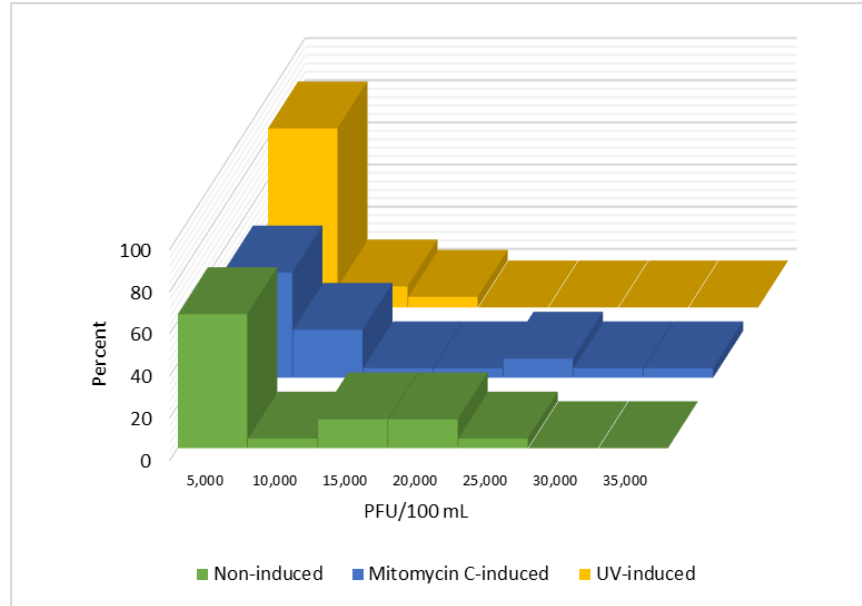
In Figure 3, around sixty percent of the male-specific non-induced coliphage counts are between 0 and 5×10^3 PFU/100 mL, constituting the major peak in the distribution. The relatively high skewness and kurtosis values of the male-specific non-induced coliphage counts along with the significant Shapiro-Wilk test p-value ($p < 0.05$) suggests a non-Normal distribution. Around fifty percent of the MMC-induced male-specific coliphage counts are between 0 and 5×10^3 PFU/100 mL, noticeably less than the non-induced coliphage counts, but with a greater percentage (around twenty percent) of counts residing between 5×10^3 and 1×10^4 PFU/100 mL. While the higher coliphage counts for the non-induced assays were around 2.5× 10^4 PFU/100 mL, the MMC-assay counts ranged up to 3.5×10^4 PFU/100 mL. Finally, around eighty-five percent of the UV-induced male-specific coliphage counts are between 0 and 5×10^3 PFU/100 mL, significantly more than non-induced and MMC-induced counts. The higher coliphage counts from the UV-induced assays only ranged up to 1.5×10^4 PFU/100 mL. Thus it can be noted that for somatic and male-specific coliphage counts, the upper range detected in UV-induced assays was always noticeably less than the upper range detected by non-induced and

MMC-induced assays. Like the somatic coliphage counts, the distribution of the male-specific

coliphages points to using a non-parametric statistical method.

To reiterate, it is often the case that non-Normal microbial concentrations require $\log_{10}$-

transformation to result in Normal distributions. Although $\log_{10}$-transforming the data improved

the skewness, kurtosis, and Shapiro-Wilk test p-values of the non-induced and MMC-induced

groups for somatic coliphages, it only improved the Normality indicators of the non-induced

group for male-specific coliphages, and even then the Kurtosis value (Kurtosis = -1.30) and

Shapiro-Wilk test p-value (Kurtosis = -1.3; Shapiro-Wilk, p = 0.1182).provided moderate

evidence against Normality for the non-induced group. After $\log_{10}$ transformation, the skewness

and kurtosis values for the MMC-induced group was -2.08 and 6.30 respectively, greater than

those of the untransformed data, with a Shapiro-Wilk test p-value of 0.0008 providing strong

evidence against Normality ($p < 0.05$). After $\log_{10}$ transformation, the skewness and kurtosis

values for the UV-induced group was -2.20 and 7.16 respectively, also greater than those of the

untransformed data, with a Shapiro-Wilk test p-value of 0.0005 providing strong evidence

against Normality ($p < 0.05$). Because $\log_{10}$-transforming the data did not consistently result in

Normally-distributed data, non-parametric statistical methods were used on the untransformed

data, beginning with the Kruskal-Wallis test.

For visual side-by-side comparison, Figures 4 and 5 depict box plots of all assays of

somatic and male-specific coliphages, respectively. In all cases, the median coliphage counts are

greater than the mean coliphage counts. The non-induced and MMC-induced somatic coliphage

box plots have similar means, though the distribution of the MMC-induced coliphages is greater,

resulting in a more elongated box. For both the assays of somatic and male-specific coliphages,

the UV-induce box plots are much narrower than their counterparts and located in the lower

PFU/100 mL ranges. The male-specific non-induced and MMC-induced box plots have long

upper whiskers, indicating the positive skew of the distributions.

Figure 4. Comparison of somatic coliphage counts from diluted sewage with and without exposure to inducing agents (NON – non-induced, MMC – mitomycin C-induced, UV – UV-induced)
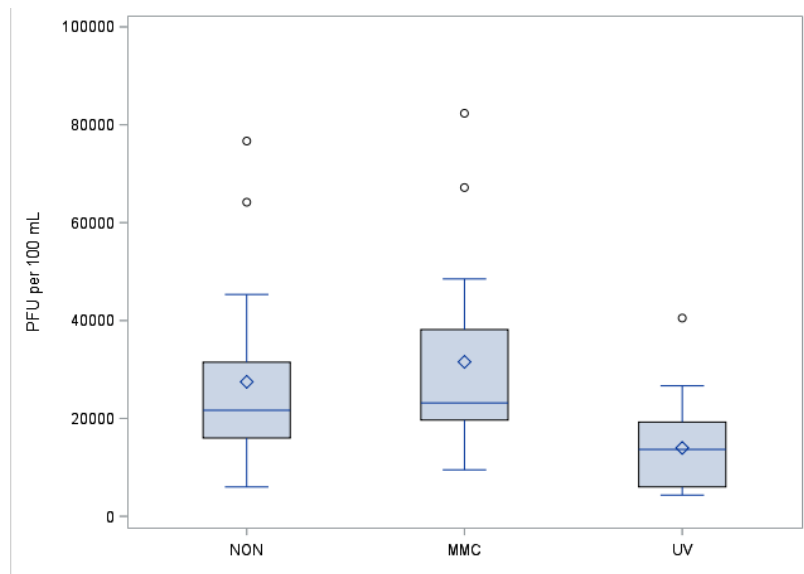
Figure 5. Comparison of male-specific coliphage counts from diluted sewage with and without exposure to inducing agents (NON – non-induced, MMC – mitomycin C-induced, UV – UV-induced)
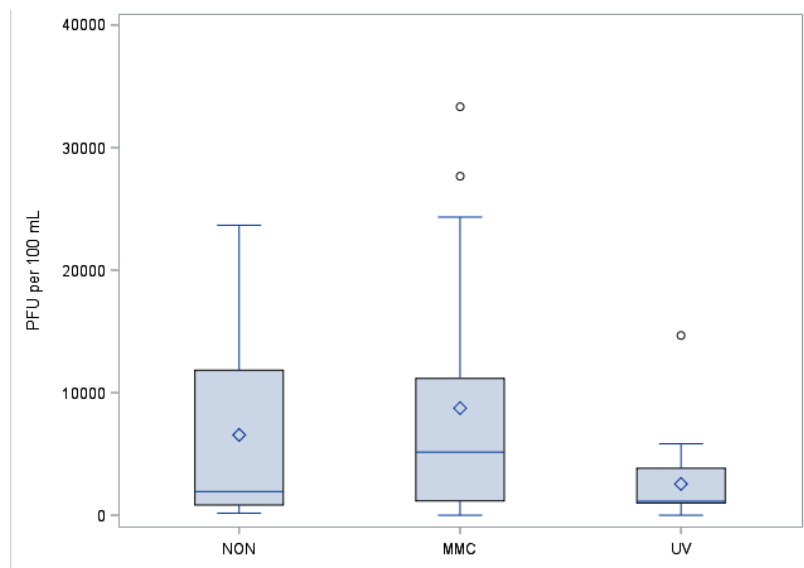
Comparison of assays of non-induced and induced coliphages

The results of the Kruskal-Wallis test for somatic and male-specific coliphage asays are available in Table 5. A significant Kruskal-Wallis test indicates that at least one of the samples stochastically dominates one other sample, but does not identify where or for how many pairs of groups this dominance occurs. According to Table 5, there is significant evidence of dominance among somatic coliphages ($p < 0.05$) and moderate evidence of dominance among male-specific coliphages ($p = 0.06$).

Table 5. Kruskal-Wallis test results for somatic and male-specific coliphage assays

	Somatic coliphages	**Male-specific coliphages**
Chi-Square	15.6273	5.5337
DF	2	2
Pr > Chi-Square	0.0004	0.0629

The previous section revealed that UV-induced counts were always less than non-induced counts for somatic and male-specific coliphages, suggesting an issue with UV prophage induction. Because of this, *post hoc* analysis was done using Dunn's multiple comparisons test. Wilcoxon signed rank tests were also done between non-induced and MMC-induced assays only for somatic and male-specific coliphages to reveal information on prophage induction. The Wilcoxon signed rank test gives a *p*-value only, providing evidence that population medians differ, and provides no direct estimate of the magnitude of any effect. The Dunn's multiple comparisons test *p*-values are the *p*-values for the comparison of the induced counts versus the non-induced counts. The *p*-values of the two tests are given in Table 6 along with the probabilities of superiority. The probability of superiority, PS_{dep} is the probability that in a randomly sampled pair of scores (one matched pair scores) the score from Condition B, the condition which most frequently has the higher score, will be greater than the score from

Condition A, the condition which most frequently has the lower score (Grissom and Kim 2012). In this case, Condition B is the MMC-induced assay and Condition A is the non-induced assay. Because of the difficulty in determining power and effect size for non-parametric, paired data, it is recommended to report PS_{dep} instead (Grissom and Kim 2012).

Table 6. Comparison of assays run with and without exposure to inducing agents for counts of somatic and male-specific coliphages estimated using EPA Method 1602

| Coliphage type	Inducing agent	Dunn's multiple comparisons test ($p =$)	Wilcoxon signed rank Test ($p =$)	Probability of superiority, PS_{dep} ($p =$)
Somatic	Mitomycin C	>0.05	<0.0001	0.90
	UV	<0.05	0.0141	0.25
Male-specific	Mitomycin C	>0.05	0.032	0.64
	UV	>0.05	0.0014	0.15

Using Dunn's multiple comparisons test, only UV-induced somatic coliphage counts were significantly different from its corresponding non-induced counts ($p < 0.05$). For all other induced versus non-induced comparisons, the p-values were greater than the alpha level of 0.05, meaning that Dunn's multiple comparisons test provides no strong evidence that the coliphage counts differ with induction. However, the software used to run the Dunn's multiple comparisons test (GraphPad Instat 3) does not report the exact p-value if the value is greater than the alpha level of 0.05. If the p-values are not too much greater than 0.05, then there may be moderate evidence that the induced counts do differ from the non-induced ones. Thus the Wilcoxon signed rank test was done using SAS 9.4 to continue the exploration for statistical evidence that the induced counts differ from the non-induced ones. Using Wilcoxon signed rank test, all induced coliphage counts were significantly different from their corresponding non-induced counts ($p <$

0.05). The PS_{dep} for the MMC-induced somatic and male-specific coliphage counts is 0.90 and 0.64, respectively. This means that MMC-induced somatic coliphage counts were higher than the non-induced counts 90% of the time while the MMC-induced male-specific coliphage counts were higher than the non-induced counts 64% of the time. The PS_{dep} for the UV-induced somatic and male-specific coliphage counts is 0.25 and 0.15, respectively.

Samples exposed to mitomycin C had consistently higher counts of somatic coliphages than samples not exposed to an inducing agent (Figure 6). The MMC-induced samples had higher counts in 19 of 21 trials (90%) and the non-induced samples had higher counts in two trials (9.5%). The average difference between MMC-induced counts and non-induced counts was 3.6×10^3 PFU/100 mL. To get the average percent increase of MMC-induced somatic coliphage counts from non-induced counts, the average difference was divided by the mean of the non-induced somatic coliphages (2.75×10^4 PFU/100 mL). Using this method, the average percent increase between MMC-induced and non-induced somatic coliphage counts is 13%.

Out of 22 tests for male-specific coliphages, 16 (73%) had higher counts in the MMC-induced samples and six (27%) had higher counts in the non-induced samples (Figure 7). The average difference between the MMC-induced counts and the non-induced counts was 2.2×10^3 PFU/100 mL. The mean non-induced male-specific coliphage count was 6.6×10^3 PFU/100 mL, resulting in an average percent increase of 33%.

Figure 6. Differences between induced and non-induced counts of somatic coliphages using mitomycin C as an inducing agent
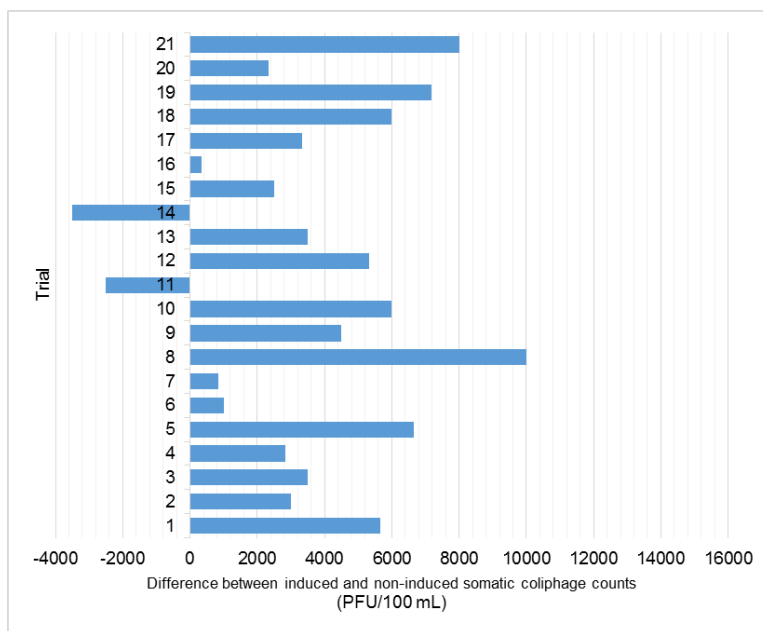
Figure 7. Differences between induced and non-induced counts of male-specific coliphages using mitomycin C as an inducing agent
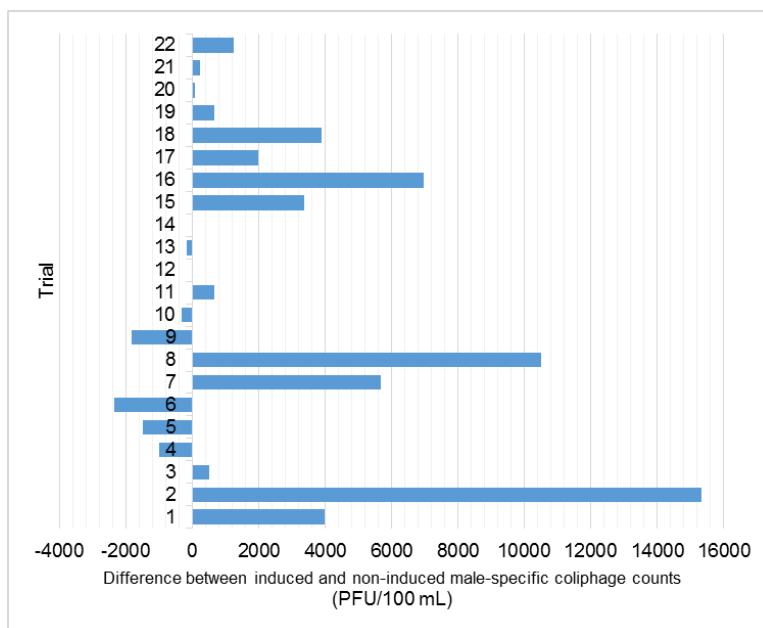

A linear regression was used as a second method to calculate percent increase from non-induced coliphage counts to MMC-induced coliphage counts. For Figures 8 and 9, the x-axes

represent the non-induced coliphage counts for somatic and male-specific coliphages respectively while the y-axes represent the MMC-induced coliphage counts for somatic and male-specific coliphages respectively. The linear regression equations and coefficient of determination (R^2) are presented in each figure in the left-hand corner. The black line is the line of equivalence ($y = x$).

The high R^2 values of both regression models indicate a strong goodness of fit. Additionally the residual plots of both linear regression models did not indicate any noticeable pattern, suggesting that errors are homoscedastic and randomly distributed and that our linear equations are appropriate models for the given data. According to the regression coefficient (slope) from Figure 8, for a 100 mL sample of diluted sewage in this study, a 1 PFU increase in the non-induced somatic coliphage counts is associated with an expected 1.006 increase in the MMC-induced somatic coliphage counts. Thus, inducing the sample with MMC results in a 0.6% increase in somatic coliphages. This was calculated by subtracting 1 from 1.006 and multiplying the difference by 100. For somatic coliphages, the 90% confidence interval (CI) of the slope is [0.9358, 1.075]. Because the 90% CI includes the null value of 1, this means that there is not enough evidence given by the linear regression model to statistically support the 0.6% increase in somatic coliphage counts after induction by MMC. According to the slope from Figure 9, for a 100 mL sample of diluted sewage in this study, a 1 PFU increase in the non-induced male-specific coliphage counts is associated with an expected 1.196 increase in the MMC-induced male-specific coliphage counts. Thus, inducing the sample with MMC results in a 20% increase in male-specific coliphages. For male-specific coliphages, the 90% CI of the slope is [1.001, 1.391]. Because the 90% CI does not include the null value of 1, this suggests that there is moderate evidence that induction by MMC results in an increase of male-specific coliphages.

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Figure 8. Linear regression of non-induced coliphages as the independent variable and mitomycin C-induced coliphages as the dependent variable, for somatic coliphages (Black line is line of equivalence where $y = x$)
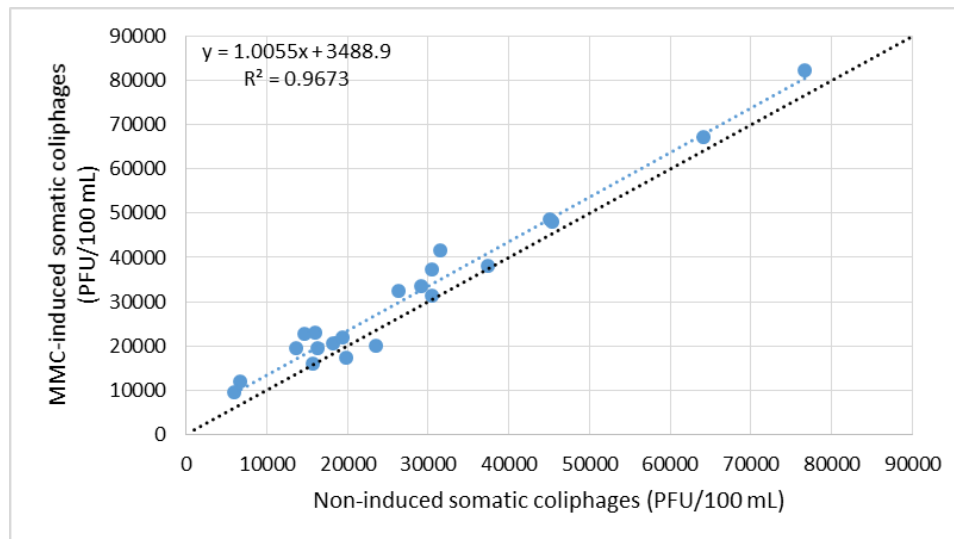
Figure 9. Linear regression of non-induced coliphages as the independent variable and mitomycin C-induced coliphages as the dependent variable, for male-specific coliphages (Black line is line of equivalence where $y = x$)
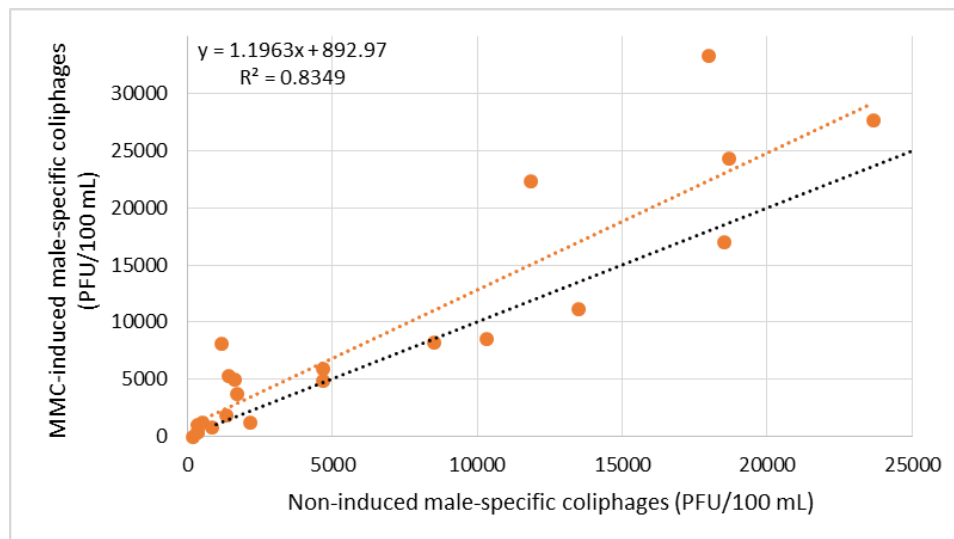
The average percent increase between non-induced and MMC-induced coliphage counts calculated from Figures 6 and 7 result in higher values than the percent increase values calculated from the linear regression model. The results of both methods are summarized in Table 7. Although the average percent increases were higher than those calculated from the

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linear regression model, the linear regression model includes 90% CIs which gives insight on the precision of the parameter estimate (in this case the parameter estimate is the slope). For example, the 90% CI of the slope of the regression of male-specific coliphages suggests that the unobservable true percent increase is potentially between 0.1% and 39%.

Table 7. Comparison of percent increase from non-induced counts to MMC-induced counts calculated by two methods

Type of coliphage	Method	
	Average percent increase	**Percent increase calculated from the linear regression model**
Somatic coliphages	13%	0.6%
Male-specific coliphages	33%	20%

Ongoing precision and recovery results

Ongoing precision and recovery from the positive control assays showed a 118 percent recovery for male-specific and 114 percent recovery for somatic, which is within the acceptable range set by EPA Method 1602 (EPA 2001).

CHAPTER 6: DISCUSSION

There is a small but significant difference between non-induced and MMC-induced coliphage counts for assays of somatic and male-specific coliphages (Wilcoxon signed rank, $p = 0.05$). Overall, the high positive skewness values in nearly all assay groups suggests that the incidences of higher, induced coliphage counts inflates the coliphage means, meaning that the medians are likely the better measures of central tendency for this dataset. This supports the decision to use a non-parametric, paired statistical test for the analysis of the results. The natural variability of coliphages in diluted sewage resulted in large standard errors and standard deviation. This, coupled with non-Normality, lowers the power of the statistical test and reduces the ability to estimate the magnitude of an effect. The UV-induced assays frequently resulted in lower coliphage counts than the non-induced assays, meaning that either the experimental setup for UV failed to detect the true coliphage population, free and induced, or the UV dose was too great and resulted in sample mortality. It is likely that the UV induction step, adapted from Muniesa and Jofre, requires improvement rather than the UV dose was too great because the UV dose used was based on literature values and was relatively small in comparison to other studies (2007). Further work should use a collimated beam UV dosing system, unavailable during this study, to improve UV dose accuracy and precision by limiting heterogeneity of the delivered UV dose (Bolton and Linden 2003). If a collimated beam UV dosing system is not available, an alternative step would be to focus on improving the UV induction step with a bench lamp to better capture phages from lysogeny, perhaps by swirling during treatment as well as before and

after to reduce phage aggregation, by adjusting the overall petri dish sample volume that undergoes UV radiation testing, or by exploring other UV doses.

On average, fewer male-specific coliphages were detected than somatic coliphages (about a magnitude of ten), but the male-specific ratio of the MMC-induced and non-induced medians was greater (MMC-induced male-specific coliphage median is 2.66 that of the non-induced, MMC-induced somatic coliphage median is 1.32 that of the non-induced). However, the Wilcoxon signed rank test does not confirm whether this magnitude of effect is statistically significant, only that there is a statistically significant difference between the population medians. To better understand the magnitude of effect, the percent increase of coliphages from non-induced coliphages to MMC-induced coliphages was investigated using two methods. The different results from both methods indicates a lack of precision and suggests that the percent increase of coliphage counts by MMC induction may be very small, potentially negligible. Further studies should aim to find improved and agreeing methods to calculate a more precise percent increase value.

Though a statistically significant difference between MMC-induced and non-induced coliphage counts was detected in diluted sewage using EPA Method 1602, the difference may not be generalizable to lysogenic contribution in environmental waters that are different in type and composition from diluted sewage. Based on the literature review, it is likely that lysogenic contribution is very small, but the focus of this study is whether a significant difference detected by EPA Method 1602 could be important, especially with regard to criteria or threshold levels set by government regulations. For example, emergency closures of harvest areas for molluscan shellfish may be opened based on coliform levels in the water or detected coliphage levels, which cannot exceed either background levels or 50 PFU/100 g of shellfish sample (NSSP

2011). It is possible that phages in a lysogenic stage of bacterial infection could cause harvest areas to open prematurely due to an underestimation of coliphage counts or that lysogenic phages that enter a lytic cycle could increase coliphage counts over a regulatory threshold. It is not clear if such an increase in coliphages would be indicative of increased numbers of pathogenic viruses or health risks.

This study, like other similar ones included in the review of literature, suggests that MMC is a better inducer of prophages than UV (Chibani-Chennoufi et al. 2004; Weinbauer and Suttle 1996; Jiang and Paul 1997; Muniesa and Jofre 2007). MMC is also easier to use and standardize compared to UV which can vary in many parameters such as peak intensity of a given lamp or distance between the lamp and the sample being induced. In the literature, MMC doses were presented in a more straightforward manner while UV doses often had to be calculated using a given peak intensity and a time interval. UV disinfection used in wastewater treatment may result in prophage induction and thus a release of lysogenic coliphages that may persist and be discharged to surface waters, releasing water that may be out of compliance into the environment. In general, MMC and UV-C are not commonly found in other environmental waters where lysogenic bacteria may dwell. Therefore the use of such inducing agents may not be indicative of what occurs in the environment. The ozone layer of the atmosphere absorbs most UV-C radiation, so UV-B and UV-A radiation may be more sensible to study in future research. UV-A radiation is the largest contributor of terrestrial solar radiation and though it is weakly absorbed by DNA, it can still result in photoproducts such as cyclobutane-type pyrimidine dimers, 6-4-photoproducts, and DNA strand breaks though in much smaller yields compared to UV-B and UV-C radiation (Kiefer 2007). Jiang and Paul found that natural lysogenic populations were sensitive to a variety of inducing agents, including natural ones such as

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sunlight, temperature, pressure, and aromatic and aliphatic hydrocarbons, but with variable results (1996). Future studies should continue to explore inducing agents that are naturally found in the environment.

The percent of positive tests (also known as the probability of superiority) observed during this study were higher than those reported in Muniesa and Jofre (2007): 90 percent for assays of MMC-induced somatic coliphages and 25 percent for assays of UV-induced somatic coliphages compared to 30 percent and 15 percent respectively. One explanation could be that the difference in results is due to the different types of samples tested. In this study, only diluted sewage was tested while the study by Muniesa and Jofre tested river water and sea water as well as diluted sewage (2007). The use of only diluted sewage is considered a strength of this study. For Muniesa and Jofre, the analysis of other water samples decreased the final percent of positive tests because the river and sea water samples rarely resulted in prophage induction while many of the raw sewage samples did experience prophage induction (2007). An alternative explanation as to why the probability of superiority was greater in this study than in the Muniesa and Jofre study lies in the possible communication among viruses in guiding lysis and lysogeny decisions. A recent study found that phages of the SPbeta group infecting *Bacillus* use a small-molecule communication system to coordinate lysis-lysogeny decisions (Erez et al. 2017). During host cell infection, the phage produces a communication peptide that is released into the medium, the concentration of which is measured by progeny phages. If the concentration of the communication peptide is sufficiently high, the phage is more likely to lysogenize (Erez et al. 2017). Although it is unknown whether coliphages utilize similar communication peptides, it is possible that removing free phages and incubating the resuspended bacteria overnight may result

not only in the induction of prophages into the lysis cycle but also in the induction of prophages into the lysogenic cycle, ultimately resulting in an underestimation of coliphages.

One limitation of this study was that the methodology used to induce environmental lysogenic bacteria may have detected phages that were not lysogenic due to the assumption that all plaques were due to lytic infection. In other coliphage studies on prophage induction, free phages were removed before re-suspending and inducing the sample bacteria. The culture was then allowed to incubate before coliphage enumeration. This is done to insure that the coliphages detected are from the lysogenic cycle rather than the lytic cycle. The methodology of this study meant that plaques from sources other than lytic and lysogenic infection could not be separated out. Although these false positive plaques would have occurred in non-induced and induced assays so that the difference between the two assays may have cancelled out the false positive effect, they still contribute to a high background level of lytic phages that made the lysogenic phage results more ambiguous and more difficult to analyze. However, the methodology of this study was chosen to better represent standard direct plating methods that would be used for monitoring ambient waters. Additionally, other standard methods based on enrichment presence/absence would be less likely to be affected by temperate coliphages. Another limitation to this study and others like it included in the review of literature is that the phage burst size, the number of phages produced per infected cell, is likely smaller in the non-sewage environmental waters than in the laboratory due to limited nutrient availability in the environment (Chibani-Chennoufi et al. 2004). Many phages in the environment are also not inducible while others are inducible with different efficiencies using alterative inducing agents (Casjens 2003). Additionally, the results of this study may not be generalizable to surface water or other geographic areas. However, a notable strength of this study is the multiple experiments on the

same type of environmental sample, particularly compared to the Muniesa and Jofre study which had a smaller number of cases per environmental sample type: as few as one and no more than fourteen (2007). Finally, the power analysis method for nonparametric, paired data used in this study was relatively simplistic meaning that continued studies on this topic should look into more appropriate power analysis or effect size methods to strength their statistical analysis.

CHAPTER 7: CONCLUSION

Although there were complications with the UV-induced assays, this study found a significant difference between MMC-induced coliphage counts and non-induced counts. The magnitude of the MMC induction effect was different for somatic and male-specific coliphages and was higher for the male-specific coliphages than the somatic coliphages. The results also support previous studies in stating that MMC is likely a better inducer of prophages than UV, if only for its ease-of-use and more standardized dose range. Future studies should run the induction method described in this study in parallel with ones outlined in previous studies that remove free phages, induce and incubate resuspended bacteria, and enumerate coliphages from that culture. Additionally, to better understand environmental dynamics of lysogeny, more research is needed on naturally occurring inducing agents and their effect on prophages from environmental bacteria. . Understanding whether temperate phages affect coliphage counts is important because even slightly elevated numbers of coliphages can be important with regards to criteria levels set by government regulations. For example, Type 2 reclaimed water in North Carolina must have a daily maximum coliphage count of less than 25 PFU/100 mL. It is important to note that because the contribution of temperate coliphages to total coliphage counts is likely small, criteria levels of higher coliphage counts will possibly be affected more than criteria levels of lower coliphage counts, such as the monthly geometric mean requirement of 5 PFU/100 mL for Type 2 reclaimed water in North Carolina. An underestimation of coliphages from neglecting to detect temperate phages could possibly lead to an incorrect compliance status

Type 2 reclaimed water quality. Similar scenarios would be possible if thresholds of coliphage

counts were adopted for monitoring ambient surface waters.

APPENDIX A. UV DOSE CALCULATION

For the given lamp (Spectroline XX-15F), the peak intensity at 10 inches above the surface is 1550 µW cm^{-2} (Spectronics Corporation 2013). The required time to achieve a dose of 45 J m^{-2} using this lamp was calculated using the exposure time equation below.

$$Peak\ intensity = 1550\ \mu W\ cm^{-2} \times \frac{1\ W}{1 \times 10^6 \mu W} \times \left(\frac{100\ cm}{1\ m}\right)^2 = 15.\ W\ m^{-2} = 15.5\ J\ s^{-1} m^{-2}$$

$$Desired\ dose\ per\ area = 45\ J\ m^{-2}$$

$$Exposure\ time = \frac{Desired\ dose\ per\ area}{Peak\ intensity} = \frac{45\ J\ m^{-2}}{15.5\ J\ s^{-1} m^{-2}} = 2.9\ s$$

The standard deviation of ± 0.3 s is based on the average human visual reaction time (Gust et al. 2009). This calculation does not take into account the age of the UV lamp bulbs. As the bulbs age, the filaments inside may oxidize, reducing the amount of current that can go through the filaments. This may result in a lower UV output overall. This means that the actual UV dose used in this study may have been less than 45 J m^{-2}.

These calculations steps were used to report the UV doses from other studies, available in Table 2.

APPENDIX B: MALE-SPECIFIC COLIPHAGE COUNT DATA

Trial	Non-induced coliphage counts (PFU/100mL)	MMC-induced coliphage counts (PFU/100mL)	UV-induced coliphage counts (PFU/100mL)
1	23667	27667	N/A
	18000	33333	N/A
2	1333	1833	1000
	2167	1167	1000
3	18500	17000	4667
	13500	11167	3667
4	18667	24333	14667
	11833	22333	5333
5	10333	8500	5833
	8500	8167	4000
6	500	1167	1000
	833	833	1000
7	168	<1.7	168
	336	336	<1.7
8	1633	5000	1483
	1167	8133	1033
9	1700	3700	1217
	1417	5300	1067
10	333	1000	667
	333	417	167
11	4667	4917	1667
	4667	5917	1417

Some duplicates have matching coliphage counts. This is an artifact of the way that plaques were counted and then extrapolated to larger volumes (100 mL). For example, in Trial 3 of the somatic coliphage assays (See Appendix C), the non-induced coliphage counts were 183 total plaques for both duplicates. After calculation, this results in 30,500 PFU/100 mL for both duplicates as well.

APPENDIX C: SOMATIC COLIPHAGE COUNT DATA

Trial	Non-induced coliphage counts (PFU/100mL)	MMC-induced coliphage counts (PFU/100mL)	UV-induced coliphage counts (PFU/100mL)
1	76667	82333	N/A
	64167	67167	N/A
2	45000	48500	6000
	45333	48167	7333
3	30500	37167	17333
	30500	31500	15167
4	37333	38167	16167
	31500	41500	15167
5	29167	33667	12167
	26333	32333	6500
6	18167	75500*	21000
	19833	17333	17833
7	6667	12000	5667
	6000	9500	6000
8	23500	20000	26667
	19333	21833	20667
9	15667	16000	24667
	16333	19667	40500
10	13667	19667	5167
	16000	23167	4333
11	18167	20500	6667
	14667	22667	4500

*Outlier, dropped during analysis

Some duplicates have matching coliphage counts. This is an artifact of the way that plaques were counted and then extrapolated to larger volumes (100 mL). For example, in Trial 3 of the somatic coliphage assays, the non-induced coliphage counts were 183 total plaques for both duplicates. After calculation, this results in a final count of 30,500 PFU/100 mL for both duplicates as well.

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