

**REVIEW OF COLIPHAGES AS POSSIBLE  
INDICATORS OF FECAL CONTAMINATION  
FOR AMBIENT WATER QUALITY**

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## **NOTICES**

This document has been drafted and approved for publication by the Health and Ecological Criteria Division, Office of Science and Technology, United States (U.S.) Environmental Protection Agency (EPA), and is approved for publication. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

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## Acronyms and Abbreviations

AOP	advanced oxidation processes
AOR	adjusted odds ratio
BOD	biochemical oxygen demand
C	Celsius
CDOM	colored dissolved organic matter
CFU	colony forming unit
CI	confidence interval
CLAT	culture latex agglutination and typing
CT	disinfectant concentration multiplied by contact time
cm	centimeter
DNA	deoxyribonucleic acid
ds	double-stranded
EPA	Environmental Protection Agency
<i>E. coli</i>	<i>Escherichia coli</i>
Famp	<i>E. coli</i> resistant to streptomycin and ampicillin (host)
FCV	feline calicivirus
FIB	fecal indicator bacteria
F-specific	male-specific or F+ coliphage (“F” refers to the genetic fertility factor that is required for bacteria to produce a sex pilus necessary for conjugation)
GE	gastroenteritis
GI	genogroup I
GII	genogroup II
GIII	genogroup III
GIV	genogroup IV
HCGI	highly credible gastrointestinal illness
ICC	integrated cell culture
ISO	International Standards Organization
kDa	kilodalton
L	liter
mg	milligram
MJ	megajoule
mJ	millijoule
mL	milliliter
mm	millimeter
mM	millimolar
MNV	murine norovirus
MPN	most probable number
MST	microbial source tracking
µm	micrometer
NEEAR	National Epidemiological and Environmental Assessment of Recreational Water
nm	nanometer
NOAEL	no observed adverse effect level
NoV	norovirus
NTU	nephelometric turbidity unit

OR	odds ratio
PCR	polymerase chain reaction
PFU	plaque forming units
QA/QC	quality assurance/quality control
QMRA	quantitative microbial risk assessments
qPCR	quantitative polymerase chain reaction
RNA	ribonucleic acid
RR	relative risk
RT-PCR	reverse transcriptase polymerase chain reaction
RT-qPCR	reverse transcriptase quantitative polymerase chain reaction
SAL	single agar layer
SD	standard deviation
ss	single-stranded
U.S.	United States
UV	ultraviolet
W	Watt
WERF	Water Environment Research Foundation
WRF	Water Research Foundation
WWTP	wastewater treatment plant

## **1. Introduction**

### **1.1. Context and Purpose**

The United States (U.S.) Environmental Protection Agency (EPA) recommended the use of the fecal indicator bacteria (FIB) *Escherichia coli* (*E. coli*) and enterococci to determine the level of fecal contamination present in environmental waters and to establish the 2012 Recreational Water Quality Criteria (RWQC), which protect the designated use of primary contact recreation (U.S. EPA, 2012). The purpose of this review is to summarize the scientific literature on coliphage properties to assess their suitability as indicators of fecal contamination in ambient water. This review covers background information on coliphage characteristics and enumeration methods (Section 2); their relationship with human health risks in epidemiological studies (Section 3); their occurrence and associations with pathogens in the environment (Section 4); and the fate and transport of coliphages in the environment (Section 5) and during wastewater treatment (Section 6). Appendix A describes the literature search strategy and summarizes the results of literature search.

At this time, EPA is considering the use of F-specific and somatic coliphages, as possible viral indicators of fecal contamination in ambient water. Coliphages are a subset of bacteriophages that infect *E. coli*. Other types of bacteriophages [i.e., those that infect *Enterococcus* and various *Bacteroides* species (spp.)] have also been evaluated for their use as indicators of fecal contamination. While some information on other types of bacteriophages is presented, this review primarily focuses on coliphages because there is more literature available on their occurrence, fate, and epidemiological relationships. Additionally, two standardized enumeration methods published by EPA are available for both coliphages.

### **1.2. Background**

For over a century, FIB (i.e., total coliforms, fecal coliforms, *E. coli*, fecal streptococci, and enterococci) have been used to detect sewage contamination in water in order to protect the public from waterborne pathogens associated with fecal material (e.g., bacteria, protozoa, and viruses) (Kehr et al., 1941; NRC, 2004). The use of FIB as indicators of sewage contamination facilitated tremendous gains in public health protection, particularly by indicating the likely presence of bacterial pathogens such as *Vibrio cholerae* (which causes cholera) and *Salmonella typhi* (which causes typhoid fever). Although advances in wastewater treatment over the last half century have facilitated gains in public health, it has been suggested that viral pathogens are the leading causative agents of recreational waterborne illnesses (Jiang et al., 2007; Sinclair et al., 2009). Unfortunately, because bacteria respond to water treatment processes and environmental degradation processes differently than viruses, traditional FIB may not be the best indicators of viral pathogens associated with fecal contamination. This review considers coliphages as possible indicators of fecal contamination in ambient water. Because FIB have long been used for managing water quality, much of this review compares coliphages to other commonly used FIB, such as *E. coli* and enterococci.

EPA conducted a series of prospective cohort epidemiological studies at multiple locations from 1972 to 1979 to better understand the relationship between FIB and swimming-associated illnesses (Cabelli et al., 1982; Dufour, 1984). Symptoms of the swimming-associated illnesses

included acute, self-limiting gastroenteritis (GE) with a short incubation period and duration. From their studies, Cabelli et al. (1982) concluded that human noroviruses (NoV) or rotaviruses were the most likely cause of the symptoms.<sup>1</sup> Soller et al. (2010) reached similar conclusions regarding the causative agent of the illnesses observed in the EPA National Epidemiological and Environmental Assessment of Recreational Water (NEEAR) study. Additionally, numerous studies have identified the presence of viruses in wastewater treatment effluent, often when traditional FIB are non-detectable (Kageyama et al., 2003; da Silva et al., 2007; Haramoto et al., 2007; Kitajima et al., 2009; Kuo et al., 2010; Simmons et al., 2011).

The human viruses most frequently associated with recreational waterborne illnesses are NoV, adenoviruses, human enteroviruses, rotaviruses, astroviruses, and hepatitis E, with NoV responsible for the large majority of viral-based gastrointestinal illnesses (U.S. EPA, 2009a). For example, Sinclair et al. (2009) found that 18 of the 27 (67%) reported viral outbreaks in ambient recreational water (does not include pools) from 1951 to 2006 were due to NoV, and 2 (7%) were due to adenovirus. As viruses are an important cause of recreational waterborne illness, it has been suggested they may also be appropriate indicators of fecal contamination in water.

Currently, there are limitations associated with using individual pathogenic viruses as indicators. For one, the measurement of densities of individual pathogenic viruses in water is expensive and time consuming, as culture-based techniques to propagate them can take over a week. Secondly, human NoV has only recently been cultured and methods for culture-based quantification of environmental water samples have not been developed yet (Papafragkou et al., 2013; Jones et al., 2014; Thorne and Goodfellow, 2014). NoV viral ribonucleic acid (RNA) can be detected and amplified through reverse transcriptase polymerase chain reaction (RT-PCR) (a semi-quantitative method) and RT-quantitative PCR (RT-qPCR, a quantitative method) (Kageyama et al., 2003; Trujillo et al., 2006; Atmar et al., 2008; Tajiri-Utagawa et al., 2009; Cashdollar et al., 2013). However, polymerase chain reaction (PCR) methods do not differentiate between infective and non-infective viruses. Human enteric adenoviruses are also difficult and slow to culture, and therefore are frequently detected using integrated cell culture (ICC) RT-PCR assays, which are semi-quantitative. Methods for these assays are improving, but they are still technically difficult and relatively slow to produce results (i.e., days) (Rodríguez et al., 2013; Polston et al., 2014). Therefore, numerous authors have proposed using bacteriophages (viruses that infect bacteria) as an indicator of human enteric viruses in water impacted by fecal contamination (Hilton and Stotzky, 1973; Gerba, 1987; Havelaar, 1987; Sobsey et al., 1995; Chung et al., 1998; Contreras-Coll et al., 2002; Hot et al., 2003; Skrabler et al., 2004a, b; Mocé-Llivina et al., 2005). In particular, coliphages, or viruses that infect *E. coli*, have been the most thoroughly investigated for this purpose.

Coliphages, particularly F-specific (also known as “male-specific” or “F+ phage”) and somatic coliphages, have been proposed as more reliable indicators of human viral pathogens associated with fecal contamination than FIB (Gerba, 1987; Palmateer et al., 1991; Havelaar et al., 1993;

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<sup>1</sup> Cabelli et al. (1982) suggested that “human rotavirus and/or the parvo-like viruses” were the etiological agents. In the 1970s the virus now called NoV was described morphologically as “picorna or parvovirus-like” (Kapikian et al., 1972).

Duran et al., 2002; Rose et al., 2004; Skrabber et al., 2004a, b).<sup>2</sup> This is based on their greater similarity to human enteric viruses in their physical structure, composition, and morphology, survivability in the environment, and persistence in treatment processes compared to FIB (Funderburg and Sorber, 1985; Havelaar et al., 1993; Gantzer et al., 1998; Grabow, 2001; Nappier et al., 2006). For example, F-specific RNA coliphages are morphologically similar to enteroviruses, caliciviruses, astroviruses, and hepatitis A and E viruses, and some somatic coliphages are similar to adenovirus (King et al., 2011). Coliphages can also be detected and quantified by simple, inexpensive, rapid, and reliable methods (Gerba, 1987; Havelaar, 1987). Although they are abundant in domestic wastewater, raw sewage sludge, and polluted waters (Havelaar et al., 1990; Debartolomeis and Cabelli, 1991; Leclerc et al., 2000; Mandilara et al., 2006), coliphages are present at lower densities in fresh feces than in wastewater (Dhillon et al., 1976; Osawa et al., 1981; Calci et al., 1998; Gantzer et al., 2002; Long et al., 2005). They originate almost exclusively from the feces of humans and other warm-blooded animals and can undergo limited multiplication in sewage under some conditions (i.e., high densities of coliphages and susceptible host *E. coli* at permissive temperatures) (Sobsey et al., 1995; Grabow, 2001). Coliphages (detected by EPA Method 1601, 1602, or approved equivalent methods) are one of the fecal indicator organisms that can be selected for microbial monitoring of groundwater systems (U.S. EPA, 2006).

### **1.3. General Attributes of an Ideal Indicator of Enteric Viral Degradation**

Methodological constraints limit reliable enumeration of individual pathogens in water (see Section 2). Because pathogen enumeration methods are not advanced enough at this time for use in routine water quality monitoring, FIB have been used to detect the presence of fecal contamination. Important attributes of an ideal indicator for fecal contamination include the following (NRC, 2004; Bitton, 2005):

- the indicator should be a member of the intestinal microflora of warm-blooded animals (see Section 2);
- the indicator should be present when pathogens are present and absent in uncontaminated samples (see Section 4);
- the indicator should be present in greater numbers than the pathogen (see Sections 4 and 6);
- the indicator should be at least as resistant as the pathogen to environmental factors (see Section 5) and to disinfection in water and wastewater treatment plants (WWTPs) (see Section 6);
- the indicator should not multiply in the environment (see Section 2);
- the indicator should be detectable by easy, rapid, and inexpensive methods (see Section 2);
- the indicator should be nonpathogenic (see Section 2);
- the indicator should be correlated to health risk (see Section 3); and
- the indicator should be specific to a fecal source or identifiable as to source of origin (microbial source tracking [MST] is not included in this review).

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<sup>2</sup> Coliphages are broken into two groups, F-specific (also referred to as male-specific or F+) RNA or DNA coliphages and somatic coliphages. Both infect *E. coli*; somatic coliphages infect *E. coli* cells through their outer membrane and F-specific coliphages infect *E. coli* via the pilus appendage, found on the surface of some types of bacteria for conjugative or motile functions.

While there is no true “ideal” indicator that fits all of the criteria above, coliphages exhibit most of these attributes, including the following:

- they are part of the intestinal microflora of warm-blooded animals (Sobsey et al., 1995; Grabow, 2001);
- they are present in greater numbers than pathogens (Havelaar et al., 1990; Debartolomeis and Cabelli, 1991; Leclerc et al., 2000);
- they are detectable by easy and rapid (1 day or less) methods (Wentsel et al., 1982; Gerba, 1987; Havelaar, 1987); and
- they are nonpathogenic (Grabow, 2001; Pillai, 2006; Jończyk et al., 2011).

Coliphages partially meet some of the other criteria, including the following:

- they co-occur with pathogens in water in some studies (for example, Havelaar et al., 1993; Jiang et al., 2001; Ballester et al., 2005; Pillai, 2006; Wu et al., 2011);
- they are at least equally resistant as some viral pathogens to environmental factors and to disinfection in water and WWTPs (Havelaar, 1987, 1990; Yahya and Yanko, 1992; Nasser et al., 1993; Gantzer et al., 1998; Sinton et al., 2002; Hot et al., 2003; Bitton, 2005; Lodder and de Roda Husman, 2005; Pillai et al., 2006; Charles et al., 2009; Bertrand et al., 2012; Seo et al., 2012; Silverman et al., 2013);
- they undergo very limited to no multiplication in the environment (Grabow et al., 1980; Grabow, 2001; Luther and Fujioka, 2004; Muniesa and Jofre, 2004; Jofre, 2009; Jończyk et al., 2011); and
- they have been shown to correlate with health risk in some studies (Lee et al., 1997; Colford et al., 2005, 2007; Wiedenmann et al., 2006; Abdelzaher et al., 2011).

Additionally, while not the focus of this review, assays for bacteriophages have been developed to identify some sources of origin (Pina et al., 1998; Brion et al., 2002; Schaper et al., 2002a; Cole et al., 2003; Noble et al., 2003; Payan et al., 2005; Savichtcheva and Okabe, 2006; Stewart-Pullaro et al., 2006; Ebdon et al., 2007, 2012; Lee et al., 2009; Wolf et al., 2010; Gómez-Doñate et al., 2011; Jofre et al., 2011; Lee et al., 2011; Nnane et al., 2011; Boehm et al., 2013).

This review evaluates the potential for coliphages to be useful as viral indicators of fecal contamination. The above attributes are considered in more detail throughout the review.

## **2. Bacteriophage Characteristics**

Bacteriophages (commonly referred to as phages) are viruses that infect bacteria. Phages were first described as a component of the human microbiome in the early 1900s and are nonpathogenic. They exist for all known bacterial species, and a wide variety have been isolated. Based on their size and morphology, bacteriophages are classified into 13 different phylogenetic families (Pillai, 2006). Generically, the bacteriophage virion (entire virus particle) consists of either double-stranded (ds) or single-stranded (ss) RNA or deoxyribonucleic acid (DNA), a protein capsid, and in some cases, a lipid membrane envelope (Pillai, 2006). Bacteriophages under evaluation as indicators of fecal contamination are nonenveloped, like many viral pathogens of interest. See Tables 1 and 2 below for more details.

In recent years, bacteriophages that infect *E. coli*, *Enterococcus*, and various *Bacteroides* spp. have been considered as possible indicators of fecal contamination (Chung and Sobsey, 1993; Grabow et al., 1995; Jofre et al., 1995; Bradley et al., 1998; Gantzer et al., 1998; ISO, 1999; Duran et al., 2002; Lucena et al., 2003; Mandilara et al., 2006; Bonilla et al., 2010; Santiago-Rodriguez et al., 2010; Vijayavel et al., 2010; Purnell et al., 2011). The majority of research on using bacteriophages as fecal indicators has been conducted on coliphages, which are bacteriophages that infect *E. coli* (U.S. EPA, 2001a). Coliphages, specifically F-specific and somatic coliphages, are the primary focus of this document and are described in detail below.

### **2.1. Origin and Replication**

Bacteriophages are considered the most abundant form of “life” on earth and can be found in all environments where bacteria grow, including in soil, water, and inside other larger organisms (e.g., humans) harboring host bacteria (e.g., *E. coli*) (Clokie et al., 2011; Dutilh et al., 2014; Díaz-Muñoz and Koskella, 2014). However, these viruses only reproduce inside metabolizing bacterial hosts and are thus considered obligate intracellular parasites that cannot multiply independently in any environment outside of the host bacterial cell (Grabow, 2001; Brüssow et al., 2004; Jończyk et al., 2011). For replication to occur in a given environment, such as in recreational waters, their host must be both viable in that environment (Grabow, 2001; Bitton, 2005; Jofre, 2009) and susceptible to bacteriophage infection (Wiggins and Alexander, 1985; Woody and Cliver, 1995, 1997). Bacteriophages use the host cell’s ribosomes, protein-synthesizing machinery, amino acids, and energy generating systems to replicate. Some bacteriophage species possess fewer than 10 genes and use essentially all of the host’s cellular functions to replicate. In contrast, other bacteriophages have 30 to 100 genes and are less dependent on the host. For example, larger bacteriophage may not require host genes for DNA replication because their own genomes contain the necessary genes (Grabow, 2001).

Bacteriophage replication includes the following steps:

- 1) adsorption: the virion attaches itself to a host cell;
- 2) penetration: the genome enters the host cell;
- 3) viral synthesis: the host cell manufactures viral components;
- 4) maturation: the components are assembled into intact new virions; and
- 5) release: virus particles leave the infected cell (Goldman and Green, 2009).

The host-specificity of bacteriophages is determined by protein molecules that serve as receptor sites on the surface of potential host bacteria. Only specific bacteriophages will recognize and attach to these specific bacterial receptor sites. Attachment leads to infection of the bacterium host as described above (Grabow, 2001).

Coliphages are generally found in the gut and are excreted in the feces of humans and other warm-blooded animals. Coliphages are present in large numbers in sewage (approximately  $10^7$  plaque forming units [PFU] per milliliter [mL]) (Ewert and Paynter, 1980; Lucena et al., 2004; Lodder and de Roda Husman, 2005). They have been investigated for years as possible viral indicators of fecal contamination (Simkova and Cervenka, 1981; Gerba, 1987; Havelaar et al., 1993; Sobsey et al., 1995; Chung et al., 1998; Contreras-Coll et al., 2002; Cole et al., 2003; Hot et al., 2003; Lucena et al., 2003; Mocé-Llivina et al., 2005; Brezina and Baldini, 2008; Wu et al., 2011). Coliphages can be divided into seven major morphological groups, or families; four of which contain somatic coliphages and three of which contain F-specific coliphages (Cole et al., 2003; Mesquita et al., 2010). Somatic coliphages infect *E. coli* cells through their outer membrane; F-specific coliphages infect *E. coli* via the pilus appendage, found on the surface of many types of bacteria. Studies indicate that somatic coliphages are excreted at higher levels than F-specific RNA coliphages and that somatic coliphages are likely to be more persistent in water than F-specific RNA coliphages (Grabow, 2001; Schaper et al., 2002a; Lee and Sobsey, 2011). Both F-specific and somatic coliphages, including their taxonomy, are described below.

Somatic coliphages are an abundant group of bacteriophages in feces and encompass DNA bacteriophages that infect coliform bacteria, including *E. coli*, via the outer membrane. The bacteriophage families *Myoviridae*, *Siphoviridae*, *Podoviridae*, and *Microviridae* have somatic coliphage representatives (Hayes, 1968; Grabow, 2001). *E. coli* strains that are used for propagating somatic coliphages include *E. coli* CN13 and *E. coli* WG5 (Muniesa et al., 2003). Coliphage strain  $\Phi$ X174 from the *Microviridae* family is a model somatic coliphage that is widely used in laboratory settings. Coliphages in the *Microviridae* family have circular ds DNA. Coliphages in the *Myoviridae*, *Siphoviridae*, and *Podoviridae* families have linear ds DNA. For more information on coliphage families, see Section 2.2 (Table 1) below.

Male-specific, or F-specific, coliphages are another broad group of coliphages that infect Gram-negative bacteria, including *E. coli*, which possess a plasmid coding for an F, or sex, pilus (Vinjé et al., 2004). F-specific coliphages are in the bacteriophage families *Inoviridae*, *Leviviridae*, and *Tectiviridae* (Cole et al., 2003; Lute et al., 2004; Ogorzaly et al., 2009; Mesquita et al., 2010). F-specific coliphages in the *Inoviridae* family are filamentous, ssDNA phages, whereas F-specific coliphages in the *Leviviridae* family are small, icosahedral, ssRNA phages and F-specific coliphages in the *Tectiviridae* family are cubic capsid (icosahedral) with linear dsDNA and no tail (Cole et al., 2003; Mesquita et al., 2010). Based on serological cross-reactivity, replicase template activity, and phylogenetic analysis, the F-specific RNA coliphages in the *Leviviridae* family have been further broken down into genogroups GI, GII, GIII, and GIV (Vinjé et al., 2004). In general, GII and GIII F-specific RNA coliphages are mainly found in environments associated with human waste, and GI and GIV F-specific RNA coliphages are mostly associated with animal waste, although these associations are not absolute (Schaper et al., 2002a; Cole et al., 2003; Vinjé et al., 2004). Several host strains of bacteria are used to enumerate F-specific coliphages in water samples, including *E. coli* resistant to streptomycin and ampicillin ( $F_{amp}$ ) and

*Salmonella enterica* serovar *Typhimurium* WG49 (Stm WG49).<sup>3</sup> Common laboratory strains of F-specific coliphages include MS2 (GI), GA (GII), Qβ (GIII), and SP (GIV) (Vinjé et al., 2004).

Despite being frequently detected in the environment, data indicate that somatic and F-specific coliphages rarely, if ever, replicate in *E. coli* under environmental conditions (Contreras-Coll et al., 2002; Jofre, 2009). Lack of replication in the environment is partially because coliphages do not replicate below a bacterial host density of  $10^4$  colony-forming units per mL (Wiggins and Alexander, 1985; Woody and Cliver, 1997). Additionally, Woody and Cliver (1997) demonstrated that the F-specific RNA coliphage Qβ cannot replicate in *E. coli* in nutrient-poor environments, and Cornax et al. (1991) asserted that the low survivability of the *E. coli* bacterial host in marine environments does not support the replication of coliphages.

F-specific coliphages have not been observed to multiply in *E. coli* suspended in water (Grabow, 2001). As described above, F-specific coliphages require the presence of F-pili on the host bacteria for infection to occur. In addition to requiring high densities of bacterial hosts for replication, the F-pili production requires optimum temperatures between 30 and 37°C (°C) with F-pili production decreasing rapidly below temperatures of 25°C (Franke et al., 2009). Additionally, most environmental isolates of *E. coli* have not been observed to produce pili even at elevated temperatures and are generally considered unsuitable hosts for F-specific RNA coliphages (Luther and Fujioka, 2004). F-specific coliphages can replicate in *E. coli* in certain water environments if fertility fimbriae are present and when the temperature is at least 30°C. However, replication under these conditions is unlikely as environmental conditions are not likely to support fertility fimbriae production (Grabow et al., 1980). However, some argue that F-specific RNA coliphages may reproduce under environmental conditions according to the “mud puddle hypothesis.” This hypothesis argues that the presence of animal waste lagoons and stagnant small puddles in a watershed may provide an environment for the generation of coliform bacteria and F-specific and somatic coliphages (Jiang et al., 2007; Reyes and Jiang, 2010). However, additional research to test whether the coliphages detected on environmental *E. coli* strains can also infect the *E. coli* strains used in laboratory assays is needed.

## 2.2. Morphology

Bacteriophages are incredibly diverse in size, morphology, surface properties, and composition. Table 1 briefly describes the structure and morphology of the seven bacteriophage families that include coliphages.

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<sup>3</sup> Stm WG49 contains an *E. coli* plasmid that codes for sex pili.

**Table 1. Morphology of a subsection of bacteriophages.**

Type	Family (Examples)	Nucleic acid	Structure
Somatic coliphages	<i>Myoviridae</i> (T2, T4)	Linear dsDNA	Nonenveloped, contractile tail, consisting of a sheath and central tube
Somatic coliphages and <i>Bacteroides</i> bacteriophages	<i>Siphoviridae</i> ( $\lambda$ , T1, T5)	Linear dsDNA	Nonenveloped, long noncontractile tail
Somatic coliphages	<i>Podoviridae</i> (T3, T7, P22)	Linear dsDNA	Nonenveloped, short noncontractile tail
Somatic coliphages	<i>Microviridae</i> ( $\Phi$ X174)	Circular dsDNA	Nonenveloped, isometric
F-specific DNA coliphages	<i>Tectiviridae</i> (PR772)	Linear dsDNA	Nonenveloped, cubic capsid (icosahedral), no tail
F-specific RNA coliphages (Genogroups I, II, III, IV)	<i>Leviviridae</i> (MS2, Q $\beta$ , F2)	Linear ssRNA	Nonenveloped, isometric
F-specific DNA coliphages	<i>Inoviridae</i> (M13)	Circular ssDNA	Nonenveloped, filamentous

Source: Pillai, 2006; Mesquita et al., 2010; Jończyk et al., 2011

Coliphages within families are sometimes grouped by similar traits or have unique characteristics, which is important because morphology affects the susceptibility of viruses to inactivation in the environment. Some specific structural characteristics, such as tails, large capsids, and lack of an envelope, can be associated with greater resistance to external factors, such as thermal degradation and degradation in water (Ackermann et al., 2004; Jończyk et al., 2011). For example, most coliphages belong to the ‘T group’ and have a tail structure. ‘T even’ coliphages possess a contractile sheath. T1 and T5 coliphages have long tails without contractile sheaths, whereas T3 and T7 coliphages have very short tails (Pillai, 2006; Jończyk et al., 2011). While morphology is linked with viral family and type, differences in morphology within these classifications have also been observed. For more details on viral properties that affect inactivation and environmental persistence of coliphages, see Section 5.0.

As illustrated in Table 2, similar to coliphages (shown in Table 1), many waterborne human enteric viruses are nonenveloped and display a range of nucleic acid structures. The virion of both coliphages and human enteric viruses consists of either ds or ss RNA or DNA (Pillai, 2006; Jończyk et al., 2011; King et al., 2011). Specifically, F-specific RNA coliphages are morphologically similar to enteroviruses, caliciviruses, astroviruses, and hepatitis A and E viruses, and somatic coliphages are more similar to adenovirus (King et al., 2011).

**Table 2. Morphology of human enteric viruses that may be transmitted in aquatic environments.**

Genus & Common name(s)	Nucleic acid	Structure
<i>Astrovirus</i> Astrovirus	Spherical ssRNA	Nonenveloped
<i>Calicivirus</i> Norovirus	Spherical ssRNA	Nonenveloped
<i>Coronavirus</i> Coronavirus	Linear ssRNA	Enveloped
<i>Enterovirus</i> Poliovirus, Coxsackievirus A & B, Echovirus	Linear ssRNA	Nonenveloped
<i>Enterovirus</i> Hepatitis A	Spherical ssRNA	Nonenveloped
<i>Hepevirus</i> Hepatitis E	Spherical ssRNA	Nonenveloped
<i>Mastadenovirus</i> Adenovirus	Linear dsDNA	Nonenveloped
<i>Parvovirus</i> Parvovirus	Linear ssDNA	Nonenveloped
<i>Reovirus</i> Reovirus	Linear dsRNA (segmented)	Nonenveloped
<i>Rotavirus</i> Rotavirus	Spherical dsRNA (segmented)	Nonenveloped
<i>Torovirus</i> Torovirus	Linear ssRNA	Enveloped

Source: Bosch, 1998; King et al., 2011

### 2.2.1 Morphological Properties Affecting Persistence

Coliphages can be inactivated, or made noninfective by various environmental factors, including temperature (Feng et al., 2003), pH (Feng et al., 2003), salinity (Sinton et al., 2002), sunlight (Sinton et al., 1999), and ultraviolet (UV) light (Sang et al., 2007). Viral inactivation occurs when viral components (nucleic acids, proteins, lipids) are destroyed. Therefore, characteristics that influence survival include coliphage morphology, including size and surface properties (Jończyk et al., 2011).

Of greatest importance, surface conformations, such as whether the virus is enveloped or nonenveloped, affects virus inactivation. Due to their nonenveloped nature, NoV, poliovirus, coxsackievirus, and echovirus are presumed to be highly resistant to environmental degradation and chemical inactivation (Bae and Schwab, 2008). The lipid content of a viral envelop renders the virus more sensitive to environmental stress including desiccation and heat, and are generally believed to be less resistant to inactivation than non-enveloped viruses (Rosenthal, 2009). Coliphages are nonenveloped and are resistant to environmental degradation and chemical inactivation similar to other enteric nonenveloped viruses (Havelaar, 1987; Havelaar et al., 1990; Yahya and Yanko, 1992; Nasser et al., 1993; Gantzer et al., 1998; Sinton et al., 2002; Hot et al., 2003; Ackermann et al., 2004; Bitton, 2005; Lodder and de Roda Husman, 2005; Pillai et al., 2006; Jończyk et al., 2011; Bertrand et al., 2012; Seo et al., 2012; Silverman et al., 2013).

Of additional consideration, differences in tail structure as well as capsid size and structure affect bacteriophage survival. For example, Ackermann et al. (2004) found that tailed bacteriophages were the most stable in adverse conditions, but found no difference in stability among bacteriophages with contractile, noncontractile, or short tails. Bacteriophages with a large capsid (100 nanometers [nm] in diameter) were found to have better preservation rates than bacteriophages with a smaller capsid (60 nm in diameter) (Ackermann et al., 2004). Lee and Sobsey (2011) found small diameter *Microviridae* to be among the most persistent of several tested somatic coliphages in water. Romero et al. (2011) attributed differences in solar inactivation rates between MS2 and rotavirus to their differing protein capsid structure and genomes, which the authors conclude may be responsible for observed differences in reactivity with individual reactive oxygen species. Overall, it is difficult to make generalizations given the complexity of interactions between physical characteristics and factors that affect bacteriophage survival.

While there are differences in survival among viruses of different families, there are also differences in survival among viruses within the same family (Sobsey and Meschke, 2003; Nappier et al., 2006). A study that estimated the survival of several virus families and genera, including adenovirus, poliovirus, and coxsackievirus, found that survival varied by virus type (Mahl and Sadler, 1975). *Siphoviridae* with flexible tails are the most persistent in freshwater environments under adverse conditions (Muniesa et al., 1999). Additionally, coliphages within the same family and with similar structural similarities do not necessarily share the same survival characteristics (Jończyk et al., 2011). For example, results from laboratory studies showed that different F-specific RNA coliphages differ in their survival in water (Brion et al., 2002; Schaper et al., 2002b; Long and Sobsey, 2004; Nappier et al., 2006). There is also demonstrated variability within taxonomic types (Brion et al., 2002).

### **2.3. Detection Methods**

Currently a variety of methods are available to detect bacteriophages. These include culture-based methods and “rapid” methods (defined as one day or less) which include immunology- and molecular-based methods. Each type of method has advantages and disadvantages (see Table 3). Plaque assays are a typical culture-based technique used for enumerating infectious virus particles (ISO, 1995, 2000, 2001; Grabow, 2001; U.S. EPA, 2001a, b; Eaton et al., 2005; Rodríguez et al., 2012a). Additionally, there are three bacteriophage methods published by the International Organization for Standardization (ISO) for F-specific RNA bacteriophages, somatic coliphages, and bacteriophages infecting *Bacteroides fragilis* (*B. fragilis*) (ISO, 1995, 2000, 2001). Rapid methods include immunology based methods (i.e., culture latex agglutination and typing [CLAT]), molecular methods (multiple types of PCR), and Fast Phage (a modified rapid version of EPA Method 1601) (Brussaard, 2004, 2009; Fong and Lipp, 2005; Kirs and Smith, 2007; Love and Sobsey, 2007; Gentilomi et al., 2008; Salter et al., 2010; Rodríguez et al., 2012b).

#### **2.3.1 Culture-Based Methods**

Standardized culture-based methods are available in both the United States and the European Union for the detection of coliphages in water (ISO, 1995, 2000, 2001; U.S. EPA, 2001a, b;

Eaton et al., 2005). The ISO methods have been optimized and tested through interlaboratory comparison (Mooijman et al., 2001, 2002, 2005; Muniesa and Jofre, 2007). The ISO Standard Method 9224A-F provides protocols for detecting or enumerating coliphages (Eaton et al., 2005). Two methods for coliphage monitoring in groundwater were approved by EPA in 2001 (U.S. EPA 2001a, b). These methods include EPA Method 1601 (two-step enrichment process) and EPA Method 1602 (single agar layer [SAL] method). EPA Methods 1601 and 1602 have undergone multi-laboratory validation (U.S. EPA 2003a, b). The results of these inter-laboratory comparisons support the use of these methods in the determination and enumeration of F-specific and somatic coliphages in groundwater (U.S. EPA, 2003a, b). These methods are approved in 40 Code of Federal Regulations Part 136 and can be used for detection of coliphages in wastewater.<sup>4</sup> These culture-based methods have been applied to rivers, estuaries, drinking water, surface water, storm water, and wastewater (Havelaar, 1987; Davies et al., 2003; Borchardt et al., 2004; Lucena et al., 2004; Sobsey et al., 2004; Ballester et al., 2005; Lodder and de Roda Husman, 2005; Nappier et al., 2006; Stewart-Pullaro et al., 2006; Bonilla et al., 2007; Locas et al., 2007, 2008; Gomila et al., 2008; Love et al., 2010; Francy et al., 2011; Rodríguez et al., 2012a).

EPA Method 1601 describes a qualitative two-step enrichment procedure for coliphages and was developed to help determine if groundwater is affected by fecal contamination (U.S. EPA, 2001a). However, this validated<sup>4</sup> procedure determines the presence or absence of F-specific and somatic coliphages in groundwater, surface water, and other waters (U.S. EPA, 2003a). Method 1601 may be used as a quantitative assay of coliphage densities in a most probable number (MPN) format (spot-plating). The Method 1601 protocol directs that a 100 mL or 1 liter (L) groundwater sample be enriched with a log-phase host bacteria (*E. coli* F<sub>Amp</sub> for F-specific coliphages and *E. coli* CN-13 for somatic coliphages) for coliphages. After an overnight incubation, samples are put on to a lawn of host bacteria specific for each type of coliphage, incubated, and examined for circular lysis zones, which indicate the presence of coliphages. For quality control purposes, both a coliphage positive reagent (enumerated sewage filtrate or pure cultures of F-specific RNA coliphage MS2 or somatic coliphage ΦX174) water sample and a negative reagent water sample (method blank) are analyzed for each type of coliphage with each sample batch. This method is considered more sensitive than EPA Method 1602, a SAL procedure discussed below (U.S. EPA, 2001a), due to the larger sample volumes used in 1601 (100 mL to 1 L) compared to Method 1602 (100 mL). In total, EPA Method 1601 requires 28 to 40 hours for a final result, depending on incubation times (Salter et al., 2010).

The EPA Method 1602 SAL procedure can be used to quantify coliphages in a sample. The Method 1602 protocol directs that a 100 mL water sample may be assayed by adding the log-phase host bacteria (*E. coli* F<sub>Amp</sub> for F-specific coliphage and *E. coli* CN-13 for somatic coliphage) and 100 mL of double-strength molten tryptic soy agar to the sample. The sample is then thoroughly mixed and the total volume is poured into multiple plates. After an incubation of 16 to 24 hours, circular lysis zones (plaques) are counted and summed for all plates from a single sample. The quantity of coliphages in a sample is expressed as PFU per 100 mL. For quality control purposes, both a coliphage-positive reagent (enumerated sewage filtrate or pure cultures of F-specific RNA coliphage MS2 or somatic coliphage ΦX174) water sample and a negative

<sup>4</sup> [http://water.epa.gov/scitech/methods/cwa/methods\\_index.cfm](http://water.epa.gov/scitech/methods/cwa/methods_index.cfm)

Method validation is defined as a process that demonstrates the suitability of an analytic method for its intended purpose (U.S. EPA, 2009b).

reagent water sample (method blank) are analyzed for each type of coliphage with each sample batch. In total, EPA Method 1602 typically requires an overnight incubation (18 to 24 hours) up to 3 days, but results can be obtained in as few as 8 to 10 hours (Salter et al., 2010).

There are also methods for coliphage detection that use membrane filters to concentrate coliphages from a water sample (Sobsey et al., 1990; Sobsey et al., 2004; Eaton et al., 2005). Volumes of water of 100 mL and greater can be concentrated on a membrane filter after addition of salts and or pH adjustments. Coliphages can then be eluted off the filter and used in one of the standard assays above, or they can be enumerated directly on the membrane filter (Eaton et al., 2005). For direct filter assays, a single assay dish is utilized for each coliphage-adsorbed filter. This significantly reduces the time and materials required. However, extraneous material on the filter can interfere with the plaque assay. Both 47-millimeter (mm) membrane and 90-mm membrane filters have been used and the membrane filtration method can be used to detect both F-specific and somatic coliphages.

One study evaluated the use of a single *E. coli* host (*Escherichia coli* host strain CB390) for the simultaneous detection of both somatic and F-specific coliphages (Guzmán et al., 2008). This host could be useful for detecting total coliphages. However, more independent and multi-laboratory validation of this method is needed. Rose et al. (2004) used *E. coli* C-3000 (ATCC #15597), which they report can host both somatic and F-specific coliphages.

EPA is currently evaluating a membrane filtration culture method and may also evaluate an ultrafiltration culture method for use in coliphage enumeration. The intralaboratory (single laboratory) method validation study is underway.

### **2.3.2 Rapid Methods**

Recently, multiple methods have been published that are faster than EPA Methods 1601 and 1602. Each method has advantages and disadvantages in terms of speed, accuracy, form of results (i.e., quantitative, qualitative, infectivity of virus), and level of training and equipment required. The rapid methods are outlined below in more detail.

#### **Polymerase Chain Reaction Methods**

The most common type of molecular method used to detect coliphages is PCR. PCR is a method of amplifying nucleic acids and involves cycling the reaction mixture through temperatures that allow for denaturing, annealing, and extension of new DNA fragments or amplicons. With each cycle, specific DNA fragments targeted by primers are doubled. This exponential amplification of DNA fragments allows samples with very small numbers of target sequences to be amplified into an amount of DNA that can be visualized on an agarose gel (Innis et al., 1990). Depending on the type of information needed (quantitative, qualitative), different types of PCR are used and are described in more detail below. Currently, there are no universal primers for the detection of coliphages, but primers are available for individual coliphage strains.

**RT-PCR:** RT-PCR is used to determine the presence of RNA or RNA viruses, such as F-specific RNA coliphages. The viral RNA is first reverse transcribed into complementary DNA, which is used as a template for the PCR reaction (Fong and Lipp, 2005; Kirs and Smith, 2007).

**Quantitative (q) PCR and RT-qPCR:** Both qPCR and RT-qPCR assays, which detect and quantify the amount of nucleic acid present, have been developed for the quantification of coliphages (Smith, 2006). These assays can determine the amount of a given coliphage present in a given sample (Yong et al., 2006; Kirs and Smith, 2007; U.S. EPA, 2007, 2010; Gentilomi et al., 2008). These PCR assays often detect only a subgroup of the total coliphages that would be quantified by plaque assays. Most recently, PCR has been performed on digital microfluidic platforms (Hua et al., 2010; Jebrail and Wheeler, 2010; Mark et al., 2010) and has been used to detect bacteriophages (Tadmor et al., 2011) and coliphages (Reitinger et al., 2012). Digital PCR on microfluidic chips promises to be a fast and accurate high-throughput technique to determine phage genome quantification.

**Multiplex PCR:** Multiplex PCR (also including multiplex qPCR, RT-PCR, and RT-qPCR) was developed to detect multiple target sequences in the same reaction tube. Thus, multiplex PCR can detect more than one type of phage in one sample (U.S. EPA, 2007, 2010). For example, RT-qPCR only quantitatively detects one type of coliphage per tube (i.e., GII F-specific RNA coliphage) while multiplex RT-qPCR quantitatively detects multiple phage targets per tube (i.e., GI, GII, and GIII F-specific RNA coliphages) (Kirs and Smith, 2007).

### Culture Latex Agglutination and Typing

The CLAT method has been validated for the detection of coliphages from fecal contamination in beach waters (Griffith et al., 2009; Wade et al. 2010) and combines a two-step enrichment process and latex agglutination serotyping to monitor for the presence of coliphages (Love and Sobsey, 2007; Rodríguez et al., 2012a). This rapid antibody-based method detects F-specific coliphages in water samples in 5 to 24 hours. Samples are generally scored as positive based on formation of clumps visible on the agglutination card after 60 seconds. Absence of such clumps signifies negative samples (Love and Sobsey, 2007). The assay is relatively inexpensive as reagents can be stored at ambient temperatures for months, unlike the reagents used for PCR-based assays (Love and Sobsey, 2007).

### Fast Phage Modified Method 1601

A modification to EPA Method 1601 called Fast Phage has been described by Salter et al. (2010). This modification incorporates the use of shelf-stable, ready-to-use reagents in a simplified format. Within the Fast Phage method, isopropyl- $\beta$ -D-1-thiogalactopyranoside is used as an enrichment medium to induce transcription of the host *E. coli lac* operon. Lysis of *E. coli* by coliphages is coupled with *lac* operon expression. Therefore, a large amplification and a rapid extracellular beta-galactosidase enzyme release during coliphage-induced lysis of the infected host are reported in comparison to the growing, uninfected host (Salter et al., 2010). Fast Phage is approved under EPA's Alternative Test Procedure program for detection of coliphages in groundwater (Salter and Durbin, 2012).

**Table 3. Advantages and disadvantages of methods to detect coliphages.**

<b>Method</b>	<b>Advantages</b>	<b>Disadvantages</b>
Culture EPA Method 1601	<ul style="list-style-type: none"> <li>• Qualitative (presence/absence);</li> <li>• Differentiates between F-specific and somatic coliphages;</li> <li>• Infectivity is determined;</li> <li>• More sensitive than Method 1602 (depending on sample volume: Method 1601 with &gt;100 mL is more sensitive than Method 1602 and Method 1601 with &lt;100 mL is less sensitive than Method 1602); and</li> <li>• Inexpensive.</li> </ul>	<ul style="list-style-type: none"> <li>• Not validated as a quantitative assay; and</li> <li>• Requires 24 hours–3 days for results.</li> </ul>
Culture EPA Method 1602	<ul style="list-style-type: none"> <li>• Both qualitative and quantitative (PFU);</li> <li>• Differentiates between F-specific and somatic coliphages;</li> <li>• Infectivity is determined; and</li> <li>• Inexpensive.</li> </ul>	<ul style="list-style-type: none"> <li>• Requires 16–24 hours for results; and</li> <li>• May be less sensitive than Method 1601 (depending on sample volume: Method 1601 with &gt;100 mL is more sensitive than Method 1602 and Method 1601 with &lt;100 mL is less sensitive than Method 1602).</li> </ul>
SM9224F Membrane Filtration	<ul style="list-style-type: none"> <li>• Both qualitative and quantitative (PFU);</li> <li>• Differentiates between F-specific and somatic coliphages;</li> <li>• Infectivity is determined;</li> <li>• Similar material requirements to EPA Methods 1601 and 1602; and</li> <li>• Greater than 100 mL volume samples can be evaluated, which increases sensitivity in ambient waters.</li> </ul>	<ul style="list-style-type: none"> <li>• Requires 16–24 hours for results; and</li> <li>• May have recovery loss due to filtration and elution steps; and</li> <li>• Turbidity in the sample may interfere with plaque identification.</li> </ul>
PCR/(reverse-transcriptase) RT-PCR	<ul style="list-style-type: none"> <li>• Rapid (~2–10 hours);</li> <li>• Increased sensitivity compared to culture methods; and</li> <li>• Can test for specific types of DNA (PCR) or RNA (RT-PCR) phages.</li> </ul>	<ul style="list-style-type: none"> <li>• Qualitative only;</li> <li>• Infectivity (live vs. dead) not determined;</li> <li>• Inhibitors may be present in the environmental matrix; and</li> <li>• Expensive (PCR equipment) and quality assurance/quality control (QA/QC) expertise required.</li> </ul>
qPCR/RT-qPCR (quantitative)	<ul style="list-style-type: none"> <li>• Rapid (~2–10 hours);</li> <li>• Quantitative;</li> <li>• Increased sensitivity compared to culture methods; and</li> <li>• Can test for specific types of DNA (qPCR) or RNA (RT-qPCR) phages.</li> </ul>	<ul style="list-style-type: none"> <li>• Infectivity not determined;</li> <li>• Inhibitors may be present in the environmental matrix; and</li> <li>• Expensive (qPCR equipment) and QA/QC expertise required.</li> </ul>
Multiplex qPCR/RT-qPCR	<ul style="list-style-type: none"> <li>• Rapid (~2–10 hours).</li> <li>• Increased sensitivity compared to culture methods; and</li> <li>• Can quantitatively distinguish between F-specific DNA (qPCR) and RNA (RT-qPCR) subgroups in one reaction tube.</li> </ul>	<ul style="list-style-type: none"> <li>• Infectivity not determined;</li> <li>• Inhibitors may be present in the environmental matrix;</li> <li>• Expensive (qPCR equipment) and QA/QC expertise required; and</li> <li>• Multiple sets of primers and probes in the multiplex qPCR reactions may cross-react, creating issues in method specificity (Batra et al., 2013).</li> </ul>

<b>Method</b>	<b>Advantages</b>	<b>Disadvantages</b>
CLAT	<ul style="list-style-type: none"> <li>• Same day results (2–24 hours);</li> <li>• Detects F-specific coliphages and has been applied to some somatic coliphage groups (Lee, 2009);</li> <li>• Can differentiate among F-specific genogroups;</li> <li>• Detects infectious coliphages;</li> <li>• Inexpensive;</li> <li>• Field portable; and</li> <li>• When used in an enrichment-CLAT format it is as sensitive as EPA Methods 1601 and 1602 (Love, 2007).</li> </ul>	<ul style="list-style-type: none"> <li>• Not quantitative unless implemented in an MPN format (Love and Sobsey, 2007).</li> </ul>
Culture Fast Phage	<ul style="list-style-type: none"> <li>• Results within 16–24 hours;</li> <li>• Differentiates between F-specific and somatic coliphages;</li> <li>• Infectivity is determined; and</li> <li>• Considered “equivalent” to EPA Method 1601 for groundwater monitoring by EPA’s Alternate Test Procedures program.</li> </ul>	<ul style="list-style-type: none"> <li>• Qualitative only; and</li> <li>• Requires laboratory equipment, reagents (Fast Phage kit), and training.</li> </ul>

Note: CLAT and PCR can be field portable, but all the quantitative methods require laboratory facilities.

Sources: ISO, 1995, 2000, 2001; Grabow, 2001; U.S. EPA, 2001a, b, 2007, 2010; Brussaard, 2004; Fong and Lipp, 2005; Kirs and Smith, 2007; Love and Sobsey, 2007; Gentilomi et al., 2008; Brussaard, 2009; Salter et al., 2010; Rodríguez, et al., 2012a, b; Salter and Durbin, 2012.

### 3. Epidemiological Relationships

Since the 1950s, epidemiological studies have been performed to evaluate relationships between fecal indicators and recreational swimming-associated illnesses in surface waters. The incidence of symptoms associated with gastrointestinal, eye, ear, and respiratory illnesses has been found to be higher in swimmers than in nonswimmers in ambient waters (Prüss, 1998; Wade et al., 2003; Zmirou et al., 2003).

Over the past several decades, EPA has conducted numerous epidemiological studies in both marine and freshwaters to evaluate the relationship of water quality indicators and human health risks. The results of an epidemiological study conducted by Cabelli et al. (1982) found that densities of enterococci in marine and freshwaters correlated with incidences of swimming-associated gastrointestinal illness, whereas densities of *E. coli* were correlated with swimming-associated gastrointestinal illness only in freshwaters. EPA's NEEAR study found that the occurrence of gastrointestinal illness in swimmers was positively associated with exposure to levels of enterococci enumerated by EPA's *Enterococcus* qPCR Method 1611 in marine and freshwater (Wade et al., 2008, 2010; U.S. EPA, 2012). The correlation between gastrointestinal illness and culturable enterococci in the NEEAR studies was positive, but not as strong as the relationship between illness and enterococci enumerated by qPCR. The odds of gastrointestinal illness was higher among swimmers compared to non-swimmers on days were coliphages were detected, but the associations did not achieve statistical significance (Wade et al., 2010). The statistical power was limited due to the relatively few positive results. In addition, only data on coliphage presence or absence in 100 mL volume samples were used for the analysis even though quantitative data may be available. Thus, further analyses of these data may be needed to fully understand the results of the study.

In 1982, Cabelli et al. suggested that viruses were a primary cause of gastrointestinal illness, in agreement with quantitative microbial risk assessment (QMRA) modeling that used data from the NEEAR freshwater study (Soller et al., 2015). QMRA modeling demonstrated that the illnesses reported during the NEEAR study were consistent with a virus that had an incubation period similar to NoV (Soller et al., 2015). However, NoV has not been confirmed as the cause of illness in these primary contact recreators. Interestingly, adenovirus (detected by qPCR) has been positively associated with gastrointestinal illness at a freshwater beach in Ohio (Lee, 2011).

A consistent association between FIB (*E. coli* and enterococci) and illness has not been reported at all beaches where epidemiological studies have been conducted (Colford et al., 2007). This may be due partially to the fact that FIB in surface waters can come from sources other than wastewater, such as rainfall, plants, runoff, animals, and human shedding. In some subtropical and temperate climates, bacteria, such as *E. coli* and enterococci, can multiply in the environment, giving a false impression of an increase in fecal pollution (Solo-Gabriele et al., 2000; Yamahara et al., 2009). Additionally, compared to non-spore-forming FIB, human enteric viruses have been found to be more persistent in water environments and more resistant to physical antagonism, such as heat (55°C) (Lee and Sobsey, 2011). There are clear advantages to having alternative indicators (e.g., other than *E. coli* or enterococci) that have the following attributes compared to *E. coli* and enterococci: The alternative indicators are more closely associated with viral gastrointestinal illnesses (e.g., that are present in intestinal microflora of

humans); they do not come from other, non-fecal related sources; they offer improved detection methods; they do not multiply in the aquatic environment; and they are more closely linked to the pathogens of concern (i.e., often present when viruses are present and absent in uncontaminated samples and as resistant to environmental factors as some viral pathogens).

Numerous studies have been conducted to determine whether both somatic and F-specific coliphages are associated with fecal contamination (Chung and Sobsey, 1993; Mocé-Llivina et al., 2005; Love and Sobsey, 2007). However, only a limited number of epidemiological studies have evaluated the use of coliphages as an indicator of human fecal contamination in recreational water. These results are summarized below in chronological order. When available, data on *E. coli* and enterococci are also presented for comparative purposes.

### **3.1 Von Schirnding et al. (1992)**

Von Schirnding et al. (1992) conducted a prospective cohort study at two marine beaches in South Africa with 733 participants (including adults and children). Beach 1 was described as moderately impacted by human sources of fecal contamination, including septic tank overflows, feces-contaminated river water, and stormwater runoff. Beach 2 was considered to be less impacted by known sources of fecal contamination. Participants were recruited at the two beaches. Those who entered the water above their waist were considered “swimmers” and those who entered the water up to their waist or who did not enter the water were designated as “nonswimmers.” A telephone follow-up call 3 to 4 days later recorded symptoms that developed after the beach visit. The symptoms were grouped as gastrointestinal (i.e., diarrhea, vomiting, stomachache, and nausea), respiratory (i.e., sore throat, cough, cold, runny/stuffy nose), and skin symptoms.

Water samples were collected on study days at three locations at each beach, both before and during maximum swimming activity. The following indicators were evaluated using culture-based methods: fecal coliforms, enterococci, staphylococci, somatic coliphages, and F-specific coliphages. The density of fecal coliforms and enterococci was statistically significantly higher at Beach 1 than at Beach 2 (median levels of fecal coliforms: 76.5 colony forming units (CFU) per 100 mL at Beach 1 and 8.0 CFU per 100 mL at Beach 2; median levels of enterococci: 51.5 CFU per 100 mL at Beach 1 and 2.0 CFU per 100 mL at Beach 2). Insignificant densities of staphylococci and coliphages were detected at both beaches.

The rates for gastrointestinal, respiratory, and skin symptoms (but not other symptoms including wheezing, earache, rashes, allergy, headache, backache) were higher for swimmers than nonswimmers at Beach 1, but the differences were not statistically significant. The relative risks (RR) of symptoms when comparing swimmers and nonswimmers were 2.45 (95% confidence interval [CI]: 0.55–10.9) for gastrointestinal symptoms, 3.28 (95% CI: 0.76–15.26) for respiratory symptoms, and 4.06 (95% CI: 0.52–31.72) for skin symptoms. The differences were not statistically significant for children younger than 10 years of age or for adults.

The authors suggested that a possible explanation for the higher rates of symptoms among swimmers than nonswimmers at Beach 1 was that the main sources of contamination were likely the bathers themselves or the sanitary facilities at the informal settlements close to the study beaches. This conclusion is supported by the known sources of contamination at Beach 1 and the

fact that insignificant densities of F-specific coliphages were detected at Beach 1 (because coliphages are more closely associated with sewage and septage than direct human fecal inputs).

At Beach 2, higher rates of respiratory symptoms were observed among nonswimmers than swimmers (but the differences were not statistically significant). The authors suggested that this apparent anomaly may reflect the presence of a respiratory outbreak in the community (and thus children perceived as sick were restricted from swimming by the parents), but because the numbers were low and not statistically significant, these findings should not be over-interpreted.

Overall, the authors felt that the study findings suggested a relationship between swimming-associated illness and water quality, but that larger study sizes (4,000 subjects) would be needed to detect statistically significant differences.

### **3.2 Lee et al. (1997)**

Lee et al. (1997) studied the risk of gastrointestinal illness associated with white-water canoeing and rafting in a cohort study of 473 canoeists and rafters using an artificial white-water course fed by the River Trent in England. The River Trent is a lowland river that receives considerable volumes of treated sewage and, during heavy rainfall, untreated sewage from storm overflows. The study was conducted on 11 nonconsecutive days between March and December 1995. Participants were recruited on the day of the study and given a questionnaire about their activities on the course, previous use of the course, medical history, and food eaten in the previous week. A second questionnaire (to be returned by prepaid postage 1 to 2 weeks after the study) included questions on the range of symptoms (respiratory tract, gastrointestinal, ear and eye, and general symptoms), date of onset and duration, additional water sports conducted (including at same course), and food eaten in the week after visiting the course. Gastrointestinal illness was defined as either vomiting or diarrhea (four or more loose stools in 24 hours), or fever combined with nausea, stomach pain, or loose bowels.

On each study day, water was tested hourly for levels of *E. coli*, enterococci (fecal streptococci), sulfite-reducing clostridia, F-specific RNA coliphages (using ISO method 10705-1), and culturable enteroviruses.

The study found a statistically significant association between risk of gastrointestinal illness and density of F-specific RNA coliphages. When comparing the exposure ranges of 26 to 32 PFU per 10 mL and 69 to 308 PFU per 10 mL to the reference levels of 1 to 3 PFU per 10 mL, the RR of gastrointestinal illnesses was 2.6 (95% CI: 1.3–5.2) and 2.8 (95% CI: 1.3–6.0), respectively.

Other variables significantly associated with increased risk of gastrointestinal illness were ingestion of water (RR = 1.9, 95% CI: 1.0–3.6 for swallowing two or more times compared to none), accidentally swimming in slalom course (RR = 2.3, 95% CI: 1.2–4.3), and eating and drinking before changing clothes (RR = 2.1, 95% CI: 1.1–4.0). Being a regular user of the course was associated with a reduced risk of gastrointestinal illness (RR = 1.6, 95% CI: 0.8–3.3) for one to six times per year compared to none; and RR = 0.3 (95% CI: 0.1–0.7) for seven or more uses compared to none).

The authors stated the observed association between fecal streptococci and *E. coli* levels and gastrointestinal illness risk was not seen after controlling for the stronger association seen with F-specific coliphages. The authors concluded that this study demonstrates the value of F-specific RNA coliphages as indicators of human fecal contamination associated with risk of gastrointestinal illness in recreational water.

### **3.3 Medema et al. (1995) and Van Asperen et al. (1998)**

Medema et al. (1995) conducted a pilot study to determine the relationship between microbiological freshwater quality parameters and the occurrence of health complaints among triathletes ( $n = 314$ ) using run-bike-runners as controls ( $n = 81$ ). Information on the occurrence of health complaints during the competition and in the week thereafter was collected through a written questionnaire. The authors did not link reported illnesses to water quality, other than to report the water quality during the time of the triathlon. The geometric means of FIB were 170 *E. coli* CFU 100 per mL and 13 fecal streptococci CFU per 100 mL. F-specific RNA coliphages geometric mean was 5.6 PFU per 100 mL. Enteroviruses were present at densities of 0.1 PFU per L. Triathletes reported higher rates of symptoms than run-bike-runners: gastrointestinal (7.7 versus 2.5%), respiratory (5.5 versus 3.7%), skin/mucosal (2.6 versus 1.2%), general (3.5 versus 1.2%), and total symptoms (14.8 versus 7.4%) in the week after the event. Approximately 75% of triathletes reported ingesting water during the swim event.

Van Asperen et al. (1998) extended the Medema et al. (1995) study over two summers. In a prospective cohort design, they followed 827 triathletes and 773 run-bike-run controls. A mailed detailed questionnaire collected data about age, sex, general health, medical, and race history, exposure to surface freshwaters in the week before and after the race, and occurrence of gastrointestinal complaints 2 days before, during, and 6 days after the race. Triathletes were also asked about goggle and wetsuit use during the race and if they ingested water during the swimming portion of the race. Four different GE endpoints were defined as follows:

- GE\_UK: (diarrhea AND three or more bowel movements per day) OR vomiting OR (nausea AND fever);
- GE\_US: vomiting OR (diarrhea AND fever) OR (nausea AND fever) OR (stomach pains AND fever);
- GE\_NL-1: (diarrhea AND three or more loose stools movements per day) AND (at least two of fever OR nausea OR vomiting OR stomach pains); and
- GE\_NL-2: diarrhea OR nausea OR vomiting OR stomach pains.

On each exposure day, water samples were collected along the swimming course. Samples were analyzed for densities of *E. coli*, thermotolerant coliforms, fecal streptococci, enteroviruses, and reoviruses, F-specific RNA coliphages, *Salmonella*, *Campylobacter*, *Aeromonas*, *Plesiomonas shigelloides*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*. The geometric mean (ranges) of the microorganisms during triathlons were: thermotolerant coliforms 78 CFU per 100 mL (0.6 to 650 CFU per 100 mL), *E. coli* 204 CFU per 100 mL (11 to 2,600 CFU per 100 mL), fecal streptococci 16 CFU per 100 mL (0.2 to 1,800 CFU per 100 mL), enteroviruses 0.04 PFU per L (0.007 to 7 PFU per L), and F-specific RNA coliphages 0.7 PFU per L (0.01 to 13.6 PFU per L).

Depending on the case definition, the attack rates of GE in the week after the event were observed to be higher among triathletes than among run-bike-runners, with odds ratios (ORs) ranging from 1.6 to 2.3. The adjusted risk of GE ranged from 2.9 to 4.7, depending on the case definition. All ORs were statistically significant.

The study showed that both *E. coli* and thermotolerant coliforms were associated with risk of gastrointestinal illness after bathing in freshwaters. The authors noted that levels of *E. coli* were higher than the levels of thermotolerant coliforms, and that the densities of *E. coli* were much more closely correlated with illness rates than the densities of thermotolerant coliforms. A relationship between health and fecal streptococci, enteroviruses, and F-specific RNA coliphages was not observed (Van Asperen et al., 1998).

### 3.4 Wiedenmann et al. (2006)

Wiedenmann et al. (2006) conducted a randomized control epidemiological study at five freshwater bathing beaches in Germany. The probable or possible sources of fecal contamination at these sites varied, but included raw and treated municipal effluent, agricultural runoff, and contamination from water fowl. Only one of the five lakes had a known point-source of contamination. A cohort of 2,196 participants (including adults, children, and teenagers) was recruited from the local population and randomized into bathers and nonbathers. Two to three days prior to exposure, participants were interviewed and underwent a brief medical examination. Bathers were exposed to water for 10 minutes and were asked to immerse their heads at least three times. Nonbathers made no contact with the water. One week after exposure, all participants were interviewed and underwent medical inspection of the throat, eyes, and ears. Three different GE endpoints were defined as follows:

- GE\_UK: (diarrhea AND three or more bowel movements per day) OR vomiting OR (nausea AND fever) OR (indigestion AND fever);
- GE\_UK-wf: (GE\_UK without consideration of stool frequency: diarrhea OR vomiting OR (nausea AND fever) OR (indigestion AND fever); and
- GE\_NL-2: diarrhea OR nausea OR vomiting OR stomach pains.

Water samples were collected at 20-minute intervals from swimming and nonswimming zones during the study period. The following microbiological parameters were evaluated: *E. coli*, enterococci, *Clostridium perfringens*, aeromonads, pyocyanine-positive *Pseudomonas aeruginosa*, and somatic coliphages. The median densities (ranges) of the microorganisms were: 20 somatic coliphages PFU per 100 mL (10 to 3,780 PFU per 100 mL; method ISO 10705-2); 136 *E. coli* CFU per 100 mL (4.7 to 5,344 CFU per 100 mL), 37 intestinal enterococci CFU per 100 mL (3.0 to 1,504 CFU per 100 mL), 15 *Clostridium perfringens* CFU per 100 mL (9 to 260 CFU per 100 mL), 8,200 aeromonads CFU per 100 mL (600 to 31,400 CFU per 100 mL), and 10 *Pseudomonas aeruginosa* CFU per 100 mL (10 to 100 CFU per 100 mL).

For somatic coliphages, the no observed adverse effect level (NOAEL) was 10 PFU per 100 mL for the two less stringent (broader) illness definitions (GE\_UK-wf and GE\_NL-2) and 150 PFU per 100 mL for the most stringent (most narrowly defined) illness definition (GE\_UK). The RRs of GE\_NL-2, GE\_UK-wf, and GE\_UK when comparing bathing in waters with somatic coliphage levels above the NOAEL, with nonbathing were statistically significant and ranged from 1.8 (95% CI: 1.2–2.6), 2.5 (95% CI: 1.5–4.0), and 4.6 (95% CI: 2.1–10.1), respectively. For

all three illness definitions, swallowing water with somatic coliphage levels above the NOAEL resulted in a significantly higher attributable risk of illness than not swallowing water.

The authors concluded that reasonable estimates for NOAELs at an average bathing intensity site are 100 *E. coli* CFU per 100 mL, 25 enterococci CFU per 100 mL, and 10 somatic coliphages PFU per 100 mL. Wiedenmann et al. (2006) concluded that a NOAEL approach would be practical for setting recreational water standards. The authors suggested that somatic coliphages would be appropriate alternative fecal indicators that could be used to set standards for freshwater just as well as *E. coli* and enterococci, especially in tropical climates, where *E. coli* and enterococci may be less reliable as indicator organisms.

### **3.5 Colford et al. (2005, 2007)**

Colford et al. (2005, 2007) conducted a prospective cohort epidemiological study at six beaches near Mission Bay, California in 2003. The study cohort consisted of nearly 8,000 participants. The authors reported that MST evaluation at Mission Bay suggested that only a minor portion of fecal input was from human point sources during the study period.

Water quality was monitored using traditional FIB enumeration methods (culturable enterococci, fecal coliforms, and total coliforms) and a subset of samples was also evaluated using: (1) new methods for measuring traditional FIB (chromogenic substrate or qPCR), (2) *Bacteroides*, (3) coliphages (somatic and F-specific coliphages), and (4) human enteric viruses (adenovirus and NoV). F-specific and somatic coliphages were detected and quantified in 1 L volumes of water by a modification of EPA Method 1601 for enrichment and spot plating that provides a MPN estimate of coliphage density. Roughly 68% of the samples had detectable levels of somatic coliphages and maximum densities were observed near 36 MPN per 100 mL. F-specific coliphages were detected in 11% of the samples and maximum densities reached only one MPN per 100 mL. No NoV was found and adenovirus was found only in one sample. The observed geometric means for enterococci (measured by qPCR) and fecal coliforms were 65 estimated number per 100 mL and 25 MPN per 100 mL, respectively.

Interviewers recorded which water sampling site was closest to the location of the individual or family on the beach. Participants were asked to complete a questionnaire prior to their departure from the beach. The questionnaire assessed possible exposures at the beach, and exposures or illnesses experienced during the previous two to three days. A follow-up telephone interview was conducted 10 to 14 days after the study to gain information on health outcomes. Health outcomes measured in the investigation included gastrointestinal illness, respiratory symptoms, and skin ailments. Two definitions of highly credible gastrointestinal illness (HCGI) were measured. One (HCGI-1) was defined as (1) vomiting; (2) diarrhea and fever; or (3) cramps and fever. The second (HCGI-2) was defined as vomiting plus fever. Multivariate analysis was conducted to assess relationships between health outcomes and degree of water contact or levels of water quality indicators. These analyses were adjusted for confounding covariates such as age, gender, and ethnicity.

Of the measured health outcomes, only skin rash and diarrhea were consistently significantly elevated in swimmers compared to nonswimmers. For diarrhea, this risk was strongest among children 5 to 12 years old. No correlation was found between increased risk of illness and levels

of *Bacteroides* or *Enterococcus*, as detected using rapid methods (qPCR) or for somatic coliphages. A significant association was observed between the levels of F-specific coliphages and HCGI-1, HCGI-2, nausea, cough, and fever. The adjusted odds ratio (AOR) was 1.26 (95% CI: 1.06–1.48) for HCGI-1; 1.43 (95% CI: 1.13–1.82) for HCGI-2; 1.34 (95% CI: 1.16–1.55) for nausea; 1.22 (95% CI: 1.02–1.48) for cough; and 1.25 (95% CI: 1.09–1.44) for fever. Colford et al. (2005, 2007) suggested that these associations be interpreted cautiously because only a small number of participants were exposed to the water at times when F-specific coliphages were detected.

### 3.6 Wade et al. (2010)

Wade et al. (2010) enrolled 6,350 participants in prospective cohort epidemiological studies conducted at three marine beaches. These beaches were located in Mississippi (Edgewater Beach), Rhode Island (Goddard Beach), and Alabama (Fairhope Beach), and were known to be impacted by discharge from nearby WWTPs. The study in Mississippi was conducted in 2005 and studies in Rhode Island and Alabama were conducted in 2007. Upon study enrollment, participants were interviewed to gather information on exposure and health status and completed a questionnaire prior to exiting the beach for the day. Based on their activities for the day, participants were divided into cohorts that included swimmers and nonswimmers. Swimming was defined as body immersion (i.e., immersion to the waist or higher). Nonswimmers were considered unexposed to recreational water. Health endpoints evaluated during the study included upper respiratory illness (defined as any two of the following: sore throat, runny nose, cough, cold, or fever), earache, eye irritation, rash, and gastrointestinal illness (defined as any of the following: (1) diarrhea (three or more loose stools in a 24-hour period); (2) vomiting; (3) nausea and stomachache; or (4) nausea or stomachache, and interference with regular activities (missed regular activities as a result of the illness).

Water samples were collected in duplicate at three different time points along three transects perpendicular to the shoreline on each study day. A total of 1,242 water samples were collected. Water samples were tested for a variety of indicators, including a faster test for F-specific coliphages based on a CLAT assay, which also distinguishes F-specific RNA coliphages and F-specific DNA coliphages. F-specific coliphages were also evaluated using a modified version of EPA Method 1601, called the 24-hour SPOT assay. Samples were tested for *Enterococcus* spp. using EPA Method 1600 (a culture-based method) and *Enterococcus* and *Bacteroidales* by qPCR.

Wade et al. (2010) reported that 56% (100 of 222) of samples at Fairhope Beach and 65% (203 of 425) of samples at Goddard Beach were positive for F-specific coliphages by the modified EPA Method 1601.<sup>5</sup> Fewer samples were positive for F-specific coliphages by the CLAT assay. At Fairhope Beach, 4% (8 of 228) and 6% (14 of 224) of samples were positive for F-specific RNA and F-specific DNA coliphages, respectively. At Goddard Beach, 7% (31 of 425) and 9% (37 of 423) of samples were positive for F-specific RNA and F-specific DNA coliphages, respectively. The AOR of gastrointestinal illness was significantly higher among swimmers compared to nonswimmers on days when F-specific RNA (AOR = 1.80, 95% CI: 1.22–2.66) or

<sup>5</sup> Edgewater Beach, data collection was stopped several days early due to the effects of Hurricane Katrina. Bacteriophage results were not reported for Edgewater Beach.

F-specific DNA coliphages (AOR = 1.69, 95% CI: 1.16–2.47) were detected by the CLAT assay or F-specific coliphages were detected by a modified 1601 method (AOR = 1.70, 95% CI: 1.12–2.57). An increased, but not statistically significant risk of gastrointestinal illness among swimmers was observed for a 1-log<sub>10</sub> increase in each of the three F-specific coliphages measured. F-specific coliphages measured by the modified 1601 method were not associated with gastrointestinal illness among swimmers. Other illnesses (i.e., respiratory illness, earache) did not show a relationship with the presence of coliphages.

The risks of both gastrointestinal illness and diarrhea were significantly associated with exposure to *Enterococcus* and *Bacteroidales* (enumerated using qPCR). F-specific coliphages, using the modified 1601 method, had a positive correlation with gastrointestinal illness in marine waters, but like culturable enterococci, the association was not significant over the full range of water quality (Wade et al., 2010).

### 3.7 Abdelzaher et al. (2011)

Abdelzaher et al. (2011) performed a randomized control exposure epidemiological study to evaluate water quality and daily cumulative health effects for bathers at a nonpoint source subtropical marine recreational beach in Miami, Florida. Study participants were randomly assigned to either the ‘bather’ or the ‘nonbather’ categories. Those assigned to the bather category were asked to spend 15 minutes in the water and nonbathers were asked to spend 15 minutes on the beach only. The daily number of bathers varied over the course of the study, with a total of 652 bathers (daily average = 43, daily range = 29–55). Similarly, for nonbathers, the total number was 651 (daily average = 43, and daily range = 25–60).

Health effects considered during the study included gastrointestinal illness, skin ailments, and respiratory illness. Gastrointestinal illness was defined as all cases of vomiting or diarrhea, or all reported cases of indigestion or nausea accompanied by a fever. ‘Diarrhea’ was defined as having three or more runny stools within a 24-hour period.

Water samples were categorized as “daily composite samples,” which were combined water samples collected throughout each sampling day either by bathers or study staff. Bather-collected samples were analyzed for a variety of indicator organisms, including enterococci (using three detection methods: membrane filtration, chromogenic substrate, and qPCR), fecal coliform, *E. coli*, *Clostridium perfringens* (all measured by membrane filtration), somatic and F-specific coliphages (measure by EPA Method 1602), human- and dog-associated MST markers (*Bacteroides thetaiotaomicron*, BacHum-UCD, HF8, and DogBac), human polyomavirus, and the *esp* gene of *Enterococcus faecium*. Pathogens evaluated in the study included: *Staphylococcus aureus*, *Vibrio vulnificus*, the protozoa *Cryptosporidium* spp. and *Giardia* spp., NoV, enterovirus, and hepatitis A virus. Investigator-collected composite samples were used for pathogen analysis using traditional large-volume concentration methods.

Average daily excess illness percentage rates (calculated by subtracting the daily illness rates for nonswimmers from that for swimmers) for gastrointestinal, skin, and acute febrile respiratory illness were 2.0% (standard deviation [SD] = 3.3), 5.6% (SD = 4.7), and 1.2% (SD = 2.9), respectively. No statistically significant correlations between health outcomes and any of the indicator organisms, including coliphages, were identified in this investigation.

Somatic coliphages were detected (range 0.3 to 1.7 PFU per 100 mL) on four of 15 days tested. On three of five days where the greatest level of excess gastrointestinal illness occurred, somatic coliphages were detected. Although no statistically significant associations between water quality and illness were observed, the authors state that this overlap is suggestive of a potential correspondence between the presence of somatic coliphages and increased risk of gastrointestinal illness. Given the low number of positive samples and that F-specific coliphages were not detected in any of the samples, no apparent association between this potential indicator and health outcomes was observed in this study. The authors suggest that a possible reason F-specific coliphages were not detected may be due to the small volume of water for each sample (100 mL) compared to other studies, such as Colford et al. (2005, 2007) who used 1 L samples, thereby increasing detection limits.

### **3.8 Griffith et al. (personal communication, 2015)**

The Southern California Coastal Water Research Project Authority conducted two prospective cohort studies at California beaches (Avalon Bay (Avalon), Doheny State Beach (Doheny)) in 2007 and 2008. Both Avalon and Doheny were impacted by faulty sanitary sewer infrastructure, which allowed microbial contamination to reach the beach via groundwater.

The studies enrolled 8,226 swimmers across the two beaches and each swimmer's water exposure was recorded. Water samples were collected several times per day at multiple locations at each beach and analyzed for up to 30 target indicators using more than 50 different methodologies. Interviewers contacted participants by phone 10 to 14 days later and recorded symptoms of gastrointestinal illness occurring after their beach visit. Regression models were used to evaluate the association between water quality indicators and gastrointestinal illness among swimmers at each beach.

In these two studies, F-specific coliphages measured by EPA Method 1602 had a stronger association with health outcomes than did culturable enterococci measured by EPA Method 1600 at Doheny and Avalon beaches. When all environmental conditions were considered in aggregate at Doheny, the OR for F-specific coliphages was 1.9 and statistically greater than 1 ( $p<0.05$ ), whereas the OR for enterococci was only 1.2 and not significant ( $p>0.05$ ). At Avalon, the OR for F-specific coliphages was 1.9 compared to less than 1.1 for enterococci, though neither was significantly different than 1.0 ( $p>0.05$ ). Under highrisk conditions, F-specific coliphages were significantly associated with gastrointestinal illness ( $p<0.05$ ) and the estimated OR was more than double that for culturable enterococci at both Avalon and Doheny. Associations were also found between F-specific coliphages and adenovirus observed at Doheny Beach (Love et al. 2014). The authors noted that when the contamination source is primarily human fecal material, indicators like F-specific coliphages are better predictors of the health risk.

### **3.9 General Conclusions from Epidemiological Studies**

Eight epidemiological investigations have evaluated the relationship between swimming-associated illness and presence of coliphages. Studies specifically evaluating the link between levels of somatic or F-specific coliphages and incidence of illness resulting from exposure to fresh and marine waters are summarized in Table 4.

With the exception of one small study (Von Schirnding et al., 1992), all of the epidemiological investigations that evaluated coliphages detected somatic or F-specific coliphages. There is considerable heterogeneity in the results of these studies, even within similar designs. For example, some studies found a significant association between the levels of F-specific coliphages and two definitions of gastrointestinal illness, cough, fever, and nausea, but found no association between increased risk of illness and levels of somatic coliphages, *Bacteroides*, or *Enterococcus* (Colford et al., 2005, 2007). Similarly, when comparing swimmers to nonswimmers on days when F-specific RNA or DNA coliphages were detected, Wade et al. (2010) found statistically significant increases in risk of gastrointestinal illness. In these cases, F-specific coliphages were potentially useful indicators.

On the other hand, at a marine recreational beach in Miami with no known point source of contamination, Abdelzaher et al. (2011) detected somatic coliphages (range 0.3 to 1.7 PFU per 100 mL) on four of 15 days tested, three of which were on days characterized by the highest excess gastrointestinal illness. However, F-specific coliphages were not detected in any of the samples, and no statistically significant correlations between water quality and illness were found. In this case somatic coliphages may have been useful, but F-specific coliphages were not useful, due to being below the detection limits of methods.

Overall, the epidemiological evidence is suggestive of a potential relationship between coliphages and human health. In more than half the studies (Lee et al., 1997; Colford et al., 2005, 2007; Wiedenmann et al., 2006; Abdelzaher et al., 2011, Wade et al., 2010), the presence of coliphages was associated with swimming-associated gastrointestinal illness. Wade et al. (2010) found that the AOR of gastrointestinal illness was higher among swimmers compared to nonswimmers on days when F-specific RNA and DNA coliphages were detected. These studies suggest that somatic (Wiedenmann et al., 2006) and F-specific coliphages (Lee et al., 1997; Colford et al., 2005, 2007; Wade et al., 2010; Griffith et al., personal communication, 2015) hold potential as feasible alternative water quality indicators in marine and freshwaters, with and without point-source contamination (Lee et al., 1997; Colford et al., 2005, 2007; Wiedenmann et al., 2006; Wade et al., 2010; Abdelzaher et al., 2011). As mentioned in Section 1.3, a good indicator should be correlated to health risk. Evaluation of the results of these eight epidemiological studies suggests that overall the studies support coliphages as potential indicators of gastrointestinal illness from recreational exposures.

**Table 4. Summary of epidemiological studies.**

Study, Sample size, Water type	Indicators evaluated	Results	Supports coliphages as water quality indicator?
Von Schirnding et al. (1992), n = 733, marine	<b>Somatic coliphages and F-specific coliphages,</b> fecal coliforms, enterococci	Low densities of coliphages were detected at both beaches. Rates for gastrointestinal, respiratory, and skin symptoms were higher for swimmers than nonswimmers at Beach 1, but the results were not statistically significant.	Colophage levels were too low to evaluate.
Lee et al. (1997), n = 473, fresh	<b>F-specific RNA coliphages, <i>E. coli</i>, enterococci, culturable enteroviruses</b>	Statistically significant association between risk of gastrointestinal illness and density of F-specific RNA coliphages. The observed association between fecal streptococci and <i>E. coli</i> levels and risk of gastrointestinal illness was not seen after controlling for the stronger association seen with F-specific coliphages.	Yes; F-specific coliphages
van Asperen (1998), 827 triathletes and 773 run-bike-run controls, Fresh	<b>F-specific coliphages, <i>E. coli</i>, fecal streptococci, thermotolerant coliforms, enteroviruses</b>	Risk of gastrointestinal illness increased significantly at levels with thermotolerant coliforms ( $\geq 220$ CFU per 100 mL) or <i>E. coli</i> ( $\geq 355$ CFU per 100 mL), compared to lower levels ( $\leq 120$ CFU per 100 mL tolerant coliforms or $\leq 238$ CFU per 100 mL for <i>E. coli</i> ). No exposure-response relationship observed for F-specific coliphages, fecal streptococci, or enteroviruses.	No
Wiedenmann et al. (2006), n = 2,196, fresh	<b>Somatic coliphages, <i>E. coli</i>, enterococci</b>	Significantly increased RR of gastroenteritis for bathing in waters with somatic coliphage levels above the NOAEL (10 PFU per 100 mL) versus nonbathing.	Yes; somatic coliphages
Colford et al. (2005, 2007), n = 8,000, marine	<b>F-specific coliphages (qPCR), somatic coliphages, culturable enterococci, fecal coliforms, total coliforms, adenovirus, and NoV</b>	Significant association between the levels of F-specific coliphages and HCGI-1, HCGI-2, nausea, cough, and fever.	Yes; F-specific coliphages
Wade et al. (2010), n = 6,350, marine	<b>F-specific RNA coliphages (CLAT), F-specific DNA coliphages (CLAT), F-specific coliphages (modified EPA Method 1601), enterococci</b>	Significantly higher risk of gastrointestinal illness comparing swimmers with nonswimmers on days when coliphages were present.	Yes; F-specific coliphages
Abdelzaher et al. (2011), n = 652, marine	<b>Somatic coliphages, enterococci, fecal coliforms, <i>E. coli</i></b>	No statistically significant correlations between health outcomes and any indicator organisms, including somatic coliphages.	Somatic coliphages detection overlaps with highest illness days.
Griffith et al. (personal communication, 2015)	<b>F-specific coliphages (Method 1602); enterococci, and 30 target indicators with 50 different methodologies.</b>	F-specific coliphages (measured using EPA Method 1602) had a stronger association with health outcomes than EPA Method 1600 at the two beaches studied.	Yes; F-specific coliphages

## **4. Occurrence in the Environment**

Coliphages, including F-specific DNA, F-specific RNA coliphages, and somatic coliphages have been detected and proposed as indicators of fecal contamination in a variety of environments. Most studies investigating coliphages as fecal indicators focused on environments that may be contaminated with human or animal fecal matter, such as water entering or exiting sewage treatment facilities, stormwater, natural lakes, rivers, streams, groundwater, seawater, and beach sand (Zaiss 1981; Sogaard, 1983; Payment et al., 1988; Araujo et al., 1997; Paul et al., 1997; Gantzer et al., 1998; Davies et al., 2003; Bonilla et al., 2007; Charles et al., 2009; Haramoto et al., 2009, 2011; Payment and Locas, 2011; Wu et al., 2011).

Studies investigating the presence of coliphages and viruses in different types of environmental waters are described below (Section 4.1). A review of the literature shows that generalizations across studies are difficult because the detection of microorganisms from fecal contamination, including viruses and coliphages are inconsistent and dependent on a number of important factors (WHO, 2001). Generally, when any two studies on coliphages and viruses are compared, there are differences between the type of detection method used – both for the coliphages and the pathogen. In addition to different detection methods, the differences between studies might include the following: type of coliphage tested (i.e., somatic, F-specific DNA, F-specific RNA); specific pathogens tested; number of samples taken; volume of sample taken; level of contamination; type of environment from which samples were taken; location of the environment; resistance of the coliphages and pathogens to environmental stressors and growth; transport characteristics of the coliphages and pathogens; carriage rates and shedding patterns of the coliphages and pathogens among host populations; presence of host populations; waste management practices; rainfall; time of year; and statistical analyses used (WHO, 2001; Bonilla et al., 2007; Wu et al., 2011). Given these differences along with the variable occurrence of viruses in fecal sources, it is not surprising that the presence of fecal indicators including coliphages and the presence of enteric viruses varies between studies.

### **4.1. Associations between Coliphages and Viruses**

Some studies have reported an association between the presence of coliphages and human viruses (Havelaar et al., 1993; Jiang et al., 2001; Ballester et al., 2005), while other studies have found no association between their presence (Ibarluzea et al., 2007; Jiang et al., 2007; Boehm et al., 2009; Viau et al., 2011b). Meta-analyses of peer-reviewed studies looking at the occurrence of microbial indicators and pathogens, including coliphages and viruses, can give an overview of the field.

In one recent study, Wu et al. (2011) analyzed a broad range of 540 indicator-pathogen pairs from studies conducted between 1970 and 2009 in a variety of water environments including: rivers, lakes, reservoirs, ponds, estuaries, costal and marine waters, and wastewater (Wu et al., 2011). Groundwaters, treated drinking waters, and sand/sediments were not included in the study. The data were analyzed using a logistic regression model adjusted for indicator classes, pathogen classes, water types, pathogen sources, sample size, the number of samples with pathogens, the detection method, year of publication, and statistical method. The association is presented as an OR, where an OR greater than one signifies that the presence of the indicator is associated with the presence of the pathogen. Not surprisingly, no single indicator was

significantly correlated with all the pathogens evaluated.<sup>6</sup> Coliphages (F-specific and somatic together) and F-specific coliphage densities were more likely to be correlated with pathogens than the other traditional indicators (*E. coli*, enterococci, and fecal coliforms) (Wu et al., 2011). The associations between coliphages and pathogens were not statistically significant (OR = 1.29, *p*-value = 0.186 and OR = 1.27, *p*-value = 0.625, respectively). See Table 5 below for specific OR and *p*-values between different categories of coliphages or indicators and pathogens in water. Silva et al. (2010) also found that in water samples collected from 16 beaches along the Portuguese coast there was no relationship between viral detection (hepatitis A and NoV) and the European regulatory-based bacterial indicators total coliform, fecal coliform, *E. coli*, and fecal enterococci.

**Table 5. Number of cases and outcome of the logistical regression analysis of the association between coliphages and pathogens in water.**

Fecal indicator	Number of cases <sup>a</sup>		OR Value	<i>p</i> -Value	95% Confidence limits
	Uncorrelated	Correlated			
Coliphages <sup>b</sup>	45	40	<b>1.29</b>	0.186	0.82 – 2.05
F-specific coliphages	24	16	<b>1.27</b>	0.625	0.48 – 3.35
F-specific RNA coliphages	15	8	0.75	0.518	0.31 – 1.80
Somatic coliphages	20	10	0.70	0.364	0.32 – 1.52
<i>E. coli</i>	29	11	0.52	0.070	0.25 – 1.06
Enterococci	34	12	0.47	0.032	0.24 – 0.94
Fecal coliforms	78	48	0.84	0.405	0.56 – 1.27

Source: Based on Table 2 in Wu et al. (2011).

<sup>a</sup> An individual case of an indicator-pathogen pair represents a statistical analysis of a published dataset of one indicator type with one pathogen type where the methods of statistical analysis, correlation coefficients, and *p*-values were reported.

<sup>b</sup> Includes F-specific and somatic coliphages.

OR values above 1 are in bold.

Studies have evaluated the association between pathogens and different subsets of coliphages (i.e., somatic, F-specific DNA and RNA) and report variable results which are influenced by the environments in which the studies are conducted (Ballester et al., 2005; Savichtcheva and Okabe, 2006; Payment and Locas, 2011). For example, Wu et al. (2011) report that no indicator-pathogen pairs were significantly associated, except for F-specific coliphage-adenovirus pairs (OR = 25.5, *p*-value = 0.019) (see Table 6 below). Wu et al. (2011) also found that the association between indicators and pathogens is significantly stronger in brackish and saline water than in freshwater. Therefore, the papers in this chapter are separated into those studies conducted in freshwater and those conducted in saline or brackish water. Because Wu et al. (2011) conducted a meta-analysis, which is summarized above, that includes most of the studies comparing coliphages to human viruses, only a few of the illustrative studies that compare coliphages to human viruses in ambient water are summarized in Sections 4.1.1 and 4.1.2 below.

<sup>6</sup> Individual articles evaluated different pathogens. Pathogens (and pathogen genes) paired with indicators included *Giardia*, *Cryptosporidium*, *Campylobacter*, *Helicobacter pylori*, *Salmonella*, shiga toxin genes, *Pseudomonas aeruginosa*, *Aeromonads*, *Vibrio*, *Staphylococcus aureus*, hepatitis A virus, adenoviruses, astroviruses, NoVs, sapoviruses, enteroviruses, human enteric viruses, filamentous fungi, yeasts, and *Candida albicans*.

**Table 6. Logistic regression of the association between indicators and different pathogens in water.**

Pathogens	Indicators			
	F-specific coliphages OR value ( <i>p</i> -value); [95% Confidence Limits]	Somatic coliphages OR value ( <i>p</i> -value); [95% Confidence Limits]	<i>E. coli</i> ; [95% Confidence Limits]	Enterococci; [95% Confidence Limits]
Adenoviruses	<b>25.5</b> ( <i>p</i> = 0.019); [1.72, 377.92]	<b>1.25</b> ( <i>p</i> = 0.862); [0.10, 15.50]	NR	NR
<i>Cryptosporidium</i> spp.	NR	0.74 ( <i>p</i> = 0.791); [0.08, 6.97]	NR	0.73 (0.700); [0.14, 3.70]
Enteroviruses	<b>1.2</b> ( <i>p</i> = 0.810); [0.27, 5.29]	NR	<b>1.19</b> (0.869); [0.16, 8.99]	0.87 (0.858); [0.18, 4.23]
<i>Giardia</i> spp.	NR	<b>1.06</b> ( <i>p</i> = 0.965); [0.09, 12.42]	NR	<b>1.06</b> (0.950); [0.18, 6.36]

Note: Data are from Wu et al. (2011).

Numbers in the table are the OR values followed by the *p*-values in parentheses. OR values above 1 are in bold.

NR (not reported) indicates that the data were not included in the paper.

Pathogens and indicators are listed in alphabetical order.

### Effects of human virus detection methods on associations between fecal indicators and pathogens

As briefly described above in Section 2.3, there are currently numerous methods to detect human viruses. These include culture methods, molecular methods, and a combination of the two (Fong and Lipp, 2005). Similar to coliphage detection methods, each method has advantages and disadvantages, which in turn affect the type of data collected, including both quantity and the type(s) of virus(es) detected. An overview of the strengths and weaknesses of each enteric virus detection method is shown below in Table 7. For example, according to Mocé-Llivina et al. (2005), genomic techniques used to detect human enteroviruses and other human viruses have detection rates from 7 to 70% and are not always consistent with the values of other methods for enumerating the same organisms. Reasons for the variability between PCR and culture-based techniques are due in part to: (1) PCR does not distinguish between infectious and noninfectious viruses (i.e., live and dead viruses); (2) the high sensitivity of PCR may contribute to artifacts, which could result in false positives; and (3) natural inhibitors in the environment may reduce or block PCR amplification resulting in false negatives or under-representation of infectious viruses (Fong and Lipp, 2005; Mocé-Llivina et al., 2005). It is important to keep in mind that differences in enteric virus detection methods (see Table 7) combined with differences in coliphage detection methods (see Table 3) may greatly affect the presence, absence and/or strength of correlations found between coliphages and enteric viruses.

**Table 7. Comparison of common methods for the detection of pathogenic human enteric viruses from environmental sources.**

Method	Advantages	Disadvantages
Cell culture	<ul style="list-style-type: none"> <li>Provides quantitative data; and</li> <li>Infectivity can be determined.</li> </ul>	<ul style="list-style-type: none"> <li>Lengthy processing time (takes days to weeks);</li> <li>Relatively more expensive than PCR; and</li> <li>Not all viruses from environmental samples can grow in cell culture (e.g., NoV).</li> </ul>
PCR (RT-PCR)	<ul style="list-style-type: none"> <li>Rapid;</li> <li>Can be quantitative (e.g., end point analysis); and</li> <li>Increased sensitivity and specificity compared to cell culture.</li> </ul>	<ul style="list-style-type: none"> <li>Usually qualitative;</li> <li>Inhibitors may be present in the environmental matrix; and</li> <li>Infectivity cannot be determined<sup>a</sup>.</li> </ul>
Nested PCR (semi-/heminested)	<ul style="list-style-type: none"> <li>Increased sensitivity compared to conventional PCR; and</li> <li>Can replace PCR confirmation steps, such as hybridization.</li> </ul>	<ul style="list-style-type: none"> <li>Qualitative only;</li> <li>Inhibitors may be present in the environmental matrix;</li> <li>Potential risk of carryover contamination when transferring PCR products; and</li> <li>Infectivity cannot be determined<sup>a</sup>.</li> </ul>
Multiplex PCR and Multiplex RT-PCR	<ul style="list-style-type: none"> <li>Several types, groups, or species of viruses can be detected in a single reaction; and</li> <li>Saves time and cost compared to PCR.</li> </ul>	<ul style="list-style-type: none"> <li>Difficult to achieve equal sensitivity for all targeted virus species, groups, or types;</li> <li>May produce nonspecific amplification in environmental samples;</li> <li>Inhibitors may be present in the environmental matrix; and</li> <li>Infectivity cannot be determined<sup>a</sup>.</li> </ul>
qPCR/RT-qPCR	<ul style="list-style-type: none"> <li>Provides quantitative data;</li> <li>Confirmation of PCR products is not required (saves time); and</li> <li>Can be done in a closed system, which reduces risk of contamination compared to nested PCR.</li> </ul>	<ul style="list-style-type: none"> <li>The lower limit of quantification is higher than the lower limit of detection, so qPCR can be considered less sensitive than presence/absence PCR;</li> <li>Can be more affected by inhibitors present in the environmental matrix than culture methods; and</li> <li>Infectivity cannot be determined<sup>a</sup>.</li> </ul>
ICC-PCR and ICC-RT-PCR	<ul style="list-style-type: none"> <li>Improves detection of infectious viral pathogens compared to conventional cell culture;</li> <li>Detects viruses that do not produce cytoplasmic effects in cell culture; and</li> <li>Provides results in half the time required for conventional cell culture.</li> </ul>	<ul style="list-style-type: none"> <li>Less time-efficient and more costly than direct PCR detection;</li> <li>Carryover detection of DNA of inactivated viruses inoculated onto cultured cells is possible; and</li> <li>Cannot be used for viruses that cannot be cultured.</li> </ul>

Note: Table modified from Table 2 in Fong and Lipp (2005).

<sup>a</sup> Can determine infectivity if conducted in combination with ICC. See row on ICC-PCR and ICC-RT-PCR in table for more details.

#### **4.1.1 Coliphage – Virus Associations in Freshwater**

In studies that evaluated the association between the occurrence of coliphages and viruses in freshwater, results have varied. For example, Espinosa et al. (2009) found a strong association between F-specific coliphages and enterovirus ( $p$ -value = 0.0182), but a weak relationship with coliphages and rotavirus ( $p$ -value = 0.1502) and astrovirus ( $p$ -value = 0.4587) in high-altitude surface water.

In a four-year study of surface source waters using 10 testing locations in the Netherlands, Lodder et al. (2010) found a significant association between densities of coliphages (F-specific and somatic) and enteroviruses, but not between coliphages and other viruses (NoV, rotavirus, and reovirus) or between the other viruses (NoV, rotavirus, and reovirus). NoV and rotavirus were detected in 45% and 48% of the samples, respectively. Infectious enterovirus and reovirus were detected in approximately 80% of the tested samples. Somatic and F-specific coliphages were detected in 100% and 97% of the samples, respectively. In the two samples where no F-specific coliphages could be detected, enteroviruses were present, and in one sample and rotavirus and NoV was also detected. Lodder et al. (2010) concluded that their results do not support a role for coliphages as indicators of source water quality, however, they also conclude that coliphages may be useful for determining treatment efficiencies.

Payment and Locas (2011) used 20 years of sampling data from their laboratory to examine the association between pathogens and multiple microbial indicators, including coliphages, in sewage, surface water, and groundwater. Although the authors review data for several water types, coliphage associations with pathogens were investigated in groundwater. Their analysis of 242 samples from 25 municipal groundwater well sites indicated that somatic and F-specific RNA coliphages were not predictive of virus presence or absence. This was due in part to the low numbers of coliphages present in the samples and their infrequent detection (Payment and Locas, 2011).

Viau et al. (2011b) found no significant association between the presence of F-specific coliphages and adenovirus, enterovirus, NoV GI, and NoV GII in tropical coastal streams. Additionally, Hot et al. (2003) found no significant association between the density of somatic coliphages and the presence of viral pathogens (RT-PCR detection of the genome of hepatitis A virus, NoV GI and GII, astrovirus, rotavirus, and infectious enteroviruses) in concentrated surface river water samples. In the 68 samples taken over 12 months, genomic detection of human pathogenic viruses was not statistically associated with the levels of somatic coliphages in surface water (Hot et al., 2003). For more information on the detection methods used, see Table 8 below.

#### **4.1.2 Coliphage-Virus Associations in Saline or Brackish Water**

The associations between coliphages and viruses in saline or brackish waters are also varied. Jiang et al. (2001) found that in urban runoff- impacted coastal waters, the presence of human adenovirus was significantly associated with the presence of F-specific coliphages (Jiang et al., 2001). Mocé-Llivina et al. (2005) found that in seawater samples at public beaches, somatic coliphages were the best indicators of enteroviruses out of all of the indicators tested (F-specific coliphages, total coliforms, fecal coliforms, and enterococci) as they were found in higher

numbers than other fecal indicators, including F-specific coliphages, and their amounts were indicative of enterovirus levels (Mocé-Llivina et al., 2005). Similarly, Ballester et al. (2005) found that in samples of marine water, both F-specific and somatic coliphages were significantly associated with adenoviruses, and F-specific coliphages were also significantly associated with rotavirus and enterovirus. Neither type of coliphage was significantly associated with the presence of astroviruses (Ballester et al., 2005). The amounts of coliphages and viruses varied by season. From seasonal and proximity data, it appeared that coliphages were more associated with viral presence than *E. coli* and that F-specific coliphages had the highest association with viral presence (Ballester et al., 2005).

In contrast, in a study of the occurrence and distribution of FIB (total coliform, fecal coliform, and *Enterococcus*), F-specific coliphages, human adenovirus, and enterovirus in freshwater streams and an estuary, Jiang et al. (2007) found a strong association between the occurrence of FIB and F-specific coliphages, but no association between the presence of F-specific coliphages and human adenovirus or enterovirus. Jiang et al. (2007) found that the detection of human viruses depends on a seasonal and freshwater-to-saltwater distribution pattern that was the opposite of that of FIB and coliphages. For more information on the detection methods used, see Table 8 below. Similarly, Boehm et al. (2009) did not find an association between the presence of coliphages, including somatic and F-specific DNA and F-specific RNA coliphages, and human enterovirus or adenovirus in marine waters in Avalon Beach, California (Boehm et al., 2009).

A summary of the above papers, detection methods, and quantitative data (when available) are presented below in Table 8. A systematic literature review was not conducted, so the studies shown in Table 8 are only a subset of the studies that likely exist.

**Table 8. Summary table of coliphages – virus correlations in ambient water.**

Study	Water type (Location)	Coliphages detected	Coliphage detection method	Viruses detected	Virus detection method	Occurrence findings
Baggi et al. (2001)	Fresh (Switzerland) (upstream of WWTP)	Somatic coliphages (means: 1.9 and 3- $\log_{10}$ PFU per mL) F-specific coliphages (range of means: 1.5–3- $\log_{10}$ PFU per mL)	ISO 10705-1	Enteroviruses, rotaviruses, and hepatitis A (41–44% of samples positive)	RT-PCR plus nested PCR	Coliphages associated with viruses. FIB not associated with viruses.
Jiang et al. (2001)	Marine Coastal waters impacted by urban run-off (Southern CA)	Somatic coliphages (5.3–3,332 PFU per L) F-specific (5.5–300 PFU per L)	EPA Method 1601	Adenovirus (880–7,500 genomes per L)	Nested PCR	The presence of human adenovirus was significantly associated with F-specific coliphages.
Hot et al. (2003)	Fresh river (France)	Somatic coliphages (range of densities: $4 \times 10^2$ – $1.6 \times 10^5$ PFU per L)	ISO 10705-2	Cell culture: total culturable enteroviruses (later determined to be poliovirus type3) Molecular methods: hepatitis A virus (1 positive /68 total), astrovirus (2/68), NoV GI (0 detects), NoV GII (1/68), rotavirus (0 detects), and enterovirus (2/68).	Cell culture and RT-PCR followed by Southern Blot	No significant association was observed between the density of somatic coliphages and the presence of infectious enteroviruses, or enterovirus genomes.
Skraber et al. (2004b)	Fresh river (France)	Somatic coliphages (Mean: $3.06 \cdot \log_{10}$ PFU/100 mL)	ISO 10705-2	<i>Enterovirus</i> spp. and NoV GII (34 samples out of 90 (38%) were positive for enterovirus (13%) and/or NoV GII (27%) genome)	<i>Enterovirus</i> spp.: cell culture, ICC-RT-PCR, and RT-PCR NoV GII: RT-PCR	The number of samples positive for pathogenic viral genome increased with increasing densities of somatic coliphages.
Ballester et al. (2005)	Marine Coastal water impacted by WWTP discharge (Boston, MA)	Somatic and F-specific coliphages	EPA Method 1602	Human astrovirus, enteroviruses, rotavirus, and adenovirus types 40 and 41	ICC-nPCR, ICC-RT-nPCR	The presence of enteric viruses and adenovirus was significantly associated with the presence of F-specific coliphages and somatic coliphages. Only F-specific coliphages were significantly associated with the presence of rotavirus and enterovirus.

Study	Water type (Location)	Coliphages detected	Coliphage detection method	Viruses detected	Virus detection method	Occurrence findings
Betancourt and Rose (2005)	Wetland and proposed sources for wetland restoration (Florida, USA)	F-specific coliphages (5 PFU per 100 mL reported for one wetland lake sample)	Agar overlay method and enrichment protocol developed by B. Yanko	Enteric viruses (detected in 14/28 samples)	Cell culture	Not discussed by the authors but low levels of occurrence in the sample set indicate association is unlikely.
Mocé-Llivina et al. (2005)	Marine coastal water impacted by urban run-off (Barcelona, Spain)	Somatic coliphages (9–12,240 PFU per 100 mL)  F-specific coliphages (0–84 PFU per 100 mL)	ISO 10705-1 ISO 10705-2	Culturable enteroviruses (0–158 PFU per 10 L)	Cell culture methods: standard plaque assay, double-layer plaque assay, VIRADEN method, RT-PCR, and RT-nPCR	Receiver operating characteristic curves of “numbers of enteroviruses in 10 L of seawater” indicated that the numbers of somatic coliphages (and enterococci) most accurately predicted the numbers of cultivable enteroviruses.
Westrell et al. (2006)	Fresh river impacted by WWTP (The Netherlands)	F-specific coliphages In 2001: Range: 6–7400 PFU per L  In 2002–2003: Peak: 5,100 Median: 1,300 PFU per L	ISO 10705-1	NoV  In 2001: January peak: 240 PCR detectable units per L  In 2002–2003: Peak: 2,000–3,000 Mean: 12–1,700 PCR detectable units per L	RT-PCR	Peaks in NoV did not coincide with those of enteroviruses, F-specific coliphages, or turbidity.
Jiang et al. (2007)	Marine and fresh coastal estuary (Newport Bay, CA)	F-specific coliphages	EPA Method 1601	Adenovirus, enterovirus	RT-PCR (enterovirus), nested PCR (adenovirus)	The seasonal and freshwater-to-saltwater distribution pattern of human viruses is the opposite of FIB and coliphages.
Boehm et al. (2009)	Marine sewage impacted beach (Avalon, CA)	F-specific DNA and RNA coliphages, somatic coliphages	Membrane filtration	Adenovirus, enterovirus	RT-PCR (enterovirus), nested PCR (adenovirus)	No association between coliphages and adenovirus or enterovirus.
Espinosa et al. (2009)	Fresh high-altitude surface water (Mexico City, Mexico)	Not specified (but likely F-specific coliphages)	Double layer culture (K12 Hfr host)	Enterovirus, rotavirus, astrovirus	RT-PCR	Coliphages showed strong association with enterovirus, but weak association with other enteric viruses.

Study	Water type (Location)	Coliphages detected	Coliphage detection method	Viruses detected	Virus detection method	Occurrence findings
Lodder et al. (2010)	Fresh rivers (The Netherlands)	Somatic coliphages (1.1 to 114,156 PFU per L), F-specific coliphages (0.12 to 14,403 PFU per L)	ISO 10705-1 ISO 10705-2	Enterovirus (present in 75% of samples (range, 0.0033 to 5.2 PFU per L)) Reovirus (83% of samples (0.0030 to 5.9 PFU per L),	Cell culture using RT-PCR and ICC-RT-PCR	A significant association was observed between the densities of the two coliphages and enteroviruses.
Payment and Locas (2011), using data taken from Locas et al. (2007, 2008)	Fresh groundwater (Canada)	Somatic and F-specific RNA coliphages	EPA Methods 1601 and 1602	Cell culture and immunoperoxidase: total culturable human enteric viruses Molecular methods: NoV, adenovirus types 40 and 41, enteroviruses, and reoviruses types 1, 2, and 3	Cell culture, immunoperoxidase, ICC-PCR, ICC-RT-PCR, and RT-PCR	Somatic and F-specific RNA coliphages were not predictive of virus presence or absence. Coliphages were present only in low numbers and less frequently than bacterial indicators.
Viau et al. (2011b), using data presented in Viau et al. (2011a)	Fresh, brackish and marine tropical coastal streams and estuaries (Hawaii)	F-specific coliphages (present in 85/88 samples, $\log_{10}$ mean $1.2 \pm 0.8$ per 100 mL)	Membrane filtration and double agar layer	Adenovirus (present in 13/88 samples, 0.8 to 4.2 gene copies per 100 mL) Enterovirus (5/88 samples, 0.4 to 4.8 gene copies per 100 mL) NoV GI (19/88 samples, 1.2 to 1,441 gene copies per 100 mL) NoV GII (11/88 samples, 0.9 to 62.4 gene copies per 100 mL)	qPCR, RT-qPCR	There were no associations between occurrence of viruses and fecal indicator densities (including coliphages).
Love et al. (2014)	Marine recreational beaches	F-specific coliphages (median concentrations at both beaches 0.3 MPN per 100 mL) Somatic coliphages (median concentrations were 4.9 and 3.1 MPN per 100 mL)	Modified version of modified version of EPA Method 1601	Adenovirus (25.5% of water samples at Doheny Beach and in 9.3% at Avalon Beach) NoV (22.3% of water samples at Doheny Beach and 0.7% at Avalon Beach)	Adenovirus: nested PCR NoV: nested RT-PCR	The presence of F-specific coliphages was positively associated with the probability of detecting adenovirus. NoV was not significantly associated with either type of coliphages.

<b>Study</b>	<b>Water type (Location)</b>	<b>Coliphages detected</b>	<b>Coliphage detection method</b>	<b>Viruses detected</b>	<b>Virus detection method</b>	<b>Occurrence findings</b>
Rezaeinejad et al. (2014)	Urbanized catchment waters (freshwater) in tropical Singapore	F-specific coliphages (mean concentration = $1.1 \times 10^2$ PFU per 100 mL) Somatic coliphages (mean concentration = $2.2 \times 10^2$ PFU per 100 mL)	EPA Method 1602	Adenovirus (mean = $9.4 \times 10^1$ gene copies/L) Astrovirus (mean = $2.9 \times 10^2$ gene copies/L) NoV GII (mean = $3.7 \times 10^2$ gene copies/L) Rotavirus (mean = $2.5 \times 10^2$ gene copies/L)	Adenovirus: real time PCR Astrovirus, rotavirus, NoV G I and GII: real time RT-PCR	F-specific coliphages were positively associated with NoV densities.

VIRADEN method = “virus adsorption enumeration” based on the direct enumeration of viruses adsorbed into nitrate-acetate cellulose membranes.

Note: Bacterial hosts for somatic coliphages include: WG5, CN13, *E. coli* 036; bacterial hosts for F-specific coliphages include: Stm WG49, *E. coli* F<sub>amp</sub>, K12 Hfr.

## 5. Environmental Factors and Fate

The ability of coliphages (including different coliphage genogroups) and other enteric viruses to survive in environmental media varies widely (Callahan et al., 1995; Reyes and Jiang, 2010; Jończyk et al., 2011; Bertrand et al., 2012). As described previously, the effect of environmental factors on coliphage survival is associated with morphology, where some specific structural characteristics, such as tails, large capsids, and lack of an envelope have been shown to be associated with greater resistance to external factors (Ackermann et al., 2004; Jończyk et al., 2011). Researchers have investigated the survival of coliphages and enteric viruses under a variety of environmental conditions. Studies have examined the effects of physical stress (e.g., temperatures and sunlight), biological antagonists (e.g., microbial predation and enzymatic degradation), and chemical antagonists (e.g., disinfection). This section focuses on physical and biological antagonists in natural aquatic environments, mechanisms of inactivation, and where data are available, compares inactivation rates of somatic, F-specific and *Bacteroides* bacteriophages to inactivation of human enteric viruses. Chemical treatment and other disinfection methods are discussed in Section 6 (Wastewater Treatment).

### 5.1. Temperature

Temperature is an important factor in viral ecology as it plays a fundamental role in attachment, penetration, multiplication, occurrence, and viability (Sobsey and Meschke, 2003; Pradeep Ram et al., 2005; Jończyk et al., 2011). Many studies have examined the effect of temperature on the survival of different viruses in aquatic environments. Both enteric viruses and coliphages have been reported to survive longer and occur more frequently at lower temperatures in natural environments and decay more rapidly at higher temperatures (i.e., seawater, river, and groundwater) (Long and Sobsey, 2004; Fong and Lipp, 2005). Below is a brief summary of the evidence of the effects of temperature on human enteric virus and coliphage inactivation in aquatic systems.

Bertrand et al. (2012) conducted a meta-analysis of the effects of temperature on the inactivation of enteric viruses and bacteriophages in food and water. The study collected 658 data points from 76 published studies and analyzed the effects of virus type, matrix (simple or complex), and temperature (<50 and ≥50°C) on virus survival. A simple matrix included: (1) synthetic media; (2) drinking water; and (3) groundwater. A complex matrix included: (1) freshwater; (2) natural seawater; (4) sewage; (4) soil; (5) dairy products; (6) food; and (7) urine (Bertrand et al., 2012). The study determined that, overall, virus inactivation was faster at temperatures ≥50°C than at temperatures <50°C and that virus inactivation was less sensitive to temperature change in complex matrices than in simple matrices (Bertrand et al., 2012). The somatic coliphage ΦX174 was highly persistent under all temperatures and matrices tested.

Studies reported differences in survival among different F-specific coliphage groups across temperature gradients. For example, Long and Sobsey (2004) reported that at 4°C, GI and GII F-specific RNA coliphages were detectable for over 100 days, GIII F-specific RNA coliphages were detectable for 3 weeks, and GIV F-specific RNA coliphages were reduced to the limit of detection after 10 days (Long and Sobsey, 2004). Of the F-specific DNA coliphages, all strains were detectable after 110 days at 4°C (Long and Sobsey, 2004). The authors also noted that the GI F-specific RNA coliphage MS2 and F-specific DNA coliphage M13 demonstrated a longer

survival in environmental waters than other F-specific coliphage species (Long and Sobsey, 2004).

Temperature can also affect survival of somatic and *Bacteroides* bacteriophages in aquatic systems. Lee and Sobsey (2011) estimated the temperature inactivation of four types of somatic coliphages in laboratory tests using both reagent grade water and surface water. The authors found that T4 (*Myoviridae* family), ΦX174 (*Microviridae* family), and λ (*Siphoviridae* family), survived better than T1 (*Siphoviridae* family), and T7 (*Podoviridae* family), at low temperatures (4°C) and high temperatures (25°C). Chung and Sobsey (1993) found that *B. fragilis* coliphages survived comparable to or better than hepatitis A, poliovirus, and rotavirus (measured using cell culture) in seawater exposed to low (5°C) and high (25°C) temperatures.

Reported comparisons between decay rates of F-specific RNA coliphages and human enteric viruses, or proxies to human enteric viruses, indicate that decay rates of both vary by temperature and water conditions. For example, in their two studies, Allwood et al. (2003, 2005) compared the survival of GI F-specific RNA coliphage MS2, feline calicivirus (FCV), and *E. coli* at 4°C, 25°C, and 37°C in chlorinated and dechlorinated water. In dechlorinated water at 4°C and 25°C, MS2 survived three times longer than both *E. coli* and FCV, whereas they had similar survival rates at 37°C (Allwood et al., 2003).

Similarly, Romero et al. (2011) found that porcine rotavirus and GI F-specific RNA coliphage MS2 had relatively low inactivation rate constants in the dark from 14 to 42°C, 10-fold increases in inactivation rates at 50°C and between 10- and 60-fold increases in inactivation rates at 60°C. In a similar experiment, Seo et al. (2012) compared the decay rates of murine NoV (MNV) and GI F-specific RNA coliphage MS2 over a temperature range of 24 to 85°C. They found that decay rate of MS2 was lower than MNV between 24°C and 60°C and that both were rapidly inactivated by temperatures >60°C (Seo et al., 2012). For more details on the decay rates at different temperatures, see Table 9 below.

### **Synergistic effects between temperature and other environmental factors**

The importance of temperature as a determinant of coliphage survival has been found to vary between freshwater and saltwater environments. For example, Reyes and Jiang (2010) noted that temperature is more important in influencing coliphage occurrence in freshwater environments than in saltwater environments (See Section 5.3 for more information on salinity). The importance of temperature as a determinant of virus survival is also dependent on the presence of sunlight. Romero et al. (2011) found that temperature played an important role in sunlight-mediated inactivation. For example, degradation rates of both GI F-specific coliphage MS2 and porcine rotavirus were higher for the same temperatures under different light conditions (full solar spectrum and only UVA and visible light) as compared to in the dark (Romero et al., 2011) (See Section 5.2 for more information on sunlight). Hurst et al. (1989) showed that temperature effects on inactivation of enterovirus was dependent on the water sources used as the aqueous phase in experiments.

### **Summary**

In summary, conclusions drawn in multiple studies indicate that the coliphages are equally persistent to, or more persistent than enteric viruses. Bertrand et al. (2012) found that somatic coliphage ΦX174 was highly persistent under all matrices and temperatures tested, and at higher temperatures, somatic and F-specific coliphages were classified as the most persistent as compared to enteric viruses. These data are consistent with the results of Allwood et al. (2003, 2005) and Seo et al. (2012). Combined, these data indicate that coliphages may be conservative surrogates for the behavior of enteric viruses under a range of temperatures (meaning they persist as long or longer than human viruses). Table 9 presents the decay rates of different types of coliphages, other fecal indicators, and human viruses.

## 5.2. Sunlight

Sunlight is also an important factor leading to virus inactivation (Sobsey and Meschke, 2003; Fong and Lipp, 2005; Jończyk et al., 2011). Sunlight that reaches Earth's surface is composed of medium and long wavelength UV light [UVB (280 to 320 nm); UVA (320 to 400 nm)], visible light (400 to 700 nm), and longer wavelengths (Love et al., 2010). There are three proposed types of virus inactivation caused by the UV wavelengths in light: endogenous direct, endogenous indirect, and exogenous indirect (Silverman et al., 2013). While UV radiation is utilized in wastewater treatment processes, this application uses primarily UVC wavelengths (which do not reach Earth's surface due to the ozone layer) and will be discussed in Section 6 on wastewater treatment. This section will focus on inactivation of viruses due to natural or simulated sunlight. Below is a brief summary of the evidence of the effects of sunlight on human enteric viruses and coliphage inactivation in aquatic systems.

**Table 9. Comparison of mean exponential decay rates of coliphages, fecal indicators and human viruses in different media at different temperatures.**

Study	Allwood et al. (2003)			Allwood et al. (2005)			Nasser (1993)			Long and Sobsey (2004)		Lee and Sobsey (2011)		Seo et al. (2012)	
Temperature	4°C	25°C	37°C	4°C	25°C	37°C	10°C	20°C	30°C	4°C	20°C	4°C	25°C	24°C	37°C
<b>Organism</b>	<b>Mean Exponential Decay Rate, <i>k</i> (d<sup>-1</sup>)<sup>a</sup></b>														
<i>E. coli</i>	0.30	0.40	0.77	0.23	0.20	0.14	0.03	0.18	0.20						
FCV	0.32	0.44	1.15	0.15	0.58										
GI F-specific RNA coliphage MS2	0.09	0.12	0.85	0.32	0.35	0.37				0.02	0.16			0.05	0.20
F-specific coliphages							N/O	0.08	N/O						
Human adenovirus							0.12	0.15	0.18						
Poliovirus type 1							0.01	0.14	0.21						
F-specific DNA coliphages										0.02– 0.23	0.13– 1.44				
GI F-specific RNA coliphages										0.02– 0.03	0.16– 0.20				
GII F-specific RNA coliphages										0.09	0.38				
GIII F-specific RNA coliphages										0.55	1.59				
GIV F-specific RNA coliphages										0.55	2.71				
GV F-specific RNA coliphages										0.63	2.88				
Somatic coliphage T1										0.10	1.15				
Somatic coliphage T4										<0.01	0.07				
Somatic coliphage T7										0.10	1.15				
Somatic coliphage ΦX174										0.02	0.15				
Somatic coliphage λ										0.01	0.12				
MNV												0.82	2.43		
<b>Study Medium</b>	Dechlorinated tap water	Dechlorinated tap water	Raw wastewater, 30-day incubation	Lake water	Lake water	Dulbecco's Modified Eagle's Medium									

N/O = die off was not observed, empty cells = not reported; <sup>a</sup>The mean exponential decay rate *k* may be used in the exponential decay equation:  $N_t = N_0 e^{-kt}$

Alternatively, *k* may be used in the base 10 exponential decay equation as  $N_t = N_0 10^{-kt/\ln(10)}$

## **Effects of sunlight on coliphage decay rates and decay rates of other fecal indicators**

Love et al. (2010) found a correlation between the size of the genome and the inactivation rate of environmental isolates of somatic coliphages in sunlight: Larger genomes were correlated with higher inactivation rates. They also found that F-specific RNA coliphages were significantly more resistant to sunlight inactivation than the F-specific DNA coliphages over an 8-hour period (Love et al., 2010). Overall, they found that under full-spectrum-simulated sunlight, inactivation rates varied more widely for ssDNA and dsDNA viruses than for ssRNA viruses, and that differences in virus inactivation rate were not just a function of nucleic acid type, but also genome length and morphology (Love et al., 2010).

Sinton et al. (1999) studied the inactivation rates of sewage-isolated somatic coliphages, F-specific coliphages, *B. fragilis* bacteriophages, and fecal coliforms by solar radiation in sewage-seawater mixtures. Overall, their data showed that sunlight conditions resulted in faster decay rates of all indicators as compared to dark conditions and that, under all conditions, somatic and F-specific coliphages had lower decay rates than *B. fragilis* bacteriophages and fecal coliforms (Sinton et al., 1999). The authors also found that colder water resulted in slower decay rates than warmer water under all light and dark conditions tested (Sinton et al., 1999).

In their follow-up study, Sinton et al. (2002) investigated the inactivation rates of waste stabilization pond effluent isolated fecal coliforms, enterococci, *E. coli*, somatic coliphages, and F-specific RNA coliphages by solar radiation in freshwater (Table 10 below). Overall, their data showed that, for all indicators, sunlight conditions resulted in faster decay rates than dark conditions and that under both light and dark conditions, somatic and F-specific RNA coliphages had smaller decay rates than *E. coli*, enterococci, and fecal coliforms (Sinton et al., 2002). Sinton et al. (2002) also found that F-specific RNA coliphages were inactivated by a wide range of wavelengths, whereas somatic coliphages were mainly inactivated by UVB wavelengths (318 nm).

**Table 10. Mean exponential decay rates of coliphages and fecal indicators in fresh river water contaminated with raw sewage or effluent under different light conditions.**

Organism	Source of contamination	Dark	Summer	Winter	Summer	Winter
		$k_D$ (/hour) <sup>a</sup>	$k_s$ (m <sup>2</sup> /MJ) <sup>b</sup>		$k_L$ (/hour) <sup>a</sup>	
Fecal coliforms	Wastewater effluent	0.02	0.09	0.08	0.26	0.14
	Raw sewage	0.01	0.28	0.22	0.70	0.30
<i>E. coli</i>	Wastewater effluent	0.02	0.08	0.07	0.25	0.11
	Raw sewage	0.02	0.29	0.24	0.70	0.33
Enterococci	Wastewater effluent	0.02	0.28	0.11	0.77	0.16
	Raw sewage	0.01	0.14	0.14	0.36	0.18
Somatic coliphages	Wastewater effluent	0.01	0.08	0.05	0.20	0.08
	Raw sewage	0.00	0.10	0.09	0.28	0.14
F-specific RNA coliphages	Wastewater effluent	0.01	0.07	0.05	0.17	0.08
	Raw sewage	0.00	0.08	0.07	0.18	0.12

Source: Sinton et al. (2002)

<sup>a</sup>The mean exponential decay rate,  $k_D$  and  $k_L$ , may be used in the exponential decay equation:  $N_t = N_0 e^{-kt}$ .

Alternatively,  $k_D$  (decay in the dark) and  $k_L$  (decay in the light) may be used in the base 10 exponential decay equation as  $N_t = N_0 10^{-kt/\ln(10)}$ . Note that  $k_L$  may be used only in equivalent solar insolation conditions as the study.

<sup>b</sup>The mean solar inactivation rate  $k_s$  may be used in the exponential decay equation:  $N_t = N_0 e^{-ksI_t}$ , where I is the solar irradiance.

### Effects of sunlight on decay rates of enteric viruses and coliphages

Individual enteric viruses and coliphages also have different levels of resistance to sunlight. For example, Love et al. (2010) observed that in seawater under sunlit conditions, the decay rates of adenovirus 2 and GI, GII, GIII and GIV F-specific RNA coliphages were similar and slower than the decay rates of F-specific DNA coliphages, somatic coliphages, and poliovirus type 3 (Love et al., 2010). These results are consistent with field experiments under conditions of similar sunlight intensity (Love, et al., 2010).

Romero et al. (2011) used both full spectrum sunlight and a combination of UVA and visible light to determine the decay rates of GI F-specific RNA coliphage MS2 and porcine rotavirus at temperatures ranging from 14 to 50°C (see Table 11 below). Under dark conditions, decay rates were not detected for either virus between 14 and 42°C whereas at 50°C, low decay rates were detected for both (Romero et al., 2011). Under full spectrum sunlight, the decay rates ( $K_{obs}$ ) of both viruses increased and those for GI F-specific RNA coliphage MS2 were below those of porcine rotavirus (Romero et al., 2011). Under a combination of UVA and visible light, both viruses had low, approximately constant degradation rates between 14 and 42°C, whereas at 50°C the rates increased slightly (Romero et al., 2011). The very low levels of degradation of both MS2 and porcine rotavirus in the absence of UVB were consistent with previous studies indicating that the majority of sunlight degradation of viruses in water is due to UVB light (Sinton et al., 2002; Romero et al., 2011). These results are consistent with the findings of Fisher et al. (2011) who found that in phosphate buffered saline, GI F-specific RNA coliphage MS2 was resistant to UVA but highly sensitive to UVB wavelengths.

Silverman et al. (2013) compared the inactivation rates of poliovirus type 3, adenovirus type 2, and GI F-specific RNA coliphages (MS2 and PRD1) under dark and full simulated sunlight conditions in four different types of environmental water (seawater from two marine beaches, river estuary water, coastal wetland, and coastal wetland collected near cattail plants) and in phosphate-buffered saline (see Table 11). They found that all dark control inactivation rates were less than those obtained from experiments conducted under full-spectrum simulated sunlight for all three viruses in all five types of water (Silverman et al., 2013). Additionally, they found that decay rates of GI F-specific RNA coliphages under full-spectrum simulated sunlight were significantly below those of poliovirus type 3 in all five types of water and less than or equal to those of adenovirus type 2. The authors conclude that GI F-specific RNA coliphages are a conservative surrogate for predicting poliovirus type 3 and adenovirus type 2 decay in all five types of water tested (Silverman et al., 2013).

**Table 11. Comparison of mean exponential decay rates of coliphages and human viruses under different light conditions.**

Medium/ Conditions	Romero et al. (2011)										Silverman et al. (2013) <sup>b</sup>	
	20 mg C/L of riverine natural organic material (full-spectrum sunlight) <sup>a</sup>										Full- spectrum sunlight	
	Microorganism		14°C	23–26°C		34°C		42°C		50°C		Temperature not provided
			k <sub>D</sub> (/h)	k <sub>L</sub> (/h)	k <sub>D</sub> (/h)	k <sub>L</sub> (/h)						
MS2		ND	4.00	ND	4.23	ND	4.49	ND	5.00	0.50	NS	0.10
Rotavirus		ND	7.31	ND	8.58	ND	8.63	ND	9.42	0.33		
Poliovirus (Type 3)											NS	0.08
Adenovirus (Type 2)											NS	0.11

ND = Nondetect

NS = Not significantly different from zero

empty cells = not reported

mg = milligrams

<sup>a</sup>The mean exponential decay rate k<sub>D</sub> and k<sub>L</sub> may be used in the exponential decay equation: N<sub>t</sub> = N<sub>0</sub>e<sup>-kt</sup>.

Alternatively, k<sub>D</sub> (decay in the dark) and k<sub>L</sub> (decay in the light) may be used in the base 10 exponential decay

equation as N<sub>t</sub> = N<sub>0</sub>10<sup>-kt/ln(10)</sup>. Note that k<sub>L</sub> may be used only in equivalent solar insolation conditions as the study.

<sup>b</sup>The decay rates reported in Silverman et al. (2013) are from water collected from Tijuana River estuary (Imperial Beach, California) at the end of the ebb tide.

### Synergistic effects between sunlight and other environmental factors

Several studies have found synergy between sunlight and other environmental factors in the inactivation rates of viruses, such as the presence of organic matter or particulate matter, sunlight, and salinity. For example, inactivation of viruses may be greater in waters with organic matter that produces reactive oxygen species (Kohn et al., 2007; Love et al., 2010; Romero et al., 2011). However, the presence of flora, fauna, and dissolved and particulate matter may also increase viral survival by blocking or absorbing photons from passing through water (Bitton et al., 1979; Romero et al., 2011). Please refer to Sections 5.4 and 5.5 for more information on microbial activity and organics, respectively. The synergy between sunlight and temperature appears to play a role in the inactivation of viruses. For example, Romero et al. (2011) concluded that temperature is a critical factor in the sunlight-mediated inactivation of GI F-specific

coliphage MS2. Please refer to Section 5.1 for more information on temperature. Differential inactivation of coliphages by sunlight can also occur in saltwater versus freshwater. For example, Sinton et al. (1999, 2002) found that salinity had a synergistic effect with sunlight. Specifically, sunlight inactivation increased with increasing salinity. For more information on salinity please see Section 5.3.

## **Summary**

In summary, data indicated that human enteric viruses and coliphages have faster decay rates under conditions of full sunlight as compared to in the dark (Sinton et al., 1999, 2002; Romero et al., 2011). Reported decay rates varied by virus, amount and wavelengths of light (UVA, UVB), temperature, and aquatic conditions (salt or freshwater), however, several studies indicated that coliphage decay rate is generally lower than enteric virus or FIB decay rate in various sunlight conditions (Sinton et al., 2002; Love et al., 2010; Romero et al., 2011; Silverman et al., 2013). Thus, coliphages may be a conservative surrogate for predicting virus decay due to sunlight.

### **5.3. Salinity**

The types and concentrations of salts found in natural waters differ depending on the type of water. Generally, seawater is considered to be 35 parts per thousand salt. Chloride (Na) and sodium (Cl) are the most prevalent ions and account for more than 85% of the salt content by mass (Murray, 2004). Concentrations of these ions (Na and Cl) are significantly lower in freshwaters, and vary depending on type and source of water (Murray, 2004).

Salts, or salinity, can influence viral survival in aquatic environments. Salinity can either increase or decrease degradation rates of viruses depending on the type and concentration of salt, the temperature, and the specific virus (Hurst and Gerba, 1980; Gutierrez et al., 2010; Mylon et al., 2010; da Silva et al., 2011; Nguyen et al., 2011; Seo et al., 2012). It has been hypothesized that monovalent salts provide strong steric and electrosteric stabilization of GI F-specific coliphage MS2, whereas divalent salts have been found to cause MS2 aggregation (Mylon et al., 2010; Nguyen et al., 2011). Similar results have been shown for rotavirus and NoV G1.1 (Gutierrez et al., 2010; da Silva et al., 2011). Aggregation of viruses can make it difficult to measure their infectivity, as plaque assays result in underestimates (e.g., a single PFU may be comprised of clumps of virus particles). Additionally, osmotic shock through rapid changes in osmotic pressure can trigger inactivation of coliphages via direct oxidization, which can cause capsid degradation and dispersion, tail fragmentation, and release of viral nucleic acids into the aquatic environment (Jończyk et al., 2011). This section will describe the effects of salinity on viral degradation. Below is a brief summary of the evidence of the effects of salinity on human enteric viruses and coliphage inactivation in aquatic environments.

### **Effects of salinity on decay rates of coliphages**

Sinton et al. (1999, 2002) found that salt water affected the decay rates of F-specific and somatic coliphages under both dark and sunlight exposed conditions. Sinton et al. (1999) studied the inactivation rates of sewage-isolated somatic coliphages and F-specific DNA and RNA coliphages in sewage-seawater mixtures. Sinton et al. (2002) studied the inactivation rates of somatic and F-specific RNA coliphages isolated from waste-stabilization pond effluent under

both dark and sunlight exposed conditions in river water, simulated estuarine water (50% river water, 50% seawater), and seawater. Under dark conditions, both somatic and F-specific coliphages had lower decay rates in river water (somatic coliphages ( $k_D = 0.008 \text{ h}^{-1}$ ), F-specific RNA coliphages ( $k_D = 0.014 \text{ h}^{-1}$ )) than in sea water (somatic coliphages ( $k_D = 0.044 \text{ h}^{-1}$ ), F-specific RNA coliphages ( $k_D = 0.044 \text{ h}^{-1}$ )) (Sinton et al., 1999, 2002). Degradation rates of somatic coliphages increased 5.5 fold in salt water compared to river water under dark conditions whereas F-specific RNA coliphages rates increased 3.1 fold under the same conditions. These data indicate that somatic coliphages are less stable in seawater than F-specific RNA coliphages under the tested conditions.

Somatic coliphages were more sensitive to salt water under sunlight conditions as well. For example, Sinton et al. (2002) determined the degradation rates of somatic coliphages and F-specific RNA coliphages isolated from waste-stabilization pond effluent under full sunlight conditions in freshwater and 50:50 water and seawater. For somatic coliphages degradation rates were  $k_S = 0.079 \text{ m}^2 \text{ megajoules (MJ)}^{-1}$  in river water,  $k_S = 0.129 \text{ m}^2 \text{ MJ}^{-1}$  in 50:50 water and  $k_S = 0.184 \text{ m}^2 \text{ MJ}^{-1}$  in sea water. Similarly, F-specific RNA coliphages rates were:  $k_S = 0.086 \text{ m}^2 \text{ MJ}^{-1}$  in river water,  $k_S = 0.092 \text{ m}^2 \text{ MJ}^{-1}$  in 50:50 water and  $k_S = 0.123 \text{ m}^2 \text{ MJ}^{-1}$  in sea water (Sinton et al., 2002). Degradation rates of somatic coliphages increased 2.3 fold in salt water compared to river water whereas F-specific RNA coliphages rates increased 1.4 fold under the same conditions. These data indicate that somatic coliphages are more sensitive to salt water than F-specific RNA coliphages under these conditions.

Overall, the authors concluded that as salinity increases, inactivation of coliphages increases as well (Sinton et al., 2002). In particular, inactivation of F-specific RNA coliphages obtained from sewage increased with salinity, but the trend in F-specific RNA coliphages obtained from stabilization ponds was less pronounced (Sinton et al., 2002). These conclusions are in agreement with those of Savichtcheva and Okabe (2006) who found that F-specific RNA coliphages were more sensitive to sunlight inactivation at high salinity.

Seo et al. (2012) investigated the differences in tolerance of MNV and GI F-specific RNA coliphage MS2 to different concentrations of NaCl (0.3, 1.3, 3.3, and 6.3% NaCl) at three different temperatures, 24°C, 37°C, and 50°C. Their results show that there are complex interactions between salt concentration and temperature for both of the viruses, with several differences between the two. They found that MS2 was more resistant to NaCl than MNV at all concentrations of NaCl and temperatures tested (Seo et al., 2012). At 24°C, MS2 did not show any reduction in infectivity at any of the NaCl concentrations and at higher temperatures, NaCl seem to have a protective effect (Table 12; Seo et al., 2012).

Hurst and Gerba (1980) compared the decay of poliovirus, echovirus, coxsackievirus and simian rotavirus in estuarine and freshwater during two different years. Decay was quicker in estuarine water relative to freshwater in one year, and decay was similar in the waters in the second years suggesting factors other than salinity may have been contributing to viral decay.

## **Synergistic effects between salinity and other environmental factors**

Several studies have found synergy between salinity and other environmental factors in the inactivation rates of viruses. For example, Seo et al. (2012) found an interaction between temperature and salt concentration. Depending on the specific virus, incubation in high concentrations of NaCl at high temperatures could either reduce virus infectivity (MNV) or increase virus infectivity (GI F-specific RNA coliphage MS2) as compared to lower concentrations of salt at the same temperature. The susceptibility of MNV to all concentrations of NaCl increased rapidly at 37°C and 50°C, whereas at the same temperatures, GI F-specific RNA coliphage MS2 was more stable at higher NaCl concentrations (1.3 to 6.3% NaCl) than at low concentrations (0.3% NaCl) (Seo et al., 2012). The authors hypothesized that the high NaCl concentration may “protect against thermally induced capsid opening or stabilize the viral protein-RNA complex” (Seo et al., 2012). For more information on the effects of temperature on virus degradation, see Section 5.1. Other studies have reported synergistic effects between salt and natural organic and inorganic matter (Mylon et al., 2010). Mylon et al. (2010) found that GI F-specific RNA coliphage MS2 aggregated at lower concentrations of Ca<sup>2+</sup> in the presence of 10 mg/L Suwannee River organic matter (100 millimolar (mM) Ca<sup>2+</sup>) as compared to just Ca<sup>2+</sup> (160 mM Ca<sup>2+</sup>). Lukasik et al. (2000) observed that mono-, di-, and trivalent salts (NaCl, MgCl<sub>2</sub>, and AlCl<sub>3</sub>) either promoted or interfered with adsorption of GI F-specific RNA coliphage MS2, somatic coliphage ΦX174, and poliovirus type 1 to different types of filters at different pH levels. For more information on adsorption to organic and inorganic matter, please see Section 5.5 below.

## **Summary**

In summary, both enteric viruses and coliphages are affected by salinity, the specific effects of which vary depending on the type of virus and the type and concentration of salt, as well as temperature. In terms of aggregation, multiple studies have found that monovalent cations are either ineffective at, or are less effective at causing aggregation of coliphages (F-specific RNA coliphage MS2) and enteric viruses (NoV GI.1 and rotavirus) than divalent cations (Gutierrez et al., 2010; Mylon et al., 2010; da Silva et al., 2011; Nguyen et al., 2011) and aggregation can affect the number of PFUs measured in a sample. In terms of decay rates, Seo et al. (2012) found that MS2 had lower decay rates than MNV at all NaCl concentrations tested (0.3 to 6.3%) at three different temperatures (24°C, 37°C, and 50°C). Table 12 below shows the decay rates from Seo et al. (2012).

**Table 12. Comparison of mean exponential decay rates of coliphages and MNV at different concentrations of salt and at different temperatures.**

°C	NaCl%	Organism	
		MNV	GI F-specific coliphage MS2 k(d <sup>-1</sup> )
24	0.3	1.17	0.05
	1.3	3.03	0.05
	3.3	2.96	0.05
	6.3	2.73	0.05
37	0.3	2.54	0.19
	1.3	4.44	0.18
	3.3	4.11	0.21
	6.3	4.11	0.11
50	0.3	61.40	10.80
	1.3	72.08	3.15
	3.3	122.80	5.54
	6.3	118.42	4.09

Source: Seo et al. (2012)

Higher k = faster decay

#### 5.4. Predation and Enzymatic Degradation

Inactivation of viruses can occur via predation or release of virucidal agents from endogenous microorganisms in environmental waters (Sobsey and Cooper, 1973; Fujioka et al., 1980; Ward et al., 1986). Many bacteria produce proteolytic enzymes that are capable of inactivating viruses, including human enteric viruses, by degradation of protein capsids (Bae and Schwab, 2008). In seawater, viriplankton are postulated to be inactivated in part by enzymatic attack and predation (Finiguerra et al., 2011). One study found that the presence/absence of microorganisms is a more important factor than temperature on virus survival in groundwater (Wetz et al., 2004). Other studies have shown that association with biofilms can also affect the inactivation of enteric viruses and coliphages. Helmi et al. (2011) found that poliovirus, GI F-specific RNA coliphage MS2, and somatic coliphage ΦX174 densities in drinking water biofilms decreased after 6 days due to inactivation and detachment, but previous research has found that biofilms protect viruses from inactivation (Skraber et al., 2007). While the effects of microbial antagonism and enzymatic degradation on coliphages are not as well studied as the effects on human enteric viruses, these processes are thought to inactivate them as well. For example, studies examining coliphages in waste stabilization ponds have shown that while sunlight is the major cause of inactivation, predation may also play a role (da Silva et al., 2008). Below is a brief summary of the evidence of human enteric virus and coliphage predation- and enzymatic degradation-mediated inactivation in aquatic systems.

#### Effects of predation and enzymatic degradation on decay rates of coliphages

In a study examining the role of aquatic plants in freshwater and salt water wetlands on the survival of waterborne coliphages, Karim et al. (2008) found that the presence of wetland vegetation significantly increased the inactivation of GI F-specific RNA coliphage MS2. The authors hypothesized that the presence of aquatic plants may enhance rhizosphere bacterial

populations, which increase coliphage inactivation due to the presence of metabolites or the presence of proteolytic substances released by microbes or plants (Karim et al., 2008).

Finiguerra et al. (2011) investigated the light-independent mechanisms of inactivation of somatic coliphage T4 (marine host: PWH3a) and coliphage P1 (enteric host: *E. coli* B) in seawater. They found that decay rates of both coliphages were reduced in particle-free seawater (<2 micrometers [ $\mu\text{m}$ ]) as compared to seawater containing nanoplankton (<10  $\mu\text{m}$ ) and the lowest decay rates were found in ultra-filtered seawater (<10 kilodaltons [kDa]). The authors concluded that inactivation of these coliphages is accelerated by naturally occurring particles, which include living organisms and heat-labile colloids and macromolecules >10 kDa (Finiguerra et al., 2011).

### **Effects of predation and enzymatic degradation on decay rates of human viruses**

A number of studies have examined the effect of microbial activity on enteric virus survival in aquatic systems. Direct predation of enteric viruses can occur via engulfment or ingestion by bacteria, protozoa, helminthes, and other aquatic organisms (Sobsey and Meschke, 2003). Fujioka et al. (1980) demonstrated that inactivation of enteric viruses (poliovirus type 1, coxsackievirus B4, and echovirus 7) in marine and estuarine waters is associated with the natural microbial community. Microbial activity has also been shown to decrease persistence of rotaviruses in raw and treated freshwaters (Raphael et al., 1985) and hepatitis A in mixed septic tank effluent (Deng and Cliver, 1995). Toranzo et al. (1982) confirmed the ability of bacteria to release virucidal agents by isolating marine bacteria that had marked activity against poliovirus (net 2-log<sub>10</sub> inactivation or greater within 6 to 8 days), coxsackievirus B-5, and echovirus 6. Sobsey and Cooper (1973) showed that microbial activity in waste stabilization pond water contributed to poliovirus inactivation. Similarly, Herrmann et al. (1974) showed that enteroviruses decayed more quickly in lake water compared to sterilized lake water. Ward et al. (1986) also showed that proteolytic bacterial enzymes were responsible for echovirus inactivation in freshwater.

Wetz et al. (2004) studied the inactivation rate of poliovirus in filtered natural seawater, unfiltered natural seawater, artificial seawater, and deionized water at 22 and 30°C. They found that the highest rates of virus inactivation occurred in unfiltered natural seawater at both temperatures tested. Prior to spiking they exposed all of the water in their experiments to >14 hours of UV light (to kill the indigenous microorganisms). Because the indigenous microorganisms were killed, the authors hypothesized that direct microbial inactivation of the viruses was highly unlikely and degradation was likely caused by a release of cellular proteases, nucleases, and other enzymes (Wetz et al., 2004).

### **Synergistic effects between predation and enzymatic degradation and other environmental factors**

Several studies have identified synergy between predation and enzymatic degradation and other environmental factors in the inactivation rates of viruses. There is some evidence that when viruses, including enteric viruses and coliphages, adsorb to particles, the associated particle may offer them some protection from predation (Fong and Lipp, 2005; Weaver and Sinton, 2009; Finiguerra et al., 2011). For more information on adsorption to organic and inorganic matter, see

Section 5.5 below. There are also synergistic effects between enzymatic degradation and temperature. For example, Wetz et al. (2004) found a synergistic effect between temperature and enzymatic degradation of poliovirus in natural seawater, as degradation rates were higher at 30°C than at 22°C. For more information on effects of temperature, see Section 5.1 above.

## Summary

Microbial predation and enzymatic degradation are both important mechanisms of virus inactivation in natural waters. Both predation and enzymatic degradation have been shown to increase human virus degradation rates in freshwater, salt water, treated water and septic system effluent (Fujioka et al., 1980; Toranzo et al., 1982; Raphael et al., 1985; Deng and Cliver, 1995; Wetz et al., 2004). While there are fewer data for coliphages, there is some evidence that microbial predation and enzymatic degradation do contribute to virus inactivation in natural waters. Due to lack of data, it is not currently possible to compare degradation rates of enteric viruses and coliphages by microbial predation or enzymatic degradation in natural waters.

### 5.5. Organic and Inorganic Matter

Aquatic environments contain both organic and inorganic matter. Inorganic matter consists of materials made from nonbiological sources and do not contain carbon (except for CO<sub>2</sub> and CH<sub>4</sub>). These include metals, chemicals, sand, clay, salts, and ions. Natural organic matter consists of materials that are made from biological sources and contain carbon. These include exudates from organisms and the materials that are produced from their decay. Organic matter in water is a diverse mixture of organic compounds ranging from macromolecules to low molecular-weight compounds (USGS, 2013). Organic matter is capable of both attenuating light (thus decreasing photoactivation rates) and producing reactive oxygen species (thus increasing photoactivation rates) (Silverman et al., 2013). Depending on the absolute amount of sunlight that reaches the virus and the amount of reactive oxygen species produced, the overall effect of organic matter can either result in decreased or increased viral photoinactivation rates.

Viruses in the environment are often associated with particulate matter, which has a major effect on persistence and transport in the environment (Gerba, 1984). For example, clay surface exchange capacity and particle size and shape affect the virus-adsorption activity of a clay (Carlson et al., 1968). Laboratory-based predictions suggest that as many as 99% of viruses in coastal waters should be adsorbed to naturally occurring colloids and particles (Finiguerra et al., 2011). If the resultant aggregate is dense and large, it can settle out of the water column (Characklis et al., 2005; Shen et al., 2008). If the aggregate is less dense, viruses may remain more mobile in the environment (Characklis et al., 2005).

The isoelectric point of the virus dictates its overall charge at a given pH, ionic strength, and water chemistry and thus affects virus adsorption. For example, reoviruses adsorb primarily to negatively charged sites on clay, while T1 and T7 coliphages adsorb to positively charged sites at environmentally relevant pHs (Gerba, 1984). Stotsky et al. (1980) found that adsorption to clay by reovirus (the family to which rotavirus belongs) and somatic coliphages (T1 and T7) increased the persistence of the viruses in lake water (Stotsky et al., 1980, as cited in Sobsey and Meschke, 2003).

This section will focus on inactivation of viruses due to interactions with organic and inorganic matter. Below is a brief summary of the evidence of the effects of organic and inorganic matter on human enteric viruses and coliphage inactivation in aquatic systems.

### **Effect of organic and inorganic matter on decay rates of coliphages**

Finiguerra et al. (2011) investigated the effects of particulate, dissolved, and colloidal organic and inorganic material in seawater on the inactivation rate of somatic coliphage T4. They determined that a significant fraction of viral inactivation (39–65%) can be attributed to passive sorption to living and inert planktonic particles (sterile debris was produced from cultivated phytoplankton species; 0.2 to 10 µm) (Finiguerra et al., 2011). The lowest decay rates were in oxidized filtrate from a 10 kDa tangential filtration system. The authors identified virucidal material between 10 kDa and 0.2 µm in size that is resistant to autoclaving. They concluded that inorganic solutes may be the primary inactivating mechanism in the dissolved fraction (Finiguerra et al., 2011).

### **Effects of organic and inorganic matter on decay rates of enteric viruses and coliphages**

LaBelle and Gerba (1980) found that adsorption to marine sediment increased the time required for 99% inactivation from 1 hour to greater than 4 days for poliovirus and from 1.4 days to greater than 6 days for echovirus. Another study found that enteroviruses associated with marine solids are infectious for longer (19 days) than unassociated enteroviruses in the water column (9 days) (Griffin et al., 2003). Shen et al. (2008) estimated somatic coliphage P22 inactivation rates to be in the range 0.27 to 0.57 per day (0.12 to 0.25 log<sub>10</sub> per day) with the highest inactivation rate found in samples with high suspended solids concentration, relatively low dissolved organic carbon content, and sediment with high clay content.

Chung and Sobsey (1993) found both temperature- and sediment-dependent differences between the decay rates of the five viruses tested: F-specific coliphages, *B. fragilis* phages, hepatitis A, poliovirus, and rotavirus. The effect of sediment differed among the viruses. Sediment protected poliovirus and human adenovirus at 5°C and 25°C and F-specific coliphages at 25°C, whereas it accelerated inactivation of rotavirus at both temperatures. *B. fragilis* phage survival was not affected by sediment at either temperature (Chung and Sobsey, 1993). Interestingly, at 5°C, all of the viruses had increasing levels of association with the sediment fraction over a 60-day period, except for hepatitis A, which had approximately constant rates over the entire period. Association with the sediment did not correlate with inactivation rates (Chung and Sobsey, 1993). All five of the viruses tested had faster decay rates at 25°C than at 5°C (Chung and Sobsey, 1993). Under the conditions tested, F-specific coliphages had similar decay rates to poliovirus in sediment at 25°C and in seawater at 5°C, and rotavirus in sediment at 5°C (Chung and Sobsey, 1993).

Silverman et al. (2013) found that the presence of photosensitizers (presumably colored dissolved organic matter [CDOM]) in five different natural waters, had different effects on human virus and bacteriophage photoinactivation in different waters exposed to full spectrum, simulated sunlight. In four of the five natural waters, the inactivation rate of poliovirus type 3 was significantly slowed relative to a clear, buffered control. In one of the five natural waters,

the inactivation of PRD-1 (which infects *Salmonella* LT2) was significantly slowed. In three of five waters, adenovirus and GI F-specific coliphage MS2 inactivation was significantly faster than the clear control buffer. The authors also examined inactivation rates in UVB-blocked simulated sunlight to gain insight into the mechanisms of photoinactivation of the different viruses. The authors concluded that exogenous mechanisms (reaction reactive species formed by photosensitizers in the water column) contributed significantly to inactivation of the viruses other than poliovirus type 3 for which endogenous processes are likely dominant.

### **Synergistic effects between organic and inorganic matter and other environmental factors**

Several studies have found synergy between organic and inorganic matter and other environmental factors in the inactivation rates of viruses. Sunlight has been shown to have synergistic effects with CDOM present in the water matrix, the effects of which vary depending on the type of virus, the amount of UVB attenuated by the CDOM, and the number and concentration of damaging radicals produced (Silverman et al., 2013). Please see Section 5.1, 5.2, and 5.3 for more information sunlight, temperature, and salinity. Viral adsorption to biofilms, sediment and organic matter can protect viruses from inactivation or expose viruses to detrimental microbial activity. Please see Section 5.4 for more information on biofilms and predation and degradation by microbes.

### **Summary**

In summary, depending on the specific environmental conditions coliphages may be a conservative surrogate for the inactivation of human enteric viruses. The presence of organic and inorganic matter affects inactivation of enteric viruses and coliphages in aquatic systems. Both organic and inorganic matter have been shown to either increase or decrease degradation rates, depending on the type of the virus and the nature of the organic matter (Chung and Sobsey, 1993; Silverman et al., 2013). For example, several groups found that poliovirus, echovirus, and enterovirus adsorption to sediment or solids decreased inactivation of the viruses (LaBelle and Gerba, 1980; Griffin et al., 2003), whereas others have found that inactivation rates increased in samples with high suspended solids and sediment with high clay content (Shen et al., 2008). While it is impossible to compare coliphages with all human enteric viruses under all conditions, Silverman et al. (2013) found that GI F-specific coliphage MS2 was a conservative surrogate for poliovirus type 3 and human adenovirus type 2 (i.e., GI F-specific coliphage MS2 had a slower decay rate than the human viruses) in five environmental waters with varying levels of photosensitizing molecules both in the dark and in full sunlight.

### **5.6. Environmental Factors Impacts Summary**

Some studies have found that coliphages are more resistant to environmental stressors than human viruses, but such findings are highly contextual and dictated by a host of local environmental conditions. The inactivation kinetics of coliphages is also relative. In general, temperature, pH, sunlight, CDOM and the association with solids are some of the most important factors influencing survival of coliphages (Schaper et al., 2002b). Table 13 summarizes these environmental factors and their mechanisms of inactivation for human enteric viruses and coliphages.

**Table 13. Summary of environmental factors influencing viral inactivation in aquatic environments.**

Factor	Coliphages	Effects		References
		NoV and other human enteric viruses	Conclusions	
<b>Physical</b>				
Temperature	<ul style="list-style-type: none"> <li>Variable decay rates among strains; wild isolates more stable than laboratory strains.</li> <li>F-specific RNA coliphages are more resistant to decay at low temperatures than high temperatures.</li> <li>Somatic coliphage ΦX174 is highly persistent under all temperatures from 0°C–100°C in a variety of matrices tested.</li> <li>Somatic and F-specific coliphages highly persistent at higher temperatures.</li> <li>At 4°C, GI and GII F-specific RNA coliphages are detectable for over 100 days, GIII F-specific RNA coliphages detectable for 3 weeks and GIV F-specific RNA coliphages reduced to the limit of detection after 10 days.</li> </ul>	<ul style="list-style-type: none"> <li>Different viruses have different decay rates at the same temperature.</li> <li>MNV more stable than human adenovirus and human rotavirus at 0°C and 50°C in a variety of media.</li> </ul>	<ul style="list-style-type: none"> <li>Viruses decay at faster rates at higher temperatures. More stable at 4°C, than at 20°C, and at &lt;50°C more stable than at ≥50°C in a variety of media.</li> <li>Salinity and sunlight have synergistic effects at temperatures ranging from 0°C to 100°C (but in general, coliphage ΦX174 has lower decay rates than rotavirus, adenovirus, and MNV)</li> <li>Somatic and F-specific coliphages are classified as the most persistent of the viruses at higher temperatures.</li> <li>At 4°C and 25°C GI F-specific RNA coliphage MS2 has lower decay rates than FCV and similar survival rates at 37°C in dechlorinated water. GI F-specific RNA coliphage MS2 has four-fold lower decay rates than MNV at 24°C and 37°C and three-fold lower decay rates at 50°C and 60°C.</li> </ul>	Hurst et al., 1980; Chung and Sobsey, 1993; Nasser et al., 1993; Skrabber et al., 2002; Allwood et al., 2003, 2005; Savichtcheva and Okabe, 2006; Lee and Sobsey, 2011; Bertrand et al., 2012; Seo et al., 2012

Sunlight	<ul style="list-style-type: none"> <li>Different viruses have different decay rates under the same sunlight conditions. Genome size of somatic coliphages is correlated with decay rate.</li> <li>F-specific RNA coliphages are more resistant to sunlight than F-specific DNA coliphages in clear seawater.</li> <li>Inactivation rates vary more widely for ssDNA and dsDNA viruses than for ssRNA viruses based on nucleic acid type, genome length, and morphology.</li> </ul>	<ul style="list-style-type: none"> <li>Different viruses have different decay rates under the same sunlight conditions.</li> <li>Poliovirus type 3 has faster decay rates than human adenovirus type 2 under full sunlight in four different environmental waters.</li> </ul>	<ul style="list-style-type: none"> <li>Virus inactivation rates are higher in sunlight conditions than in the dark.</li> <li>UVB wavelengths are the most damaging.</li> <li>Synergistic effects with temperature, salinity, organic, and inorganic matter.</li> <li>Direct damage to protein capsid and genetic material and indirect inactivation due to reactive oxygen species and other free radicals.</li> <li>In full sunlight in seawater, the decay rates of human adenovirus type 2 and F-specific coliphages (MS2, Fi, Q<math>\beta</math>, and Sp) are similar.</li> <li>The decay rates of F-specific DNA coliphage M13 and poliovirus type 3 are also similar.</li> <li>Decay rates for porcine rotavirus are two- to three-fold higher than decay rates for GI F-specific RNA coliphage MS2 when tested between 14°C and 42°C.</li> <li>GI F-specific RNA coliphage MS2 is a conservative surrogate for decay of poliovirus type 3 and human adenovirus type 2 in four types of environmental water.</li> </ul>	Bitton et al., 1979; Sinton et al., 1999, 2002; Sobsey and Meschke, 2003; Duizer et al., 2004; Fong and Lipp, 2005; Love et al., 2010; Jónczyk et al., 2011; Lee and Sobsey, 2011; Romero et al., 2011; Silverman et al., 2013
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<b>Chemical</b>			
Salinity	<ul style="list-style-type: none"> <li>GI F-specific RNA coliphage MS2 does not aggregate in high concentrations of monovalent cations but does aggregate in high concentrations of divalent cations.</li> <li>Concentrations of 1.3 to 6.3% NaCl were protective of GI F-specific RNA coliphage MS2 at 37°C and 50°C.</li> <li>Salt water compared to freshwater affects the decay rate of F-specific coliphages and somatic coliphages under both dark and sunlight-exposed conditions.</li> <li>F-specific coliphages are more tolerant in salt water than in freshwater in the dark.</li> <li>Somatic coliphages are more tolerant of salt water than freshwater under sunlight conditions.</li> </ul>	<ul style="list-style-type: none"> <li>NoV GI.1 aggregates with both mono- and divalent cations, rotavirus aggregates with divalent cations.</li> <li>In seawater, FCV has an initial reduction (due to salt content), but retains infectivity over a month.</li> </ul>	<ul style="list-style-type: none"> <li>Salinity either increases or decreases degradation rates of viruses based on type and concentration of salt and specific virus.</li> <li>Salinity can affect viral adsorption to organic and inorganic matter.</li> <li>There are synergistic effects with salinity and temperature and organic and inorganic matter.</li> <li>At 24°C, 37°C, and 50°C, GI F-specific RNA coliphage MS2 is more resistant to 0.3–6.3% NaCl concentrations than MNV.</li> </ul>

Organic and Inorganic Matter	<ul style="list-style-type: none"> <li>39 to 65% of viral inactivation of coliphage T4 due to living and inert planktonic particles 0.2 to 10 <math>\mu\text{m}</math>.</li> <li>Both somatic and F-specific coliphages adsorb to particles <math>&lt;5 \mu\text{m}</math> in size.</li> <li>Somatic coliphages attach preferentially to particles <math>&lt;2 \mu\text{m}</math> in size.</li> <li>Sediment protects F-specific coliphages at 25°C, but not at higher temperatures.</li> <li>Under full sunlight in environmental waters containing CDOM, GI F-specific RNA coliphage MS2 degradation is dominated by exogenous mechanisms.</li> </ul>	<ul style="list-style-type: none"> <li>Adsorption to clay increases persistence in lake water for reovirus (the family to which rotavirus belongs).</li> <li>Inactivation of poliovirus and echovirus decreases with adsorption to marine sediment.</li> <li>Enteroviruses are associated with particulate matter protected from degradation.</li> <li>Sediment protects poliovirus and human adenovirus at 5°C and 25°C.</li> <li>Sediment accelerates inactivation of rotavirus at 5°C and 25°C.</li> <li>Under full sunlight, in environmental waters containing CDOM, poliovirus type 3 degradation is dominated by endogenous mechanisms, human adenovirus type 2 degradation dominated by exogenous mechanisms.</li> </ul>	<ul style="list-style-type: none"> <li>Organic and inorganic material impact viral degradation rates.</li> <li>Organic matter either decreases or increases viral deactivation rates.</li> <li>Viruses adsorb to suspended particulate matter.</li> <li>Synergistic effects among sunlight, temperature, pH, and salinity.</li> <li>F-specific coliphage decay rates are similar to poliovirus in sediment at 25°C and in seawater at 5°C, and rotavirus in sediment at 5°C.</li> <li>MS2 is a conservative surrogate for decay of poliovirus type 3 and human adenovirus type 2 in different types of environmental waters.</li> </ul>	Chung and Sobsey, 1993; Griffin et al., 2003; Sobsey and Meschke, 2003; Characklis et al., 2005; Kohn et al., 2007; Shen et al., 2008; Finiguerra et al., 2011; Romero et al., 2011; Silverman et al., 2013
<b>Biological</b>				
Predation and Enzymatic Degradation (including biofilms)	<ul style="list-style-type: none"> <li>Wetland vegetation increases inactivation of GI F-specific RNA coliphage MS2.</li> <li>Somatic coliphage T4 inactivation in seawater is accelerated by naturally occurring particles <math>&gt;10 \text{ kDa}</math>.</li> <li>Biofilms protect viruses from inactivation: somatic coliphage <math>\Phi X174</math> and GI F-specific RNA coliphage MS2 densities in drinking water biofilms decrease after 6 days.</li> </ul>	<ul style="list-style-type: none"> <li>The presence of microbial activity in raw and treated freshwaters decrease rotavirus persistence.</li> <li>Highest poliovirus inactivation rates in unfiltered natural seawater compared to filtered natural seawater, artificial seawater, and deionized water.</li> <li>NoV is more persistent in biofilm samples and viral genomes persist longer in biofilm than in wastewater.</li> </ul>	<ul style="list-style-type: none"> <li>Both predation and enzymatic degradation increase virus degradation rates in different types of water.</li> <li>Virus adsorption to biofilms is protective, but microbial activity in biofilms causes virus inactivation and degradation.</li> <li>Synergistic effects with temperature and the presence of organic and inorganic matter.</li> <li>No data on direct comparisons of predation effects on human enteric viruses and coliphages.</li> </ul>	Fujioka et al., 1980; Toranzo et al., 1982; Raphael et al., 1985; Deng and Cliver, 1995; Wetz et al., 2004; Skrabber et al., 2007; Karim et al., 2008; Shen et al., 2008; Skrabber et al., 2009; Weaver and Sinton, 2009; Finiguerra et al., 2011; Helmi et al., 2011

## 6. Wastewater Treatment

Treated wastewater is a source of viruses in ambient water (Kageyama et al., 2003; da Silva et al., 2007; Haramoto et al., 2007; Kitajima et al., 2009; Kuo et al., 2010; Simmons et al., 2011). This section provides a broad overview of how coliphages, human enteric viruses, and FIB behave during various wastewater treatment processes. This section does not evaluate engineering technologies or provide specifics on treatment processes. Rather, the overall context is to evaluate whether coliphages could be better than traditional FIB at indicating removal or inactivation of human enteric viruses during wastewater treatment.

Coliphages have been considered useful microorganisms for evaluating wastewater treatment efficacy (Duran et al., 2003; Lucena et al., 2004; Bitton, 2005). Because coliphages and human enteric viruses have similar morphological and structural characteristics (see Section 2.2), often co-occur in feces, and often share fate and transport characteristics (see Section 5.0), the reduction of human enteric viruses and coliphages may follow similar patterns during wastewater treatment depending on the method of pathogen removal (Havelaar et al., 1993; Turner and Lewis, 1995; Rose et al., 2004). These shared attributes of viruses also suggest that coliphages would be better indicators for human enteric viruses than traditional FIB in wastewater. This section discusses colophage behavior during wastewater treatment and compares it to other enteric viruses (with a focus on NoV) and FIB.

Somatic coliphages have been reported to outnumber F-specific coliphages in both treated and untreated wastewater sources (Grabow et al., 1993; Gantzer et al., 1998; Grabow, 2001; Aw and Gin, 2010). The lower density of indigenous F-specific coliphages is a potential limitation of their use as an indicator. The range of colophage densities, specifically the lower end found in influent is highly variable. For example, within the influent for six WWTPs, Rose et al. (2004) found somatic and F-specific coliphages from  $10^3$  to  $10^6$  PFU per 100 mL (host strain ATTC 15597), and F-specific coliphages at  $10^2$  to  $10^8$  PFU per 100 mL (host strain ATTC 700891). In a study of eight WWTPs in Canada that serve 20,000 to 60,000 people, Payment and Locas (2011) found F-specific coliphages in influent in a range of  $10^2$  to  $10^6$  PFU per 100 mL. Because bacteria in biological treatment systems are not in logarithmic growth, it is unlikely that F-specific coliphages replicate during treatment (Rose et al., 2004).

Wastewater treatment processes are often categorized as primary, secondary, tertiary and advanced treatment, and disinfection. There are a variety of different secondary treatment unit processes that can produce different qualities of water. Tertiary treatment has different purposes and definitions depending on the State, and outside the United States. Definitions can vary widely. In addition, natural treatment systems, such as waste stabilization ponds, are commonly used to provide treatment that is roughly similar to primary and secondary treatment. There are a wide variety of technologies available for wastewater treatment, and almost all WWTPs in the United States include secondary treatment and some type of disinfection. This section focuses on the removal or inactivation of coliphages and enteric viruses during the various steps of wastewater treatment.

It is important to understand that treatment efficacy depends on the quality of the effluent prior to disinfection (particularly turbidity or UV transmittance), pH, temperature, the type of

chlorination (free or combined), chlorine dose and contact time, UV dose, and a number of other factors (Asano et al., 2007). Given the importance of the specifics of the treatment processes, it is difficult to draw generalized conclusions from studies that do not provide adequate information on the treatment specifics. For example, efficacy of chlorination depends on the type of chlorination, the contact time, and the specific nature of the secondary treated water. Coliphages can be resistant to some chlorination practices (Havelaar, 1987; Sobsey, 1989; Havelaar et al., 1990; Yahya and Yanko, 1992; Nasser et al., 1993; Gantzer et al., 1998; Bitton, 2005; Harwood et al., 2005), but sequential chlorination (the free chlorine portion) can provide up to 6-log<sub>10</sub> removal (LACSD, 2013).

Ideally, to examine the question of how coliphages, FIB, and enteric viruses compare during wastewater treatment, a study would include the following design attributes:

- enumeration of indigenous somatic and F-specific coliphages, FIB, and one or more human viruses (not addition of a laboratory generated stock of virus);
- density in influent and effluent;
- calculated log<sub>10</sub> reduction values;
- detailed information on the treatment processes that were applied including information on discharge requirements that would impact level of treatment; and
- full-scale wastewater treatment facilities (not pilot and bench scale studies).

Although most of the literature found for this review did not include all of the above attributes, the studies that provided the most relevant information are discussed in more detail (Rose et al., 2004; Harwood et al., 2005; Aw and Gin, 2010; Keegan et al., 2012). It is beyond the scope of this review to conduct a meta-analysis for synthesizing data from the different studies, so each study is discussed individually. Given that this is a broad, high-level review and treatment details are lacking in most of the studies, the nuances of wastewater treatment diversity are not discussed.

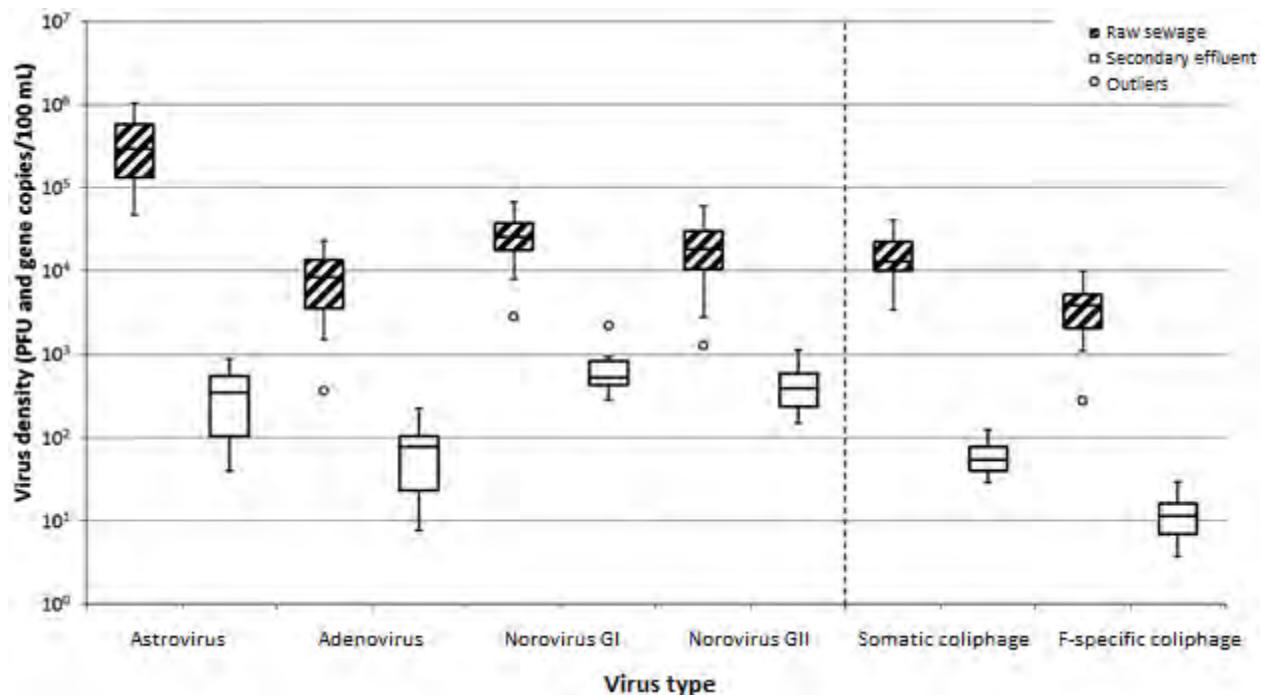
The Water Environment Research Foundation (WERF) conducted an evaluation of the reduction of pathogens, FIB, and alternative indicators (including somatic and F-specific coliphages) at six WWTPs that produced tertiary recycled water by collecting samples five times (approximately once every 2 months) over the course of a year (Rose et al., 2004). Samples were obtained from the WWTPs at various stages of the treatment process and the microorganism density was evaluated by culture dependent methods. A comparison of the log<sub>10</sub> reductions of all coliphages and enteroviruses across all facilities indicated that the combination of primary and biological secondary treatment results in a ~2-log<sub>10</sub> reduction of coliphages and enteroviruses, filtration results in a ~0.5-log<sub>10</sub> reduction of both coliphages and enteric viruses, and disinfection results in a ~0.5-log<sub>10</sub> reduction of coliphages, and little to no reduction of enteroviruses (Rose et al., 2004). The lower average reduction of enteric viruses from disinfection at all six plants was concluded to be partially due to the fact that enteroviruses were below detection limits in 69% of the samples, and samples with no detection were recorded as being at the detection limit (Rose et al., 2004). Coliphages were closer in log<sub>10</sub> reductions to enteroviruses than traditional FIB. Whereas coliphages and enteroviruses both had a cumulative reduction of ~3 to 4 log<sub>10</sub>, FIB had a cumulative reduction of ~5 to 6- log<sub>10</sub>.

The study found that the densities of coliphages and viruses in the influent samples from the different WWTPs were not significantly different; whereas the densities in final effluent were significantly different among WWTP (Rose et al., 2004). These data indicate that differences in effluent densities were related to the treatment processes employed in each WWTP. Although no correlation of the density of coliphages and enteroviruses was found, the authors suggest that it is possible to predict the absence of enteroviruses based on colophage levels. Levels less than 10 colophage PFU per 100 mL (either F-specific coliphages, or F-specific combined with somatic coliphages) were indicative of effluents with no detectable cultivatable enteroviruses (Rose et al., 2004). While Rose et al. (2004) reported  $\log_{10}$  reductions, they did not provide detailed information on the treatment processes.

Harwood et al. (2005) evaluated the same data reported in Rose et al. (2004). F-specific coliphages were detected in 100% of the influent samples at densities ranging from  $10^3$  PFU per 100 mL to  $10^8$  PFU per 100 mL. Although enteroviruses were above detection limits in 31% of the disinfected effluent samples, coliphages and enteroviruses co-occurred in only 13% of the disinfected effluent samples. The authors reported a weakly significant relationship between the presence or absence of enteroviruses and coliphages in disinfected wastewater effluent (Harwood et al., 2005).

Aw and Gin (2010) reported that, when comparing raw sewage to secondary effluent at a plant in Singapore (where wastewater is treated using activated sludge processes), on average, somatic and F-specific coliphage densities were reduced by  $2.4\text{-}\log_{10}$  and NoV GI and GII were reduced by  $\sim 2\text{-}\log_{10}$ . Specifically, somatic coliphages were reduced from  $1.8 \times 10^5$  to  $10^2$  PFU per 100 mL, F-specific coliphages from  $4.3 \times 10^4$  to  $10^2$  PFU per 100 mL, NoV GI from  $3.2 \times 10^5$  to  $7.1 \times 10^3$  gene copies per 100 mL and NoV GII from  $2.3 \times 10^5$  to  $5.2 \times 10^3$  gene copies per 100 mL. Coliphages were quantified by infectivity assays and NoV were quantified by qPCR amplification. PCR amplification can amplify both infectious and noninfectious virus particles and may therefore overestimate the number of infectious NoV particles. The authors found significant correlation between levels of somatic coliphages and adenoviruses, and between F-specific coliphages and NoV GII in raw sewage samples (Aw and Gin, 2010).

Figure 1 shows example reductions for three WWTPs in Singapore (secondary effluent - activated sludge). Somatic and F-specific coliphages had on average  $2.4\text{-}\log_{10}$  reduction, and were reduced at a similar rate as enteric viruses, adenovirus, and astrovirus. NoV reductions were less, but assays were based on qPCR results evaluating both viable and nonviable NoV (Aw and Gin, 2010).



**Figure 1. Comparison of coliphages and enteric viruses in raw sewage and secondary effluent.**

Somatic and F-specific coliphages (PFU per 100 mL) and enteric viruses (gene copy number per 100 mL) isolated from raw sewage ( $n = 18$ ) and secondary effluent ( $n = 18$ ). The box represents 50% of the data values. The line across the inside of the box represents the median value, and the lines extending from the box represent the 95% CIs. Outliers are represented by circles. Hashed boxes are raw sewage, and open boxes are secondary effluent (adapted from Aw and Gin, 2010).

Keegan et al. (2012) investigated the required chlorine and chloramine contact times for inactivating enterovirus (Coxsackie B5) and adenovirus 2. Enterovirus and adenovirus 2 were cultured and added separately to wastewater with varying turbidity levels (0.2, 2, 5, and 20 nephelometric turbidity unit [NTU]) and pH (7, 8, and 9) at 10°C. The spiked samples were exposed to different chlorine/chloramine concentrations and contact times to determine the contact times for up to 4-log<sub>10</sub> virus inactivation. Results demonstrated that increasing contact times are needed with increased turbidity and increased pH. For both viruses, a 4-log<sub>10</sub> inactivation was possible even at the highest turbidity tested (20 NTU). The authors indicated that the results of the study will be used in the development of new wastewater disinfection guidelines for Australia.

Regulatory agencies in Australia use coliphages as indicators for wastewater treatment efficacy. When evaluating a WWTP, the South Australian and Victorian Departments of Health use minimum removal values as defaults for each treatment process, unless it has been demonstrated that a greater inactivation is achievable in the system (Keegan et al., 2012). Table 14 shows the log<sub>10</sub> reductions for wastewater treatments used by the South Australian and Victorian

Departments of Health. Note that coliphage removals are more similar to human virus removal than *E. coli* or bacterial pathogen removal for many treatments.

**Table 14. Log<sub>10</sub> removals of enteric viruses and indicator organisms.**

Treatment	Indicative Log <sub>10</sub> Removals <sup>a</sup>			
	Viruses (including adenoviruses, rotaviruses and enteroviruses)	Coliphages	<i>E. coli</i>	Bacterial pathogens
Primary treatment	0–0.1	N/A	0–0.5	0–0.5
Secondary treatment	0.5–2.0	0.5–2.5	1.0–3.0	1.0–3.0
Dual media filtration with coagulation	0.5–3.0	1.0–4.0	0–1.0	0–1.0
Membrane filtration	2.5–>6.0	3.0–>6.0	3.5–>6.0	3.5–>6.0
Reverse osmosis	>6.0	>6.0	>6.0	>6.0
Lagoon storage	1.0–4.0	1.0–4.0	1.0–5.0	1.0–5.0
Chlorination	1.0–3.0	0–2.5	2.0–6.0	2.0–6.0
Ozonation	3.0–6.0	2.0–6.0	2.0–6.0	2.0–6.0
UVC light	>1.0 adenovirus >3.0 enterovirus, hepatitis A virus	3.0–6.0	2.0–>4.0	2.0–>4.0

Sources: Australian Guidelines for Water Recycling (2008) and Keegan et al. (2012)

<sup>a</sup> Reductions depend on specific features of the process, including detention times, pore size, filter depths, and disinfectant. The default values are accumulated across the treatment train processes. Each row shows only the reduction for that treatment step.

## 6.1. Primary Treatment

Primary treatment of wastewater involves settling of solids in settling tanks and results in different reduction rates of different microbe groups. Viruses are too small to settle and are only removed during primary treatment if they are attached to larger particles. The settling velocities of individual bacteria and protozoan cysts are low compared to the retention time of sedimentation tanks; thus, their removal is also enhanced by attachment to larger particles. As a result, the removal efficiencies of microorganisms is a function of their association with wastewater particles. Asano et al. (2007) report that typical removal is <0.1- to 0.3-log<sub>10</sub> for fecal coliforms, 0.1- to 1.0-log<sub>10</sub> for *Cryptosporidium*, <1-log<sub>10</sub> for *Giardia*, and <0.1-log<sub>10</sub> for enteric viruses. Additionally, Lucena et al. (2004) determined the density of bacterial indicators (e.g., fecal coliforms, enterococci, and sulfate-reducing bacteria) and bacteriophages (e.g., somatic coliphages, F-specific coliphages, and *B. fragilis*-specific bacteriophages) that are present in incoming wastewater and effluent after primary treatment (for secondary treatment see below) from treatment plants in Argentina, Columbia, France, and Spain. The average reductions for the various indicators during primary settling ranged from 0.3- to 0.5-log<sub>10</sub> units. Irrespective of the geographical location, no significant difference in the reduction of any of the indicator microorganisms was observed (Lucena et al., 2004). In the same study, the addition of lime had a significant effect on F-specific RNA coliphage removal, which approached 2-log<sub>10</sub> units, but not on somatic coliphage removal (Lucena et al., 2004). At another WWTP in Ireland, after primary treatment the mean reduction of F-specific RNA coliphages was 0.32-log<sub>10</sub> (SD ± 0.55-log<sub>10</sub>) (Flannery et al., 2012). Finally, Ottoson (2005) investigated the reduction of microorganisms and indicators at multiple WWTPs in Sweden, and found that during primary treatment, somatic

coliphages were reduced by  $0.8\text{-log}_{10}$  ( $SD = 0.4$ ) and F-specific coliphages were reduced by  $1.3\text{-log}_{10}$  ( $SD = 0.7$ ) (Ottoson, 2005).

## 6.2. Secondary Treatment

Secondary treatment of wastewater involves the use of a natural population of bacteria, such as the mixed liquor flocs in activated sludge treatment or the biofilm on trickling filters, to decrease biochemical oxygen demand (BOD), organic material, and in some cases nutrients (depending on the design). In activated sludge treatment, aeration is necessary to support the growth of the aerobic heterotrophic bacteria that consume the soluble organic material in the wastewater. Although secondary treatment is not designed to remove pathogens, removal of indicator organisms and pathogens often occurs.

Secondary treatment results in different  $\log_{10}$  reduction values for different microorganisms and depends on the specifics of the secondary treatment. In a widely used general resource book (Water Reuse), Asano et al. (2007) report that the typical range of removal is 0 to  $2\text{-log}_{10}$  for fecal coliforms,  $1\text{-log}_{10}$  for *Cryptosporidium*,  $2\text{-log}_{10}$  for *Giardia*, and 0- to  $2\text{-log}_{10}$  for enteric viruses. In addition, Asano et al. (2007) report that secondary treatment using activated sludge results in a mean reduction of  $1.83\text{-log}_{10}$  for GI F-specific RNA colophage MS2. The Australian Guidelines for Water Recycling report  $\log_{10}$  reduction ranges of 1- to  $3\text{-log}_{10}$  for *E. coli*, 0.5- to  $2.5\text{-log}_{10}$  for coliphages, and 0.5- to  $2\text{-log}_{10}$  for enteric viruses (NRMMC-EPHC-NHMRC, 2008). A study of WWTPs in Argentina, Colombia, France, and Spain found that secondary treatment reduced somatic coliphages, *Bacteroides fragilis* bacteriophages, and F-specific coliphages between  $1.0\text{-}1.6\text{-log}_{10}$  units (Lucena et al., 2004). In a study of WWTPs in Switzerland, Baggi et al. (2001) found that three WWTPs with mechanical, biological, and chemical processes provided 0.6- to  $0.8\text{-log}_{10}$  reductions for F-specific and somatic coliphages. A fourth WWTP with mechanical, biological, and chemical processes, plus sand filtration provided 1- to  $4.4\text{-log}_{10}$  reductions for F-specific and somatic coliphages (Baggi et al., 2001).

While some coliphages and human virus removal occurs during secondary treatment, they are still typically detectable in non-disinfected secondary effluent. Aw and Gin (2010) detected somatic coliphages and F-specific coliphages along with adenoviruses, astroviruses, and NoVs in 100% of the secondary effluent samples tested (Figure 1). Somatic coliphages and F-specific coliphages were present in secondary effluent at 100 PFU per 100 mL (Aw and Gin, 2010). In six WWTPs secondary effluents, Rose et al. (2004) found that somatic and F-specific coliphages ranged from 10 to  $10^5$  PFU per 100 mL, enterococci from  $10^3$  to  $10^5$  CFU per 100 mL, and enteroviruses from 10 to  $10^2$  MPN per 100 mL. However, in 27% of the secondary effluent samples, enteroviruses were below the detection limits. In five Australian WWTPs, Keegan et al. (2012) found coliphages, adenoviruses, rotaviruses, reoviruses, NoV, and enteroviruses in secondary effluent (Table 15).

**Table 15. Virus densities in secondary treated wastewater samples from five Australian WWTPs.**

Sample location	Microorganism					F-specific RNA coliphages PFU/L
	Adenovirus genome/L	Enterovirus genome/L	Reovirus genome/L	NoV genome/L	Rotavirus genome/L	
Bolivar	1.59 x 10 <sup>5</sup>	0	3.7 x 10 <sup>8</sup>	2.7 x 10 <sup>5</sup>	1.63 x 10 <sup>4</sup>	5.0 x 10 <sup>5</sup>
Bolivar 2	9.3 x 10 <sup>6</sup>	0	0	2.0 x 10 <sup>3</sup>	>6.0 x 10 <sup>3</sup>	3.7 x 10 <sup>4</sup>
Glenelg	2.8 x 10 <sup>5</sup>	0	1.16 x 10 <sup>8</sup>	2.3 x 10 <sup>4</sup>	1.05 x 10 <sup>4</sup>	5.0 x 10 <sup>3</sup>
Cairns	8.1 x 10 <sup>5</sup>	ND	ND	ND	ND	ND
Brisbane	1.7 x 10 <sup>6</sup>	ND	ND	ND	ND	ND
ACT	ND	ND	ND	4.0 x 10 <sup>3</sup>	ND	21

Source: Keegan et al. (2012)

Performed in triplicates with mean results shown in the table.

ND = not detected.

Some other studies also measured  $\log_{10}$  reduction values for coliphages and enteric viruses, but did not provide enough information on treatment design and operations to understand how these reductions might apply in other WWTPs. For example, Lodder and de Roda Husman (2005) found that secondary treatment resulted in the reduction of 1.8- $\log_{10}$  for NoV, 1.6- $\log_{10}$  for F-specific coliphages, and 1.1- $\log_{10}$  for somatic coliphages. Ottoson et al. (2005) found that secondary treatment mean reductions from multiple WWTPs in Sweden were 1.73- $\log_{10}$  (SD = 0.6) for F-specific coliphages and 1.04- $\log_{10}$  (SD = 0.3) for somatic coliphages, which were similar to reductions of enteroviruses 1.3- $\log_{10}$  (SD = 0.7), and NoVs 0.89- $\log_{10}$  (SD = 0.3). FIB had higher  $\log_{10}$  reduction values; enterococci was reduced 2-  $\log_{10}$  (SD = 0.5) and *E. coli* was reduced 2.3-  $\log_{10}$  (SD = 0.6) (Ottoson et al., 2005). Flannery et al. (2012) measured the densities of FIB, F-specific coliphages, and NoV GI and GII in both influent and final effluent at a WWTP. Treatment included preliminary processing by screening and grit removal followed by treatment with a conventional activated sludge system, including primary sedimentation, aeration, and secondary clarification, but no further treatment details were provided. A comparison of influent to secondary effluent found that mean culturable F-specific coliphage densities were reduced by 2.13- $\log_{10}$ , NoV GI gene copy densities were reduced by 0.8- $\log_{10}$ , NoV GII gene copy densities were reduced by 0.92- $\log_{10}$ , and *E. coli* densities were reduced by 1.49- $\log_{10}$  (Flannery et al., 2012).

Appendix B is a compilation of studies that investigated coliphage and NoV densities before, during, and/or after wastewater treatment. It includes mostly non-disinfected secondary effluent, but some disinfected effluents are also included as noted in the table. The Appendix B information is focused on NoV compared to coliphages, because of NoV's importance as an enteric pathogen. NoV is the leading etiological agent of gastrointestinal illness in the United States, and of an estimated 36.4 million cases of domestically acquired gastrointestinal illness, NoV causes an estimated average of 20.8 million cases annually (Scallan et al., 2011).

Some of the studies reviewed in this section evaluated correlations between coliphages and enteric viruses to determine the usefulness of coliphages as surrogates for human viral presence in non-disinfected secondary effluent. Gantzer et al. (1998) showed a significant correlation between the density of coliphages and infectious enteroviruses in secondary effluent and the

correlation between the density of somatic coliphages and the presence of the enterovirus genomes ( $p$ -value <0.0001). No enteroviruses were isolated in secondary effluent (without disinfection) when the somatic coliphage density was between 100 and 10,000 PFU per L (Gantzer et al., 1998). Although the treatment specifics were different, these results are similar to those in Rose et al. (2004), who found that coliphage levels less than 10 PFU per 100 mL in final disinfected effluent contained no detectable cultivatable enteric viruses (Rose et al., 2004). The threshold level in the WERF study is based on tertiary disinfected effluent and not non-disinfected secondary effluent (Rose et al., 2004). Ottoson et al. (2006) found there was no significant correlation between the reduction of coliphages or FIB compared to viruses (enteroviruses and NoV) in secondary treated wastewater. Flannery et al. (2012) also found no correlation between the densities of *E. coli* and F-specific coliphages with either NoV GI or NoV GII levels in effluent wastewater ( $r < 0.07$  in all instances).

### **6.3. Wastewater Treatment Ponds**

Wastewater treatment ponds, also known as waste stabilization ponds or lagoons, are shallow synthetic basins that treat sewage in a single or series of anaerobic, facultative or maturation ponds. Aeration and encouragement of aquatic life are other possible features of wastewater treatment ponds. Verbyla and Mihelcic (2015) analyzed virus removal data from 71 different systems. They found weak to moderate correlation between virus removal and hydraulic retention time. For each  $\log_{10}$  reduction of viruses a geometric mean of 14.5 days of retention (95<sup>th</sup> percentile was 54 days of retention) was required. GI F-specific RNA coliphage MS2 coliphage is considered to be the best surrogate for studying sunlight disinfection in wastewater treatment ponds. Inactivation of coliphages by solar radiation in lagoons and ponding systems tends to be seasonal, with the most effective inactivation occurring in summer months (Davies-Colley et al., 2005; Blatchley et al., 2007). Sunlight inactivation of viruses is discussed in Section 5.2 and is compared to UVC inactivation in Section 6.5.4.

The open water wetland is similar to a maturation pond, but instead of having planktonic algae, the algae are part of a biomat on the bottom of the pond. Silverman et al. (2015) found that removals of F-specific and somatic coliphages were similar in a pilot-scale system. Based on laboratory and modeling work, they determined that GI F-specific RNA coliphage MS2 was inactivated more slowly than poliovirus under summer conditions, but more rapidly under winter conditions. More research is needed to determine how the relative inactivation rates of indigenous coliphages (F-specific and somatic coliphages) and other enteric viruses change seasonally in open water wetlands.

### **6.4. Tertiary Treatment and Advanced Treatment**

Tertiary treatment typically refers to particle removal processes (e.g., granular media filtration, cloth filtration, or membrane filtration) that are employed before final disinfection. The amount by which viruses (and other pathogens) are reduced by filtration varies depending on filter characteristics, operating practices, microbial properties, including size, surface properties, and degree of association with other microorganisms or particles, and water quality variables (Levine et al., 2008). Tertiary treatment may also refer to chemical or biological nutrient removal processes (e.g., targeting nitrogen and/or phosphorus), although these processes are sometimes considered part of secondary treatment. Literature reports for treatment plants employing nutrient

removal were included in Section 6.2. Advanced treatment trains, which can be applied to filtered tertiary effluents, can be used to further purify water for indirect or direct potable reuse. Advanced treatment typically involves advanced oxidation processes (AOPs) and dense membranes (nanofiltration and reverse osmosis) that target the removal of pathogens and trace organic contaminants (Leverenz et al., 2011; NRC, 2011; Gerrity et al., 2013). Membrane processes are reviewed here. Disinfection processes, including UV, ozone, free chlorine, combined chlorine, and AOPs are described in detail in Section 6.5.

**Depth filtration** involves the use of granular media (e.g., sand, anthracite, garnet, or activated carbon) in single (mono-media) or layered configurations (multi-media). Microorganism removal differs based on a variety of factors, including water quality, the type and size of granular media, the filtration velocity, and the use of coagulant and/or polymer. Typical removals from depth filtration are reported to be 0 to 1-log<sub>10</sub> for fecal coliforms, 0 to 3-log<sub>10</sub> for *Cryptosporidium*, 0- to 3-log<sub>10</sub> for *Giardia*, 0- to 1-log<sub>10</sub> for enteric viruses and ~0.14- to 2-log<sub>10</sub> for coliphages (Rajala et al., 2003; Hijnen et al., 2004; Zanetti et al., 2006; Asano et al., 2007; Hijnen and Medema, 2007). Asano et al. (2007) report that tertiary treatment using depth filtration results in a mean reduction of 0.29-log<sub>10</sub> for GI F-specific RNA coliphage MS2, and Zanetti et al. (2006) found that tertiary sand filtration resulted in a mean reduction of 0.31-log<sub>10</sub> for *E. coli* and 0.14-log<sub>10</sub> for somatic coliphages.

Rajala et al. (2003) conducted both laboratory and pilot-scale experiments on rapid sand filtration of wastewater effluent from WWTPs in Finland. In the laboratory experiment, the rapid sand filtration reduced coliphages by 0.15- to 0.26-log<sub>10</sub> (30–46%) at a hydraulic load of 5 meters per hour and 0.13- to 0.27-log<sub>10</sub> (23–38%) at a hydraulic load of 10 meters per hour. In the pilot experiments (hydraulic loads range 7.7 to 10 meters per hour), coliphages were reduced by 0.66- to 1.5-log<sub>10</sub> (7–97%), depending on the plant (Rajala et al., 2003). Based on pilot-scale filter studies on rapid depth filtration, Williams et al. (2007) found that the removal efficiency of GI F-specific RNA coliphage MS2 (seeded into secondary effluent) was similar to that of *E. coli* and total coliforms (~ 0.8 log<sub>10</sub> at a loading rate of 12.2 meters per hour). The removal efficiency of MS2 was more sensitive to the coagulant dose, compared to the indicator bacteria. In an experimental rapid sand filtration setup, virus size (based on ΦX174, MS2, and T4 coliphages) was the only factor that influenced retention and the larger the virus, the greater the retention (Aronino et al., 2009).

Levine et al. (2008) conducted experiments to examine pathogen reduction from sand filtration of secondary effluent at five full-scale water reclamation facilities in the United States (three plants using monomedium and two plants using dual media) at peak usage over the course of a year. These are the same facilities that are reported in Rose et al. (2004). The average reductions for all five plants ranged from 0.1- to 4.2-log<sub>10</sub> for fecal coliforms, 0.3- to 1.1-log<sub>10</sub> for infectious *Cryptosporidium*, 0.7- to 1.5-log<sub>10</sub> for *Giardia*, 0.3- to 1.2-log<sub>10</sub> for culturable enteroviruses, 0.3- to 1.3-log<sub>10</sub> for F-specific coliphages, and 0.2- to 0.8-log<sub>10</sub> for somatic and F-specific coliphages (Levine et al., 2008). The authors found that the differences in average reduction rates between plants were likely due to a combination of loading rates, chemical addition practices (chlorine and coagulant), backwashing and post backwashing operating strategies, and the effectiveness of upstream biological treatment and sedimentation (Levine et al., 2008). In general, log<sub>10</sub> reductions of indicator bacteria (coliforms, enterococci, and *Clostridium*) was 2-to 9-fold greater

than the  $\log_{10}$  reduction of pathogens, suggesting that monitoring with bacterial indicators may over predict pathogen reductions. Rose et al. (2004) noted that shallow sand filters were more effective than deep-bed dual-media or monomedia filters for removal of coliphages and viruses. However, this result was affected by the fact that pre-disinfection (pre-chlorination) was used for the shallow sand filter tests but not for the deep-bed filters.

**Surface Filtration** includes mechanical sieving of secondary effluent, through cloth, metal or synthetic woven materials with a pore size of ~10 to 30  $\mu\text{m}$ . In comparative testing for 15- to 30- $\mu\text{m}$  particles, surface filtration removed more particles than granular filtration over all particle sizes tested (Olivier et al., 2003). Asano et al. (2007) reported average reductions for surface filtration of 0- to 1- $\log_{10}$  for coliform bacteria and 0- to 0.5- $\log_{10}$  for enteric viruses. These results are consistent with Levine et al. (2008), who found that cloth filtration of secondary effluent at a full-scale water reclamation facility at peak usage over the course of a year resulted in average reductions of 3- $\log_{10}$  (range: 1.9 to 4.3) for fecal coliforms, 0.5- $\log_{10}$  (range: 0.3 to 0.7) for infectious *Cryptosporidium*, 0.5- $\log_{10}$  (range: -0.4 to 1.3) for *Giardia*, 0.5- $\log_{10}$  (range: 0.3 to 0.8) for culturable enteric viruses, 0.6- $\log_{10}$  (range: -0.1 to 1.8) for F-specific coliphages, and 0.4- $\log_{10}$  (range: -0.1 to 1) for somatic and F-specific coliphages.

**Membrane filtration**, a type of advanced treatment, involves forcing wastewater through a thin membrane filtering under pressure. Membranes with different sized pores can be used, including microfilters ( $>50$  nm), ultrafilters (2 to 50 nm), nanofilters ( $<2$  nm), and reverse osmosis (polymer matrix without discrete pores; particles are excluded and uncharged molecules pass through membrane by diffusion). In general, the smaller the pore size used, the greater the reduction of pathogens and the higher the operating pressure (Asano et al., 2007). For example, Asano et al. (2007) reported that typical removal of pathogens from microfiltration are 1- to 4- $\log_{10}$  for fecal coliforms, 1- to 4- $\log_{10}$  for *Cryptosporidium*, 2- to 6- $\log_{10}$  for *Giardia*, and 0- to 2- $\log_{10}$  for enteric viruses. Ultrafiltration results in removal of 3- to 6- $\log_{10}$  for fecal coliforms,  $>6$ - $\log_{10}$  for protozoa, and 2- to 7- $\log_{10}$  for viruses. Nanofiltration results in removal of 3- to 6- $\log_{10}$  for all types of bacteria,  $>6$ - $\log_{10}$  for protozoa, and 3- to 5- $\log_{10}$  for viruses. Reverse osmosis results in reductions of 4- to 7- $\log_{10}$  for fecal coliforms, 4- to 7- $\log_{10}$  for *Cryptosporidium*,  $>7$ - $\log_{10}$  for *Giardia*, and 4- to 7- $\log_{10}$  for enteric viruses (Asano et al., 2007).

Perfectly intact nanofiltration and reverse osmosis membranes should not allow passage of any bacteria or viruses; however, leaks in seals, and membrane imperfections or damage to the membranes could allow their passage. Thus, monitoring the integrity of membranes is critical to ensuring high removal of microorganisms.

Juby (2003) found that microfiltration of screened primary effluent at a demonstration plant in California resulted in typical reductions of 4.7- $\log_{10}$  for fecal coliforms and 1.7- $\log_{10}$  for coliphages. Gomez et al. (2006) used secondary effluent from a WWTP in Spain to determine pathogen reduction rates from microfiltration and ultrafiltration. They found that microfiltration resulted in a 2.7- $\log_{10}$  reduction in fecal coliform, the removal of *E. coli* below detectable limits, and a 1.3- $\log_{10}$  reduction of coliphages, whereas ultrafiltration resulted in a 4.7- $\log_{10}$  reduction of fecal coliform, the removal of *E. coli* below detectable limits, and a 3.5- $\log_{10}$  reduction of coliphages (Gomez et al., 2006).

**Membrane Bioreactors** are a relatively new technology that combine an activated sludge bioreactor with membrane filtration, which replaces both secondary and tertiary treatment. Zhang and Farahbakhsh (2007) found that membrane bioreactor pilot plants achieved  $5.8\text{-log}_{10}$  removal of coliphages. Whereas conventional activated sludge process followed by advanced tertiary treatment (nitrifying rotating biological contactors, sand filtration, and chlorination), achieved  $5.5\text{-log}_{10}$  removal of coliphages. For membrane systems, coliphages appear to be better indicators of microbial removal efficacy (especially viral removal) probably because the pore size of most microfiltration and ultrafiltration membranes exclude fecal coliforms, but some coliphages can still pass through the membrane pores (Zhang and Farahbakhsh, 2007). In a full-scale membrane bioreactor study, Purnell et al. (2015) reported a  $5.3\text{-log}_{10}$  reduction in indigenous somatic coliphages. Indigenous F-specific coliphages were less abundant and demonstrated a  $3.5\text{-log}_{10}$  reduction. In ‘spiking’ experiments, suspended GI F-specific RNA coliphage MS2 demonstrated a  $2.25\text{-log}_{10}$  reduction (Purnell et al., 2015).

## 6.5. Disinfection

Disinfection of secondary effluent can be achieved using physical (UVC radiation) and chemical (chlorine, chloramines, and ozone) treatments. This section focuses on the physical and chemical treatments of secondary effluent and the effects of these treatments on coliphages, FIB, and enteric virus inactivation. Although solar radiation can also play a role in further disinfection of secondary effluent by lagooning (Gomila et al., 2008), lagooning is not typically considered a disinfection treatment (see Section 6.3). It is important to note that if ammonium levels are not reported, it cannot be determined whether free chlorine or combined chlorine was present during the disinfection step. This is important because many studies report on water samples from secondary disinfected effluent, but there is wide variation in what secondary disinfected effluent includes.

As mentioned previously, studies with water samples collected at full-scale WWTPs are preferred. However, pilot scale and bench-scale studies are also included when full-scale data were not available.

### 6.5.1 Free Chlorine

Chlorine ( $\text{Cl}^-$ ) is the most widely used wastewater disinfectant (Asano et al., 2007). Chlorine is an efficient disinfectant for most enteric bacteria, but is generally less efficient against viruses, protozoan cysts, and bacterial spores (Keegan et al., 2012). The effectiveness of chlorine is impacted by disinfection dose, contact time, temperature, and water quality variables (pH, turbidity, presence of ammonia and oxidant demand) (Rose et al., 2004; Asano et al., 2007). For example, above pH 7, a 10 mg per L residual chlorine resulted in  $4\text{-log}_{10}$  reduction of F2 coliphages, whereas at low pH, as little as one-fifth of this chlorine achieved the same reduction (Hajenian and Butler, 1980).

Due to the highly reactive chemical nature of free chlorine ( $\text{HOCl}$  and  $\text{OCl}^-$ ), in secondary effluents containing ammonium, it rapidly combines with ammonium to form chloramines, a form of combined chlorine (U.S. EPA, 2002; Tree et al., 2003; Asano et al., 2007). Disinfection with free chlorine can be achieved in ammonium-containing effluents if “breakpoint” chlorination is practiced, in which sufficient free chlorine is added to convert all ammonium to

nitrogen. Free chlorine is a much stronger oxidant than combined chlorine, and is more effective at inactivating pathogenic bacteria and coliphages than combined chlorine (Tyrrell et al., 1995; Duran et al., 2003; Tree et al., 2003; Keegan et al., 2012). Combined chlorine will be discussed in more detail in Section 6.5.2. It is important to note that both free chlorine and combined chlorine can result in formation of hazardous disinfection byproducts such as trihalomethanes, haloacetic acids, chlorite, and other hazardous compounds.

In a bench-scale study, Shin and Sobsey (2008) studied the inactivation of NoV by free chlorine using molecular methods and compared its inactivation to F-specific RNA coliphage MS2 and poliovirus type 1. F-specific RNA coliphage MS2 and poliovirus type 1 were measured using both culture-based and molecular methods. The authors conducted experiments using 1 and 5 mg per L free chlorine. Inactivation of NoV was similar to F-specific RNA coliphage MS2 and faster than poliovirus type 1 when densities were measured using molecular methods. They also showed that the CT (disinfectant concentration times contact time) required for NoV inactivation was not significantly different from other viruses even though the molecular methods likely overestimate the CT needed. Thus, the study authors concluded that chlorine is a useful disinfectant for NoV.

If a study evaluates disinfected effluent, but does not report ammonium levels, it cannot be determined whether free chlorine or combined chlorine was present. Of the six WWTPs evaluated in Rose et al. (2004), four used chlorine disinfection, but only one of the WWTP had ammonium levels that allowed for free chlorine. Rose et al. (2004) combined the data from the four WWTPs and found that on average, 300 minutes of contact time with chlorine (or combined chlorine) in secondary effluent resulted in a 3-log<sub>10</sub> reduction of enterococci, whereas 500 minutes of contact time were required for a 3-log<sub>10</sub> reduction of enteroviruses. Data from the WWTP with free chlorine disinfection of nitrified and filtered effluent is shown in Table 16. More studies that compare chlorine inactivation of FIB, indigenous F-specific and somatic coliphages, and enteric viruses in nitrified effluent are needed.

**Table 16. Average (and percent positive) microorganism effluent densities in a WWTP with free chlorine treatment of nitrified and filtered secondary wastewater.**

Treatment	Microorganism				
	Total coliform CFU/100 mL	Enterococci CFU/100 mL	Somatic and F- specific coliphages PFU/100 mL	F-specific coliphages PFU/100 mL	Enterovirus MPN/100 mL
Influent	3.41 x 10 <sup>7</sup> (100%)	7.36 x 10 <sup>5</sup> (100%)	2.84 x 10 <sup>5</sup> (100%)	3.14 x 10 <sup>5</sup> (100%)	1.52 x 10 <sup>4</sup> (100%)
Filtered and disinfected with free chlorine	11.3 (60%)	0.2 detection limit (0%)	10.4 (80%)	10.4 (80%)	0.3 (80%)

Source: Rose et al. (2004)

Over a 13-month period water samples were taken at Easterly WWTP in Vacaville, California and evaluated for FIB, F-specific coliphages, NoV, and other pathogens (Olivieri et al., 2012). This WWTP has bar screens, grit removal, primary clarification, secondary treatment with activated sludge, secondary clarification, nitrification, and chlorine disinfection and de-

chlorination. Log<sub>10</sub> removal of fecal coliform through secondary disinfection was a median of 6.8-log<sub>10</sub> (range 6.1- to 8.4-log<sub>10</sub>; all non-detects were set at the detection limit). In finished effluent, fecal coliforms were not detected in 50 of the 55 samples (<2 MPN per 100 mL detection limit), and the remaining 5 were at the limit of detection. For enterococci, log removals through secondary disinfection was a median of 5.8-log<sub>10</sub> (range 3.2- to 6.2-log<sub>10</sub>; all non-detects were set at the detection limit). In finished effluent, enterococci were not detected in 22 of 32 samples (<1 MPN per 100 mL detection limit). The 10 detectable enterococci samples ranged from 1 to 648.8 MPN per 100 mL. F-specific coliphages were detected in all 32 influent samples. Densities ranged from 60 PFU per 100 mL to 13,000 PFU per mL, with a median of 2,750 PFU per 100 mL. F-specific coliphages in the final disinfected and dechlorinated effluent were below the detection limit (<1 PFU/100 mL) in all but two samples. One sample was at the detection limit (1 PFU/100 mL), and the other sample was 2 PFU per 100 mL. The log removal of F-specific coliphages had a median of 3.4-log<sub>10</sub> (range 1.8- to 4.1-log<sub>10</sub>). NoV were present in the ten WWTP influent samples and not detected in eleven final disinfected and dechlorinated effluent samples (Olivieri et al., 2012). This demonstrates that free chlorine applied to nitrified effluent is quite effective at inactivating F-specific coliphages and NoV.

### 6.5.2 Combined Chlorine

As stated above, studies have shown that in secondary effluent with ammonium, the free chlorine rapidly combines with the ammonium to form chloramines (Asano et al., 2007). Combined chlorine compounds are less effective at inactivating microorganisms than free chlorine (Tyrrell et al., 1995; Duran et al., 2003; Tree et al., 2003; Keegan et al., 2012).

Tree et al. (2003) studied the chlorine-mediated inactivation of both seeded (*E. coli*, *Enterococcus faecalis*, GI F-specific RNA coliphage MS2, and poliovirus – all measured using culture-based methods) and naturally occurring (*E. coli*, enterococci, F-specific coliphages, and enterovirus – also using culture-based methods) bacterial and viral indicators in primary sewage effluent. The inactivation rates of three applied doses of free chlorine (8, 16, and 30 mg per L) were investigated in both seeded sterilized primary effluent and unsterilized primary effluent. Although free chlorine was applied, Tree et al. (2003) found that the amount of free chlorine available in effluent decreased rapidly within the first 5 minutes and then remained approximately constant for the duration of the experiments (30 minutes). In both experiments, the authors found that FIB (*E. coli* and enterococci) were inactivated more rapidly and at lower doses than the viruses (F-specific coliphages, poliovirus, and enterovirus) and that chlorine dose and time of exposure had significant effects on survival of all organisms tested in both experiments (Table 17). Both *E. coli* (laboratory-cultured and indigenous) and enterococci had linear degradation rates and were completely inactivated over the course of the experiment at all chlorine applications tested. In contrast, enteroviruses had biphasic degradation rates, with a rapid initial rate, followed by a slower inactivation rate. F-specific RNA coliphages (both laboratory-cultured and naturally occurring) only showed degradation for the first 5 minutes of exposure after which no further degradation occurred. The authors suggest that the slower rate of degradation for enteroviruses and lack of degradation of F-specific RNA coliphages after 5 minutes is likely due to the weaker effect of combined chlorine on viruses. The authors conclude that F-specific RNA coliphages are a useful and conservative model surrogate for chlorine inactivation of viruses in sewage (Tree et al., 2005).

**Table 17. Log<sub>10</sub> reduction of FIB, enteric virus, and F-specific coliphages in sewage matrix due to chlorine (adapted from Tree et al., 2003).**

Matrix	Microorganism	Applied chlorine concentration		
		8 mg/L	16 mg/L	30 mg/L
Seeded into sterilized primary sewage matrix	<i>E. coli</i>	>5 (<5 min.)	>5 (<5 min.)	>5 (5 min.)
	<i>Enterococcus</i>	1 (<5 min.)	<1 (<15 min.)	>5 (5 min.)
	<b>Poliovirus</b>	1 (after 30 min.)	<2 (after 30 min.)	4 (after 30 min.)
	<b>F-specific RNA coliphage MS2</b>	<1 (after 30 min.)	<1 (after 30 min.)	1 (after 30 min.)
Naturally occurring in raw sewage (after primary treatment)	<b>Indigenous <i>E. coli</i></b>	4 (5 min.)	4-5 (5 min.)	5 (5 min.)
	<b>Indigenous <i>Enterococcus</i></b>	>3 (15 min.)	>3 (5 min.)	>3 (5 min.)
	<b>Indigenous Enteroviruses</b>	<1 (after 30 min.)	>1 (after 30 min.)*	>1 (after 5)*
	<b>Indigenous F-specific RNA coliphages</b>	<1 (after 30 min.)	<1 (after 30 min.)	<1 (after 30 min.)

\* Because these were naturally occurring level (so lower density than seeded samples), the detection limit was reached after about a 1-log<sub>10</sub> reduction.

Note: Times in parenthesis indicate the duration of the chlorine treatment. Log reductions were estimated based on graphical information.

Duran et al. (2003) determined chlorine-mediated inactivation rates of both spiked and naturally occurring FIB, bacteriophages, and enteroviruses in secondary effluents. The authors found that after secondary effluent was exposed to 10 mg per L chlorine at the WWTP, naturally occurring FIB (*E. coli* and enterococci) were reduced at significantly higher rates than were naturally occurring viruses (F-specific RNA coliphages, somatic coliphages, *B. fragilis* bacteriophages, and enteroviruses). Specifically, mean reductions of naturally occurring microorganisms in chlorinated secondary effluent were: 2.9-log<sub>10</sub> (SD = 2.5) for fecal coliforms, 2.0-log<sub>10</sub> (SD = 0.7) for enterococci, 1.6-log<sub>10</sub> (SD = 0.6) for somatic coliphages, 0.6-log<sub>10</sub> (SD = 0.5) for F-specific RNA coliphages, 0.3-log<sub>10</sub> (SD = 0.3) for *B. fragilis* bacteriophages, and 0.4-log<sub>10</sub> for enteroviruses (no SD was given due the low number of positive samples) (Duran et al., 2003). Chlorination of secondary effluent in the laboratory resulted in similar inactivation rates as those found from chlorination at the WWTP (Duran et al., 2003). Both F-specific and somatic coliphages were inactivated more efficiently than enteroviruses. However, coliphages were closer to enterovirus log<sub>10</sub> reductions than FIB.

To determine if different types of viruses have different levels of resistance to chlorine, Duran et al. (2003) spiked secondary effluent with several bacteriophages, the vaccine strain of poliovirus type 1 Lsc 2a, and an enterovirus isolated from the environment, AR51101-1. The log<sub>10</sub> reduction of bacteriophages and enteroviruses are presented in Table 18.

**Table 18. Log<sub>10</sub> reduction of bacteriophages and enteroviruses in spiked secondary effluent after chlorination with 20 mg/L of chlorine.\***

Phage or Virus		20 Min	30 Min
Enteroviruses	AR51101-1	3.1	3.6
	Poliovirus type 1	1.3	1.6
Somatic coliphages	ΦX174	2.8	3.3
	MY2	2.3	2.8
	SS13	0.2	0.4
	SR51	2.0	2.5
	SC12	1.0	1.7
F-specific RNA coliphage	MS2	1.3	2.1
Bacteriophages specific to <i>B. fragilis</i> HSP40	B40-8	0.1	0.4

Source: Duran et al. (2003)

\*Concentration of free chlorine was 0 after 20 minutes.

Haramoto et al. (2006) determined the densities of NoV GI and GII and F-specific coliphages in the raw sewage and final effluent at a WWTP that used conventional activated sludge process followed by chlorination. When comparing raw sewage to chlorinated secondary effluent, on average, NoV GII genome copies were reduced by 3.69-log<sub>10</sub>, *E. coli* densities were reduced by 3.37-log<sub>10</sub>, total coliform densities were reduced by 3.05-log<sub>10</sub>, F-specific coliphage densities were reduced by 2.81-log<sub>10</sub>, and NoV GI genome copies were reduced by 2.27-log<sub>10</sub> (Haramoto et al., 2006).

Other studies have also demonstrated that FIB are more sensitive to chlorine than viruses. Tyrrell et al. (1995) found that combined chlorine was ineffective at inactivating F-specific coliphages (such as F-specific RNA coliphages), but was effective at eliminating vegetative bacteria from secondary effluent. Dee and Fogleman (1992) evaluated coliphage removal in a Denver treatment facility, because routine monitoring of the plant showed that coliphages were escaping the wastewater treatment processes. Monochloramine residuals were monitored in the effluent, and the contact times that coliphages had with monochloramines were measured. In the WWTP studied monochloramines alone were not capable of a 2-log<sub>10</sub> reduction in coliphage density (Dee and Fogleman, 1992). Average coliphage densities in effluent were 0.36 ± 1.03 PFU per 100 mL in the summer and 0.08 ± 0.19 PFU per 100 mL for the rest of the year (Dee and Fogleman, 1992).

In a bench-scale study, Sobsey et al. (1988) compared the inactivation of hepatitis A virus, coxsackievirus B5, F-specific RNA coliphage MS2, and somatic coliphage ΦX174 when exposed to 0.5 mg free chlorine (pH 6-10) per L and 10 mg monochloramine per L in phosphate buffer. Hepatitis A virus was inactivated quickly by free chlorine, but was relatively resistant to monochloramine. Coxsackievirus was relatively resistant to both. Somatic coliphage ΦX174 was most sensitive of the viruses to free chlorine at all pHs, except pH 10, and was the virus most sensitive to monochloramine. Inactivation of F-specific RNA coliphage MS2 by free chlorine was quicker than hepatitis A at low pH, but less rapid at higher pHs. In this study F-specific RNA coliphage MS2 was the virus most resistant to monochloromine.

Log<sub>10</sub> reduction values for all the human enteric viruses compared to coliphages do not seem to be available. Given treatment diversity, it might be impossible to rank all the enteric viruses in

order of resistance. However, it should be noted that adenoviruses seem to be more resistant to combined chlorine than enterovirus (Irving and Smith, 1981; Cromeans et al., 2010). Adenovirus-2 is one of the most resistant viruses to chloramines and adenovirus-2 has similar resistance as adenovirus 40 and 41 (Keegan et al., 2012). Reovirus may be even more difficult to remove than adenovirus and enteroviruses through secondary treatment processes (Irving and Smith, 1981). Future studies that compare the behavior of coliphages to adenovirus would be helpful for evaluating the utility of coliphages as indicators of the presence of viruses in wastewater.

### 6.5.3 Ozone

Ozone is a highly reactive chemical that damages microorganisms and reacts with water to produce hydroxyl radicals ( $\text{OH}^-$ ) that oxidize organic pollutants (Paraskeva and Graham, 2002). Like other disinfection processes, high doses of ozone can result in hazardous disinfection byproduct formation such as bromate. Additionally ozone decomposition occurs faster at higher temperatures and higher pH, which can alter disinfection efficacy (U.S. EPA, 1999). Viruses as, a group, are the most sensitive microorganisms to ozone of all the microorganisms listed on EPA's Contaminant Candidate List (U.S. EPA, 1998; Gerba et al., 2003).

Several studies have shown that in secondary effluent, ozone is more effective at inactivating coliphages than FIB (Tyrrell et al., 1995; Gehr et al., 2003; Tanner et al., 2004). For example, Gehr et al. (2003) found that a transferred ozone dose of 17 mg per L resulted in a  $3\text{-log}_{10}$  reduction of F-specific RNA coliphages, whereas a transferred ozone dose of 30 to 50 mg per L was required for a  $2\text{-log}_{10}$  reduction in fecal coliforms and resulted in less than a  $1\text{-log}_{10}$  reduction in *Clostridium perfringens* (Gehr et al., 2003). Tyrrell et al. (1995) found that secondary sewage treated with a pulse of ozone [mean residual ozone concentrations of 0.30 ppm (SD = 0.08)] resulted in approximate mean reductions of  $2.5\text{-log}_{10}$  for F-specific coliphages,  $2.25\text{-log}_{10}$  for somatic coliphages,  $1.3\text{-log}_{10}$  for fecal coliforms,  $1.2\text{-log}_{10}$  for enterococci, and  $0.2\text{-log}_{10}$  for *C. perfringens*. Lazarova et al. (1998) found that 5 minutes of contact time of a 5-mg per L dose of ozone resulted in a  $5\text{-log}_{10}$  removal of F-specific RNA coliphage MS2.

Tanner et al. (2004) investigated the effects of ozone on poliovirus, F-specific RNA coliphage MS2, *Klebsiella terrigena*, *E. coli*, heterotrophic plate count bacteria, fecal coliforms, and total coliforms, either in secondary effluent or reverse osmosis treated water. In secondary effluent, continuous ozone treatment for 1 minute resulted in an average inactivation of  $2.5\text{-log}_{10}$  for coliphages, and  $<1.5\text{-log}_{10}$  reductions for total coliforms, fecal coliforms, and heterotrophic plate count bacteria. In demand-free reverse osmosis treated water, the authors found that 1 minute in 0.2 mg ozone per L resulted in a  $>3\text{-log}_{10}$  inactivation of poliovirus and 1 minute in 0.25 mg ozone per L resulted in a  $6\text{-log}_{10}$  inactivation of F-specific RNA coliphage MS2. Tanner et al. (2004) also found that increasing the concentration of ozone reverse osmosis treated water resulted in increased inactivation of all indicator organisms tested (F-specific RNA coliphage MS2, *Klebsiella terrigena*, and *E. coli*). Table 19 presents the highest  $\log_{10}$  reductions of each indicator organism at a given ozone concentration.

**Table 19. Inactivation of FIB and F-specific RNA coliphage in ozone disinfected water.\***

Indicator	Log <sub>10</sub> inactivation	Ozone concentration (mg ozone/L)
F-specific RNA coliphage MS2	≥5.41	0.22
<i>Klebsiella terrigena</i>	4.71	0.25
<i>E. coli</i>	4.15	0.25
poliovirus	>3	0.2

Source: Tanner et al. (2004)

\*Water was spiked after receiving RO treatment.

Finch and Fairbairn (1991) found that in demand-free phosphate buffer, 1.6-log<sub>10</sub> units more inactivation was observed with GI F-specific RNA coliphage MS2 than with poliovirus type 3. The authors conclude that use of MS2 coliphage as a surrogate organism for studies of enteric virus with ozone disinfection systems can overestimate the inactivation of enteric viruses. In contrast, Shin and Sobsey (2003) documented the inactivation of MS2, Norwalk virus, and poliovirus type 1 in the presence of ozone using infectivity assays (for MS2 and poliovirus type 1) and RT-PCR (all three viruses). Using a 0.37-mg per L dose of ozone at pH 7 and 5°C, the authors found that the three viruses were inactivated approximately 3-log<sub>10</sub> within 5 minutes and hence had similar inactivation behavior when detected using molecular methods. Inactivation measured by infectivity of F-specific RNA coliphage MS2 and poliovirus type 1 agreed well with their inactivation using molecular methods. Hall and Sobsey (1993) studied the decay of F-specific RNA coliphage MS2 and hepatitis A virus in buffered water when exposed to ozone, as well as ozone and hydrogen peroxide in series. They found that both F-specific RNA coliphage MS2 and hepatitis A virus behaved similarly and were rapidly inactivated (up to 6-log<sub>10</sub> in 5 seconds) by two types of treatments.

#### 6.5.4 UVC

In contrast to oxidative disinfection processes with chemicals like free chlorine and ozone, the efficacy of UVC (hereafter referred to as UV) disinfection is not affected by conditions like temperature, pH, and the presence and concentration of reactive organic matter (UV absorbance by organic and inorganic matter can shield microorganisms from UV, but the reactive properties of the organic matter don't affect the UV, as happens with chemical disinfectants) (Oppenheimer et al., 1993; Hijnen et al., 2006). UV light is primarily absorbed by nucleic acids of microorganisms, causing harmful photoproducts such as thymine dimers on the same nucleic acid strand. If the damage is not repaired, replication is blocked, leading to subsequent inactivation of microorganisms (Ko et al., 2005). UV inactivation of microorganisms, including coliphages, is proportional to the UV fluence or dose, the product of the UV intensity and exposure time. Unlike free and combined chlorine and ozone, UV does not produce harmful disinfection byproducts (Oppenheimer et al., 1993). UVC has a shorter wavelength than UVA and UVB. UVC is filtered by the atmosphere, so does not occur in sunlight that reaches the surface of the earth. UVC is the most biologically damaging of the three UV wavelength classes and can be created artificially with UVC bulbs for treatment of water. UVC wavelengths (100 to 280 nm), also called short-wave or germicidal UV, have been shown to result in a 1.09- to 2-log<sub>10</sub> reduction of indicator bacteria and coliphages in secondary effluent (Rose et al., 2004).

In general, coliphages have been found to be more resistant to UVC light than FIB. For example, Gehr et al. (2003) demonstrated that GI F-specific RNA coliphage MS2 is more resistant to UV inactivation than fecal coliforms in effluent. Tree et al. (2005) found that F-specific RNA coliphage MS2 was more resistant to UV reduction than *E. coli* (4-log<sub>10</sub> reduction required 62.5 mJ/cm<sup>2</sup> for MS2 and 5.32 mJ/cm<sup>2</sup> for *E. coli*) in seeded, sterilized secondary effluent. Wilson et al. (1992) found that the viruses GI F-specific coliphage MS2, rotavirus, poliovirus, and hepatitis A were at least 7.1 times more resistant than the bacteria *Klebsiella terrigena*, *Legionella pneumophila*, *Salmonella typhi*, *Aeromonas hydrophila*, *E. coli*, *Campylobacter jejuni*, *Yersina enterocolitica*, *Shigella dysenteriae*, and *Vibrio cholerae*. In general, the bacteria tested were three to ten times more susceptible to UV irradiation than the viruses (Wilson et al., 1992).

One of the six WWTPs studied in Rose et al. (2004) used UV disinfection. Table 20 shows the densities of microorganisms in the filtered secondary effluent compared to the filtered secondary effluent after UV disinfection.

**Table 20. Average (and percent positive) microorganism densities in a WWTP with UV treatment of filtered secondary effluent (n=5).**

Treatment	Microorganism				
	Total coliform CFU/100 mL	Enterococci CFU/100 mL	Somatic and F-specific coliphages PFU/100 mL	F-specific coliphages PFU/100 mL	Enterovirus MPN/100 mL
Filtered secondary effluent	1.79 x 10 <sup>4</sup> (100%)	5.8 x 10 <sup>2</sup> (100%)	1.14 x 10 <sup>3</sup> (100%)	1.41 x 10 <sup>2</sup> (100%)	0.7 (40%)
Filtered secondary effluent disinfected with UV with free chlorine	11.9 (80%)	4.38 (20%)	10 detection limit (0%)	10 detection limit (0%)	0.5 detection limit (0%)

Source: Rose et al. (2004).

The National Water Research Institute (NWRI) and Water Research Foundation (WRF) *Ultraviolet Disinfection Guidelines for Drinking Water and Water Reuse* provided information on log<sub>10</sub> inactivation of viruses and FIB. They indicated that water is essentially “pathogen-free” if a 5-log<sub>10</sub> poliovirus reduction and a 7-day median total coliform density of 2.2 MPN per 100 mL is achieved. When media filtration is employed, effluent quality can vary, and particulate matter may shield pathogens from UV light to various degrees. In these cases, a reduction equivalent dose of 100 mJ (millijoules) per square centimeter (cm<sup>2</sup>) is typically adequate to inactivate total coliform to less than 2.2 MPN per 100 mL. The report also indicated a 5-log<sub>10</sub> reduction of poliovirus can be achieved with a UV dose of 50 mJ/cm<sup>2</sup> based on laboratory studies, however the 100 mJ per cm<sup>2</sup> dose is recommended to account for effluent variability.

When using membrane filtration or ultrafiltration prior to UV treatment, the impact of particles is normally eliminated. In this situation, the *Ultraviolet Disinfection Guidelines for Drinking Water and Water Reuse* noted that a 5-log<sub>10</sub> reduction in poliovirus can be achieved with a UV dose of 50 mJ/cm<sup>2</sup>, and a design UV dose of 80 mJ per cm<sup>2</sup> is suggested to account for variability in the

effluent quality for membrane filtration or ultrafiltration. When using reverse osmosis for filtration, a reduction of at least  $2\log_{10}$  for viruses can be achieved through the reverse osmosis process, and the additional  $3\log_{10}$  reduction required for poliovirus can be achieved with a UV dose of about 30 mJ per  $\text{cm}^2$ . Therefore, to account for variability in the effluent quality for reverse osmosis the design UV dose of 50 mJ per  $\text{cm}^2$  is recommended (NWRI-WRF, 2012).

UV disinfection efficiency of secondary effluent is influenced by hydraulic properties of the reactor and wastewater characteristics, such as initial density of microbes, UV absorbance, and the concentration and characteristics of suspended solids (Koivunen, 2007; NWRI-WRF, 2012). For example, organic humic acid floc particles shielded viral surrogates (F-specific RNA coliphage MS2 and somatic coliphage T4) from UV light to a greater degree than inorganic kaolin clay floc particles of similar size (diameters  $<2$  mm) (Templeton et al., 2005). However, humic acid floc particles also caused a greater reduction in  $\log_{10}$  inactivation virus than larger activated sludge particles, which suggests that particulate chemical composition (e.g., UV absorbing content) and size may be important factors in the survival of particle-associated viruses during UV disinfection (Templeton et al., 2005). Because the study did not include human viruses, more data are needed to know whether these results extend to human viruses. Table 21 presents the estimated rate constants from a study on UV inactivation of F-specific RNA coliphage MS2 (NWRI-WRF 2012).

**Table 21. UVC inactivation of F-specific RNA coliphage MS2.**

Dose (mJ/ $\text{cm}^2$ )	Surviving density (PFU/mL)	Log survival (log PFU/mL)	Log <sub>10</sub> inactivation (log PFU/mL)
0	$1.00 \times 10^7$	7.0	0.0
20	$1.12 \times 10^6$	6.05	0.95
40	$6.76 \times 10^4$	4.83	2.17
60	$1.95 \times 10^4$	4.29	2.71
80	$4.37 \times 10^3$	3.64	3.36
100	$1.20 \times 10^3$	3.08	3.92
120	$7.08 \times 10^1$	1.85	5.15
140	$1.48 \times 10^1$	1.17	5.83

Source: NWRI-WRF (2012)

Different types of somatic coliphages have different levels of resistance to UV light. For example, Lee and Sobsey (2011) estimated the UV inactivation of five types of somatic coliphages (T1, T4, T7  $\Phi$ X174,  $\lambda$ ) representing the four families (*Microviridae* ( $\Phi$ X174), *Myoviridae* (T4), *Podoviridae* (T7) and *Siphoviridae* ( $\lambda$ , T1)) in laboratory tests using both reagent-grade water and surface water. Using regression analysis, the authors predicted the UV doses (mJ per  $\text{cm}^2$ ) for a  $4\log_{10}$  inactivation of each of the somatic coliphages to be (in order of most to least resistant): 24 for  $\lambda$ , 18 for  $\Phi$ X174, 12 for T7, 11 for T1, and 4 for T4. Note that these doses are lower than what is recommended for treatment plants. Based on these results, the authors concluded that different somatic coliphage families can have very different inactivation rates and that  $\Phi$ X174 and  $\lambda$  are the most resistant to UV radiation. In addition, different wavelengths have different efficacy at coliphage attenuation. For example, GI F-specific RNA coliphage MS2 was three times more sensitive to wavelengths near 214-nm compared to the 254-nm output of low-pressure lamps in simulated drinking water (Mamane-Gravetz et al.,

2005). There is also evidence that laboratory propagated F-specific RNA coliphage MS2 is inactivated by UV at a rate that is twice that of indigenous F-specific coliphages (Oppenheimer et al., 1993). This highlights the importance of data evaluating indigenous coliphages.

Nuanualsuwan et al. (2002) evaluated coliphages and other virus inactivation in a phosphate-buffered solution and UV light treatment. The UV dose ( $\text{mJ/cm}^2$ ) required for  $1-\log_{10}$  inactivation was 47.85 for FCV, 36.50 for hepatitis A virus, 24.10 for poliovirus type 1, 23.04 for GI F-specific RNA coliphage MS2, and 15.48 for somatic coliphage  $\Phi X 174$  (Nuanualsuwan et al., 2002). The coliphages were slightly more sensitive to UV compared to the human viruses.

In contrast, other studies have found that coliphages are more resistant to UV than human viruses. For example, Wiedenmann et al. (1993) found that in a sodium chloride solution, to achieve  $4-\log_{10}$  inactivation, a three-times higher UV dose was required for GI F-specific RNA coliphage MS2 compared to hepatitis A virus. Havelaar (1987) found that, in secondary effluent, F-specific coliphages are more resistant to UV treatment than coxsackievirus, rotavirus, and poliovirus. Similarly, Tree et al. (2005) found that UV reduction of F-specific RNA coliphage MS2 was less than that of poliovirus and FCV (a surrogate for NoV) in seeded, sterilized secondary effluent. To achieve a  $4-\log_{10}$  reduction, doses ( $\text{mJ per cm}^2$ ) of 62.5 for GI F-specific RNA coliphage MS2, 27.51 for poliovirus, and 19.04 for FCV were required. In bench-scale experiments Wilson et al. (1992) found that GI F-specific coliphage MS2 was 1.9 times more resistant to UV irradiation than the viruses, rotavirus, poliovirus, and hepatitis A.

Human adenoviruses are more resistant to UV light than other waterborne (enteric) viruses with single and ds RNA genomes. The human adenovirus genome is comprised of dsDNA, which affords the virus the ability to use host cell repair enzymes to repair damage in the DNA caused by UV light (Hijnen et al., 2006). In contrast, viral genomes that are single stranded DNA cannot be repaired in host cells because there is no second strand to serve as a template for replication of the nucleic acid. Viral genomes made of RNA are not repaired efficiently because mammalian hosts do not have sufficient repair mechanisms for RNA (Eischeid et al., 2011). Thompson et al. (2003) conducted a pilot-scale study to examine the effects of UV disinfection on viruses in wastewater. In seeded tertiary treated wastewater,  $4-\log_{10}$  inactivation of poliovirus type 1 required 35  $\text{mJ per cm}^2$ , GI F-specific RNA coliphage MS2 required 100  $\text{mJ per cm}^2$ , and human adenovirus (types 15 and 2) required 170  $\text{mJ per cm}^2$ . In a buffered demand-free water  $4-\log_{10}$  inactivation of FCV required 36  $\text{mJ per cm}^2$ , GI F-specific RNA coliphage MS2 required 119  $\text{mJ per cm}^2$ , and human adenovirus-40 would have required 226  $\text{mJ per cm}^2$  (extrapolated value,  $4-\log_{10}$  reduction was not achieved) (Thurston-Enriquez et al., 2003).

### 6.5.5 UVA and UVB

Solar radiation, which consists of UVA/UVB in addition to longer wavelengths, can also be used as a disinfection method. Wastewater treatment ponds (see Section 6.3) can be used to treat sewage or to further treat secondary effluent. For example, Davies-Colley et al. (2005) constructed an outdoor (exposed to solar radiation) advanced pond system to determine the solar

inactivation of somatic and F-specific RNA coliphages and *E. coli* in secondary effluent.<sup>7</sup> Tests were conducted in both the summer and winter to determine effects of seasonal variation in solar radiation intensity. Somatic coliphages showed a 2.2-log<sub>10</sub> reduction in summer and a 0.45-log<sub>10</sub> reduction in winter, whereas *E. coli* had a greater than 4-log<sub>10</sub> removal in both seasons.

Reductions of F-specific RNA coliphages were not reported due to low native coliphage densities in the influent. The authors found that solar radiation within the UVB range (represented by measurements at 311 nm) was responsible for somatic coliphage inactivation, whereas F-specific RNA coliphages were hypothesized to be inactivated by both UVA and UVB (Davies-Colley et al., 2005). These results are consistent with Sinton et al. (2002), who found that under a variety of conditions, F-specific RNA coliphages were inactivated by a wide range of wavelengths, whereas somatic coliphages were mainly inactivated by UVB wavelengths (less than 318 nm). Davies-Colley et al. (2005) concluded that the advanced pond system is efficient at removing coliphages mainly during the summer (or in the tropics), but not in the winter due to decreased intensity of solar radiation.

Gomila et al. (2008) compared inactivation of coliphages and enteric viruses in secondary effluent that was treated by UVC radiation (laminar flow through four banks of eight lamps of 87.5 Watts [W] each) or treated in a sunlit aerobic lagooning system with a residence time of 60 days. Inactivation of somatic coliphages, F-specific coliphages, and enteroviruses was greater in a lagooning system compared to UVC treatment, as shown in Table 22. Using either a UVC radiation step in a treatment facility or solar radiation in a lagooning system yielded a greater or equal inactivation of coliphages as compared to enteric viruses. Costán-Longares et al. (2008) investigated the log<sub>10</sub> inactivation of enteroviruses between secondary effluent and different types of tertiary treatment at WWTPs in Spain. The authors found a greater than 2-log<sub>10</sub> reduction in enteric virus density from secondary treatment after lagooning (Costán-Longares et al., 2008).

**Table 22. Log<sub>10</sub> reduction in coliphages and enteric viruses in secondary effluent after lagooning in sunlight or UVC treatment.**

Microorganism	Log <sub>10</sub> reduction (lagooning)	Log <sub>10</sub> reduction (UVC treatment)
Somatic coliphages	0.8	0.5
F-specific coliphages	1.6	0.5
Enteroviruses	0.7	0.5

Source: Gomila et al. (2008)

To compare the effects of UV wavelengths present in sunlight on both coliphages and enteric viruses, Lee and Ko (2013) exposed F-specific RNA coliphages MS2 and MNV, to UVA and UVB lamps. For all experiments, viruses were suspended in either saline solution or groundwater and viral densities were measured by EPA Method 1602 (MS2) or plaque assays (MNV). UVA irradiation resulted in a negligible effect on both F-specific RNA coliphages MS2 and MNV across the dose range tested (0 to 1500 mJ per cm<sup>2</sup>). In contrast, MNV was found to be significantly more susceptible to UVB than MS2; exposure to 376 mJ per cm<sup>2</sup> UVB resulted in a

<sup>7</sup> Sewage from the Ruakura Research Centre, near Hamilton, New Zealand fed into the advanced pond system. The F-specific coliphage level was consistently low in the Ruakura sewage, so primary treated sewage from Hamilton City was “spiked” into the pond system.

$4\text{-log}_{10}$  reduction of MNV whereas 909 mJ per  $\text{cm}^2$  UVB was required for the same reduction of F-specific RNA coliphage MS2 (Lee and Ko, 2013). Duizer et al. (2004) found that caliciviruses (enteric canine calicivirus no. 48 and respiratory FCV F9) were more susceptible to UVB than coliphages as <50 mJ per  $\text{cm}^2$  UVB resulted in a  $4\text{-log}_{10}$  reduction of both viruses (as determined by cell culture) when suspended in buffer.

## 7. Conclusions

This review provides background information relevant to the use of coliphages, specifically somatic and F-specific coliphages, as indicators of fecal contamination. The following is a summary of the major conclusions of this review.

**Methods.** Coliphage enumeration methods are adequate for water quality monitoring. EPA Method 1601 may be the most useful. Rapid methods are possible and MST methods for coliphage are under development. The ability to measure both somatic and F-specific coliphages on a single host may be useful, but needs validation (Rose et al., 2004; Guzmán et al., 2008). EPA is currently evaluating a membrane filtration culture method and may also evaluate an ultrafiltration culture method for use in coliphage enumeration. The intralaboratory (single laboratory) method validation study is underway.

**Epidemiological studies.** This review summarizes eight epidemiological studies that evaluated the relationship of coliphages and gastrointestinal illness from exposure to recreational water. Five of the eight studies found a statistically significant relationship between F-specific coliphages and illness levels (Lee et al., 1997; Colford et al., 2005, 2007; Wiedenmann et al., 2006; Wade et al., 2010; Griffith et al., personal communication, 2015). One of the studies found a statistically significant increase in RR of GE in bathers when somatic coliphage levels were above the NOAEL of 10 PFU per 100 mL (Wiedenmann et al., 2006).

**Occurrence in the Environment.** Some studies have reported an association between the presence of coliphages and human viruses, while other studies have found no association between their presence in environmental waters. The results are strongly influenced by the environments in which the studies are conducted. For example, an association between indicators and pathogens has more often been reported for brackish and saline water than for freshwater. There is evidence that coliphage and F-specific coliphage densities are more strongly associated with pathogens than other traditional indicators (*E. coli*, enterococci, and fecal coliforms).

**Environmental Fate.** Coliphages might be reasonable surrogates for enteric viruses in the environment. Human viruses and coliphages both decay faster at temperatures above 50°C compared to lower temperatures. Human viruses and coliphages both decay faster in sunlight than in the dark and are most stable near neutral pH (~6 to 9), but can also survive in lower pH environments (i.e., in the gastrointestinal tract of warm-blooded animals). The effect of salinity is equivocal and some studies have shown increased and others decreased inactivation in higher salinity waters. In fresh, treated, septic, and salt water, predation and environmental factors (e.g., temperature, sunlight, pH) have been shown to increase degradation of both coliphages and enteric viruses. Organic and inorganic matter affect inactivation—both have been shown to increase or decrease decay rates, depending on the virus and the nature of the composition of the organic or inorganic matter. There are synergistic or antagonistic interactions between all these environmental stressors.

**Wastewater treatment.** For primary and secondary treatment, the removal efficiencies of FIB, F-specific coliphages, somatic coliphages, and enteric viruses are not substantially different. Disinfection is the key step in wastewater treatment for microbial inactivation. Although

disinfection efficacies vary depending on the details of the treatment process, and the characteristics of the incoming water at each step, some general conclusions are possible. UVC and ozone are most effective at virus inactivation, followed by free chlorine. Combined chlorine is not as effective at virus inactivation as the other disinfection treatments. For free chlorine (chlorination of nitrified effluents), there are insufficient data to draw conclusions about the relative inactivation efficiencies of FIB, indigenous F-specific coliphages, indigenous somatic coliphages, and enteric viruses. For combined chlorine (non-breakpoint chlorination of un-nitrified effluents), F-specific coliphages and enteric viruses are more resistant to inactivation than FIB. In laboratory studies of UVC disinfection, coliphages and enteric viruses are generally more resistant to inactivation compared to FIB. However, the inactivation rates of individual strains of F-specific and somatic coliphages, as well as enteric viruses, are variable. For example, adenovirus is highly resistant to UVC. With the exception of ozone, F-specific and somatic coliphages overall are likely to be more conservative indicators than FIB in water treated by most disinfectants.

**Overall.** Table 23 compares coliphage attributes against the currently recommended indicators of fecal contamination, *E. coli* and enterococci. Each indicator/method combination is summarized and compared against indicator attributes described in Section 1.3. While some of the same limitations exist, coliphages are likely a better indicator of viruses in fecal contamination than the current FIB (i.e., enterococci and *E. coli*).

**Table 23. Attributes of fecal contamination indicators.**

Indicator Attribute	Enterococci (e.g. EPA Method 1600)	E. coli (e.g. EPA Method 1603)	Coliphages (e.g. EPA Method 1602)
Intestinal microflora of warm-blooded animals	Yes	Yes	Yes
Present when pathogens are present and absent in uncontaminated samples	Present when fecal pathogens are present, but may also be present in nonfecally contaminated ambient water.  Not indicative of viruses in WWTP effluent.	Present when fecal pathogens are present, but may also be present in nonfecally contaminated ambient water.  Not indicative of viruses in WWTP effluent.	Present when fecal pathogens are present, but is likely absent in nonfecally contaminated ambient water.  Better surrogate for viruses than enterococci or <i>E. coli</i> in WWTP effluent.
Present in greater numbers than the pathogen (in this case, human viruses)	Depends on source <sup>a</sup>	Depends on source <sup>a</sup>	In most cases
Equally resistant as pathogens (in this case viruses) to environmental factors	Not as resistant as viruses (except for ozone).	Not as resistant as viruses (except for ozone).	Under most conditions
Equally resistant as pathogens (in this case viruses) to disinfection in water and WWTPs	Not as resistant as viruses (except for ozone).	Not as resistant as viruses (except for ozone).	Under most conditions. However, adenovirus is more resistant than coliphages and other enteric viruses to UV inactivation.
Should not multiply in the environment	Can multiply in the environment	Can multiply in the environment	Not likely enough to affect criteria levels
Detectable by means of easy, rapid, and inexpensive methods	Yes, but need EPA Method 1611 for rapid enumeration. Other easy and rapid methods are available.	Yes, but EPA method is not considered rapid (requires overnight incubation). Other easy and rapid methods are available.	Yes, but Method 1601 needs validation for quantification. Other easy and rapid methods are available.
Indicator organism should be nonpathogenic	Generally nonpathogenic <sup>b</sup>	Generally nonpathogenic. <sup>c</sup>	Nonpathogenic
Demonstrated association with illness from epidemiological studies	Yes	Yes	Yes
Specific to a fecal source or identifiable as to source of origin	Not EPA Method 1600, but MST methods being developed.	Not EPA Method 1603, but MST methods being developed.	Not EPA Method 1602, but MST methods being developed.

<sup>a</sup> In raw sewage FIB are present in greater numbers than pathogens. Viruses are less vulnerable to treatment processes than bacteria, so could survive treatment in greater numbers than bacteria.

<sup>b</sup> Enterococci can be pathogenic or antibiotic resistant in some settings, like hospitals, but generally not in ambient water.

<sup>c</sup> Enterohemorrhagic *E. coli*, specifically O157:H7, grows poorly at 44°C and is often negative for beta-glucuronidase, so is not detected by Method 1603 (Degnan and Standridge, 2006). Other pathogenic strains could be detected by EPA Method 1603.

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## **APPENDIX A: Literature Search Strategy and Summary of Literature Search Results**

The literature search strategy consisted of a number of combined approaches. The following ‘synopsis of information’ and search terms were used to search online databases, including PubMed. To supplement these searches, individual authors used free search engines on the internet to find articles pertaining to specific information needed. The titles of literature cited in specific reports, books, review articles, and conference proceedings were evaluated for relevance.

### **Synopsis of information gathered during the 2012 literature search:**

- Evaluate the use of bacteriophage as indicators of fecal contamination or wastewater treatment efficacy
- Determine the sources and persistence of bacteriophage in the environment
- Evaluate the correlation between bacteriophage occurrence and pathogens and traditional fecal indicator bacteria (FIB) in wastewater
- Evaluate properties that affect fate and transport of viruses and bacteriophage
- Describe the different methods used for detection and analysis of bacteriophage concentrations, particularly in recreational waters
- Evaluate the environmental factors (e.g., organics, temperature, pH, UV/sunlight, predation, salinity, porosity, etc.) affecting viral and coliphage degradation
- Compare degradation in WWTP for bacteriophage and enteric viruses (i.e., primary, secondary, and tertiary disinfection)

### **Initial Literature Search Strategy Conducted by Professional Librarian**

Database: PubMed

Dates: 1985-present (Search conducted on July 5, 2012)

Language: No restrictions

Retrieve: Titles and year

Results Format: Microsoft Word; EndNote

Interested in international and domestic journals and government reports.

#### Search terms:

Set 1: bacteriophage OR coliphage

AND

Set 2: Water OR illness OR health OR risk

### **Search Results from PubMed**

The PubMed search resulted in approximately 2,400 records after removing duplicates. These titles were reviewed for relevance based on the outline for the literature review and the synopsis above. From the database of titles, 391 articles were sorted as “yes” and 81 were sorted as “maybe.” Because this number of titles was still large, 125 “top” articles were selected. The 125 articles were retrieved by the EPA librarian and sent to the contractor (ICF International). The

125 articles were the starting place for the literature review. Additional targeted literature searches were required to obtain more complete information on specific topics.

## **HECD Resources**

HECD has developed a robust library of references on waterborne pathogens. These resources were included in the resources for this project. Primary authors had access to the HECD library of PDFs.

### **Supplemental Searches by Primary Authors**

#### **Primary Author 1:**

Search Engine: Google Scholar (<http://scholar.google.com/>)

Search terms: Number of Records Considered:

Bacteriophage AND temperature	20
Bacteriophage AND pH	20
Bacteriophage AND sunlight	20
Bacteriophage AND UVA	10
Bacteriophage AND UVB	10
Bacteriophage AND organic matter	20
Bacteriophage AND sediment	20
Bacteriophage AND predation	20
Bacteriophage AND degradation	20
Bacteriophage AND biofilms	15
Bacteriophage AND morphology AND survival	25
Enterovirus AND temperature	20
Enterovirus AND pH	20
Enterovirus AND sunlight	20
Enterovirus AND UVA	10
Enterovirus AND UVB	10
Enterovirus AND organic matter	20
Enterovirus AND sediment	20
Enterovirus AND predation	20
Enterovirus AND degradation	20
Enterovirus AND biofilms	15

#### **Primary Author 2:**

Date	Search Engine	Search Terms	# of Titles Reviewed	# of Articles retrieved
9-20-12	PubMed	reviews for fecal source tracking	20	2
9-20-12	PubMed	reviews for coliphages as viral indicators	20	2
9-20-12	PubMed	reviews for microbial indicators and pathogens	20	2

9-20-12	Google	coliphage environment fecal contamination	20	2
9-20-12	Google	coliphage indicator environment	20	2
9-25-12	PubMed	Policies and practices for beach monitoring	40	2
9-25-12	Google Scholar	coliphage detection in fresh water	20	2
9-25-12	Google Scholar	coliphage presence in environment review	20	2
9-25-12	Google Scholar	alternative indicators of fecal pollution	20	2
9-26-12	Google Scholar	Bacteriophage in the environment	20	4
9-27-12	Google Scholar	environmental detection coliphage	40	4
9-27-12	Google Scholar	coliphages environment	40	4
9-27-12	Google Scholar	fecal source tracking	40	4
10-1-12	PubMed	coliphage source tracking	30	2

The document was undergoing internal EPA review and external peer review throughout 2013-2014. Additional supplemental searches were conducted to address EPA internal and external peer reviewer comments. Ultimately, over 2,500 titles were reviewed for inclusion in the literature review. There are 342 citations in the final document.

The Quality Assurance Project Plan includes Information Decision Criteria for selection of cited references. The relevant Information Decision Criteria for this project from the Quality Assurance Project Plan includes:

1. More recent references were preferred over older references, unless the older reference was particularly notable, important, or widely cited.
2. Accessibility – References needed to be obtained within project time and budget constraints.
3. English language was required.
4. Scientific, peer-reviewed publications were preferred, along with others references that presented a balanced and objective tone. Government publications such as federal regulations, state regulations, standards, permits, guidance documents, and other government publications were acceptable.
5. The reference related to the scope of the information sought. In this case a document outline was available.
6. Geographic relevance – Data collected in the U.S was preferred, but data from other countries was also relevant.

7. For a given topic, a literature review citation may have been used in lieu of listing numerous primary research articles cited in the literature review. This was done when the additional detail provided by the primary citations was not needed.
8. If a particular point was already in the document and a citation was already provided, additional citations backing up this same point were not added. Redundant articles were not necessarily cited.
9. Information provided through personal communication (phone, email) was used only when another more widely obtainable source was not available for the same information. Information obtained through personal communication needed to be highly relevant.
10. Books citations were acceptable, but sources that could be more easily obtained were preferred. Book citations were preferred when the book is an important resource in the field.
11. Newspaper articles were not searched or cited.
12. Websites were not cited as primary sources of information. URLs are provided for some of the references.
13. Information and references that presented alternative points of view or conclusions to the mainstream view were given equal considerations to consensus or majority viewpoints. Both alternative and majority viewpoints and conclusions had to provide justification based on facts, employ accepted methodologies, and be grounded in the scientific method.

## APPENDIX B: Coliphage and NoV Densities during Wastewater Treatment

Table A illustrates how coliphage reduction compares to NoV reduction during wastewater treatment. To be included in Table A, the study has to include quantitative norovirus data, quantitative coliphage data, and water samples from raw sewage or effluent. The studies are listed in chronological order by year of publication.

**Table A. Coliphage and NoV densities during wastewater treatment.**

Organism	Influent (raw)	Effluent	Log reduction ( $\log_{10}$ )	Treatment	Location	Reference
NoV (RT-qPCR)	Mean $2 \times 10^5$ PCR detectable unit per L	NR <sup>a</sup>	1.8	Non-disinfected activated sludge secondary treatment (preceded by primary and phosphorus removal); No design/operational information provided to understand secondary treatment or performance.	Netherlands	Lodder and de Roda Husman, 2005
F-specific bacteriophages (ISO 10705-1)	Mean $10^6$ PFU per L	NR <sup>a</sup>	1.6			
Somatic coliphages (ISO 10705-2)	Mean $10^6$ PFU per L	NR <sup>a</sup>	1.1			
NoV GI (RT-qPCR)	0.17–260 copies per mL (range)	NR	$2.27 \pm 0.67$	Activated sludge secondary treatment and chlorination; No design/operational information provided to understand secondary treatment or performance;	Tokyo, Japan	Haramoto et al., 2006
NoV GII (RT-qPCR)	2.4–1900 copies per mL (range)	NR	$3.69 \pm 1.21$			
F-specific coliphages (ISO 10705-1)	NR	NR	$2.81 \pm 0.77$			
NoV (RT-qPCR)	Mean $3.29 \pm 0.26$ $(<2.9\text{--}3.65) \log_{10}$ MPN PCR units per L	NR	$0.89 \pm 0.26$ (0.39–1.3)	Non-disinfected chemical precipitation and activated sludge (1 plant filtered the effluent and 1 plant provided additional nitrogen removal);	Sweden	Ottoson et al., 2006
F-specific coliphages (ISO 10705-1)	NR	NR	$1.73 \pm 0.59$ (0.74–2.63)			

Organism	Influent (raw)	Effluent	Log reduction ( $\log_{10}$ )	Treatment	Location	Reference
Somatic coliphages (ISO 10705-2)	NR	NR	1.04 ± 0.32 (0.61-1.86)	No design/operational information provided to understand secondary treatment or performance.		
Somatic coliphages (ISO 10705-2)	Mean $2.9 \times 10^6$ ( $\pm 2 \times 10^6$ ) PFU per mL	Mean $2.5 \times 10^4$ ( $\pm 2.9 \times 10^4$ ) PFU per mL	Mean 2.16 ± 0.42	Activated sludge secondary treatment and chlorination; No design/operational information provided to understand secondary treatment or performance; no information if this is free chlorine, combined chlorine, ammonia concentration.	Italy	Carducci et al., 2009
Somatic coliphages (EPA Method 1602)	Mean $1.8 \times 10^5$ PFU per 100 mL	Mean $10^2$ PFU per 100 mL	2.4	Non-disinfected activated sludge secondary treatment; No design/operational information provided to understand secondary treatment or performance.	Singapore	Aw and Gin, 2010
F-specific coliphages (EPA Method 1602)	Mean $4.3 \times 10^4$ PFU per mL	Mean $10^2$ PFU per 100 mL	2.4			
NoV GI (RT-qPCR)	Mean $3.2 \times 10^5$ copies per 100 mL	Mean $7.1 \times 10^3$ copies per 100 mL	~2 <sup>b</sup>			
NoV GII (RT-qPCR)	Mean $2.3 \times 10^5$ copies per 100 mL	Mean $5.2 \times 10^3$ copies per 100 mL	~2 <sup>b</sup>			
Somatic coliphages (EPA Method 1602)	Range $9.1 \times 10^4$ to $4.5 \times 10^5$ PFU per 100 mL	Range 3 to 63 PFU per 100 mL	NR	Conventional secondary treatment with chlorine disinfection. No design/operational information provided to understand secondary treatment or performance.	Ohio, United States	Francy et al. 2011
F-specific coliphages (EPA Method 1602)	Range $3.1 \times 10^5$ to $2.2 \times 10^7$ PFU per 100 mL	Range <1 to 37 PFU per 100 mL	NR			
NoV GI (qRT-PCR)	Range 230 °C to $2.2 \times 10^3$ GC per L	Range <2.7 to 1.8 °C GC per L	NR			

Organism	Influent (raw)	Effluent	Log reduction ( $\log_{10}$ )	Treatment	Location	Reference
Somatic coliphages (EPA Method 1602)	Range $2.4 \times 10^4$ to $3.0 \times 10^6$ PFU per 100 mL	All <1 PFU per 100 mL	NR	Conventional tertiary treatment (sand filtration) with UV disinfection. No design/operational information provided to understand secondary treatment or performance.		
F-specific coliphages (EPA Method 1602)	Range $3.8 \times 10^4$ to $2.1 \times 10^5$ PFU per 100 mL	All <1 PFU per 100 mL	NR			
NoV GI (qRT-PCR)	Range <560 to $<8.3 \times 10^3$ GC per L	Range <36 to <67 GC per L	NR			
Somatic coliphages (EPA Method 1602)	Range $2.6 \times 10^4$ to $2.2 \times 10^6$ PFU per 100 mL	Range <1 to $1.1 \times 10^3$ PFU per 100 mL	NR	Membrane bioreactor (Kubota® Membrane Systems by Ovivo MBR)		
F-specific coliphages (EPA Method 1602)	Range $3.8 \times 10^4$ to $1.9 \times 10^6$ PFU per 100 mL	Range <1 to 19 PFU per 100 mL	NR	with UV disinfection. No design/operational information provided to understand secondary treatment or performance.		
NoV GI (qRT-PCR)	Range $49 \times 10^4$ GC per L	Range <1.5 to <130 GC per L	NR			
Somatic coliphages (EPA Method 1602)	Range $2.6 \times 10^4$ to $2.2 \times 10^6$ PFU per 100 mL	Range <1 to 8 PFU per 100 mL	NR	Two medium-sized Kubota™ (Osaka, Japan) system microfiltration membrane bioreactors	Ohio, United States	Francy et al., 2012
F-specific coliphages (EPA Method 1602)	Range $3.8 \times 10^4$ to $1.9 \times 10^6$ PFU per 100 mL	Range <1 to 2 PFU per 100 mL	NR	with UV disinfection. No design/operational information provided to understand secondary treatment or performance.		
NoV (qPCR, qRT-PCR)	Range $49 \times 10^4$ GC per L	Range <1.5 to <53 GC per L	NR			
Somatic coliphages (EPA Method 1602)	Range $2.4 \times 10^4$ to $3.0 \times 10^6$ PFU per 100 mL	<1 PFU per 100 mL	NR	One small-sized conventional secondary plant with tertiary treatment and UV disinfection. No design/operational information provided to		
F-specific coliphages (EPA Method 1602)	Range $1.1 \times 10^4$ to $2.1 \times 10^5$ PFU per 100 mL	<1 PFU per 100 mL	NR			

Organism	Influent (raw)	Effluent	Log reduction ( $\log_{10}$ )	Treatment	Location	Reference
NoV (qPCR, qRT-PCR)	Range $<5.6 \times 10^2$ to $<8.3 \times 10^3$ GC per L	Range <36 to <67 GC per L	NR	understand secondary treatment or performance.		
Somatic coliphages (EPA Method 1602)	Range $9.1 \times 10^4$ to $4.5 \times 10^5$ PFU per 100 mL	Range 3 to 63 PFU per 100 mL	NR	One medium-sized conventional secondary plant with chlorine disinfection. No design/operational information provided to understand secondary treatment or performance.		
F-specific coliphages (EPA Method 1602)	Range $3.1 \times 10^5$ to $2.2 \times 10^7$ PFU per 100 mL	Range <1 to 37 PFU per 100 mL	NR			
NoV (qPCR, qRT-PCR)	Range $2.3 \times 10^{2c}$ to $1.5 \times 10^3$ GC per L	Range <1.8 <sup>c</sup> to <2.7 GC per L	NR			
F-specific RNA coliphages (double agar layer)	NR	Means $5 \times 10^3$ to $5 \times 10^5$ PFU per L	NR	Secondary treated wastewater (2 WWTPs) <sup>d</sup>	Australia	Keegan et al., 2012
Somatic coliphages (double agar layer)	NR	Means $9 \times 10^4$ to $1.67 \times 10^5$ PFU per L	NR			
NoV (RT-qPCR)	NR	Means ND to $2.7 \times 10^5$ genomes per L	NR	Secondary treated wastewater (5 WWTPs)		
NoV GI (RT-qPCR)	Mean $3.32 \pm 0.64$ (range 2.05–4.76) log <sub>10</sub> density	Mean $2.53 \pm 0.57$ (range 1.26–4.06) log <sub>10</sub> density	$0.80 \pm 0.49$	Non-disinfected activated sludge secondary treatment;	Ireland	Flannery et al., 2012
NoV GII (RT-qPCR)	Mean $3.55 \pm 0.89$ (range 1.81–5.34) log <sub>10</sub> density	Mean $2.63 \pm 0.71$ (range 1.51–4.08) log <sub>10</sub> density	$0.92 \pm 0.76$	No design/operational information provided to understand secondary treatment or performance.		
F-specific coliphages (ISO 10705-1)	Mean $5.54 \pm 0.51$ (range 3.87–6.82) log <sub>10</sub> density	Mean $3.41 \pm 0.77$ (range 2.00–5.84) log <sub>10</sub> density	$2.13 \pm 0.76$			
Somatic coliphages (ISO 10705-2)	Mean $7.10 \pm 0.40$ -log <sub>10</sub> PFU per L	Mean $4.99 \pm 0.53$ -log <sub>10</sub> PFU per L	$2.11 \pm 0.40$	Rainy days: Activated sludge, chlorination. No design/operational	Italy	Carducci and Verani, 2013

Organism	Influent (raw)	Effluent	Log reduction ( $\log_{10}$ )	Treatment	Location	Reference
NoV (RT-qPCR)	Mean $5.83 \pm 2.87$ - $\log_{10}$ GC per L	Mean $5.80 \pm 2.75$ - $\log_{10}$ GC per L	$0.02 \pm 0.61$	information provided to understand secondary treatment or performance.		
Somatic coliphages (ISO 10705-2)	Mean $7.22 \pm 0.40$ - $\log_{10}$ PFU per L	Mean $5.00 \pm 0.56$ - $\log_{10}$ PFU per L	$2.21 \pm 0.46$	Dry days: Activated sludge, chlorination. No design/operational		
NoV (RT-qPCR)	Mean $5.92 \pm 2.86$ - $\log_{10}$ GC per L	Mean $6.04 \pm 2.94$ - $\log_{10}$ GC per L	$-0.11 \pm 0.34$	information provided to understand secondary treatment or performance.		
F-specific RNA coliphages (ISO 10705-1)	Mean $5.26$ - $\log_{10}$ PFU per 100 mL	Mean $2.96$ - $\log_{10}$ PFU per 100 mL	NR	Screening and grit removal, phosphate removal through ferric sulfate, secondary treatment, UV disinfection. No	United States	Flannery et al. 2013
F-specific RNA coliphage (RT-qPCR)	Mean $5.11$ - $\log_{10}$ GC per 100 mL	Mean $4.57$ - $\log_{10}$ GC per 100 mL	NR			
NoV GII (RT-qPCR)	Mean $3.87$ - $\log_{10}$ GC per 100 mL	Mean $3.61$ - $\log_{10}$ GC per 100 mL	NR	design/operational information provided to understand secondary treatment or performance.		
F-specific RNA coliphages (ISO 10705-1)	Range $5.9 \times 10^4$ to $7.5 \times 10^5$ PFU per L	Range $1.5 \times 10^3$ to $2.5 \times 10^4$ PFU per L	NR	Primary sedimentation. Influent data includes samples taken when treatment was interrupted.	Fjellfoten, Norway	Grøndahl-Rosado et al. 2014
NoV GI (qPCR)	Range $2.9 \times 10^3$ to $1.4 \times 10^6$ GC per L	Range $5.6 \times 10^3$ to $9.2 \times 10^4$ GC per L	NR	No design/operational information provided to understand secondary treatment or performance.		
NoV GII (qPCR)	Range $6.0 \times 10^4$ to $1.4 \times 10^7$ GC per L	Range ND to $2.0 \times 10^5$ GC per L	NR			
Somatic coliphages (ISO 10705-2.2)	Mean $4.9 \times 10^3$ PFU per mL	Range <0.01 to $2.5 \times 10^4$ PFU per mL	Range of Mean Reduction $0.6 \pm 0.6$ to $5.2 \pm 1.2$	Raw municipal post-screen wastewater influent and effluent from three pilot-scale sand filters with different filter material and grain size designs and one with a separate phosphorous removal unit. No design/operational	Kuopio, Finland	Kauppinen et al. 2014
F-specific coliphages (ISO 10705-1)	Mean $3.6 \times 10^3$ PFU per mL	Range <0.01 to $1.8 \times 10^4$ PFU per mL	Range of Mean Reduction $0.7 \pm 0.6$ to $5.3 \pm 1.3$			

Organism	Influent (raw)	Effluent	Log reduction ( $\log_{10}$ )	Treatment	Location	Reference
NoV GI (RT-PCR)	Mean 80 GC per mL	Range <0.5 to $1.5 \times 10^3$ GC per mL	Range of Mean Reduction $0.6 \pm 0.6$ to $2.2 \pm 0.8$		information provided to understand treatment or performance.	
NoV GII (RT-PCR)	Mean $2.3 \times 10^4$ GC per mL	Range <0.4 to $1.1 \times 10^4$ GC per mL	Range of Mean Reduction $0.5 \pm 0.2$ to $4.0 \pm 0.6$			

Density units are as reported in the cited reference.

NR = not reported (in some cases effluent or influent densities were not reported, but  $\log_{10}$  reduction was reported); Stm WG49 = *Salmonella enterica* serovar *Typhimurium* WG49 (host); ND = Not detected; GC = genome copies

<sup>a</sup> PFU reported in graphical format for influent, digester, high rate pond, algal settling pond, and maturation pond

<sup>b</sup> Estimated from figure

<sup>c</sup> Reported as estimated value extrapolated at the low end. PCR threshold cycles were past the upper limit of the standard curve.

<sup>d</sup> Information from Table 3.6 and 3.7 in Keegan et al. (2012). The Bolivar WWTP consists of primary treatment (screening, grit removal, sedimentation), activated sludge, lagoon (16 day retention), chlorination (or dissolved air flotation and chlorination); collected undisinfected samples from the lagoon influent (thus secondary effluent), lagoon effluent, and post dissolved air flotation prior to chlorination. Two Melbourne WWTPs were tested: MW1 – primary settling, ASP (not defined); MW2 – anaerobic digestion (not defined), ASP (not defined), and lagoon polishing (26 days). This study was done for a very specific focus related to water recycling.