Proceedings from the U.S. Environmental Protection Agency (EPA) Coliphage Experts Workshop March 1-2, 2016

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Disclaimer

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Acronyms

AS	activated sludge
AOR	adjusted odds ratio
AWQC	ambient water quality criteria
BAV	beach action value
C	Celsius
CA	California
CDC	Centers for Disease Control and Prevention
CFU	colony forming unit
cm	centimeters
CPE	cytopathic effects
CT	contact time
CWA	Clean Water Act
DNA	deoxyribonucleic acid
ds	double-stranded
EPA	Environmental Protection Agency
EU	European Union
FDA	Food and Drug Administration
FIB	fecal indicator bacteria
FIO	fecal indicator organism
FRNA	F-specific RNA coliphage
g	gram
GI	gastrointestinal
GM	geometric mean
GPD	gallons per day
HAB	harmful algal bloom
HECD	Health and Ecological Criteria Division
HRC	high rate clarification
ICR	Information Collection Rule
ISO	International Organization for Standardization
ISSC	Interstate Shellfish Sanitation Conference
IWA	International Water Association
L	liter
MCRT	mean cell resident time
mg	milligram
MGD	million gallons per day
mL	milliliter
mm	millimeter
MPN	most probable number
MS	male specific
MSC	male-specific coliphage (same as F-specific coliphage)
MST	microbial source tracking
m-TEC	membrane thermotolerant *E. coli* agar

MWRDGC	Metropolitan Water Reclamation District of Greater Chicago
NEEAR	National Epidemiological and Environmental Assessment of Recreational Water Study
NOAEL	no observed adverse effects level
NPDES	National Pollutant Discharge Elimination System
PCR	polymerase chain reaction
PFU	plaque forming unit
POTW	publicly owned treatment works
QMRA	quantitative microbial risk assessment
qPCR	quantitative polymerase chain reaction
RCT	randomized controlled trial
RNA	ribonucleic acid
RT-PCR	reverse transcriptase polymerase chain reaction
RWQC	recreational water quality criteria
SC	somatic coliphage
SCCWRP	Southern California Coastal Water Research Project
ss	single stranded
TMDL	total maximum daily load
U.S.	United States
UV	ultraviolet light
WERF	Water Environment Research Foundation
WRP	water reclamation plant
WWTP	wastewater treatment plant

Foreword

The goal of the *Coliphage Experts Workshop* was to obtain input on science questions related to coliphage from experts in the fields of environmental microbiology, microbial risk assessment, and environmental epidemiology to inform coliphage criteria development. The goal of the workshop was not to reach consensus, rather, it was designed to be a critical thinking and information gathering exercise. Therefore, the workshop proceedings below provide a record of the workshop presentations, discussions, and primary outcomes, but do not contain official Agency recommendations.

This document was peer reviewed in accordance with EPA's Peer Review Handbook (EPA/100/B-15/001).

Executive Summary

The United States (U.S.) Environmental Protection Agency (EPA) Office of Science and Technology convened a workshop, hereafter referred to as the *Coliphage Experts Workshop*, in March, 2016 where twelve invited technical experts in the fields of environmental microbiology, microbial risk assessment, and recreational water epidemiology met with Agency staff to engage on how best to protect public health from human viral pathogens that can be found in water that contains fecal contamination. The EPA is developing Clean Water Act (CWA) §304(a) Recreational Water Quality Criteria (RWQC) for coliphage, a viral indicator, to ensure public health is protected from water sources influenced by fecal contamination, particularly wastewater. In this Workshop EPA sought scientific insights in five topic areas: 1) the need for an enteric viral indicator; 2) coliphages as a predictor of gastrointestinal illness; 3) coliphages as an indicator of wastewater treatment performance; 4) evaluation of F-specific (also known as "male-specific") and somatic coliphages; and 5) systematic review of enteric viral densities. Participants were also asked to identify future research needs. These proceedings report the findings of the *Coliphage Experts Workshop,* as described below.

Topic 1 Need for Viral Indicator

EPA previously published a *Review of Coliphages as Possible Indicators of Fecal Contamination for Ambient Water Quality* (hereafter referred to as EPA's *Coliphage Literature Review*). The workshop participants commented on EPA's conclusion that the research literature supports that human enteric viruses are an important cause of illnesses associated with ambient recreational water exposures. Overall, it was noted that data are well described in the literature regarding viral illnesses and occurrence in recreational waters, viral-associated outbreaks, epidemiological studies, and quantitative microbial risk assessments (QMRA). The participants also identified important advantages and disadvantages when using coliphages for assessing viral fecal contamination in ambient waters compared to traditional fecal indicator bacteria (FIB).

Topic 2 Coliphages as a Predictor of Gastrointestinal Illness (GI)

Workshop participants commented on the strength of the association between coliphage and human health illness in epidemiological studies conducted in ambient recreational waters. The experts agreed that available evidence is suggestive that coliphage may be a useful indicator of GI illness, particularly at sites impacted by human fecal contamination. The participants also commented on specific characteristics that influence the association between coliphage and human health illness, principally the source and intensity of fecal contamination. The panel suggested that future epidemiological studies should include coliphages as a measured indicator and that investigators should collect larger water samples so that coliphages are more easily quantified. The participants also discussed whether specific conditions exist under which traditional FIB do not adequately protect public health. The participants noted that coliphage deserve further consideration, especially for situations with sporadic or predominately human-impacted fecal sources.

Topic 3 Coliphage as an Indicator of Wastewater Treatment Performance

Workshop participants also commented on EPA's *Coliphage Literature Review* conclusion that human pathogenic viruses can enter surface waters via wastewater treatment effluent. Experts stated that viruses can enter surface waters via wastewater treatment plant (WWTP) effluent, and noted that treatment configurations exist where viruses are reduced to levels below the sensitivity of the assays utilized. Episodic loading and periods where WWTPs exceed design flows are key causes of viral surface water contamination; these are influenced by wet weather, storm events, snow melt, and hydraulic influencers (inflow and infiltration).

Participants summarized the most important reasons coliphages might be useful indicators (or models) of the behavior of human enteric viruses in wastewater treatment and disinfection processes, and commented on EPA's *Coliphage Literature Review* conclusion that monitoring for coliphages would be more useful than enterococci and *Escherichia coli* (*E. coli*) in predicting removal of human viral pathogens during wastewater treatment.

Topic 4 Evaluation of F-specific and Somatic Coliphages

Workshop participants commented on important advantages and disadvantages of using the two types of coliphages as 1) predictors of human health illness in recreational waters and 2) indicators of wastewater treatment performance. Experts' opinions varied on whether somatic coliphages, F-specific coliphages, or both would be better for the various applications. Participants also discussed whether specific attributes of the two coliphage types or site conditions (e.g., fecal source) influence the usefulness of the indicator or would favor the use of one type of coliphage. Finally, the participants briefly discussed research conducted in other countries that are currently investigating or assessing the use of coliphages for various purposes. Specifically, academic research on the use of coliphages as indicators of water quality has been conducted in Singapore, Australia, Canada, Argentina, Columbia, Brazil, South Africa, Japan, South Korea, New Zealand, Tunisia, and the European Union (EU). The participants noted that regulatory authorities in different parts of the world, including Australia, are beginning to consider coliphages as indicators of water quality, noting they are traditionally used in the shellfish industry.

Topic 5 Systematic Literature Review of Viral Densities

EPA is planning to use a quantitative microbial risk-based approach to derive RWQC for coliphages. The risk methodology relies on densities of key viral pathogens and coliphages in raw wastewater. EPA conducted systematic literature reviews to understand and document these viral densities. The participants commented on the risk assessment approach, the information collected to date, and what additional data might be considered. Overall, there was support for how the analysis was structured and conducted. Individual experts provided search databases suggestions and considerations regarding the bootstrap and risk assessment approaches. Experts supported the study inclusion criteria EPA used and recommended that future data from the United States and other countries should be subject to the same inclusion criteria as the data currently in the analysis.

Future Research

Recommendations to address data gaps were also captured from the discussion during the two-day workshop. At the end of the workshop, participants were asked to classify the various research projects as long-term or short-term *and* high, medium, or low priority. Topic 6 lists all short and long-term research projects discussed.

Introduction

The U.S. EPA's Office of Science and Technology convened a workshop, *Coliphage Experts Workshop*, at the EPA Potomac Yard Office in Arlington, Virginia on March 1–2, 2016. Twelve invited technical experts in the fields of environmental microbiology, microbial risk assessment, and recreational water epidemiology met with Agency staff over two days.

Purpose of the Workshop

The purpose of the *Coliphage Experts Workshop* was to engage internationally recognized experts on how best to protect public health from viral contamination of water given currently available information. EPA organized the workshop into five topic areas, with 16 total charge questions. EPA views this workshop as part of an ongoing commitment to protect public health through enhancement of CWA 304(a) RWQC.

Specific goals of the workshop included:

- Obtain input on science questions from experts in the fields of environmental microbiology, microbial risk assessment, and environmental/recreational water epidemiology.
- Gather scientific insight to determine the best coliphage type (F-specific or somatic) for use in CWA 304(a) criteria.
- Define conditions where coliphages might be most useful for preventing illnesses and identifying impaired waters.
- Identify research needs that can be completed in the short-term (3 to 5 years).

Workshop Design

The workshop was designed to provide an opportunity to share and listen to ideas, not to reach consensus on any particular topic. All relevant discussion, including conflicting opinions, are included in this document. Experts provided individual views and were not asked for recommendations or agreement. Experts represented a spectrum of perspectives from academia, EPA Office of Research and Development, other federal agencies (Centers for Disease Control and Prevention [CDC] and the Food and Drug Administration [FDA]), and the wastewater industry.

Each expert participant was assigned the role of "Topic Lead" for one agenda topic. Prior to the workshop, Topic Leads were asked to prepare written responses to the charge questions for their topic. At the workshop, each Topic Lead provided a 15-minute oral summary of their responses to the charge questions. Two to four Topic Leads were assigned to each workshop topic. Following the oral presentations, the whole expert group discussed the topics and associated charge questions.

The remaining sections of this workshop proceedings document are organized parallel to the workshop agenda (Appendix A). For each topic, the charge questions are presented, followed by a topic summary, and highlights from the group discussion.

Peer reviewer comments were incorporated into these proceedings to provide additional expert views. Several more recently published studies were added, as suggested by peer reviewers.

Background

Welcome and Introductions

Elizabeth Behl, Director of the Health and Ecological Criteria Division (HECD) in EPA's Office of Water, provided opening remarks describing the purpose and importance of the workshop and welcoming and thanking the participants. She explained EPA's role in the development of RWQC is to provide national criteria that are scientifically defensible and protective of designated uses (e.g. primary contact recreational use) so that states may adopt the criteria into their Water Quality Standards to protect their waters under the CWA. States may also adopt other scientifically defensible criteria into their standards, which are final after approval by EPA. In 2012, EPA published RWQC that maintained the FIB water quality levels from the 1986 Ambient Water Quality Criteria (AWQC). Although the 2012 RWQC included supplemental tools, such as *Enterococcus* measured by quantitative polymerase chain reaction (qPCR) and the Beach Action Value (BAV), many stakeholders expressed a need for further tools for protection of recreational waters and to take advantage of the latest science. This workshop is a key milestone in the effort to address the need for viral indicators to enhance the protection of people recreating in those waters.

Dr. Sharon Nappier (HECD) presented a background perspective to help frame the workshop discussions.

The CWA establishes the basic structure for state water quality standards, including regulation of pollutant discharge into the waters of the United States. CWA 304(a) RWQC are recommendations intended to be used by states, territories, and authorized tribes adopting water quality standards to protect the designated use of primary contact recreation. RWQC are used for different purposes including:

1. Preventing illness by preventing fecal contamination and pathogens from entering surface waters through point source permits (National Pollutant Discharge Elimination System [NPDES] permits).
2. Identifying impaired waters through CWA 303(d) Listing and restoring impaired waters by developing Total Maximum Daily Loads (TMDLs).
3. Enabling states to identify potentially hazardous conditions to beachgoers by the issuance of beach notifications.

Figure 1 shows a conceptual model for recreational exposures of fecally associated pathogens. Dominant elements (or elements with the most available information for consideration in RWQC development) are captured by fully enclosed boxes. Elements with less information which are more difficult to quantify are captured in boxes with dashed lines. Fecally associated pathogens can enter surface waters via point and non-point sources. Humans can be exposed to pathogens via fresh and marine waters; sand exposure has also been linked to GI illness. The dominant exposure route is ingestion, though inhalation and dermal exposures can also occur. Receptors are both children and adults. The predominant endpoint is GI illness, but some pathogens are associated with other health endpoints.

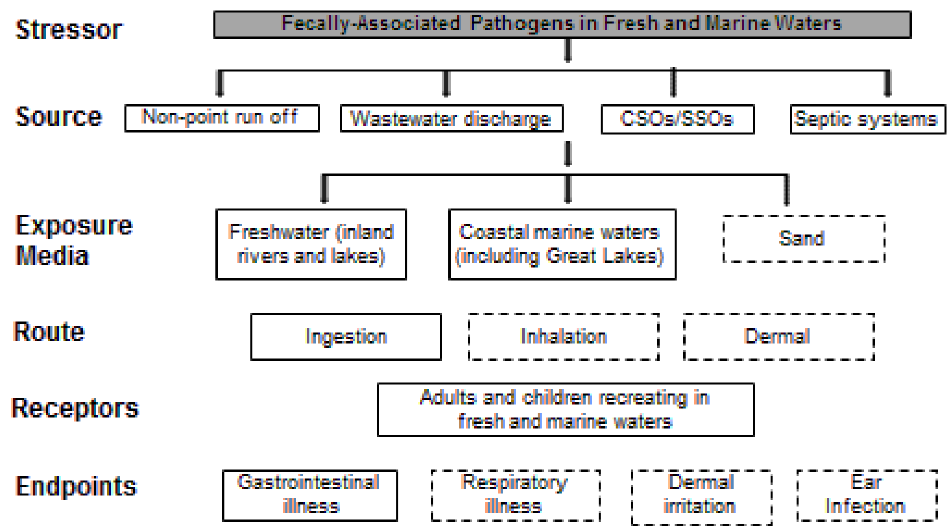

Figure 1. Conceptual model for recreational exposures

EPA's most recent RWQC, published in 2012, recommends two FIB: enterococci (for marine and freshwater) and *E. coli* (for freshwater). The magnitude, duration (30-day), and frequency are specified for both indicator types and for two different illness rates (32 and 36 National Epidemiological and Environmental Assessment of Recreational Water Study [NEEAR] GI illness per 1,000 recreators). The 2012 RWQC also include supplemental tools, such as a qPCR method for same-day notification and BAV for precautionary notification.

Application of the RWQC can both prevent illnesses and identify waters that need improved water quality. For example, the use of FIB has led to the targeting and control of bacterial pathogens in wastewater discharges. Historically, bacterial pathogens caused the most serious disease outbreaks (e.g., cholera and typhoid), and wastewater treatment improvements and discharge permits based on FIB effectively control such bacterial pathogens. More recently, quantitative microbial risk assessment (QMRA), epidemiology, and microbial water quality studies indicate that viruses may be a more significant cause of swimming-associated illnesses in human-impacted waters. For example, U.S. outbreak surveillance data collected by CDC points to noroviruses as being the leading viral pathogen responsible for untreated recreational water outbreaks (with noroviruses responsible for ~17% of untreated recreational outbreaks between 2003 and 2012 [http://www.cdc.gov/healthywater/surveillance/recreational/2011-2012-figures.html]). Current wastewater treatment processes, however, do not specifically target enteric virus removal/inactivation. Thus, viruses can enter surface waters from both treated and untreated human sources.

Cultural FIB are effective at predicting bacterial impairments of water quality, but epidemiological studies indicate they may not always predict all types of illnesses, such as those caused by viruses (e.g., norovirus and adenovirus). For example, several epidemiological studies suggest high illness rates occurring at EPA's recommended water quality levels (Marion et al., 2010; Lamparelli et al., 2015). Additionally, other epidemiological studies found statistically significant relationships between GI illness and the viral indicator coliphage (Lee et al., 1997; Colford et al., 2005, 2007; Wiedenmann et al., 2006; Wade et al., 2010; Griffith et al., 2016).

Additional advantages of using coliphage as an indicator of recreational water quality include: they are of fecal origin and thus highly concentrated in sewage; they are physically similar to human enteric viruses of concern (ribonucleic acid [RNA] coliphages are more like norovirus and somatic coliphages are more like adenoviruses); and they have similar persistence patterns to human enteric viruses of concern during treatment and in response to environmental conditions (e.g., no appreciable re-growth in ambient waters). In summary coliphages are useful models for fate and transport of human enteric viruses. Further, coliphages are non-pathogenic and are easy to measure compared to human pathogenic viruses, which have methodological constraints (e.g., length of time to obtain results after samples are taken and need to concentrate multiple liters of water). An additional advantage is that methods are available to easily measure culturable viruses, rather than only nucleic acid targets.

Coliphage has been recommended for use as a viral indicator by EPA and FDA. EPA's Ground Water Rule recommended coliphage to detect and quantify viruses in groundwater in addition to *E. coli* and enterococci. The Interstate Shellfish Sanitation Conference (ISSC) and FDA have used F-specific coliphage for shellfish bed opening decisions, after closure resulting from contamination from wastewater discharge (FDA, 2009). Also, the National Water Research Institute and the Water Research Foundation recommend use of viruses and viral indicators to measure disinfection of treated wastewater to support water reuse.

Availability of a coliphage-based RWQC could enable states to enhance public health protection from viruses in vulnerable locations by facilitating development of discharge permits to prevent viruses entering recreational or source drinking waters and by identifying impaired waters or potentially hazardous conditions at beaches.

In April 2015, EPA published *Review of Coliphages as Possible Indicators of Fecal Contamination for Ambient Water Quality* (hereafter referred to as EPA's *Coliphage Literature Review*), which included the following overarching conclusions:

- Methods — Coliphage methods are available for water quality monitoring. EPA is in the process of validating methods in ambient waters and wastewaters.
- Epidemiological Studies — Five of the eight relevant epidemiological studies report a statistically significant relationship between coliphage and GI illness levels.
- Occurrence in the Environment — Coliphages are not always significantly correlated with the presence of human viruses in environmental waters (because of source and fate differences) but are better correlated with pathogens than traditional FIB, which are the only currently recommended recreational criteria.
- Environmental Fate — Coliphages are generally good surrogates for the behavior of human enteric viruses. Behaviors between coliphages and human enteric viruses with temperature, sunlight, pH, salinity, environmental degradation, and inorganic/organic matter are similar.
- Wastewater Treatment — F-specific and somatic coliphages are more conservative indicators of viral pathogen removal overall than traditional FIB in wastewater treated by most disinfectants.

Topic 1: Need for Viral Indicator

In EPA's *Coliphage Literature Review*, EPA concluded that coliphages likely are better indicators of viruses in fecal contamination, compared to currently recommended FIB (i.e., enterococci and *E. coli*). Topic 1 addressed the overall need for a viral indicator as evidenced in epidemiological, microbial risk assessment, outbreak, and microbial water quality studies. The following charge questions were provided to the Topic Lead Experts. These experts each provided a 15-minute presentation based on their submitted written responses to the charge questions (Appendix B).

Charge Questions:

1. ***Comment on EPA's conclusion that the literature (including epidemiological, risk assessment, outbreak, and microbiological) supports that viruses are an important cause of illnesses associated with exposure to ambient recreational waters.***

2. ***Comment on EPA's conclusion that the literature supports that coliphages can be used as an indicator of human viral fecal contamination.***

3. ***What are the most important advantages and disadvantages of using coliphage for assessing human viral fecal contamination compared to traditional FIB in ambient waters?***

Topic 1 Overview

Ample evidence exists documenting that human enteric viruses are the leading cause of illnesses associated with exposure to ambient recreational waters, as was reviewed in EPA's *Coliphage Literature Review*. Supportive data are well described in the recreational water literature on viral occurrence, outbreaks, epidemiological studies, and QMRA. However, varied information exists on the importance of enteric viruses in different types of human fecal sources of contamination (i.e., wastewater, septic). Depending on the source of fecal contamination, coliphages may address the viral etiologies documented in epidemiological studies.

The individual experts agreed that coliphage would be useful as an additional indicator of fecal contamination for CWA 304(a) RWQC purposes. However, clarity is needed on the most suitable coliphage type (F-specific, somatic, or both). Additionally, epidemiological studies vary regarding the association between coliphages and illnesses. Some important factors that influence coliphage relationships in epidemiological studies might include geography, temperature, time, study design, fecal source, and other key variables. Further investigation could reveal which coliphage types are associated with specific fecal sources.

Important advantages and disadvantages of using coliphage for assessing human viral contamination in ambient waters compared to traditional FIB were identified. Key advantages include: 1) coliphages have physical, chemical, and functional characteristics that are similar to human pathogenic viruses, and thus mimic or model human pathogenic viruses; 2) coliphages and human viruses are both consistently present in large municipal sewage systems (>1 million gallons per day [MGD]); 3) coliphages have been shown to be useful in evaluating individual wastewater treatment processes, disinfection efficacy, and shellfish harvesting waters; and 4) coliphage enumeration methods are developed, inexpensive, and could be incorporated into easy-to-use commercial kits. Rapid methods (<8 hours) are available, but have not undergone multilaboratory validation in wastewater. Noted disadvantages, particularly for F-specific coliphage, include that excretion of coliphage by individuals is variable and inconsistent. The smaller the treatment system, the lower the probability of having coliphage present. A peer-reviewer noted that because viruses are more variable and dilute in the environment, concentration steps may need to be added to the current enumeration methods, so that they are more readily detectable in ambient waters. Additionally, coliphages are very diverse, consequently designing molecular methods that capture the full diversity of coliphages potentially of interest is difficult. Note, a discrepancy exists between analytical methods measuring infectiousness and those measuring the presence of viral nucleic acids. However, this disadvantage is related to how we apply methods for making risk and management decisions and is not an inherent disadvantage. A major advantage of coliphage is that culturable methods measure infectious virus particles, allowing for the prediction of removal of human viral pathogens during wastewater treatment.

Topic 1 Group Discussion

Below are additional items discussed by the entire panel of experts that are related to Topic 1's charge questions.

Sediments: The experts indicated sand and sediments support the accumulation of microbes that affect water quality. FIB accumulate and grow in the environment and can have high concentrations in sediment and sand. Coliphages need a high density bacterial host for replication and thus, do not readily regrow in the environment. The panel discussed how the data for coliphage in sediment were mixed. One expert indicated that coliphage survive longer in sediments than in water (as do FIB and pathogens). However the fact that coliphage do not easily regrow in the environment, as compared to traditional FIB, is a benefit to the use of coliphage, though this has not been studied extensively.

Coliphage types: One expert suggested it would be easiest to consider both coliphage groups and measure them simultaneously, using the bacterial host CB390, which is infected by both somatic and F-specific coliphage. In addition, they noted, deciphering which plaques are somatic coliphage and which are F-specific coliphage is easy because the somatic coliphage plaques are large and the F-specific plaques are small. Coliphage types have been confirmed by picking plaques on the bacterial host CB390. A peer-reviewer indicated that the *E. coli* strains used in typical assays are optimized for detection of coliphage associated with sewage, and that environmental coliphage may more efficiently replicate in *E. coli* isolated from the environment (Reyes and Jiang, 2010).

Variability: Experts discussed that coliphage and FIB have similar levels of variability in ambient waters and future research could further evaluate variability of coliphages compared to FIB. One expert questioned the distance from the fecal source at which one should measure the variability. Several experts agreed that more than one indicator should be employed, and determining which conditions are best for the different indicators is important. For example, F-specific coliphage seems to be more common in some geographic locations, but less common in others. In studies in Spain and Florida with warm ambient waters, F-specific coliphages are not found. Thus, F-specific coliphages might not be useful indicators for warmer waters.

Septic, municipal, and groundwater sources: Experts noted that F-specific coliphages are consistently found in municipal sewage, but not necessarily in individual septic tanks because not all individuals excrete F-specific coliphage. For example, a CDC study reported approximately 50% of tested septic tanks were positive for F-specific coliphages. However, once the tank was positive for F-specific coliphages, it tended to stay positive. One expert noted their concern about viruses from WWTPs traveling to shellfish growing areas. They agreed that, because the carrier rate of F-specific coliphages among individuals in a population is low, it is not a good indicator for sewage coming from a single house septic system or a non-point source, such as a boat. One exception is septic systems for coastal rental properties, which mimic larger municipal systems because of multiple users. Sources contaminating shellfish waters often include overboard discharges or one septic system; and in these cases, F-specific coliphage may not be effective indicators. One expert emphasized that F-specific coliphages are most effective when large municipal sewage sources impact the overlying shellfish waters. It was indicated that because traditional FIB are inactivated by chlorine disinfection, a widely used disinfectant which has little or no effect on viruses, coliphage are needed to adequately protect public health, especially in some waters.

Another expert pointed out that floods and rise in sea level may enhance surface and groundwater connections in some locations and the rise in sea level could impact many coastal states. This connection is already manifested in many areas and leads to septic system overruns. Another expert indicated that with septic systems, viruses including coliphages travel more efficiently and more extensively from on-site systems. On average, attenuation of viruses and coliphages in groundwater downgradient from a subsurface source will be less than for FIB. One expert suggested that we should be designing experiments to ask if there are sources specifically related to the different somatic coliphages and to determine which taxonomic groups of coliphages are present or predominantly associated with humans. Another expert noted source tracking strategies are needed. For example, when low-flow contamination was present, septic systems were shown to link to the human marker; and when it rains, run-off markers (i.e., human and cow) were found.

A peer-reviewer indicated that there are increased levels of enteric viruses during outbreaks and there can be seasonal differences in occurrence. For example, virus levels will increase during increased illness incidences among a population (Sinclair et al., 2009). In the case of norovirus, incidence of infection increases during the winter in temperate climates. It is unknown if coliphage would be expected to increase along with increases in the incidence of enteric virus infections in a community.

Experts further discussed sewage-contaminated groundwater as another source of fecal contamination. One expert discussed the Avalon, California (CA) beach epidemiological study site conducted by the Southern California Coastal Water Research Project (SCCWRP). At this site, the sewage infrastructure is faulty and pipes are corroded because salt water is used to flush toilets. In this instance, it was noted that viral indicators are the best for protecting people from sources that are not always obvious and where FIB are not detected. For example, at Avalon, viruses were detected in the water, in the absence of FIB, and there was a health risk, indicating that coliphage would be useful in this situation.

Topic 2: Coliphage as a Predictor of Gastrointestinal Illness

The second topic discussed was coliphage as a predictor of GI illness. In EPA's *Coliphage Literature Review*, eight epidemiological studies were reviewed. Five of the eight studies found a statistically significant relationship between coliphage and illness. Four studies found a relationship between F-specific coliphages and GI illness. One additional study found an association between GI illness and somatic coliphage and suggested a no observed adverse effects level (NOAEL) of 10 plaque forming units (PFU) per 100 milliliters (mL). Topic 2 Experts were asked to reflect on the strength of association identified in epidemiological studies along with conditions under which coliphage may better predict illness than FIB.

Charge Questions:

1. ***Comment on the overall strength of the association between coliphage and human health illness in epidemiological studies conducted in ambient recreational waters.***

2. ***Are there specific characteristics that influence the association between coliphage and human health illness (i.e., source of contamination, salinity)?***

3. ***Are there specific conditions under which traditional FIB are not adequate to protect public health (i.e., Lamparelli et al., 2015; Marion et al., 2010) and if so, comment on the potential for coliphages to be useful in those situations?***

Topic 2 Overview

Workshop participants commented on the strength of the association between coliphage and human health illness in epidemiological studies conducted in ambient recreational waters. The experts agreed that available evidence is suggestive, but inconsistent. The inconsistency may be due in part to the wide range of methods, sites, study designs, and measurement methods. A main concern noted was the frequency of studies that did not detect coliphage or had few detects. This poses a major issue for establishing exposure response associations or establishing threshold values. However, some studies do provide evidence that coliphage may be a useful indicator of GI illness under conditions where human sources of fecal contamination are likely. Future epidemiological studies should specifically include coliphage as a measured indicator.

The participants also noted that as with traditional FIB, various factors influence epidemiological relationships. In particular, the source and intensity of the fecal contamination are important. Specifically, a peer-reviewer noted most of the positive associations between coliphage and illnesses have been found at beach areas impacted by sewage pollution. Studies that collected larger water samples detected coliphage more frequently. Additionally, the type of disinfection (or lack of disinfection) used at the contamination source could influence the association between coliphage and illness (e.g., see Wiedenmann et al., 2006).

The participants discussed whether specific conditions exist under which traditional FIB do not adequately protect public health and if coliphages are potentially useful in those situations. Culturable coliphage deserve further consideration for situations with sporadic or predominately human sources where FIB are not a strong indicator or for determining how soon a beach can be reopened after a contamination event.

Topic 2 Group Discussion

Below are additional items discussed by the group that are related to Topic 2's charge questions.

Viral illnesses: The experts discussed whether viruses are causing illnesses in epidemiological studies. In addition to the studies presented by the topic leaders (Appendix B), one expert mentioned a study of bathing beaches impacted by stormwater in Sydney Harbor that supported a viral etiology. In this study, norovirus was isolated both from children and the stormwater drain (Ferson et al., 1993). Experts agreed that future epidemiological studies need to collect pathogen data, as well indicator data. One expert mentioned EPA's epidemiological study conducted at Washington Park beach and that salivary immunoassays were included, which will provide pathogen exposure information (to date the work has not been published).

Additionally, experts discussed overall health outcomes associated with recreational water exposure. Several experts agreed that the respiratory infections, such as those associated with adenovirus, are important, in addition to the GI illnesses. Epidemiological studies by Fleisher et al. (2006, 2010) found the illness burden was high for respiratory infections. One expert questioned if an indicator specific for the respiratory health endpoint is needed. Another expert noted that adenovirus may persist longer than traditional FIB in the environment and respiratory infections could be observed in the absence of GI illness. It was noted that in recreational water epidemiological studies, if participants are asked about GI symptoms, information on coughs and related respiratory symptoms is also typically collected.

Accumulation of microorganisms in the environment: The group discussed the accumulation of viruses and coliphages in the environment. It was noted that there is evidence that people are being exposed to viruses accumulating in sand. However, evaluating differences in viral exposures from recreational water versus sand is difficult to measure in epidemiological studies.

In addition to sand, it was noted that pathogenic viruses and coliphages might accumulate in algal mats and possibly during harmful algal blooms (HABs). It was postulated that some of the illnesses observed during algal blooms are not only associated with algal toxins, but may be caused by pathogens accumulating during the blooms. Another expert agreed that some bacteria may potentially accumulate and multiply in vegetative material, particularly when surface waters receive effluent treated only by seasonal disinfection (rather than year-round disinfection).

Salinity: One expert asked if any conclusions can be drawn regarding the effects of salinity based on available data. A peer-reviewer noted that there are reports on coliphage distribution along a salinity gradient, but it is not clear that the pattern observed is due to source or due to die-off. Another expert offered that epidemiology cannot address certain characteristics related to survival of the indicator, such as salinity. One expert suggested going back to beaches where epidemiological studies have been conducted previously. However, another expert cautioned that many confounders exist when sampling for indicators at the same beach at a different time. For example, the impacts from municipal sewage, bather population, or WWTP performance could be different. A large amount of data on those parameters would need to be collected for comparisons between years.

Epidemiological study design attributes and future site needs: The group discussed various aspects of epidemiological study designs and why the Wiedenmann et al. (2006) study was so successful at detecting an association between GI illness and coliphage. Wiedenmann et al. (2006) included six locations and more than 2,000 individuals, including children as young as four years of age. The fecal sources impacting the site were untreated human point sources, which are rich with indicators, and the study participants had variation in exposure to different levels of indicators. Individual experts noted the randomized controlled trial (RCT) study design has benefits when well-designed.

Additionally, sanitary surveys and water quality monitoring are important for choosing a beach site for an epidemiological study. A certain percentage of samples should be positive for the indicators of interest. Additional monitoring could be conducted at WWTPs to determine the loading coupled with calculations of dilution at the beach. Monitoring the sites for inclusion in possible epidemiological studies could be done in 12 months. The precipitation over the previous year, including overall drought or wet weather conditions, should be recorded. The larger patterns can also influence study results, beyond just the daily measures. Experts noted that animal contributions, in particular birds, are confounding factors that need to be considered in epidemiological studies.

Several experts suggested having a national mobile application for enrolling individuals to self-report health after going to a beach and concurrently mobilizing a national team to do a water quality assessment. Another expert indicated an epidemiological study of California surfers did use a mobile application for collecting data from surfers, which may be useful in future studies (Arnold et al., 2017).

Sufficient information: The group discussed whether the epidemiological studies supported moving forward with coliphage criteria. One expert said there is not enough information to discard the idea of developing coliphage criteria. Another noted an epidemiological dose-response relationship for developing a guideline value for predicting risk is not available. However, it was noted at the workshop that EPA is considering a risk assessment-based approach to deriving the criterion value. Another expert suggested the group consider what was sufficient for decision making previously and not be biased by the abundance of information currently available on FIB.

One expert recalled that some of the first epidemiological studies were conducted by EPA in the 1970s. At that time, EPA looked at the best candidate indicators and the study authors (Cabelli et al., 1982) concluded that enterococci was best. At the time, it was recommended to EPA to evaluate coliphage in the future. This expert proposed that if the goal is to advance knowledge and understanding, a variety of phages should be studied in the future.

A peer-reviewer pointed out that direct relationships between the incidence of illness and coliphage may never be possible because of the limitations of any epidemiological study. However, this does not preclude their eventual use as a measure of recreational water quality as more reflective of the risk of viral infection, as compared to traditional bacterial indicators.

Topic 3: Coliphage as an Indicator of Wastewater Treatment Performance

Topic 3 Experts evaluated coliphage as an indicator of wastewater treatment performance. In EPA's *Coliphage Literature Review*, EPA summarized indicator attributes and treatment removal efficiencies of FIB, coliphages, and human enteric viruses. EPA concluded that coliphages are likely a better indicator of viruses across wastewater treatment, compared to the currently recommended FIB (i.e., enterococci and *E. coli*).

Charge Questions:

1. ***Comment on EPA's conclusion that human pathogenic viruses are entering surface waters via wastewater treatment effluent.***
2. ***Summarize the most important reasons that coliphages might be useful models or indicators of the behavior of enteric viruses in wastewater treatment and disinfection processes.***
3. ***Comment on EPA's conclusion that monitoring for coliphages would be more useful than enterococci and E. coli in predicting human viral pathogens in wastewater treatment effluent.***

Topic 3 Overview

The experts agreed that human pathogenic viruses are entering surface waters via wastewater treatment effluent. Treatment configurations exist, however, where pathogens are at levels below detection. In addition, the national variability and national distribution of pathogens coming out of WWTPs is not known. Episodic loading and when WWTPs exceed design flows are important events, which are influenced by wet weather, storm events, snow melt, and hydraulic influences.

The most important reasons that coliphages might be useful models or indicators of the behavior of human enteric viruses in wastewater treatment and disinfection processes include: 1) coliphages are more similar to human enteric viruses than FIB, and 2) coliphages are consistently present in municipal sewage, thus providing a baseline for looking at log reductions by different treatment processes under varied conditions; and 3) the literature suggests that coliphage and human viruses have similar reductions during wastewater treatment, however not all disinfection processes and treatment configurations have been evaluated. For example, there is some evidence that coliphage log reductions reflect human virus log reductions during wastewater treatment better than enterococci, particularly for chloramines, free chlorine, and ultraviolet light (UV) treatment. For ozonation, coliphage and viral pathogens are inactivated at lower doses, compared to enterococci. However, not all coliphage react the same to treatments. It was additionally noted that F-specific coliphage have been demonstrated to work well for assessing shellfish safety in waters impacted by wastewater treatment. In particular, they perform much better than traditional FIB as treatment indicators when WWTPs are operating at flow capacities above their design level. In these cases, FIB may be within permit limits, but F-specific coliphage increase steadily as flow gets higher and higher above design capacity.

Topic 3 Group Discussion

Below are additional items discussed by the group that are related to Topic 3's charge questions.

Non-culturable viruses: Experts discussed the fact that some pathogens are culturable in cells, allowing for the evaluation of infectivity, while others, such as norovirus, are not readily culturable. When assessments are limited to evaluating culturable viral assays, only a small portion of the pathogens are quantified. For example, in the case of shellfish, one expert noted that evaluating only infectious viruses fails to capture norovirus, but it is known that many of the illnesses are caused by noroviruses. Recently norovirus has been cultured, but the method has not been developed into a quantitative assay for either clinical or environmental samples. One expert noted that FDA compared reduction of viable coliphage to numbers of norovirus detected with molecular tools. Norovirus RNA signals are found in effluent and reductions across treatment correlate with coliphage, but information on the infectivity of norovirus in the effluent is lacking. However, one expert noted norovirus could be useful for direct pathogen measurement and an index of other fecalborne pathogens.

Disinfection: Regarding UV treatment, UV inhibits the replication function of viral RNA, but leaves viral capsids intact. In theory, if some RNA viruses are inactivated by UV, then others should react similarly because the target is the same. One expert discussed his studies on viral, protozoan, and bacterial pathogens in UV and chlorine treated effluent. If oxidized tertiary effluent is treated with UV and chlorine, *E. coli*, enterococci, coliphages, and *Clostridium perfringens* are not detectable (per 100 mL); however molecular analyses detected adenovirus and norovirus nucleic acids, and *Giardia* and *Cryptosporidium* were detected microscopically as immunofluorescent (oo)cysts. Unfortunately, it is unclear if the molecular and immunofluorescent microscopy assay results represent viable organisms. Experts discussed several ongoing projects that include reoviruses, secondary treatment, and UV disinfection.

Another expert noted qPCR signals are very good at evaluating the activated sludge step and the physical processes related to removal of viruses from wastewater. Disinfection needs to be evaluated separately, however, because the molecular signal can persist when the pathogen is inactivated (versus physically removed). Disinfection, in particular, has not been thoroughly studied, and disinfection processes vary in their effectiveness against inactivating different pathogen classes.

Laboratory/small-pilot studies: The group discussed full-scale versus laboratory bench-model systems as data sources. One expert discussed the challenge in obtaining data from full-scale plants. Several experts noted that in the short-term, laboratory, bench-top data, and small pilot studies can be conducted cheaply and quickly, and can compare different coliphages, other viruses, culture, and qPCR methods against various unit treatment processes. Experts described the utility of previous bench-scale studies on MS2 coliphage and hepatitis A virus treated with chlorine and monochloromine. Several experts countered that seeded and indigenous viruses show different persistent patterns to treatment, and it can be difficult to translate the bench-scale information conducted on limited strains to full-scale operations (Gerba et al., 2015). Limitations were noted regarding imitation of microbial clumping when seeding samples. One expert expressed the importance of normalizing the metrics (such as descriptions of flow rate and facility treatment capacity) used across treatment plants so it is easier to link unit treatments to standardized log viral reductions.

Similar reductions of coliphages and human viruses: Experts agreed that reductions of coliphages overall are more similar to pathogenic virus reductions than bacteria across treatment processes, and viruses are in general more resistant to disinfection than bacteria. However, there are disinfectants where the reverse is true (i.e., ozone).

The group discussed where coliphage have demonstrated to be useful. One expert offered that although FIB can identify catastrophic failures of treatment processes, F-specific coliphage can also identify more subtle upsets with treatment processes. For example, FDA's shellfish data indicate that sometimes when WWTPs meet all fecal coliform discharge permits, culturable F-specific coliphage can still be detected. If human viral pathogen densities occur at up to 10^9 particles per liter in raw sewage, and the WWTP facility provides only 2 to 4 viral log reductions, then norovirus can still occur in effluent (infectious and non-infectious).

Overall, the group agreed that if forward progress is to be made, pathogen indicators representing viruses and protozoa shold be considered in future criteria recommendations. Specifically, coliphages and *C. perfringens* spores would be useful for addressing the persistence of these microbial groups.

Continuing advancements: A peer-reviewer pointed out that changes in wastewater treatment are occurring. Particularly in the Western United States, denitrification and enhanced phosphorus removal is becoming more common as wastewater treatment plants upgrade. These upgrades have resulted in enhanced removal of human enteric viruses over conventional activated sludge and more effective disinfection with chlorine (Schmitz, et al., 2016). Data are not yet available to determine whether coliphages behave in a similar manner. Separately, the composition of raw wastewater is also changing as low-flush toilets, water efficient washing machines, and use of cold water rather than hot water for washing changes the dilution and survival of pathogens in sewage (Gerba et al., 2017).

Topic 4: F-Specific Coliphage versus Somatic Coliphage

Topic 4 Experts were tasked with looking at the different types of coliphages and providing an assessment of whether one type is more useful than the other. EPA's *Coliphage Literature Review* provides background information relevant to the use of both F-specific and somatic coliphages, as indicators of viral fecal contamination.

Charge Questions:

1. ***What are the most important advantages and disadvantages of using these two types of coliphages as (a) predictors of human health illness in recreational waters; (b) indicators of wastewater treatment performance?***

2. ***Are there specific attributes of these coliphages or conditions (i.e., source) that influence the usefulness of the indicator, or would favor the use of one type of coliphage over the other?***

3. ***EPA is aware that other countries have considered coliphages for various purposes. Please provide summaries and commentaries for any of these efforts you deem helpful.***

Topic 4 Overview

The two types of coliphages (F-specific and somatic) were compared as (a) predictors of human health illness in recreational waters and (b) indicators of wastewater treatment performance. Experts noted that the application drives the preference, and both somatic and F-specific have advantages. Among experts, opinions ranged on whether somatic, F-specific coliphage, or both would be better for the various applications. Experts agreed that bacterial host selection for coliphage assays is important, and it was noted that a single available cell host can capture both coliphage types.

Epidemiological evidence is suggestive of relationships between both groups of coliphages and GI illness. For measuring log reductions in wastewater treatment, some individual experts indicated somatic coliphages are consistently more numerous in wastewater and thus provide the most dynamic range in log reduction by wastewater treatment. Somatic coliphages, as a group, also have the most diverse viron types to represent the broad range of human enteric viruses. Under some conditions, they persist longer in the environment, and thus may provide a more useful conservative surrogate role compared to F-specific coliphages. However, others noted that F-specific RNA coliphages are present in sufficiently high densities (approximately 10^6/liter) in raw sewage and they behave more similarly to the RNA human viruses of concern. Additionally, there are situations where F-specific coliphages have been seen to outnumber somatic coliphage, such as reclaimed water with high UV treatment, clay sediments, and groundwater from an alluvial gravel aquifers.

The experts agreed that there is diversity within both coliphage groups, and data are sparse on how the full diversity of these coliphage groups behave during wastewater treatment. Unfortunately, while many published studies provide information on either somatic or F-specific coliphages, most studies do not include both types. Beyond occurrence information, there are even fewer studies that investigate environmental factors or viral attributes that could be different for somatic coliphages compared to F-specific coliphages. From a methodological perspective, somatic coliphages are easier to count in plate assays because the plaques can be more easily visualized by their larger size and clarity.

The participants briefly discussed other countries where coliphages have been studied for various purposes including The Netherlands (Havelaar et al., 1986), Singapore (Liang et al., 2015; Vergara et al., 2015), Australia (Keegan et al., 2012; Charles et al., 2009), Canada (Payment et al., 1988), Argentina (Lucena et al., 2003), Columbia (Lucena et al., 2003; Venegas et al., 2015), Israel (Alcalde et al., 2003; Armon et al., 1995), South Africa (Grabow et al., 1986, 2001; Momba et al., 2009), Japan (Hata et al., 2013), New Zealand (Wolf et al., 2008), Tunisia (Yahya et al., 2015), China (Fu et al., 2010) and the EU (Contreras-Coll et al., 2002; Lucena et al., 2003; Ibarluzea et al., 2007; Araujo et al., 1997; Arraj et al., 2005, Blanch et al., 2004).

Internationally, bacteriophages have been included in guidelines affecting water reclamation in Australia (Queensland Government, 2005) and biosolids applied in agriculture in Australia and Colombia (Western Australia Government, 2012; República de Colombia, 2014). Additionally, two drafts of regulatory documents regarding drinking and reclaimed water including coliphages are being circulated for discussion in the European Union.

Topic 4 Group Discussion

Below are additional items discussed by the group that are related to Topic 4's charge questions.

Training and ease of method: The group discussed the importance of focusing on laboratory training. WWTP operators are concerned about adopting coliphage methods because they prefer the simplicity of Colilert and Enterolert. If coliphage analysis can be done with pre-packaged methods, like the Easyphage kit (from Scientific Methods), then fewer objections exist. Most commercial systems are currently too complicated. One expert indicated that companies do not develop pre-packaged kits until the methods are needed for regulatory monitoring. The individual experts agreed that kits provide quality control. Of importance is that the level of precision is meaningful for the level of decision making needed. Simple and complete kits can equal more success in multi-laboratory studies. Another expert noted that many laboratories test both recreational and shellfish waters. This expert recommended using videos and other modern outlets to provide training and convey the process practically to reduce variability. This expert felt that there were lessons learned from how the qPCR method was originally presented. Because researchers focused on problems and extensive protocols, the method was not accessible to the broader community. For new methods, simplicity and training are important. One expert noted that somatic coliphages are easier for training because F-specific coliphage have smaller plaques. The group agreed about the value and importance of training and implementation of a consistent program.

Lessons learned from shellfish programs: The expert group agreed that lessons learned from the shellfish program would be useful for EPA's evaluation of coliphage in recreational waters. The shellfish community took 30 years to transition from Most Probable Number (MPN) to a direct membrane Thermotolerant *E. coli* Agar (m-TEC) method, demonstrating that transitions can be slow. The U.S. shellfish program includes regional laboratory training where laboratory technicians practice the method. One expert noted that the EU shellfish program found a strong correlation between the occurrence of F-specific coliphages in shellfish and noroviruses. Some EU researchers recommended F-specific coliphage for shellfish water monitoring, but the idea was not implemented because it would have downgraded many waters.

Personal sampling devices and composite samples: The group discussed composite samples and personal sampling devices in epidemiological studies. One expert noted that taking samples at different times during the day is difficult logistically, thus integrated composite samples are preferred. Researchers have not yet developed user-friendly, tamper-proof personal samplers, thus there is an opportunity for engineers to design samplers that can be put on individuals to better represent exposure. Another expert indicated that the California Surfer Study (Arnold et al., 2017) intended to provide personal sampling devices to the surfers enrolled in the epidemiological study, but a small enough device was not available. Another expert added that the situation is complex, and added that tracer dye studies should be conducted to allow for placement of sentinel monitoring stations close to the fecal sources.

Environmental replication: There was discussion of the possibility of replication of coliphage in the environment. The individual experts agreed that phages would not appreciably replicate in the environment because they need a concentrated bacterial host population for replication (Jofre, 2009). However, a peer-reviewer expert noted microbial ecology overall is very complicated and that coliphage replication is possible. One expert posed the question of whether one could distinguish release of phage from bursting cells versus actual phage replication. One expert thought there might be a way to look at packaging versus presence of replicons. Another expert thought that growth curves could differentiate between bursting cells and replication, but was not aware that anyone has sewage-related data. Another expert suggested looking beyond sewage at other places of potential replication such as *Cladophora* mats. Another expert indicated that he published a paper on indigenous phage survival in sewage, looking at survival in seawater and the effects of sunlight and whether coliphages can grow in shellfish. This expert observed that coliphage could grow in shellfish spiked with the *E. coli* host (Famp) at elevated temperatures, such that the shellfish were essentially incubators. However, this expert indicated that levels of hosts in wastewater are orders of magnitude higher than anything that is growing on *Cladophora* mats, thus replication in mats would be unlikely. A peer-reviewer indicated that storm drains could be another potential place of coliphage replication. During Southern California's dry, summer season, storm drains can accumulate very high concentrations of *E. coli* and can reach temperatures needed for coliphage replication.

Somatic and F-specific coliphages: One expert suggested that we know less about somatic than F-specific coliphages, but more hosts are possible for somatic coliphages. It was noted that the F-specific coliphage methods perform more homogenously than the somatic coliphage methods.

Topic 5: Systematic Literature Review of Viral Densities

EPA has developed ambient water quality criteria using risk-based approaches for chemicals. EPA is considering using a quantitative microbial risk-based approach to derive recreational criteria for coliphage. The risk methodology relies on identification of densities of key viral pathogens and coliphages in raw wastewater. EPA has conducted systematic literature reviews to understand and document these viral densities. In this session, approaches EPA is considering for the risk assessment, the systematic literature review, and the non-parametric method for building distributions of virus occurrence in wastewater influent were presented (Appendix B). An updated version of this research was published after the workshop (Eftim et al., 2017).

Charge Questions:

1. ***Comment on the information collected on viral densities to date.***
2. ***Are there any additional data that should be considered?***

Topic 5 Overview

There was overall support for how the systematic literature review and analysis was structured and conducted. Experts recommended that any future data from the United States and other countries should meet the same inclusion criteria as the data currently in EPA's analysis.

Topic 5 Group Discussion

Below are additional items discussed by the group that are related to Topic 5's charge questions.

<u>Systematic Review</u>: Transparency regarding the criteria used for data inclusion is very important because it addresses the quality of the data and the importance of access to raw data. Both the criteria used to screen the papers and methodology used for bootstrapping, would provide the public with valuable information The bootstrapping methodology could be used for other types of datasets, like the Chicago River study (MWRDGC, 2008; Aslan et al., 2011) with culture and qPCR data.

<u>*Data suggestions*</u>*:* Experts offered several suggestions on data inclusion, such as the use of: 1) the Embase database for literature searches; 2) publication date for use as a proxy for study quality; 3) publications other than those written in English; 4) and non-peer reviewed reports, such as those from sanitation districts. However, they recommended that unpublished data would need to pass the study inclusion criteria.

<u>*Bootstrap analysis suggestions*</u>: Experts offered several comments on the bootstrap analysis of data, such as 1) possible error may be introduced by normalizing small volumes to liters; 2) a threshold for minimum sample size for the bootstrapping approach should be employed; and 3) significant figures should be adjusted in the presentation.

<u>*Criterion risk assessment approach suggestions*</u>: Experts offered several comments on the risk assessment approach presented. One expert noted inclusion of culture-based reovirus because infectious reoviruses pass through wastewater treatment. Reovirus is a good index of virus behavior during treatment because they do not cause illnesses, but are excreted in human feces.

One expert noted that culture data provide greater certainty when making health risk estimates, compared to molecular data, because culture date provide information on infectivity. If the goal is to predict human health risks, then methods that indicate infectivity are useful. Additionally, log reductions from molecular data are less reliable through treatment. For example, log reductions based on molecular data are less than the log reductions of culturable coliphages. However, another expert indicated that FDA did a meta-analysis that looked at efficacy of WWTPs to reduce total coliphage and genome copies of norovirus. He acknowledged that genome copies were very beneficial in identifying reductions of viruses during wastewater treatment. The numbers were variable based on sampling location and season, but they found that WWTP provided a 2 to 3 log reduction of genome copies (Pouillot et al., 2015).

Ultimately, it was noted that the difficulty of using molecular methods, like qPCR, to estimate log reductions is not relevant to the risk assessment approach presented because distributions of pathogens were modeled for influent only, which is before treatment process are applied. One researcher indicated that a study in Sydney, Australia looked at over 1,000 samples of effluent from primary and tertiary treated water. In that data set, presence/absence of adenoviruses, enteroviruses and reoviruses by cell culture were compared, indicating reoviruses as generally the most resistance infectious human enteric viruses in environmental waters (Ashbolt et al., 1993), as also reported in a recent review (Betancourt and Gerba, 2016). Furthermore, adenoviruses have been shown to be more prevalent than noroviruses in bathing waters (Wyn-Jones et al., 2011). While there appears to be no constant ratio of total viruses (qPCR) to cell culture infectious viruses (Aslan et al., 2011), in groundwaters the most stable to least stable appears to be: coliphage PhiX174 (0.5 d^{-1}) > adenovirus 2 > coliphage PRD1 > poliovirus 3 > coxsackie virus B1 (0.13 d^{-1}), whereas the order for qPCR results was: norovirus genogroup II > adenovirus > norovirus genogroup I > enterovirus (Charles et al., 2009).

Coliphage methods are culture-based and should be collected and used for the analysis. Experts suggested that EPA should proceed with the presented risk assessment approach to support criteria and noted that QMRA has been used in Australia for standards. The QMRA methodology is a good pathway forward on how to evaluate human enteric viruses as agents of global concern, such as norovirus.

Future Research

Sixteen research ideas were captured during the two-day meeting. The workshop organizers then asked the expert participants to indicate whether these research ideas were long-term or short-term *and* high, medium, or low priority. This was not a consensus activity, rather experts individually prioritized projects. At the conclusion of the workshop, the results of th exercise were collated by workshop organizers to determine the order of preferred priority for both the short-term and longer-term projects.

Short-term projects in order of preferred priority:

- Evaluate many indicators and pathogens using small model (bench-scale) studies to understand treatment efficacy for a variety of processes.
- Model persistence of coliphage related to many different factors.
- Compare variability of coliphage to traditional FIB, including temporal and spatial differences depending on source and distance from source.
- For epidemiological studies, although GI illness is the most plausible endpoint, the health endpoints with the highest burden of health impacts should also be considered (such as respiratory illness).
- Evaluate existing data to identify specific pathogens causing swimming-associated illnesses.
- Study coliphage diversity from different household sewage sources using metagenomics and a variety of host cells.
- Collect more data on FIB and coliphage in wastewater effluent.

Longer-term projects in order of preferred priority:

- Develop guidance on standard epidemiology methods so studies can be more easily compared. Also encourage researchers to make data publicly available for meta-analyses.
- Support groups who are interested in conducting epidemiological studies.
- Determine which coliphage are associated with animal and which with humans for microbial source tracking purposes.
- Determine if coliphage replicate in algal mats.
- Investigate what is happening with coliphage in sewage, including differences in survival and whether phage are replicating or increasing because cells burst.
- Conduct a community analysis of bacteriophage in algal mats, biofilms, and other natural environments that influence source waters.
- Support building better maps for sanitary surveys, including locations of fecal sources.
- Support development of phone applications for use in epidemiological studies, so citizens can self-report.
- Monitor beaches for coliphage to help identify sites for future epidemiological studies

Closing Statements

Following discussion of all topics and charge questions, all experts were asked to state what they thought were the most relevant messages from the workshop. The participants' individual comments are below:

- The time has come to move away from using FIB to determine the efficacy of WWTPs. Although there are data gaps associated with coliphage, a viral indicator should be used. The old science is based on typhoid and not on the greatest virus of concern today—norovirus.

- In examining the difficulties with the available epidemiological data, it is important to ensure coliphage are useful in the context of TMDLs as a process indicator of log reductions. As long as samples are collected close to the source of fecal contamination, confidence can be placed in using coliphage.

- Norovirus is a major burden of disease that needs to be recognized and addressed, however possible. Coliphage can be a useful tool and provides a better model or surrogate for norovirus than FIB.

- Development of simple, user-friendly detection methods that can be readily implemented is important for the implementation of coliphage criteria. It is also important to understand coliphage types and densities in raw sewage and effluent (see: Sobsey et al., 2014). Near-term studies doing parallel detection of coliphages by both infectivity (culture) and molecular methods in the same samples would provide valuable information on their ratios in untreated and treated wastewaters and in ambient recreational waters. Doing the same for culturable human enteric viruses at the same time would provide even better information for greater insights into the potential usefulness of molecular methods to provide information predictive of infectivity and human health risk.

- A global problem is the management of human and animal excreta. The problem grows as populations increase. It is attached to every ecosystem service. Risk frameworks and discussions of wastewater treatment in a One Water multi-barrier approach for health are important. The CWA does not specify risk-based and evidence-based assessments, but these are important tools for identifying how to best manage wastewater treatment in the future. Excreta management has not moved forward, but wastewater treatment has. The questions that need to be answered are: how much wastewater treatment is needed for virus removal in the "One Water Framework" and how can coliphage inform the decision?

- There needs to be an Alternate Test Procedure process for coliphages, so it can be published as an EPA standard method. When characterizing coliphages, microbial source tracing should be used to identify the source(s), especially when no human sewage is identified (by HF183 or HumM2 microbial source tracking [MST] markers), as the health risk may be substantially less.

- Coliphage is very valuable as an additional tool for managing water quality. In dry areas, as for example Spain, water is recycled and it is important to have water quality indicators that address multiple uses, including coliphages. With warmer climate, water reuse will be more frequent.

- I am glad to see EPA moving towards measuring viruses, even if it is only coliphage. The strength of coliphage in epidemiology studies is not strong, possibly due to method-related issues. Additional studies will be needed to determine the efficacy of coliphage as a general indicator of recreational water quality, especially at non-wastewater impacted beaches.

- I am optimistic about where this is going, but I wonder if we have really addressed the conditions where coliphage are better than FIB. I think there are situations where that is possible, but we need to be clear about that moving forward. Epidemiology will not provide a lot of information and only supplemental information. We will need to consider how genomic copies can be used in risk assessments

- More research is needed to fully evaluate whether coliphage would be better indicators than FIB. Changes in indicator organisms may require publicly owned treatment works (POTWs) to make substantial infrastructure investments, so we should make sure that these changes also provide substantial improvements over existing indicators for human health outcomes, and that their selection is well-supported enough to be stable for years/decades (not part of a revolving door of indicators that would create a moving target for regulatory compliance)

- This is a well-run workshop and the charge questions highlighted important health issues. The basic epidemiology paradigm is exposure and outcome. Measurement of personal and fixed exposures, coupled with the ability to capture variability, will be useful in future epidemiological studies. Additionally, identification of the pathogens that cause recreational water illnesses is key.

- Viral and bacterial pathogens by nature differ broadly in pathogen traits and modes of infectivity, so it is reasonable to conclude that a viral indicator may be of interest. Coliphage are an interesting and viable option for multiple reasons as an addition to current criteria, but direct viral pathogen detection, especially via digital polymerase chain reaction (PCR) is gaining ground rapidly and ought to be included in this process.

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Appendix A. Workshop Agenda and Participant List

U.S. Environmental Protection Agency
Coliphage Experts Workshop
March 1 – 2, 2016 | One Potomac Yard, South Bldg, Room S4370/80
2777 S. Crystal Drive, Arlington, VA 22202

Agenda

Participant List:

Name	***Affiliation***
Coliphage Experts	
Nicholas Ashbolt	University of Alberta
William Burkhardt	U.S. Food and Drug Administration
Kevin Calci	U.S. Food and Drug Administration
Jack Colford	University of California, Berkeley
Sorina Eftim	ICF
John Griffith	Southern California Coastal Water Research Project
Vincent Hill	Centers for Disease Control and Prevention
Juan Jofre	University of Barcelona, Spain
Naoko Munakata	Sanitation Districts of Las Angeles County
Rachel Noble	University of North Carolina
Joan Rose	Michigan State University
Mark Sobsey	University of North Carolina
Jeff Soller	Soller Environmental
Timothy Wade	U.S. Environmental Protection Agency

Disclaimer: This meeting is ***not*** a federal advisory committee, and EPA will not be seeking consensus or recommendations. The Coliphage Experts Workshop is an information gathering exercise and individual opinions of the experts will be captured.

Approx. Timing	Draft Agenda Item	Goal of Agenda Item
Day 1: March 1, 2016		
8:15 – 8:30 AM	**Meet in Lobby for Escort**	Participants meet in lobby; EPA escort to meeting room required. Check-in and receive nametags and meeting materials.
Welcome and Introduction		
8:45 – 9:00 AM	**Welcome and Introductions** Elizabeth Behl (EPA)	Open workshop and introduce S. Nappier. Introduce experts.
9:00 – 9:30 AM	**Introduction on RWQC Efforts** Sharon Nappier (EPA)	Provide historical context and present Coliphage related efforts today to date.
9:30 – 9:45 AM	**Overview of Workshop** Sharon Nappier Jeff Soller	Clarify understanding of scope, objectives, outputs, agenda, and schedule. Introduce the facilitator.
9:45 – 10:00 AM	**Break**	
Topic 1: Need for Viral Indicators		
Charge Questions: In April 2015, EPA published *Review of Coliphages as Possible Indicators of Fecal Contamination for Ambient Water Quality* (hereafter referred to as EPA's *Coliphage Literature Review*). In this review, EPA concluded that coliphages are likely better indicators of viruses in fecal contamination, compared to currently recommended fecal indicator bacteria (FIB) (i.e., enterococci and *E. coli*). 1. Comment on EPA's conclusion that the literature (including epidemiological, risk assessment, outbreak, and microbiological) supports that viruses are an important cause of illnesses associated with exposure to ambient recreational waters. 2. Comment on EPA's conclusion that the literature supports that coliphages can be used as an indicator of viral fecal contamination. 3. What are the most important advantages and disadvantages of using coliphage for assessing viral fecal contamination compared to traditional FIB in ambient waters?		
10:00 – 10:45 AM	**Background on Topic** John Griffith Vincent Hill Mark Sobsey	Provide information and individual responses to the topic charge questions.
10:45 – 11:30 AM	**Discuss Charge Questions** Facilitator: Jeff Soller	Discuss charge questions for this topic.
11:30 – 11:45 AM	**Discussion Summary**	
11:45 – 1:15 PM	**Lunch on your own**	

Topic 2: Coliphage as a Predictor of Gastrointestinal Illness		
Charge Questions: In EPA's *Coliphage Literature Review*, eight epidemiological studies were reviewed. Four of the eight studies found a statistically significant relationship between F-specific coliphages and gastrointestinal (GI) illness. One additional study found a statistically significant association between GI illness and somatic coliphage and suggested a no observed adverse effects level (NOAEL) of 10 plaque forming units (PFU) per 100 milliliters (mL). 1. Comment on the overall strength of the association between coliphage and human health illness in epidemiological studies conducted in ambient recreational waters. 2. Are there specific characteristics that influence the association between coliphage and human health illness (i.e., source of contamination, salinity)? 3. Are there specific conditions under which traditional FIB are not adequate to protect public health (i.e., Lamparelli et al., 2015[a]; Marion et al., 2010[b]) and if so, comment on the potential for coliphages to be useful in those situations? [a]Lamparelli, C.C., Pogreba-Brown, K., Verhougstraete, M., Zanoli Sato, M.I.Z., de Castro Bruni, A., Wade, T.J., Eisenberg, J.N.S. 2015. Are fecal indicator bacteria appropriate measures of recreational water risks in the tropics: A cohort study of beach goers in Brazil? Water Research 87: 59-68. [b] Marion, J.W., Lee, J., Lemeshow, S., Buckley, T.J. 2010. Association of gastrointestinal illness and recreational water exposure at an inland U.S. beach. Water Research 44(16): 4796-4804.		
1:15 – 2:00 PM	**Background on Topic** Tim Wade Jack Colford	Provide information and individual responses to the topic charge questions.
2:00 – 2:45 PM	**Discuss Charge Questions** Facilitator: Jeff Soller	Discuss charge questions for this topic.
2:45 – 3:00 PM	**Discussion Summary**	
3:00 – 3:45 PM	**Break**	
Topic 3: Coliphage as an Indicator of Wastewater Treatment Performance		
Charge Questions: In EPA's *Coliphage Literature Review*, EPA summarized indicator attributes and treatment removal efficiencies of FIB, coliphages, and enteric viruses. EPA concluded that coliphages are likely a better indicator of viruses across wastewater treatment, compared to the currently recommended FIB (i.e., enterococci and *E. coli*). 1. Comment on EPA's conclusion that human pathogenic viruses are entering surface waters via wastewater treatment effluent. 2. Summarize the most important reasons that coliphages might be useful models or indicators of the behavior of enteric viruses in wastewater treatment and disinfection processes.		

3. Comment on EPA's conclusion that monitoring for coliphages would be more useful than enterococci and *E. coli* in predicting human viral pathogens in wastewater treatment effluent.		
3:15 – 4:00 PM	**Background on Topic** Bill Burkhardt and Kevin Calci Naoko Munakata Joan Rose	Provide information and individual responses to the topic charge questions.
4:00 – 4:45 PM	**Discuss Charge Questions** Facilitator: Jeff Soller	Discuss charge questions for this topic.
4:45 – 5:00 PM	**Discussion Summary**	
5:00 PM	**Adjourn Day 1**	
6:00 PM	**Group Dinner (Optional)**	

Approx. Timing	Draft Agenda Item	Goal of Agenda Item
Day 2: March 2, 2016		
8:00 AM	**Meet in Lobby for Escort**	Participants meet in lobby; EPA escort to meeting room required.
Welcome and Introduction		
8:15 – 8:30 AM	**Announcements** Sharon Nappier Audrey Ichida Jeff Soller	Logistical announcements
Topic 4: F-Specific Coliphage vs Somatic Coliphage		
Charge Questions: EPA's *Coliphage Literature Review* provides background information relevant to the use of both F-specific and somatic coliphages, as indicators of viral fecal contamination. EPA is considering these two possible viral indicators for use in future recreational water quality criteria (RWQC). 1. What are the most important advantages and disadvantages of using these two types of coliphages as (a) predictors of human health illness in recreational waters; (b) indicators of wastewater treatment performance? 2. Are there specific attributes of these coliphages or conditions (i.e., source) that influence the usefulness of the indicator, or would favor the use of one type of coliphage over the other? 3. EPA is aware that other countries have considered coliphages for various purposes. Please provide summaries and commentaries for any of these efforts you deem helpful.		
8:30 – 9:15 AM	**Background on Topic** Rachel Noble Juan Jofre Nick Ashbolt	Provide information and individual responses to the topic charge questions.

Approx. Timing	Draft Agenda Item	Goal of Agenda Item
9:15 – 10:00 AM	**Discuss Charge Questions** Facilitator: Jeff Soller	Discuss charge questions for this topic.
10:00 – 10:15 AM	**Discussion Summary**	
10:15 – 10:30 AM	**Break**	
Topic 5: Systematic Literature Review of Viral Densities		
Charge Questions: EPA has developed ambient water quality criteria using risk-based approaches for chemicals. EPA is considering using a quantitative microbial risk-based approach to derive RWQC for coliphage. The risk methodology relies on densities of key viral pathogens and coliphages in raw wastewater. EPA has conducted systematic literature reviews to understand and document these viral densities. The relevant information collected to date will be reviewed and summarized. 1. Comment on the information collected to date. 2. Are there any additional data that should be considered?		
10:30 – 11:00 AM	**Background on Topic** Sorina Eftim	Provide additional information to facilitate the topic's discussion.
11:00 – 11:45 AM	**Discuss Charge Questions** Facilitator: Jeff Soller	Discuss charge questions for this topic.
11:45 – 12:00 PM	**Discussion Summary**	
12:00 – 1:15 PM	**Lunch on your own**	
Future Research		
Charge Questions: EPA is in the process of developing future RWQC for Coliphage. EPA anticipates a draft publication will be available in 2017. 1. What are the key uncertainties regarding the development of future RWQC for Coliphage? 2. Please describe research that could be completed in a relatively short time period to address these key uncertainties, which would support the development of future RWQC for Coliphage?		
1:15 – 2:00 PM	**Discussion of future research** Facilitator: Jeff Soller	Discuss research that could be completed in the next 1 to 2 years. A list of future research needs will be tracked during all topic discussions and revisited during this session.
2:00 – 3:00 PM	**Concluding Statements and Final Remarks** Jeff Soller Sharon Nappier	Discuss conclusions from the workshop and next steps. Revisit take-home messages from each topic.
3:00 PM	**Adjourn Day 2**	

Appendix B. Expert's Written Responses to Charge Questions

This appendix includes the written responses to the charge questions that the Experts submitted to EPA prior to the workshop. The Expert's responses are presented without editorial modifications or proof reading.

Topic 1: Need for Viral Indicators

Topic Leads:

- John Griffith
- Vincent Hill
- Mark Sobsey

Charge Questions:

In April 2015, EPA published Review of Coliphages as Possible Indicators of Fecal Contamination for Ambient Water Quality (hereafter referred to as EPA's Coliphage Review). In this review, EPA concluded that coliphages are likely better indicators of viruses in fecal contamination, compared to currently recommended fecal indicator bacteria (FIB) (i.e., enterococci and *E. coli*).

1. Comment on EPA's conclusion that the literature (including epidemiological, risk assessment, outbreak, and microbiological) supports that viruses are an important cause of illnesses associated with exposure to ambient recreational waters.

2. Comment on EPA's conclusion that the literature supports that coliphages can be used as an indicator of viral fecal contamination.

3. What are the most important advantages and disadvantages of using coliphage for assessing viral fecal contamination compared to traditional FIB in ambient waters?

<u>Response</u>: John Griffith

Charge Question 1: Overall, the literature supports that viruses are an important cause of GI illness from exposure to recreational waters. However, despite a great deal of effort, it has been difficult to establish a direct correlative relationship between viruses and illness in epidemiological studies. This like due to the fact that human pathogenic viruses are inherently difficult to measure in environmental waters and epidemiological studies must as a practical matter, rely on a fixed time and sampling point for water quality assessment, while swimmers and bathers move freely about. Thus, the exposure to viruses at the point where water samples are collected for viral analysis are only a rough proxy for the actual exposure of an individual swimmer.

The most compelling evidence for viruses as the etiological agent of GI illness from contact with recreational water comes from the modeling exercise conducted by Soller et al. (2015), which determined that the onset of GI illness observed in the NEEAR study most closely resembled that of norovirus infection. While not based on actual measurements, this work is supported by empirical data from a study conducted at Surfrider Beach in Malibu, CA, in which the authors concluded that the onset of diarrhea in swimmers most closely resembled that expected of norovirus infection (Arnold et al., 2013).

While it is plausible that norovirus is the likely cause of GI illness in the above-mentioned studies, the lack of direct correlative evidence supporting these assertions in troubling. Without such evidence, it is difficult to ascertain how much GI illness to attribute to norovirus or to know whether or not there are other as yet unknown viruses that are at least in part responsible. Norovirus was unknown until fairly recently and only became detectable with the advent of molecular measurement methods. It seems equally plausible that there may be additional viral agents capable of producing similar symptoms that have yet to be characterized by virologists, but may play a role in the GI illnesses observed in the swimmers in these studies. Further epidemiological studies which utilize advanced detection technologies, such as digital droplet PCR, capable of enumerating norovirus at the low concentrations found in environmental waters are needed to answer this question.

Charge Question 2: Overall, the literature supports the conclusion that coliphages may be used as an indicator of viral fecal contamination. Although the studies cited had disparate designs, differences in the type of exposure (e.g. swimming vs. rafting) and differed as to whether water exposure was to fresh or marine water, coliphages were positively correlated with illness in more than half of the studies cited.

While coliphage were often correlated with illness in swimmers, an unresolved issue is how to choose which coliphages to measure and which measurement method to use. The studies cited in EPA's review target a variety of different types of coliphage using an equally large number of methods. Without additional research this issue cannot be resolved. One of the hallmarks of a reliable fecal indicator organism (FIO) is that the measurement methods produce equivalent results. The literature as well as personal experience show that different coliphage measurement methods often produce different results in terms of their observed relationship to water contact health risk. These issues will need to be resolved and measurement methods standardized before coliphage can be considered a reliable water quality indicator.

Charge Question 3: Perhaps the biggest advantage of coliphage as an water quality indicator versus traditional FIB is that they are more likely to mimic the fate and transport mechanisms of human enteric viruses in the environment than are bacteria. This is important because viruses, not bacteria, are posited to cause >90% of waterborne illnesses (Soller et al., 2015).

Coliphage have two main attributes that make them a superior water quality indicator over traditional FIB. The first is that they are not as easily removed by typical wastewater treatment regimens as FIB, making them a more viable indicator of the presence of “disinfected” wastewater plumes. This is because everything about traditional wastewater treatment facilities is geared toward removing and inactivating FIB in order to bring concentrations in the final effluent, with dilution, into compliance with regulations. Unfortunately, this treatment regimen does not remove or inactive enteric viruses or coliphage with similar efficiency. To understand why this is so, one only need look to the fact that bacteria and viruses are very different in terms of their biology. Bacteria are living cells that must maintain cellular integrity to survive. They expend energy for growth, locomotion and to maintain homeostasis under often hostile environmental conditions and are easily inactivated or killed by UV light, oxidation and other common water treatment practices. In contrast, viruses, including coliphage, are defined as bordering on the living and non-living. Like all viruses, coliphage require a specific host for reproduction, are non-motile and are not as easily inactivated by common wastewater treatment processes as are FIB. Thus it is that the relative concentrations of active viruses compared to FIB in wastewater effluent are often higher in treated wastewater than in the untreated water entering the plant. These attributes potentially make coliphage a much better indicator for human enteric viruses in wastewater than are FIB.

The second advantage of coliphage over FIB is that it is more likely to share the same fate and transport as human enteric viruses, both in terms of dispersion in the environment and decay characteristic in sunlight. This is especially important when viruses may be discharged into water bodies with groundwater contaminated by leaking sanitary infrastructure. For example, in a epidemiology study conducted at Doheny State beach, FIB concentrations were only correlated with GI illness on the few days when sewage contaminated water was flowing from San Juan Creek. Despite this, water contact continued to be correlated with GI illness throughout the study period, even though levels of FIB were exceedingly low (Griffith et al., 2016). Further, coliphage was positively associated with the presence of human adenovirus and had a stronger relationship to GI illness than did *Enterococcus* under high-risk conditions. A retrospective investigation at Doheny State Beach revealed a degraded sanitary collection system which was hydrologically connected to the beach.

Despite a stronger relationship to GI illness in some studies, coliphage do have some disadvantages compared to FIB. On disadvantage is that it is more expensive and labor intensive to measure coliphage than traditional FIB. A second disadvantage is that no one coliphage type or measurement method has yet distinguished itself as superior to all others in epidemiology or laboratory studies. Finally, there is recent evidence in the literature to suggest that coliphage may be able to replicate in the environment in warmer freshwater environs (Ravva and Sarreal, 2016). While this should not exclude its use in marine and cold water bodies, it does call into question the conventional wisdom that coliphage cannot reproduce in the environment.

<u>Response</u>: Vincent Hill

Charge Question 1: I agree with EPA's general conclusion that there is sufficient scientific and epidemiological evidence that viruses are an important cause of illnesses associated with exposure to ambient recreational waters. However, the evidence base for this conclusion is more robust with respect to gastrointestinal illness than respiratory, skin or other illnesses. U.S. outbreak surveillance data points to noroviruses as being the leading viral pathogen responsible for untreated recreational water outbreaks (with noroviruses responsible for ~17% of untreated recreational outbreaks between 2003 and 2012 [http://www.cdc.gov/healthywater/surveillance/recreational/2011-2012-figures.html]), although recreational water-associated disease outbreaks in the U.S. have also been reportedly caused by adenoviruses and hepatitis A virus. While relatively few risk assessments have focused on viruses in recreational waters, several have reported higher than tolerable risk values for general viral infection and adenovirus in particular (in Lake Michigan; Wong et al., 2009), hepatitis A virus (in South Africa; Venter et al., 2007), but some (e.g., Kundu et al., 2013, van Heerden et al., 2005) have found no elevated risk (for adenovirus in both studies).

With respect to the eight epidemiological studies discussed in the EPA review, the conclusions that can be drawn from these studies is mixed. Three of the eight studies provide no statistically significant support for the association of coliphages with increased risk of disease after recreational water bathing. Of the other five studies, only two provide strong support for such associations (Wiedenmann et al., 2006 for somatic phages and Griffith et al., 2016 for F-specific phages). Three studies provide some support, but each of these with important caveats. First, the study by Lee et al. (1997) should be reconsidered by EPA as to whether it has sufficient scientific standing to provide guidance for EPA's decision-making process. One issue with using Lee et al. (1997) as a reference is that the manuscript was published in *Water Science & Technology* from an International Water Association (IWA) Health-Related Water Microbiology conference proceedings and was not subject to formal peer review. EPA epidemiologists and statisticians should review this manuscript, preferably by providing comments and questions for the study authors to answer, to obtain additional information regarding study methods (e.g., the statistical methods are described in one sentence) In addition, while Lee et al. (1997) appears to provide solid support for the correlation between F-specific RNA (FRNA) coliphages and gastrointestinal illness, the authors used *Salmonella* Typhimurium WG49 apparently without pretreating the water samples with *S*. Typhimurium WG45 to removal somatic *Salmonella* phages (as was recommended by EPA's Stetler and Williams, 1996 and Handzel et al., 1993). Thus, Lee et al. (1997) used a non-standard methodology (by current standards) that appears to have resulted in yielding data for total coliphages. In the second of the three studies that provide some support for coliphages as disease risk indicators, Wade et al. (2010) provides suggestive evidence, but no statistically significant associations were found for coliphages (as they were for enterococci by qPCR). The reason given for this was an insufficient number of coliphage detections. However, Wade et al. (2010) did report that the adjusted odds ratio (AOR) of GI was significantly higher on days when F-specific phages were detected, so this finding provides some support, but no support for establishing a NOAEL (as was reported in the Wiedenmann et al., 2006 study). Similarly, while the Colford et al. studies (2005; 2007) reported statistically significant associations between the levels of F-specific coliphages in marine beach water and several categories of reported illness, but the authors cautioned that the conclusions should not be strongly interpreted because the statistical association were based on relatively few F-specific coliphage detections (max concentration of 1 MPN/100 mL and detections in only 11% of samples). I am afraid that this (relative infrequency of detection vs. FIB) is going to be a recurring theme for coliphages as reliable indicators of fecal contamination and risk of illness.

Charge Question 2: My experience with coliphages during research on animal waste systems, domestic sewage systems, decentralized wastewater systems, and fecally impacted receiving water is that coliphages are useful as indicators of treatment system efficacy for virus removal and inactivation, but coliphages may not be reliable indicators of fecal contamination in ambient waters unless these waters receive wastewater (including stormwater) loadings from large population centers and (in the case of F-specific phages) large-volume samples (~1 L) are tested. The coliphage detection frequency and concentration data from the EPA-reviewed epidemiological studies also bear this out: somatic coliphages are typically present in surface waters at levels that are ~ 4-10 times lower than *E. coli* (and also lower than enterococci), with concentrations of F-specific coliphages being even lower. Somatic coliphages have the advantage that they can be reliably detected in 100-mL sample volumes, but most studies indicate that reliable detection of F-specific coliphages requires analysis of ≥ 1 L, and even then the chances of detection may be affected by human population size and person-person excretion variability. As part of a study we recently published (Schneeberger et al., 2015), we analyzed two single-family home septic tanks 13 times over the course of 2 years for a suite of fecal indicators. We detected somatic coliphages multiple times (5 of 13 samples) in one family's septic tank, but not in the other family's septic tank (only 1 detection in 13 samples), and detected F-specific coliphages only once between the two tanks (data not published). We also studied the presence of coliphages in larger scale decentralized wastewater reuse systems. As the size of the system increased, the chance of detecting coliphages (especially F-specific coliphages) in influent to the treatment systems increased (see table below).

Site ID	Facility Type	Scale of reuse	F-specific phage Detection frequency (Geometric Mean (GM) PFU/100 mL)	Somatic phage Detection frequency (GM PFU/100 mL)
A	Resort; golf course community with two hotels and small commercial; 900 customers	Large-scale multi-subdivision development; ~50,000 to 500,000 gallons per day (gpd) flow depending upon season	4/4 (8700)	4/4 (4×10^4)
B	Resort; residential and commercial resort community; 475 customers	Large-scale subdivision & commercial district; ~30,000 to 300,000 gpd flow depending upon season	4/4 (3×10^4)	4/4 (1×10^5)
C	Resort; retirement community; small; "residential" condo	Medium-scale condo complex; ~5,000 to 50,000 gpd depending upon season	3/3 (3500)	3/3 (1×10^5)

D	Seasonal; high school and middle school complex	Small-scale:large flow range due to school schedule	1/3 (*E. coli* GM = 7 x 10^4 /100 mL)	3/3 (6400)
E	Small cluster system serving 3 homes and a business	Small-scale : ~1,000 gpd design flow	1/3 (*E. coli* GM = 4 x 10^6)	3/3 (3000)
F	High-rise building; 35-story; > 250 units; "residential"	Medium-scale on-site; "sewer mining"; sewer backup; > 20,000 flow	1/3 (*E. coli* GM = 1 x 10^6)	3/3 (2900)
G	High-rise family with commercial aspects; >290 units	Medium-scale on-site; "sewer mining"; sewer backup; > 20,000 flow	0/4 (*E. coli* GM = 1 x 10^5)	2/4

In a past outbreak investigation (O'Reilly et al., 2007) we measured somatic and F-specific coliphages, along with FIB and a suite of pathogens, in ground water samples that were suspected to be impacted by onsite wastewater systems and associated with the outbreak. We detected *E. coli* in 6 of 13 ground water samples. We measured somatic coliphages in 3 of the 6 ground water samples that were positive for *E. coli* (and in one ground water sample that was negative for *E. coli*). We detected F-specific coliphages in only 1 of the 13 ground water samples (in a sample containing *E. coli* at 118 colony forming units (CFU)/100 mL and somatic coliphages at 3 PFU/100 mL). We did not detect F-specific coliphages in a ground water sample found to contain *E. coli* at 420 CFU/100 mL, somatic coliphages at 92 PFU/100 mL, and which was positive for adenovirus and enterovirus).

We have also observed the difficulty in detecting F-specific phages in an ongoing large-scale study of irrigation ponds, where we have only detected F-specific coliphages in 4 of 110 irrigation pond samples when we analyzed only 100 mL. When we analyzed 50-L water samples concentrated by ultrafiltration, we detected F-specific coliphages in 23 of 110 irrigation pond samples (corresponding to an approximate sample analysis volume of 15 L). Median *E. coli* for this set of surface water samples was 12 CFU/100 mL (*E. coli* detected in 96 of 110 samples), so fecal contamination of these ponds has been relatively low (and highly variable).

Charge Question 3: One the primary advantages is that coliphages are viruses present in fecal waste as part of normal gut microflora and they demonstrate similar physical properties and fate and transport characteristics as enteric viral pathogens (e.g., based on waste treatment studies showing them to be more resistant to treatment processes than FIB). They are not metabolically active, like FIB are, and have thus far not been shown to replicate in the environment as has been reported for *E. coli* (e.g., in sub-tropical climates) and enterococci (especially in conjunction with plant matter in water systems). Aspects of coliphages vs. FIB that are important include:

Fecal Indicator	Advantages	Disadvantage
E. coli	Generally present in all warm-blooded animals Excreted at relatively high levels; relatively higher concentrations in natural water vs. other indicators Narrowly-defined indicator (i.e., a bacterial species) conducive to molecular detection Standardized analytical methods for 100-mL samples available in numerous formats	Easily inactivated by disinfection systems; faster die-off in the environment Cannot tolerate salt water May replicate under warm, organic-rich conditions No rapid (< 8-h), quantitative viability method available
Enterococci	Generally present in all warm-blooded animals Excreted at relatively high levels, though lower levels than *E. coli* Relatively narrowly-defined indicator (i.e., few dominant species) conducive to molecular detection Salt water tolerant Standardized analytical methods for 100-mL samples available in numerous formats	May be more readily inactivated by disinfection systems; faster die-off in the environment (however, they are more resistant to inactivation than *E. coli* and may show similar survival as coliphages. Replicate under organic-rich conditions, especially in presence of plant matter No rapid, quantitative viability method available
Somatic coliphages	Higher excretion frequency on population basis than F-specific phages Higher excretion levels and concentrations in r and impacted water than F-specific phages Lower inactivation rates than FIB in treatment systems and environment Rapid P/A method available	Not as readily detected as FIB in impacted waters No rapid, quantitative viability method available Operational group comprised of multiple phage families complicates some analyses (e.g., molecular)
F-specific coliphages	Can provide animal versus human fecal waste source information Lower inactivation rates than FIB in treatment systems and environment Rapid P/A method available	Not excreted by all warm-blooded animals, including humans Excreted at lower levels than somatic phages and FIB; likely need to assay at least 1 L for chance of consistent detection in surface waters No rapid, quantitative viability method available Operational group comprised of multiple phage families complicates some analyses (e.g., molecular)

<u>Response</u>: Mark Sobsey

Charge Question 1: There is ample but not rich evidence documenting that human enteric viruses are important causes of illnesses associated with exposure to ambient recreational waters. Several lines of evidence based on the available scientific literature support this position as was reviewed in the EPA report. The three main types of evidence, specifically microbiological, epidemiological and risk assessment-based, are available. There is well-documented, real-word evidence relevant to exposure assessment and health effects assessment.

The exposure assessment evidence comes from the well-known and well-documented data on the occurrence of enteric and respiratory viruses in the human population, their shedding from infected human hosts and their presence in sewage and other human excreta such as sewer overflows and sewage bypasses and on-site septic systems that impact ambient waters, including those used for primary contact recreation. Virus shedding levels in feces, respiratory secretions and other excreta are often in the millions to billions per gram or mL and many of them become constituents of sewage and other sources of fecal wastes that may enter the ambient water environment. It has been well-documented for many decades that human sewage contains a wide range of human enteric, respiratory and other infectious viruses often at concentrations of hundreds to thousands of infectious units per liter and sometimes higher. When measured by molecular methods that quantify gene copies, the concentrations of these viruses are even higher. Any human enteric or respiratory virus of health concern shed by members of a population will almost certainly be present in sewage and related wastes and waste-impacted ambient waters. Since the availability of mammalian cell culture systems to detect and quantify some of these viruses, beginning in the 1950s, many of the viruses of health concern have been found and quantified in sewage and in ambient surfaces waters impacted by sewage and other human waste sources. Enteroviruses, reoviruses, rotaviruses and adenoviruses are examples of human enteric viruses have been detected regularly in sewage, discharged sewage effluents and ambient waters impacted by point and non-point human waste sources. Some of these viruses are documented causes of waterborne outbreaks. With the development of nucleic acid based molecular methods, primarily PCR and reverse transcriptase PCR (RT-PCR), in the 1990s it is now possible to detect and quantify additional enteric, respiratory and other viruses of human health concern in sewage, other human wastes and ambient waters impacted by such wastes that have difficult or so far impossible to detect and quantify by infectivity in cell culture systems. These include noroviruses, sappoviruses, astroviruses, and hepatitis A and E viruses, all of which are important because they cause human infection and illness, including documented waterborne outbreaks by some of them. All of these viruses have been detected in sewage and ambient waters.

There is credible epidemiological evidence from data of prospective cohort studies and randomized controlled trials for excess human health effects in the form various illnesses or health conditions consistent with possible and perhaps likely viral etiologies, including gastrointestinal, respiratory, eye, and skin infections, among swimmers and others with recreational water exposures. A variety of different human enteric and respiratory viruses can cause these kinds of infections and illnesses. Unfortunately, few if any studies have collected clinical samples that document the presence of human viruses or other evidence of recent viral infection such as immunological evidence, in recreational water bathers, except for a few waterborne outbreaks reported to EPA and CDC in the United States. However, based in the signs and symptoms of illness and other clinical findings, there is credible evidence to expect that at least some of the excess illness and other adverse health effects occurring in recreational bathers compared to non-bathing controls is caused by various viruses. The types of illnesses, adverse health effects and health conditions reported by swimmers are consistent with possible and perhaps likely viral etiologies.

Other studies have addressed the risks of waterborne disease from recreational exposures to viral pathogens using quantitative microbial risks assessment. Such studies have obtained data on virus concentrations in recreational water and used human infectivity dose-response data from the scientific literature to estimate the risks of viral infection and illness from recreational water exposures. Such studies provide further evidence that excess risks of infection, illness or other adverse health effects can be estimated based on measured concentrations of human viral pathogens in recreational waters.

Charge Question 2: There is ample evidence from the literature supporting coliphages as viral indicators of fecal contamination, based on them meeting the various criteria to be a suitable fecal indicator, specifically for viral pathogens. First, coliphages ARE enteric viruses that are harbored by human and other mammals and are shed fecally by these sources. They have physical, biochemical, morphological and biological properties that are the same as or similar to human enteric and respiratory viruses of health concern. Most people harbor and fecally shed coliphages of one kind or another, although the carriage rates in individual humans and animals may be somewhat variable. However, raw sewage and other fecal wastes from even small numbers of people invariably contains coliphages at typical concentrations of 100s per mL. In addition, they are applicable to and detectable in all types of water and wastewater and other relevant samples. They are often documented to be present in feces, sewage and fecally contaminated ambient waters when viral pathogens are present. In addition, their numbers are associated with amount of fecal contamination, with higher concentrations in samples that are more fecally contaminated based on other measurable properties. Coliphages outnumber viral pathogens in sewage and other fecally contaminated samples, including ambient waters. Under most conditions, coliphages do not “grow” in the environment or have non-fecal environmental sources. Although some coliphages can replicate in host bacteria in aqueous media when concentrations of both are sufficiently high, such conditions occur rarely if ever in most environmental samples, except possibly fresh raw sewage containing susceptible host bacteria. On average coliphages survive and persist as long as or greater than most human viral pathogens. However, their survival varies among the different coliphages as does the survival of different human enteric viruses, including to wastewater treatment processes. Coliphages are easily detected and quantified by simple laboratory tests in a short time, making them a practical and affordable viral indicator of human enteric viruses. Coliphages have defined characteristics that vary among the different coliphage taxonomic groups but are constant and predictable with their different groups, as are the human enteric viruses. Coliphages are also harmless to humans and other animals and therefore safe and easy to culture for the purpose of detecting and quantifying them in wastewater and water. There is some but as yet only limited evidence that the numbers of coliphages in ambient water are associated with risks of enteric illness in those exposed to them from recreational activities (that is, a dose-response relationship). However, the predictability of coliphages as viral indicators of risks of viral infection and illness from recreational water exposures requires further study to better understand and quantify these relationships.

Charge Question 3: Because coliphages are viruses that resemble human enteric viruses in a variety of relevant properties and because they meet all of the criteria of a good fecal indicator system for viral pathogens of interest and concern from recreational water exposures, they have several important advantages over and are more credible than traditional FIB. FIB do not meet as many of the desired criteria to be viral indicators of fecal contamination of recreational waters. Among other reasons, this is because coliphages are much more like human enteric virus than are FIB, they survive and persist more like human enteric viruses in wastewater, ambient waters and in response to environmental stressors and wastewater treatment process than do FIB and because unlike many of the FIB, they do not multiply, regrow or otherwise increase in numbers in sewage and environmental waters. Furthermore, some studies of wastewater treatment systems show that coliphages are reduced to lesser extents than traditional FIB by conventional primary-secondary treatment and disinfection. Therefore they are more like the human viral pathogens in their reductions by wastewater treatment systems. In some studies, coliphages and *Clostridium perfringens* spores are at higher concentrations in treated sewage effluents than are any of the traditional FIB. See Figure 1 below.

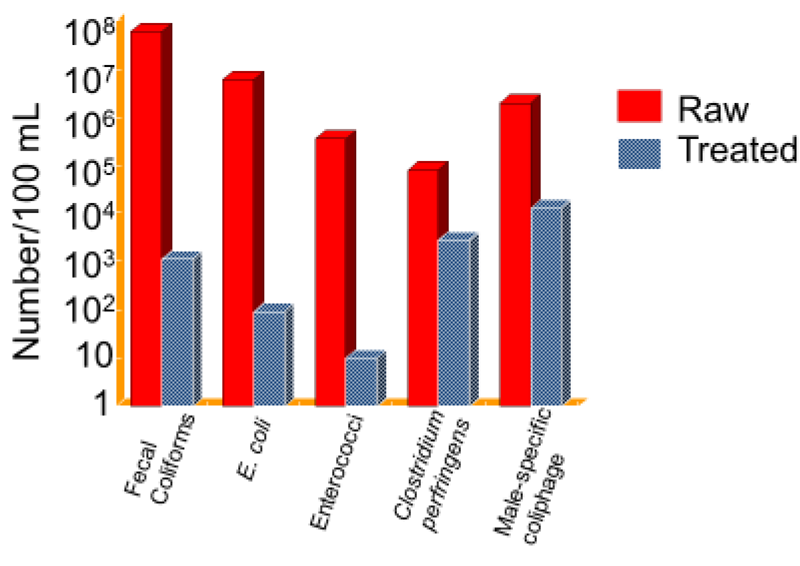

Figure 1. Microbial indicators in raw and treated wastewater (primary, secondary-treated, and chlorination; GM values of 24 bi-weekly samples)

Topic 2: Coliphage as a Predictor of Gastrointestinal Illness

Topic Leads:

- Jack Colford
- Tim Wade

Charge Questions:

In EPA's Coliphage Review, eight epidemiological studies were reviewed. Four of the eight studies found a statistically significant relationship between F-specific coliphages and gastrointestinal (GI) illness. One additional study found a statistically significant association between GI illness and somatic coliphage and suggested a no observed adverse effects level (NOAEL) of 10 plaque forming units (PFU) per 100 milliliters (mL).

1. Comment on the overall strength of the association between coliphage and human health illness in epidemiological studies conducted in ambient recreational waters.

2. Are there specific characteristics that influence the association between coliphage and human health illness (i.e., source of contamination, salinity)?

3. Are there specific conditions under which traditional FIB are not adequate to protect public health (i.e., Lamparelli et al., 2015; Marion et al., 2010) and if so, comment on the potential for coliphages to be useful in those situations?

<u>Response</u>: Jack Colford

In my opinion, the EPA has provided a thoughtfully constructed review document summarizing the published literature to date on the relationship between coliphage and human illness. I am not aware of additional published literature with evidence to address this specific question that has been omitted. The EPA review of the eight studies concludes with the statement: "Overall, the epidemiologic evidence is suggestive of a potential relationship between coliphages and human health."

Because of the limited peer-review literature addressing this question, this conclusion is based on few published results and I agree that the wording a "potential relationship" is correct given the evidence available to this point. This relationship, like all claims about associations between potential markers of illness and the illnesses themselves needs to be scrutinized critically. The charge question specifically asks about the overall strength of the association between coliphage and human health illness. When people ask epidemiologists about the "strength" of an association, the questioner is most often interested in the magnitude of the measure of association (such as the odds ratio or rate ratio or relative risk). However, equally important in the evaluation of the "strength" of an association is an examination of the potential biases that may have caused a systematic over or underestimation of the relationship. I think this potential for bias raises the most concern in the small published literature to date on the question of the coliphage/health relationship. These concerns about potential biases are important enough in my interpretation of the results to warrant a brief description of them here. The EPA has (appropriately) not attempted to synthesize the disparate results of the studies and present one summary ("meta-analytic") numeric estimate of the overall relationship.

It should be mentioned that the conduct of human health studies such as those presented is operationally difficult. The enrollment and follow-up of participants is not an easy task, the accurate measurement of coliphage and other indicators is challenging and expensive, and the analyses of the data can be complicated if not carried out in experienced hands. The potential for bias exists at each of these steps with resulting impact on the estimate of the strength (or existence) of the reported relationship. My critique of these studies begins with an acknowledgement of how difficult they are to complete and with recognition of and congratulations to the investigators who carried them out. This acknowledgement notwithstanding, I believe there are a large number of potential biases present in these studies.

Several studies found no relationship between measured coliphage and illness. The reader is left to wonder if this is simply due to the **small study sample sizes** (and resulting wide confidence intervals overlapping the null hypothesis). At times the authors, understandably, are reaching to describe their results as "positive" associations despite the lack of statistical significance (this reach is not unique to this field).

Throughout this body of work there is **a multiplicity of both exposure and outcome definitions**. In a situation such as this there is the possibility of misclassification bias (of exposure and/or outcome). Other sessions of this conference will address the question of the proper coliphage measures that should be employed, but with respect to outcome measurement I would argue that it is likely that the slightly different definitions of gastrointestinal illness and other health outcomes are reasonably aligned to allow for interpretation across studies. I think it unlikely that our interpretation of the results is impaired by this potential misclassification bias of the outcome (illness); I cannot comment yet on the potential misclassification of the exposure and will wait until I hear the presentations addressing this issue.

Very few of the studies employed the use of **negative control outcome measures**. This refers to the inclusion of symptoms and/or outcomes that could not plausibly be affected by the exposure of interest (here the exposure to coliphage). Use of negative control outcomes is a broad technique for identifying several types of bias. For example, if the swimmers and non-swimming comparison groups experienced a rate of traffic accidents after leaving the beach that showed the same strength of association with coliphage level as the association of illness to coliphage exposure, one would wonder if some sort of reporting bias was present in the data (since traffic accidents are not plausibly linked to coliphage exposure). I did not find that the authors of the reviewed studies employed negative control outcome measures.

One feature of these studies that makes it difficult to arrive at a firm conclusion about the strength of the association is the **differing choices of a counterfactual (comparison) group**. In some studies, the counterfactual for the exposed bathers is a group of non-swimmers with no water contact. In other studies, the counterfactual is a group of swimmers with lower levels of water exposure. Since non-swimmers and swimmers with low water exposure are plausibly different from each other with respect to key other variables (current health status, age, gender, etc.) the choice of which comparison group to use can impact the estimate of the strength of the association and thus bias the results.

Some of the studies employed the “gold standard” in clinical research, the **randomized controlled trial (RCT) design**. Generally, RCTs are believed to be crucial in linking exposures and outcomes because of their ability to balance characteristics in exposed and unexposed groups and to remove from the participants any choice about whether or not to undergo exposure. Although RCTs are powerful tools, I do not believe they are of critical importance for the study of this question (the association of coliphage and illness) because it is essentially impossible that swimmers and non-swimmers chose their exposure (or non-exposure) to coliphage in any way based on the coliphage levels in the water. The two RCTs presented, however, were conducted under experimental conditions that may not reflect normal beach exposure mechanisms or time...making the generalizability of the results difficult for me to interpret Additionally, the RCTs were, due to the costs and logistics required, quite small with respect to the numbers of subjects enrolled. As described above, these small sample sizes contributed to large confidence intervals which made the detection of statistically significant results more challenging even if truly present.

It is difficult to assess from the materials presented to us whether or not **publication bias** might also impact our understanding of the strength of the coliphage/illness association. It is widely known that authors who conduct studies with negative results are less likely to submit that work for publication and that journals are less likely to publish negative studies that are submitted. In the present context of these coliphage studies, publication bias could be present if authors who published recreational water studies omitted coliphage results while publishing their more traditional indicator results.

Methods in the field of epidemiology are evolving and improving – at least we hope that to be the case. A major effort is underway in the field to adhere to much more rigorous guidelines with respect to **scientific replication**. These guidelines include, for example, the prior publication of analysis plans to help ensure that authors don't "cherry-pick" the results they choose to present in their published work. Current replication standards also suggest that investigators make de-identified datasets available for public download and independent replication. To the best of my knowledge, none of these authors (myself included) followed these steps in their work because it was not common practice in the era in which these studies were conducted. Hopefully, this will occur with subsequent research on this topic. Such replication helps to ensure that the strength of an association stands up more firmly to outside review and to confirm that the findings are not due to error.

In epidemiology the term **"effect modification"** (or "interaction") is often used to refer to factors that influence the relationship between an exposure of interest and an outcome. For example, the (strong) effect seen between asbestos and lung cancer is modified by the presence of smoking in a way such that the presence of both smoking and asbestos is more strongly associated with lung cancer than would be expected by the "sum" of the two exposures independently. In the studies reviewed for the relationship between coliphage exposure and illness, there is some evidence to suggest that the relationship of coliphage to illness may be modified by subject age and by the presence of a point source discharge. This would have the immediate effect of calling into question whether coliphage levels alone, without specification of the age of the population and the location of the exposure, could be an accurate risk monitoring tool. Two papers presented in the review (Lamparelli and Marion) show associations between other fecal indicators and illness in disparate settings including an inland lake and a tropical beach. The association of coliphage or other viral indicators with illness in these settings cannot be determined with currently available data.

The confidence of the field in the use of fecal indicator bacteria for recreational water monitoring grew out of the replication of a large number of studies under various conditions; a similar pathway to understanding is likely to be required to properly understand the relationship between coliphage and illness. The studies to date have inserted coliphage measurement into studies of fecal indicator bacteria; there will likely need to be large studies focused on coliphage as the exposure of interest. In conclusion, the evidence presented is suggestive but not conclusive about the relationship between coliphage exposure in recreational water and human illness. Although I am not able to draw a firm conclusion about the relationship at this point, the current evidence does suggest that this is a promising relationship that deserves further evaluation because of its biologic plausibility and the attractive operational features of coliphage measurement that are being discussed in other sessions of this conference.

<u>Response</u>: Tim Wade

Charge Question 1: FIB such as *Enterococcus* and *E. coli* have been broadly and successfully used as indicators of water quality at beach sites, and have for the most part been established as predictors of swimming associated GI illness in epidemiological studies. However, in some notable circumstances, studies have failed to demonstrate an association, or the association has been weaker than expected. Moreover, as noted in EPA's Coliphage review, researchers have believed for many years that enteric viruses may be responsible for the excess illnesses commonly observed among swimmers. As a result, an indicator better suited to viral waterborne pathogens is theoretically well-justified. However, epidemiology studies of coliphage and human health in recreational waters are relatively few and generally limited in terms of scope and diversity compared to studies focusing on fecal indicator bacteria such as *Enterococcus* and *E. coli.* Despite the limited number and scope of the studies, several of those that have evaluated an association between coliphage and swimming associated illnesses have found fairly convincing evidence that coliphage presence, or in some cases density, was associated with illnesses among swimmers. In addition to the studies noted in EPA's review, a report by Pike et al. in marine waters also appeared to find an association with coliphage and gastrointestinal illness (Pike at al., 1994). Because this study was a precursor to randomized exposure studies by Fleisher and Kay (2006) presumably this study was at a site impacted by human sewage.

In evaluating the overall strength of the association, a first critical question concerns the illness endpoint. This should be based in part on the strength and consistency of observed associations, but biological plausibility should guide which endpoints are considered in the first place. Because the general idea behind using coliphages is that they may be a more specific indicator of fecal contamination, and in particular a better indicator of viral pathogens associated with feces, the most plausible endpoint for consideration is GI illness, and to a lesser extent respiratory illness. Endpoints such as skin rashes, ear and eye infections, could also feasibly be associated with fecal contamination, they are less strongly linked to viral waterborne pathogens, so should be considered secondary outcomes.

The epidemiology studies considered in the review are quite heterogeneous in terms of study population, design, location, water type (marine vs. fresh), water sampling, data analysis and illness outcome definition and assessment, which limits the ability to combine and assess the literature as a cohesive group or to make broad ranging general conclusions regarding coliphage and swimming associated illness. Even when a common endpoint, such as GI illness is considered, the specific definition can vary across studies, sometimes significantly, which can affect interpretation of the results. For example norovirus associated infections often produce a limited or no fever. Some definitions of GI illness used in epidemiology studies require fever in addition to diarrhea or other symptoms as part of an episode- this could result in norovirus associated illness episodes being underestimated or not counted.

Although the specific definitions varied, the most frequent endpoint associated with coliphage was GI illness which is consistent with the biological plausibility discussed above and the current understanding of coliphage as an indicator. However, the studies as a whole are varied and somewhat inconsistent in the findings. Critically, there is inconsistency in the types of coliphage measured- while some measured multiple types, some studies only measured somatic, some F-specific, and at least two only measured F-specific RNA coliphage. Only a few studies tested multiple coliphages, so for most studies associations with, for example somatic and F-specific coliphage cannot be compared. Often, sufficient detail is not provided, for example Von Schirnding et al. (1992) indicates “coliphages” were tested in addition F-specific bacteriophages, and does not report numbers of samples detected and range, only “insignificant densities…were detected”. Similarly, Van Asperen et al. (1998) only tested for F-specific RNA bacteriophage, and although the densities are reported, quantitative measures of illness associations are not reported, only a mention that an exposure response relationship was not observed. This limits the ability to assess trends across studies of potentially positive but non-significant trends and the possibility of combining data in a formal meta-analysis.

One of the strongest associations observed between coliphage and GI illness was Lee et al. (1997) in a study of canoeists and rafters in a man-made river which was highly contaminated by treated and untreated sewage and runoff. In this study, increased risk of GI illness were observed for canoeists and rafters exposed to F specific RNA coliphage between 230-320 PFU/100 ml and 690-3080 PFU/100 ml compared to 10-30 PFU/100 ml. Note that there is some question on whether these levels are correct, because one table reports the results as per 100 ml and the other as per 10 ml. Regardless, because of the high levels of contamination and because the study focused on canoeists and rafters, the generalizability of these findings to other sites and populations is questionable.

Wade et al. (2010) and Colford et al. (2007) each found positive associations with F-specific coliphage at marine beaches among a beachgoing population using a similar °design at a human impacted and a primarily non-human impacted beach. However, especially for Colford et al. (2007), the findings are limited due to few swimmers exposed on days when coliphage was present. Griffith et al. (2016) appears to provide some additional evidence under conditions when a human impact was likely but is impossible to fully evaluate because the study is unpublished and full details are not publicly available.

The study with perhaps the strongest and most convincing findings of an association and potential exposure-response was the randomized exposure study by Wiedenmann et al. (2006). This study found excess GI illness, or a NOAEL, above 10 somatic coliphage per 100 ml (note that this level is below the reference range for Lee et al. (1997) for F-specific RNA coliphage). Unfortunately, F-specific coliphage was not measured. Interestingly, no other studies have reported an association with somatic coliphage. Abdelzaher et al. (2011) also conducted a randomized exposure study, but somatic and F-specific coliphage were detected infrequently and no associations with illness were observed.

In summary, there is some epidemiological evidence that coliphage is associated with GI illness among swimmers in both marine and fresh recreational waters. However, overall, the evidence is rather inconsistent and a coherent synthesis is limited due to a wide range of methods, sites, study designs, measurement methods, etc. There is not, however, strong evidence to establish exposure response associations based on these studies as a whole. A main concern is the numbers of studies that did not detect coliphage or had few detects- this poses major issue for establishing exposure response associations or establishing threshold values.

Charge Question 2: The epidemiological studies alone conducted to date do not provide a clear answer to this. Positive associations have been found in a range of conditions, although inconsistently. Studies have found positive associations at both marine and freshwater beaches, and at sites impacted by human sources (Lee et al., 1997; Wiedenmann et al., 2006; Wade et al., 2010) as well as sites impacted by non-human sources (Colford et al., 2007). Comments by Griffith et al. (2016) would also indicate that human vs. non-human source is important as an association was only observed on days of "high risk", or likely human impact. As discussed above, the lack of detection or low concentrations of coliphage in some studies is concerning. Sites where coliphage are detected infrequently would influence the ability to detect an association with health, unless the conditions under which they are not-detected can be clearly understood. In these cases, coliphage may be less favorable than standard FIB which can usually be commonly detected. If, for example, the extent to which salinity or other factors impact the ability to detect coliphages, this could be a concern but I am not aware if these methodological limitations are an issue. In most cases, however, the low detections were at sites with non-point and likely non-human sources of fecal contamination.

As with most general indicators, it is expected that coliphages would be best associated with GI illness in a consistent manner at sites impacted by human sources of fecal contamination. Because they can also be found in the feces of other animals, which are unlikely to carry waterborne enteric viruses, this lack of human-specificity would likely limit their application at predominantly animal impacted sites, which seems to be supported in the study by Abdelhazer et al. (2011) and from the comments noted by Griffith et al. (2016). However, the study by Colford et al. (2007) seems to contradict this where some association was found with only coliphage and no other indicators at a beach impacted predominantly by birds.

One condition which may influence the association with coliphage and illness is the type of treatment sewage receives prior to discharge. In conditions where there is a relatively consistent source of raw or untreated sewage, it may be that coliphage adds little compared to standard FIB, though it certainly may perform at least as well. However at sites impacted by treated sewage or partially treated sewage, which disproportionally kills FIB and bacterial pathogens over viruses such as chlorination, coliphage may be a preferred indicator as it will likely better mimic the survival of viral pathogens.

Charge Question 3: The epidemiology literature provide little information to address this issue. Only a limited number of epidemiology studies have been conducted in a tropical environment and those that have did not test for coliphage. Theoretically, as several authors have speculated, coliphage may be a better indicator in tropical environments where FIB can persist, or potentially regrow in the environment, since coliphages do not. This may be especially true at tropical sites where sewage is at least partially disinfected, such as Hawaii. However, at this time this is speculation as no studies have addressed this directly. The EPA Coliphage Review cites one paper which observed no association between coliphages and pathogens in tropical coastal streams, which provides some evidence that coliphages may not in fact provide additional benefit in tropical environments, but the evidence is very limited in this area.

The conclusion of the authors of the Lamparelli et al. (2015) study was that FIB, *Enterococcus* and *E. coli* were in fact were well-associated with GI illness among swimmers in tropical environments where inputs were primarily raw or untreated sewage. Absolute illness rates differed and may have been higher than allowable under EPA regulations but the authors do not directly report swimming associated illness and differences could be a result of the study population, illness definition or study design, differences in the study population and the high level of contamination observed at these beaches. As discussed above, when impacts are a constant source of raw sewage, coliphage may add little as an indicator compared to general fecal indicators.

Marion et al. (2010) also observed associations with *E. coli* and in a subsequent paper found that human adenovirus in combination with *E. coli* explained more GI illness than either indicator alone. So although Marion et al. (2010) provides some evidence of the benefit of a viral pathogen-indicator, since they did not measure coliphage it is difficult to draw any conclusions directly regarding coliphages.

As discussed above, when the sources are treated sewage, or diffuse and mixed sources, coliphage may be a better indicator as it is less affected by disinfection and better mirrors the fate and survivability of waterborne pathogenic viruses. Although the NEEAR freshwater studies, which were conducted at sites with treated sewage, (Wade et al., 2008) did not measure coliphage, they speculated that the reason the *Enterococcus* qPCR measure was better associated with GI illness compared to culture was the persistence of the genetic material compared to the culturable measures, potentially better mirroring waterborne viruses' survival through the treatment process.

In summary, provided they can be detected, I would expect coliphage should be at least as effective as FIB in sites with a high level of human fecal contamination from untreated sources. However, if they are undetected at sites with moderate levels of contamination, this may be problematic. Finally they may provide an advantage when sites are impacted by treated sewage effluent or at sites with sporadic impacts of human fecal contamination (due to their longer persistence in the environment). However, epidemiology studies alone only provide partial and inconsistent support for these expectations.

Topic 3: Coliphage as an Indicator of Wastewater Treatment Performance

Topic Leads:

- Bill Burkhardt and Kevin Calci
- Naoko Munakata
- Joan Rose

Charge Questions:

In EPA's Coliphage Review, EPA summarized indicator attributes and treatment removal efficiencies of FIB, coliphages, and enteric viruses. EPA concluded that coliphages are likely a better indicator of viruses across wastewater treatment, compared to the currently recommended FIB (i.e., enterococci and *E. coli*).

1. Comment on EPA's conclusion that human pathogenic viruses are entering surface waters via wastewater treatment effluent.

2. Summarize the most important reasons that coliphages might be useful models or indicators of the behavior of enteric viruses in wastewater treatment and disinfection processes.

3. Comment on EPA's conclusion that monitoring for coliphages would be more useful than enterococci and *E. coli* in predicting human viral pathogens in wastewater treatment effluent.

<u>Response</u>: Bill Burkhardt and Kevin Calci

Charge Question 1: Human pathogenic viruses were documented in treated wastewater effluent three decades ago by Cabelli, Dufour, Havelaar, Gerba, Goyal, Bitton, and Sobsey to name a few. Contemporary data from da Silva et al. (2007); Flannery et al. (2012); Katayama et al. (2008); and Tian et al. (2012), are several that have not only detected but quantified Norovirus from treated wastewater effluent with adequate extraction and RT-qPCR inhibition controls. Data from those types of peer reviewed journals articles and USFDA data were the basis of the AEM manuscript entitled, *Meta-Analysis of Norovirus and Male-specific coliphage (MSC) Concentration in WWTPs* by Pouillot et al. (2015). The manuscript supports the conclusions of the EPA, by modeling the influent, pre-disinfected effluent and final effluent virus loads and demonstrating that, on average, wastewater treatment plants contribute human enteric viruses into the marine environment.

In addition, decades of outbreaks of viral gastroenteritis associated with molluscan shellfish, in many cases, supports the conclusion that treated wastewater can impact shellfish growing areas. Recently, the FDA has documented the impact of a variety of wastewater treatment designs, flows, and disinfection types on shellfish sentinels placed at various dilutions of effluent in the estuarine environment. The results were consistent with the EPA conclusion that secondary treatment with chlorination does not sufficiently reduce or inactivate enteric viruses in the final effluent discharge. Furthermore, the human enteric viral impact from the treated wastewater effluent can be quantified in the sentinel shellfish tissue.

Charge Question 2: Coliphages and more specifically MSC meet many of the “ideal indicator” requirements discussed by Berg, Cabelli and Goyal. Based on six facts, Kott (1981) proposed coliphage as indicators of human enteric viruses; 1) Coliphages are found in abundance in wastewater and polluted water, 2) The population of coliphages exceed those of enteric viruses, 3) Coliphages are incapable of reproduction outside the host organism, 4) Coliphages can be isolated and counted by simple methods, 5) The time interval between sampling and final result is shorter than that for enteric viruses, and 6) Many coliphages are more resistant to inactivation by adverse environments and disinfection than enteroviruses. Although dated they still hold true today.

One of the limitations of using coliphage as a surface water pollution indicator and trying to correlate them with the occurrence of human enteric virus is they do indeed have different ecologies. That issue is circumvented if coliphage are used directly as a treatment process indicator. MSC are well suited as an indicator of treatment because they are roughly the same shape, size and nucleic acid composition as human norovirus. Although coliphage and enteric virus tend to have a high affinity to sewage solids, most mechanical secondary treatment plants are not designed to slow the water enough to achieve greater than a two log reduction during the treatment process. FDA data demonstrate a substantial reduction in MSC from tertiary WWTPs.

MSC have been shown to be very resistant to chlorination as applied by most WWTPs. The amount of free chlorine in a high demand environment limits the efficacy. Combine that with relatively short contact times and the impact is further reduced to less than a log reduction. In contrast, a greater than two log reduction of MSC will be observed with UV disinfection on a tertiary system working at or below design flow. One of the great benefits of using MSC is that not only is the method facile, cheap and relatively quick, but it informs you of the viability of the organism. Unlike the molecular test which show the genetic material been conserved but cannot distinguish if the protein coat or receptors are intact. In general, it seems enteric virus inactivation by WWTP disinfection is more similar to MSC than anything else we have to compare as a process indicator.

Charge Question 3: Treatment standards and technologies have steadily improved from their inception. A large leap occurred in the 1950s by disinfecting effluent with chlorine to stay off Typhoid fever outbreaks linked to swimming in and harvesting shellfish from sewage impacted waters. The bacterial pathogen was mitigated from the effluent but the viral risk remained. In the following decades treatment plants standards continued to improve but FIB were still found commonly in effluent especially under higher than designed flows. Catchments had many combined sewage overflows that kept WWTP impacted waters easily identified by FIB analysis at water quality stations.

Currently, standards and design technologies can produce bacterial-free effluent under flow capacity well over their design. FDA data demonstrate it is specifically these instances that the MSC outperform the FIB in assessing the increased contribution of enteric viral load from the WWTP. As sanitary indicators *E. coli* and enterococci serve us well, but with respect to performance indicators of modern day WWTP viral reduction efficiencies, they do not come close.

Response: Naoko Munakata

This response focuses on Charge Questions #2 and 3, specifically on the behavior of various organisms across disinfection processes. Most data presented below is given numerically in the literature, but some were estimated based on graphs.

Because treatment depends on the process and the organism of interest, the following sections are organized by process. A 2008 Water Environment Research Foundation (WERF) report (Leong et al., 2008) estimated that in the United States, there are 4,450 major POTWs, defined by the EPA as facilities owned by a state or municipal entity and have an average dry weather design flow of >0.95 MGD. Of these plants, 70-75% were estimated to use chlorine or chloramines for disinfection; most of these plants likely use chloramines, because wastewater effluents typically contain ammonia, which reacts with chlorine to form chloramines. Approximately 20-25% of the facilities were estimated to use UV, approximately 3-4% did not disinfect, and 7 plants used ozone. Therefore, this report focuses on these disinfection technologies.

Chloramines

Chloramine disinfection is generally given as a function of the contact time (CT) value, which is the product of the total chlorine residual concentration and contact time. The total chlorine residual decays over time in wastewater effluents; CT values can use the added dose (which over-estimates exposure to the disinfectant), the final residual (which under-estimates exposure), or an integration (rarely used). The residual chosen can make a large difference in CT value; for example, if the added dose is 5 milligrams (mg)/L and the residual after 90 minutes is 2 mg/L, the “dose CT” is 450 mg-min/L, but the “residual CT” is 180 mg-min/L. For compliance with regulations such as the California Title 22 requirements for recycled water, the residual CT is used. For some of the data collected, both the dose and residual were given, but in others, only one value was provided. Figure 1 presents disinfection data for various organisms for both the dose and residual CT values.

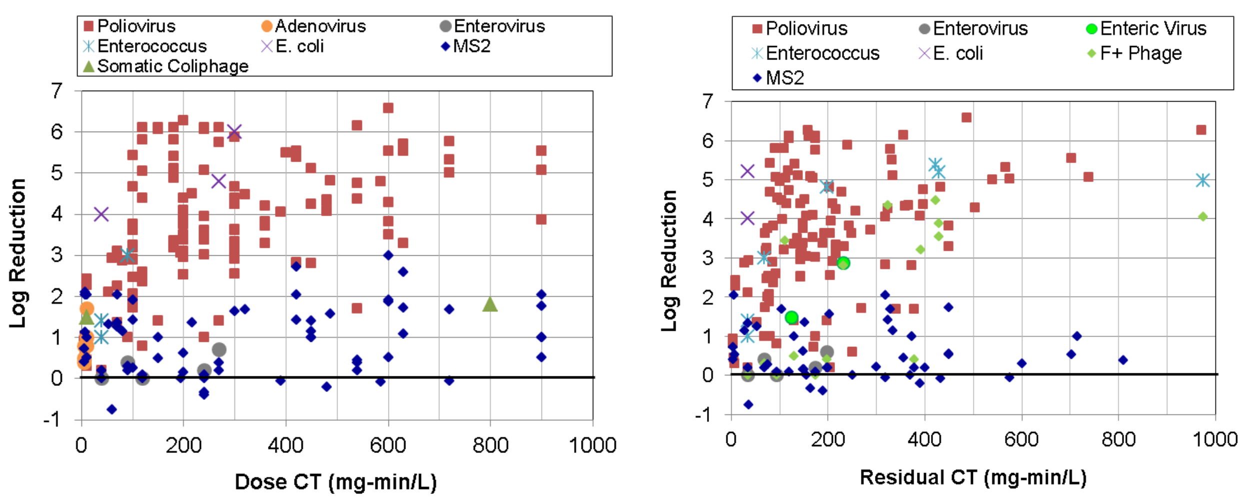

Figure 1. Inactivation of various organisms by chloramines as a function of (a) dose CT and (b) residual CT

In Figure 1, some data were not shown because they were outside the graphed time scale:

- 1(a) Somatic coliphage log reductions of 2.0-2.2 at CT values of 2700-4900 mg-min/L. (Worley-Morse, 2015).
- 1(b) MS2 log reductions of 0.3-0.8 at CT values of 1700-7500 mg-min/L (Shang et al., 2007).

Based on CT data, poliovirus disinfection appears to be highly variable. *E. coli* disinfection was similar to or slightly greater (less conservative) than poliovirus disinfection, and *Enterococcus* and F+ coliphage disinfection was within the range of poliovirus disinfection. MS2, somatic coliphage, and "enteroviruses" appear to be more resistant to chloramines than poliovirus. The enterovirus data are unexpected, because poliovirus is a member of the enterovirus genus. However, the data are from a single study that investigated seeded poliovirus and indigenous enterovirus in primary effluent, while most of the other data were collected with secondary or tertiary effluent; the authors also hypothesized that the high resistance of the indigenous virus was due to association with particles, which shielded them from the chloramines.

Overall, *Enterococcus* or F+ coliphage may be the best indicator; their disinfection was similar to poliovirus. *E. coli* appear to be less resistant than poliovirus based on the (limited) residual CT data and may therefore be non-conservative. MS2 coliphage are clearly more resistant (and therefore conservative), and it is tempting to identify them as the best indicator. However, several authors suggest that chloramines have no effect on MS2, and attribute any observed disinfection to free chlorine that exists immediately after dosing, before it reacts with ammonia to form chloramines. Consequently, MS2 may not show any disinfection by chloramines, regardless of the true level of inactivation of pathogenic viruses. The data on somatic coliphage are limited but suggest that they are similarly resistant to chloramines.

Free Chlorine

Free chlorine disinfection is also generally given as a function of the CT value. Figure 2 presents disinfection data for various organisms for the residual CT values; there were insufficient data for comparison with the dose CT values. Based on these data, MS2 appears to be a reasonable indicator of poliovirus and adenovirus disinfection; F+ coliphage appear to be more resistant.

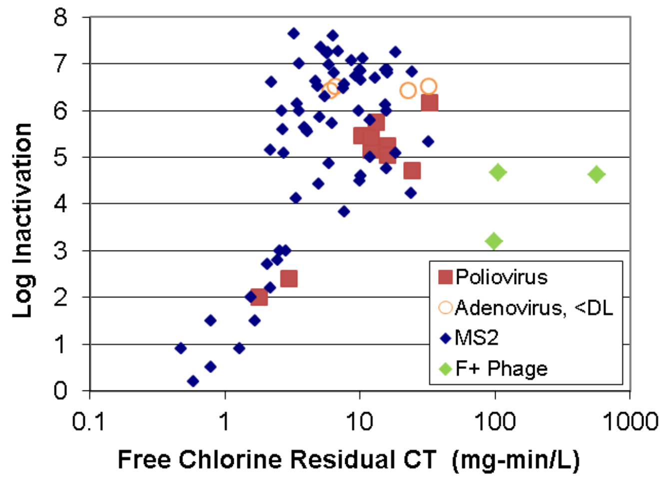

Figure 2. Inactivation of various organisms by free chlorine as a function of residual CT

UV

Over the past 10-20 years, the use of UV for disinfection has been increasing at wastewater treatment plants across the country. Figure 3 shows inactivation of various organisms by UV disinfection, primarily with low pressure UV lamps. *E. coli* was the least resistant of the organisms shown. Poliovirus, other viruses (coxsackievirus, reovirus, rotavirus, hepatitis A, Echovirus), and somatic coliphage showed similar resistance to UV; *Enterococcus* and MS2 were slightly more resistant than these organisms. Adenovirus is notoriously resistant to UV disinfection and showed a lower level of inactivation than any of the other organisms. Based on this study, *E. coli* is not an ideal indicator organism because it is not conservative relative to the pathogenic viruses. *Enterococcus*, somatic coliphage, and MS2 appear to be slightly more conservative than the pathogenic viruses, and would be better indicator organisms of most enteric viruses (except Adenovirus).

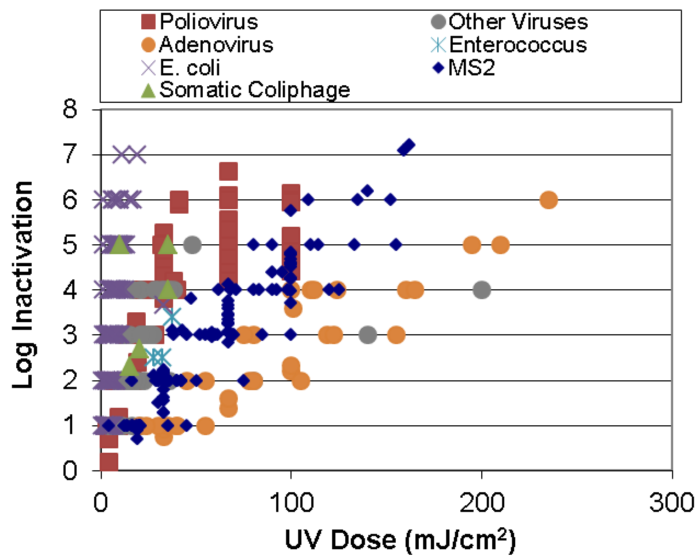

Figure 3. Inactivation of various organisms by UV disinfection.
"Other viruses" include coxsackievirus, reovirus, rotavirus, hepatitis A, Echovirus

Ozone

Ozone disinfection is often reported as a function of the transferred ozone dose, or less commonly, the applied ozone dose. In the presented study, MS2 is less resistant (conservative) than poliovirus, and therefore is not a good indicator. Enteroviruses were disinfected to below detection (>~2.8-log) at a transferred ozone dose of 5 mg/L. Thus, based on the limited data presented here, MS2 is not a good indicator organism because it is less conservative than poliovirus; *Enterococcus* may be a viable indicator.

Summary

Based on the data presented here, it is difficult to conclude that coliphage are better indicators of the behavior of enteric viruses in wastewater treatment.

- F+ coliphage (mostly tested with MS2) were slightly conservative indicators of most enteric viruses (except adenovirus) for UV disinfection, and behaved similarly to poliovirus for free chlorine. MS2 was conservative for chloramines, but may be so resistant that it shows no disinfection, regardless of the level of enteric virus disinfection; this is particularly problematic given the high percentage of POTWs using chloramines for disinfection. Finally, for ozone, MS2 was less conservative/resistant than poliovirus.
- Very limited data on somatic coliphage suggest that they may behave similarly to MS2, with little to no disinfection by chloramines, even at doses where poliovirus is disinfected. For UV, it behaved similarly to most of the enteric viruses (except adenovirus).
- *E. coli* appears to be slightly less resistant than poliovirus to chloramines, and is much less resistant than enteric viruses to UV disinfection. Therefore, it is not an appropriate indicator organism.

- *Enterococcus* may be the most promising indicator. Its behavior was similar to poliovirus for chloramines and to enteric viruses for UV (except adenovirus), and it was more conservative than enteroviruses to ozone.
- Overall, significantly more data are needed to better evaluate the ability of various organisms to serve as appropriate indicators under the different disinfection systems in use at POTWs around the country.

<u>Response</u>: Joan Rose

Charge Question 1: There is ample evidence that viruses are found in secondary effluent, yet the percentage of samples positive and concentrations are highly related to the concentrations found in raw sewage, the treatment processes and whether there is disinfection used. One of the issues is that design and operations of sewage treatment plants around the United States is highly varied.

A full scale study of six wastewater facilities in the United States concentrations of coliphage15597 ranged from 10^4 to 10^6 PFU/100 mL. The concentrations of coliphage700891 (f-amp) were more variable within each plant and ranged from 10^3 to 10^7 PFU/100 mL. Figure 1

A comparison of the concentrations of enteric viruses isolated from the untreated wastewater (MPN) ranged from about 10^2 to 10^5 MPN/100 L. Figure 2. (Enteric viruses. Samples were filtered through Virusorb 1MDS Filters (Cuno Inc.) as per EPA (1994) methodology. Filters were eluted with 1 L of 1.5% beef extract (BBL V) in 0.05 M glycine (pH 9.5, ~25°C) (EPA/Information Collection Rule [ICR]). The eluted sample was concentrated by organic flocculation and assayed for enteric viruses by the observation of cytopathic effects (CPE) on recently passed (<4 days) cell lines. MPN determinations were performed using EPA released software).

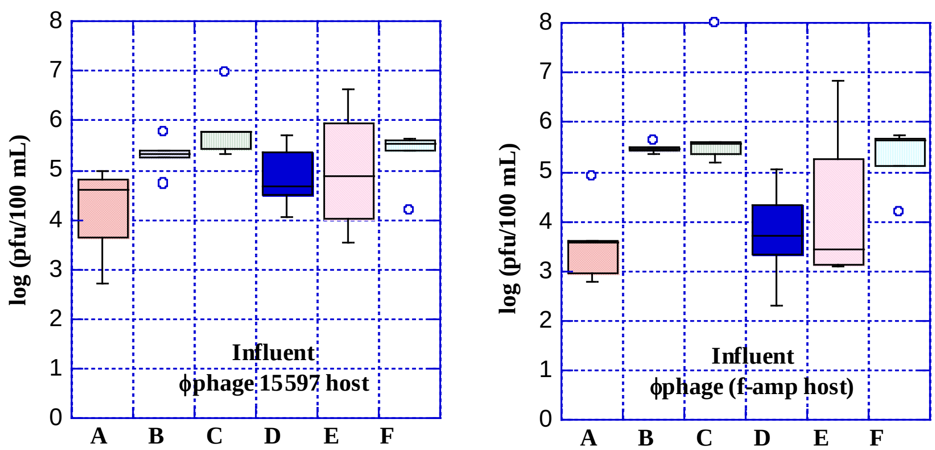

Figure 1. Coliphage in full scale raw sewage in six facilities in the United States

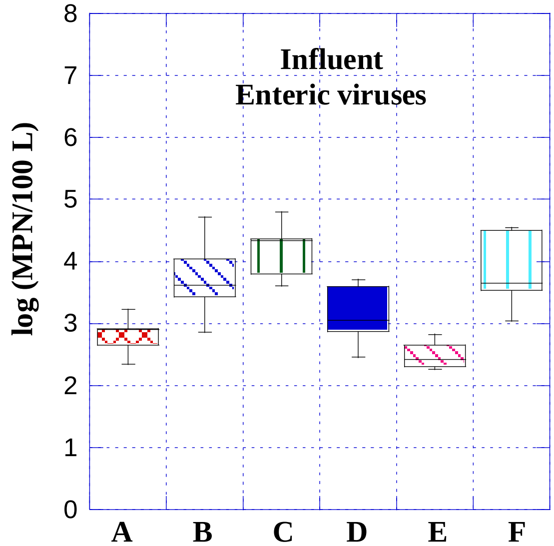

Figure 2. Cultivatable enteric viruses in raw sewage from six full scale systems in the United States

The concentrations of the ϕphage$_{15597}$ host (somatic and MS) ranged from about 10 to 10^5 PFU/100 mL and were more variable than the concentrations of the ϕphage$_{70089}$ f-amp (male-specific [MS]). For the four activated sludge facilities (A,B,C,D), coliphage levels were lowest in the secondary effluents from plant A, which has a longer mean cell resident time (MCRT, the average time that the mixed liquor suspended particles remain in the activated sludge process).

In secondary effluent the concentrations of viruses ranged from below 10 to about 103 MPN/100 L with the lowest median concentrations of enteric viruses associated with plants A, E, and F. Enteric virus detection limits for the secondary effluent samples ranged from 2.9 to 11 MPN/100 L, depending on the sample volume processed. The concentrations of enteric viruses in about 33% of the secondary effluent samples were below detection limits, with nondetects associated with all facilities, except plant C (shortest MCRT) (Figure 3).

These facilities were reclamation systems and after secondary treatment the effluent underwent sand filtration and was then disinfected so turbidities were down that might have interfered with the inactivation. After disinfection concentrations of enteric viruses were below detection limits (0.3 to 1.5 MPN/100L) in 69% of the samples. They were never detected in the disinfected effluent from facility E (UV) and rarely detected in facility B (prechlorination of filter) or D. In most cases, the detected concentrations of enteric viruses were below 1 MPN/100L (Figure 4).

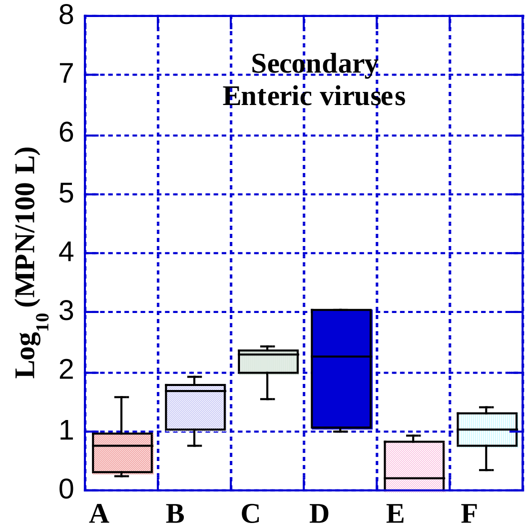

Figure 3. Enteric viruses in secondary effluent (from Rose, 2004)

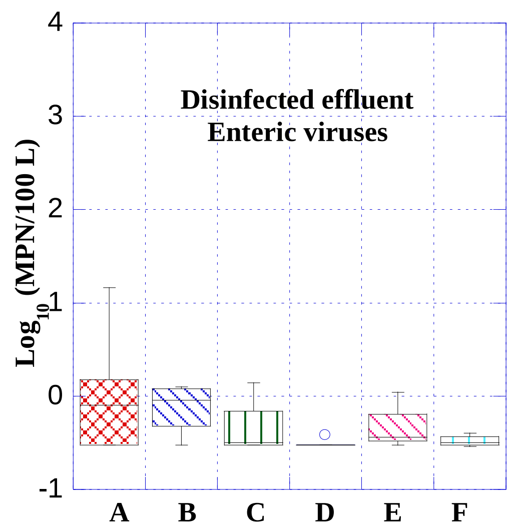

Figure 4. Enteric viruses in disinfected effluent (post filtration) (from Rose, 2004)

Many other studies have used molecular techniques and it is clear that the viral particles are present at high concentrations.

In studies in the Chicago river (Aslan et al., 2011) which received non-disinfected effluent, culturable viruses were detected in 100% of the small number of samples assayed by cell culture (13) on both BGM and A549 cell lines. The infectious virus numbers ranged from 0.12 to 33 MPN/L using BGM cells and 0.11 to 26 MPN/L using A549 cells. Average virus concentrations in limited contact recreational waters as determined by cell culture using BGM cells and A549 cells were 5.5 MPN/L and 7.6 MPN/L, respectively. For bathing beaches, average virus concentrations as determined by cell culture using BGM cells and A549 cells were 8.8 MPN/L and 2.5 MPN/L, respectively.

Charge Question 2: The viral morphology and size are associated with persistence and resistance to treatment. One of the issues is the wide range of enteric viruses and phage found in wastewater. Determining the relationships will require much more data.

We have been working with the City of Toledo on a study to examine waterborne pathogen levels in urban watersheds during wet weather events. The City of Toledo approximately doubled the wet-weather flow treatment capacity of the Bay View Water Reclamation Plant (WRP) by adding auxiliary treatment facilities that include DensaDeg® High Rate Clarification (HRC) with effluent chlorination. The objective of this study is to evaluate the disinfection efficacy of the HRC auxiliary wet weather treatment process, by analyzing pathogen removal by the HRC train in comparison to the activated sludge (AS) train.

We have found that coliphage removal was very similar to Enteric viruses in this study and it was different then the bacteria and protozoa.

Toledo rain event 5-31-2015 logarithm$_{10}$ reduction values of bacteria, protozoa, and viruses by conventional and high rate clarification treatment processes.

ORGANISM	CONVENTIONAL			HRC		
	Influent to Activated Sludge Effluent (pre-chlorination)	**Activated Sludge Effluent (pre-chlorination) to Disinfected Effluent (post-dechlorination)**	**Influent to Disinfected Effluent (post-dechlorination)**	**Influent to HRC Effluent (pre-chlorination)**	**HRC Effluent (pre-chlorination) to Disinfected Effluent (post-chlorination)**	**Influent to Effluent (post-chlorination)**
Somatic phage [a]	0.38	1.48	**1.85**	0.39	0.53	**0.93**
Male-specific phage [a]	1.21	0.64	**1.85**	0.39	0.09	**0.48**
Enterococci [a]	1.73	0.72	**2.46**	0.48	1.59	**2.07**
Campylobacter [ab]	0.00	-0.02	**-0.02**	0.02	0.00	**0.02**
Salmonella [c]	1.27	0.22	**1.48**	0.57	1.16	**1.73**
Cryptosporidium [c]	0.49	0.12	**0.62**	1.35	-0.30	**1.05**
Giardia [c]	1.20	-0.18	**1.02**	1.69	-0.38	**1.32**
Total Cultivable Virus [c]	2.84	-1.23	**1.61**	0.27	0.60	**0.87**
Adenovirus [c]	2.84	-0.84	**2.00**	-0.21	0.60	**0.39**

[a] Reduction values calculated from geometric mean values of microbial concentrations (Table 1). Non-detects calculated as detection limit.

[b] As a result of low initial concentration, reduction values will appear low. For samples returning a positive result, detection of *Campylobacter* was at or just above the limit of detection of the assay.

[c] Reduction values calculated from composite values of microbial concentrations (Table 4 and Table 6). Non-detects calculated as detection limit.

Charge Question 3: The phage in many studies have been able to address viral reductions, yet the ability to predict presence/absence or concentrations will require more data and analysis.

The presence of human enteric cultivatable viruses were correlated to phage in a coastal system in a binary regression analysis. Where the human viruses were found only after the phage reached 100 PFU/mL. This relationship may be watershed specific.

Topic 4: F-Specific Coliphages vs Somatic Coliphages

Topic Leads:

- Nicholas Ashbolt
- Juan Jofre
- Rachel Noble

Charge Questions:

EPA's Coliphage Review provides background information relevant to the use of both F-specific and somatic coliphages, as indicators of viral fecal contamination. EPA is considering these two possible viral indicators for use in future recreational water quality criteria (RWQC).

1. What are the most important advantages and disadvantages of using these two types of coliphages as (a) predictors of human health illness in recreational waters; (b) indicators of wastewater treatment performance?

2. Are there specific attributes of these coliphages or conditions (i.e., source) that influence the usefulness of the indicator, or would favor the use of one type of coliphage over the other?

3. EPA is aware that other countries have considered coliphages for various purposes. Please provide summaries and commentaries for any of these efforts you deem helpful.

Response: Nicholas Ashbolt

Charge Question 1; Point 1: What are the most important advantages and disadvantages of using these two types of coliphages as:

a) predictors of human health illness in recreational waters;
b) indicators of wastewater treatment performance?

Given that the overall review is to "…summarize the scientific literature on coliphage properties to assess their suitability as indicators of fecal contamination in ambient water" there is an apparent misunderstanding as to the value of any general fecal indicator, which coliphages are, versus "…usefulness of the indicator…" with respect to indicator uses identified in points 4.1 (a) and (b)?

Point 1(a) focusing on F-specific and somatic coliphages, the review fails to clarify that predictors of human health illness can only be identified by epidemiology studies, whereas predictors of recreator risk can be undertaken by epidemiology and/or QMRA. The second apparent misunderstanding arises from epidemiology studies in the United States largely focusing on water known to be impacted by sewage. For sewage-impacted waters is seems reasonable that enteric "viral pathogens are the leading causative agents of recreational waterborne illnesses" [p1, para 3]. However, in non-sewage(human)-impacted recreational waters, where epi data typically shows no ill effects (likely due to the lack of sensitivity in epi studies used, that can be overcome by QMRA risk estimates), clearly human enteric viruses are not likely to be the main class of pathogen causing illness. [p1, para 3 goes on to state, leaving it ambiguous as to what fecal sources by…] "This review considers coliphages as possible indicators of fecal contamination in ambient water."

> **Hence the review needs to clarify it is limited in scope to sewage-impacted recreational waters or state otherwise if that is the case. This question is fundamental to answering Topic 4.1(a)**
>
> **The fact that Abdelzaher et al. (2011) non-point study [P23] is included suggests more than just sewage-impacted is to be considered?**

As predictors of human enteric viruses, both F-specific and somatic coliphages are consistently present in sewage, hence what the review needed to do was to clarify fate and transport scenarios to compare data for these (culturable) coliphages against specific human enteric virus infectivity's (rather than a meta-analysis of publish data). Then the question (a) of predicting human health illness could be answered.

The review appears to just provide a general context background [bottom of P2 to first para of P3]; and the misleading statement [P3, para 1] "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)." This is potentially misleading as while F-specific coliphages required the F-pilus in *E. coli* (or other bacteria) to enter their host and this is thought to only be expressed at warm-blooded animal temperatures, somatic coliphages enter via the cell wall so in theory, any cold-blood animal or environmentally adapted *E. coli* or related bacterium could amplify and release somatic coliphages (which represent a very broad range of bacteriophage families).

> **We simply do not know what range of somatic coliphages may grow outside of warm-blooded animal's gastrointestinal tracts.**
>
> **This concern is backed up by the 540 indicator-pathogen pair study of Wu et al. (2011) [P27-29] where coliphages and pathogens were not significantly correlated, except for F-Specific coliphages-adenovirus pairs, which was confounded by salinity level; and by Lodder et al. (2010) [P31] both coliphage groups sig correlated with enteroviruses but not with other enteric viruses (norovirus, rotavirus and reovirus).**

Comments like [P7, para 2] "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)" portray a lack of ecological understanding, where environmental *E. coli* grow with *Cladophora glomerata* mats or in sediments at higher densities (Davies et al., 1995; Verhougstraete et al., 2010).

Yet the comment [P7, end second last para] "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." is a good point.

> **Also, F-specific deoxyribonucleic acid (DNA) coliphages are much less studied and hence poorly understood as to their environmental sources and persistence, making the broad charge question 'F-Specific coliphages' too broad – suggest the authors are generally describing F-Specific RNA coliphages.**
>
> **The term 'microflora' [e.g. used P3, 1.3 first bullet] was replaced in the 1980's with the term 'microbiota' by microbial ecologists and others, given *Bacteria* and *Archaea* are not little plants, but two separate domains of life, more distant than plants are from animals! Please correct throughout.**

[P4, bullet two] about coliphages "they are present in greater numbers than pathogens (Havelaar et al., 1990; Debartolomeis and Cabelli, 1991; Leclerc et al., 2000)" is not so clearly evident for environmental waters. For example, somatic coliphages and F-Specific RNA coliphages across U.S. sewage range 2.1-3.9 and 2.1- 3.7 $\log_{10}$ PFU/mL respectively against qPCR human adenoviruses of 1.9-3.4 genome copies/mL; but F-RNA coliphages more rapidly lost infectivity against the others, so over time in the environment higher ratios are less likely (McMinn et al., 2014). These recent U.S.-reported coliphages concentrations appear significantly lower that reported for Europe [P 6, para 2] "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)." Also, [P56, para 3] lower ranges and more variable concentrations reported for the United States and Canada by Rose et al. (2004) and Payment and Locas (2010).

> **Furthermore, seems the reviewers and authors of Abdelzaher et al. (2011) [P23, Section 3.7] were unaware that F-Specific coliphages vs somatic coliphages being more rapidly**

inactivated in warm water, as in that Florida epi study, which could have accounted for non-detects of F-Specific coliphages, whereas in the colder Avalon/Doheny waters F-Specific coliphages were detected and associated with sewage pollution and health outcome [P24 Section 3.8], but not by Boehm et al. (2009) [P32, para 2] between qPCR enteric viruses and coliphages– possible method sensitivity issues in that study? And Viau et al. (2011) [P31, para 4] found no sig correlation between F-Specific coliphages and various human enteric viruses in tropical coastal streams. Hence, as a National ambient water quality criterion, F-Specific coliphages may not be a good target group, which is a view generally backed up in the review [Environmental Factors and Fate P37-45; particularly P42, para 1 Love et al. (2010) and P43-44 Romero et al. (2011) temperature-sunlight synergistic effects].

Point 1 (b) focusing on F-specific and somatic coliphages as indicators of wastewater treatment performance

At a first pass, the review provides good evidence consistent with this reviewer's view that the highest concentration, standard method somatic coliphage group provides the largest array of viron types and highest concentrations to estimate general human enteric virus removal by wastewater treatment processes [P57-58 specifically provide this evidence]. Given all the uncertainties in pathogen assays, it seems unlikely that additional work in this area will yield a different conclusion.

As reported for Australian, coliphages are used as indicators of wastewater treatment efficacy [P59-60, by Keegan et al. (2012)]. However, what is missing in the review is the recent data from tropical Singapore on the value and development of a recreational freshwater somatic coliphages standard (Vergara, 2015; Vergara et al., 2016).

What is missing in the review is some description of the target log reduction required for safe recreational in receiving waters, so as to clarify what level of sensitivity is required to demonstrate satisfactory wastewater treatment performance, and therefore what type(s) of methods maybe applicable.

Charge Question 2: As described above, somatic coliphages provide the most dynamic range in concentration reduction by wastewater treatment and in viron types to represent a broad range of human enteric viruses. They also persist longer in the environment, so may provide a more useful conservative surrogate role compared to F-Specific coliphages.

Charge Question 3: In addition to Australian wastewater treatment identified in the review, Singapore's developing use was missed (e.g., Vergara et al., 2015)).

Response: Juan Jofre

Methodological considerations

Standardized methods, EPA and International Organization for Standardization (ISO), are available for the detection of both somatic and F-specific coliphages. ISO method for F-specific adds the protocol for differentiating F-specific RNA phages. Strains used for the detection of F-specific are *E. coli* HS and *S. typhimurium* WG49 respectively. The qualifications of the methods:

- Both methods give similar numbers of counts (Schaper and Jofre, 2000; Guzman et al., 2008)
- Strain HS seems more stable than WG49, but the stability (and re-selection) of strain WG49 is easier to check since it has explicit genetic markers whereas strain HS has not.
- Plaque reading of somatic coliphages is easier than that of F-specific.
- Somatic coliphages need a shorter time for results than F-specific phages.
- The possibility of counting both at once exist. For this purpose strain *E. coli* C3000 (Rose et al., 2004) and *E. coli* CB390 (Guzman et al., 2008) have been used as host strains. Strain C3000 detects lesser numbers of somatic coliphages as compared to strains CN13 (EPA) and WG5 (ISO). Strain CB 390 according to results reported first in Spain (Guzman et al., 2008, and already validated in the US (Sobsey et al., 2014), counts numbers similar to the sum of those detected by WG5 or CN13 and those detected by strains WG49 or F(amp) HS.
- Methods based in the detection of host lysis by determination of a molecule released by cell lysis are also available and offer the possibility of fast methods adapted to friendly use kits.

Relation of coliphages to health risk

Seven epidemiological studies conducted to evaluate the relationships between the presence of indicator bacteriophages in surface waters and swimming illnesses have been performed with disparate outcomes (U.S. EPA, 2015). Three of them only studied F-specific phages, and whereas two of them found some correlation, the third one did not. In one of the studies only considered somatic coliphages and found some relationship. In the studies where both phages were tested results are incongruent, since one of them failed to show any relationship with either somatic or F-specific phages, a second found relationship with F-specific phages and a third found the correlation with somatic coliphages.

No epidemiological studies to correlate phages and disease in drinking water have been performed. However, an overlapping between a jaundice outbreak and a high incidence of somatic coliphages in potable water occurred in a municipality of West Bengala, India in 2014 (Mookerjee et al., 2014).

Overall, the epidemiological evidence, though without sound statistical correlations, suggests a likely relationship between coliphages and human health. Also available data do not show a clear better performance of any of the two groups of bacteriophages.

Coliphages' abundance

The inputs of somatic coliphages in the environment by feces is greater than that of F-specific coliphages.

Other than feces, the main contributors of water contamination, this is raw wastewaters, secondary effluents, sludges, slurries and manure. With little exceptions, when counting in these matrixes both groups of phages by standardized methods (EPA or ISO) in the same samples, somatic coliphages overnumber F-specific phages by a factor of 5 to 10.

Thus, values of somatic coliphages in raw municipal wastewater range between $5x10^5$-10^7 per 100 mL and those of F-specific between $5x10^4$-$5x10^6$ per 100 mL (Grabow et al., 1993, Contreras-Coll et al., 2002, Lucena et al., 2003, Yahya et al., 2015, Zang and Farahbakhsh, 2007). In samples from septic tanks, somatic and F-specific coliphages were detected with values ranging from 10^6 to 10^7 PFU per 100 mL and 10^5 to 10^6 PFU per 100 ml respectively (Lucena et al., 2003).

Regarding animal contamination sources, the most reported values of somatic coliphages range from 10^4- 10^5 PFU per 100 mL in slurries and manures to $4x10^8$ in abattoir wastewaters, whereas the most reported values for F-specific bacteriophages range from 10^4 PFU per 100 mL in animal slurries and manures to $2x10^8$ in abattoir wastewaters (Grabow et al., 1993, Hill and Sobsey, 1998, Blanch et al., 2006).

The most frequent values of somatic coliphages in secondary effluents range mostly from 10^3 to 10^5 PFU per 100 mL and those of F-specific and F-specific RNA phages from 10^2 to $5x10^4$ PFU per 100 mL (Aw and Gin, 2010, Grabow, 1993, Costan-Longares et al., 2008, Zang and Farahbakhsh, 2007, Yahya et al., 2015; Gomila et al., 2008).

The concentrations of somatic coliphages and F-specific phages detected in effluents released by lagooning are variable depending on the extent of the treatment and the season. In any case, the relative proportion of somatic coliphages to F-specific RNA phages remains as in incoming water (Hill and Sobsey, 1998, Alcalde et al., 2003, Lucena et al., 2004, Gomila et al., 2008).

The same trend, this is somatic coliphages overnumbering F-specific phages, is true for primary and raw sludge. Concentrations above 10^7 PFU of somatic coliphages and well over 10^6 PFU per g of dry weight had been reported for primary and raw sludge (Guzman et al., 2007, Mandilara et al., 2006b).

Regarding surface waters, reports from diverse parts of the world regarding concentrations of both groups of phages measured by either EPA or ISO methods, after analyzing equal volumes of sample and using exactly the same procedure for phage detection in surface waters indicate numbers of somatic coliphages being greater than numbers of F-specific phages in either fresh or marine waters. With little exceptions, the gap between the numbers of somatic and those of F-specific is equal or greater than the gap in sources (Lucena et al., 2003; Rezaeinejad et al., 2014; Ibarluzea et al., 2007; Contreras- Coll et al., 2002; Choi et al., 2005; Skraber et al., 2002, Mocé-Llivina et al., 2005; Lodder et al., 2010, Love et al., 2014).

Persistence in water environments

There are numerous data reported on persistence and resistance experiments with laboratory grown model somatic coliphages and F-specific coliphages. Nevertheless, I will limit the discussion to naturally occurring coliphages.

Experiments performing *in situ* inactivation with sewage and/or waste stabilization pond effluent mixed with river or seawater in a proportion of 10 % placed in a 560 millimeter (mm) depth (300 liters) open-top chambers were done. Experiments were performed in winter (approx. 12ºC) and summer (approx. 16ºC), in the dark, and with sunlight. The calculated T_{90} for somatic coliphages ranged from 7 hours in seawater, summer and sunlight to 2303 hours in river water, winter and the dark; and the calculated T_{90} for F-specific RNA bacteriophages ranged from 4.8 hours in seawater, summer and sunlight to > 2303 hours in river water, winter and the dark (Sinton et al. 1999, 2002).

In other experiments, authors mixed raw municipal wastewater at a proportion of 2% with either river or sea water, placed the mixture in dialysis tube, with a cut-off of 14.000 Daltons, that once sealed were placed at a 20-25 centimeter (cm) depth in the same site where the river and sea water were collected (Durán et al., 2002; Mocé-Llivina et al., 2005). These sites were in the shadow during part of the day. For somatic coliphages T_{90} ranged from 53 hours in seawater and summer (>25ºC) to 385 hours in winter (6-10ºC) and river water, whereas F-specific RNA T_{90} ranged from 14 hours in seawater and summer (>25ºC) and 323 in river water and winter (6-10ºC).

Following land application of liquid pig manure Gessel et al. (2004) found that the numbers of somatic coliphages after application are always higher than the values of F-specific, and that the proportion somatic coliphages to F-specific phages rather increases in the following days.

> In my opinion, it is worth to mention that the great majority of data regarding persistence and presence of F-specific and F-specific RNA bacteriophages in receiving waters have been obtained in cold and temperate climates. However, some evidences indicating that in areas or periods with surface water temperatures higher than 25ºC the picture might be different. Thus, in the experiments of *in situ* inactivation in the summer time, the T_{90} of F-specific RNA phages becomes clearly shorter than those of somatic coliphages (Durán et al., 2002). In addition, in oxidation ponds that can be viewed as an in situ inactivation experiment, Alcalde et al. (2003) reported that in a stabilization pond series in the dry and warm desert of Judea (Israel), the F-specific bacteriophages were removed more efficiently than somatic coliphages, mostly during the summer. In the other hand, F-specific RNA bacteriophages occurring in secondary effluents are significantly more sensitive to temperatures ranging from 25ºC to 40ºC than *E. coli* and somatic coliphages (Agulló-Barceló, 2013). A pair of studies performed in the United States determined the numerical ratios between F-DNA phages and F-RNA phages in surface waters and observed that in summer the F-specific RNA phages were minority, whereas in the other seasons were more than 90% (Cole et al., 2003; Rahman et al., 2009). The abatement of F-specific RNA phages in sludge mesophilic (30-35ºC) anaerobic digestion is faster (approximately two $\log_{10}$ units versus one $\log_{10}$ unit) than that of somatic coliphages (Mandilara et al., 2006b;

Guzmán et al., 2007). Thus, in my opinion, the persistence of F-specific and F-specific RNA bacteriophages need some extra verifications before rating their persistence in warm areas.

Resistance to treatments

Primary sedimentation, flocculation-aided sedimentation, activated sludge digestion, activated sludge digestion plus precipitation and trickling filters remove both somatic coliphages and F-specific bacteriophages in numbers ranging from 50% to 99.9 %, without significant differences in the extent of reduction of the numbers of both groups of bacteriophage (Aw and Gin, 2010, Grabow, 1993, Costan-Longares et al., 2008, Zang and Farahbakhsh, 2007, Yahya et al., 2015; Gomila et al., 2008).

Depth filtration, precipitation/flocculation plus filtration and even membrane filtration does not remove differently somatic and specific coliphages found in secondary effluents (Nieustadt, 1988, Rose et al., 2004, Hill and Sobsey, 1998, Zang and Farahbakhsh, 2007, de Luca et al., 2013 , Marti et al., 2011).

Chlorination of secondary effluents in water reclamation seems to cause a major effect on somatic coliphages, but this differential effect is not great enough to change the relative proportions and somatic coliphages follow overnumbeing F-specific phages (Rose et al., 2004, Costan-Longares et al., 2008, Montemayor et al., 2008, Mandilara et al., 2006a).

Regarding radiations, UV irradiation has a significant major effect on somatic coliphages than in F-specific, and after strong UV treatments F-specific numbers surpass those of somatic coliphages (Costan-Longares et al., 2008, Montemayor et al., 2008).

Foto-oxidation (H_2O_2, TiO_2) uses to be a very effective treatment for bacteriophages, as it has been described for specific bacteriophages; but when foto-oxidizing a secondary effluent a greater elimination of F-specific bacteriophages is noticed (Agulló-Barceló et al., 2013).

Most sludge treatments required prior to its release in soil are founded in heat treatment. A major inactivation of F-specific phages can already be observed in mesophilic digestion, and all the treatments with higher temperatures increase the difference in the ratio somatic coliphages:F-specific phages (Mignotte-Cadierges et al., 2002. Guzman et al., 2007, Mandilara et al., 2006b).

Regarding the effect of soil filtration in the abatement of both groups of phages, again studies comparing both are lacking. Sinton et al. (1997) reported that in an alluvial gravel aquifer F-specific RNA phages showed greater attenuation than somatic coliphages.

General conclusion

Detection of somatic coliphages is easier than that of F-specific phages

No clear difference between somatic and F-specific phages regarding relation to risk

No clear differences regarding overall persistence in the environment (except at water temperatures over 25ºC)

No clear differences regarding overall resistance to treatments (except UV and heath)

Regarding abundance, the general trend is that somatic coliphages overnumber F-specific coliphages in the great majority of the settings, including surface, and hence recreational, waters. However, there are some matrixes such as reclaimed water in which UV plays the main role in inactivation, clayey sediments and groundwater from certain aquifers where F-specific phages have been described to become predominant.

Having these considerations in mind, the convenience of detecting both groups of bacteriophages at once has to be considered.

PROJECT IN THE EUROPEAN UNION

In 1996 we were commissioned by the European Union to lead a study aimed to determine whether methods based in bacteriophages were feasible for being applied in routine for the determination of water quality in bathing waters and to make a preliminary study of concentration of bacteriophages in a number of bathing areas through Europe (Jofre et al., 2000). More than 20 labs across Europe participated in the study. In this study, other than assessing the feasibility of methods permitted us to complete the ISO methods for the detection of bacteriophages in waters and to have.

First conclusion was that the application of all methods in routine was feasible, but that the method for detecting somatic coliphages is easier, faster and more cost effective than the methods for detecting F-specific RNA phages and phages infecting *Bacteroides* (Mooijman et al., 2005). As well some preliminary data on numbers of the different groups of bacteriophages in fresh and marine bathing waters through Europe were provided (Contreras-Coll et al., 2002).

<u>Response</u>: Rachel Noble

The discussion regarding selection of either MSC or somatic coliphage (SC) for potential criteria development for recreational waters is a complex one. MSC are typically single stranded RNA viruses (found as linear ssRNA) as Leviviridae, and when found as Inoviridae (ss DNA) or Tectiviridae (ds DNA) are DNA viruses. As such they are typically suggested to be biochemically similar to other human enteric pathogens such as noroviruses and enteroviruses. Under an electron microscope, enteroviruses such as Coxsackie virus, and MSC cannot be distinguished from one another. SC are DNA viruses, from the families Podoviridae, Microviridae, Myoviridae and Siphoviridae. Their genome can be arranged as either linear ds DNA, or circular ss DNA, they are thought to approximate the biochemical and persistence attributes of DNA-based human viral pathogens such as adenoviruses. Somatic phages have been considered in the literature to potentially replicate more readily in aquatic environments as replication does not require the F pilus interaction. In addition, somatic coliphages generally outnumber MSC in wastewater and raw water sources by about a factor of 5. To some this has been seen as a benefit (less likely to suffer from non-detect results when quantifying SC), and to some these features have been seen as strong negatives. This document is not intended as a comprehensive review of all of the past literature conducted on the two types of coliphage, rather it frames any consideration of either type or both types for criteria development in the context of the top issues featuring the most recent published (and some as yet unpublished) literature. It also highlights areas where little research has been conducted, identifying data gaps that are vital for study prior to any final decision.

1. Advantages and Disadvantages: A. Prediction of human health

In summary of studies featured in the EPA Coliphage Review. Several epidemiological studies conducted in the past decades noted a relationship with MSC. In many of those studies, somatic coliphages were not measured. There were also several studies, such as Wiedenmann et al. (2006) a relationship was observed between somatic coliphages and human health. In southern California, it was reported (Colford et al., 2007) that F+ coliphage was weakly predictive of human health outcomes. However, this result was based on a very small sample size, and there was concern by the PI group that these results should not be over-interpreted. In later epidemiological studies in Avalon and Doheny State Beach, F+ coliphage (measured using EPA Method 1602) exhibited a stronger relationship with GI symptoms than *Enterococcus* quantified by EPA 1600 at the two beaches where it was measured, but the odds ratio when considering the entire study (all risk conditions) were not statistically different from 1 for either marker. During high risk conditions, the relationships for the F+ coliphage were statistically stronger than for EPA 1600 but it must be noted that this was a small subset of the time over which the study was conducted. A molecular method, which was specific for F+ RNA Coliphage Genotype II. Interestingly, when only the high risk conditions were considered the relationship disappeared. The relationship was weak, but it was the only indicator that was statistically significantly associated with GI illness at Malibu. It should be mentioned that no indicator combinations across the studies consistently stronger predictive relationship to human health than EPA 1600. In southern California, none of the beaches are impacted by known, permitted sewage discharges, but there are confirmed human sources of fecal contamination at both Doheny and Avalon Beaches. Finally, only EPA1602 resulted in the significant relationships for F+ coliphage, not EPA1601, which calls into question the enrichment approach.

1.B. As indicators of wastewater treatment performance or as proxies of human viral pathogens (need to raise the question of quantitative comparisons of viral pathogen infectivity (cell culture) versus viral indicator infectivity (plaque formation).

There are a host of issues that prevent the ability to use coliphage enumeration as true proxies of infection by human viral pathogens. While the biochemical traits of the viruses may be similar, and therefore it is speculated that patterns of degradation and persistence would be similar, there are significant methodological hurdles to comparing human infection by viruses to coliphage infectivity as enumerated by plaque formation. The details on the methodological limitations are beyond the scope of this document, however this document will address and report relationships that have been observed in recent studies.

A completely different study design was utilized by Pouillot et al. (2015) to assess relationships of MSC loss in sewage influent versus norovirus genogroups I and II. They found that in certain types of wastewater treatment plants (e.g. lagoon systems with chlorination), norovirus loss rates were very close to those measured by MSC loss rates. Within plant systems, they observed correlations between the two groups of viruses and their loss rates. While this study was not relevant to the question at hand, it did demonstrate in a large meta-analyses context a strong correlative relationships between the mean reductions of norovirus genogroup II and MSC was observed (r=0.8). Given the scale of this data analyses, and the fact that they “judged” the quality of the reduction data by the presence of censored values, this supports the use of MSC as an index of treatment success. Whether this usefulness extends into receiving waters is still in question.

Unfortunately, to date, there have been no similar meta-analyses of somatic coliphage disinfection loss rates to this reviewer’s knowledge. Therefore, it is difficult to evaluate. However, in this framework, given that it is postulated that the main concern to public health at beaches is human enteric viruses such as norovirus, it may be that MSC are the more useful proxy between the two.

2. Differences such as source, degradation characteristics.

As far as replication in the environment, somatic coliphages have been suggested to be the most likely to replicated naturally in aquatic environments. Their replication does not require the F pilus interaction that is necessary for MSC replication. It has been previously thought that little replication occurs in the natural environment, especially at temperatures below 30C, but this has been challenged in the recent literature by Ravva and Sarreal (2016). These researchers have conducted research in waters used for produce and have lines of evidence indicating that the host utilized to assess replication in the natural system matters (environmental hosts versus laboratory provided hosts), and they have also demonstrated that fluctuations in temperatures (winter versus summer) and the presence of host during decay experiments had dramatic effects on persistence and degradation. In this published research, the study design included assessment of growth/replication of the phages in the presence of host and at different temperatures. Contrary to previously held dogma, the three of the four MSC evaluated were documented to replicate during incubation at 10 C.

There have been two recent studies that were conducted on the rates of loss of viral pathogens in relation to MSC and/or SC. In the first, as yet unpublished manuscript (Wu et al., 2016), both a first-order decay rate approach and a new Bayesian approach were used to compare rates of loss. The study showed two main results, first that SC degraded more rapidly than MSC in summer (25°C) months), and that sunlight degradation for both was more rapid than shaded. The most astounding differences in this particular study were seen between winter and summer months. In the winter condition, SC still degraded more rapidly than MSC, but the overall rates of degradation were less than one-fourth that which had been measured in the summer conditions.

Other considerations in the recently reported literature include a publication by Ogorzaly et al. (2010) that demonstrated that adenoviruses remained infective for much longer than their studied MSC counterparts, even when studied at 20°C. This study indicated that the selection of MSC alone as an indicator, at least of the highly prominent adenovirus group, was not suitable.
From Virobathe, Wyer et al. (2012), demonstrated relationships between human adenoviruses and somatic coliphage. MSC were not part of this assessment. So no further comparison is possible.

Other recently featured articles highlight issues that play into the process of examining correlative relationships between viral indicators and viral pathogens. Jones et al. (2014) compared 5 currently published methods for recovery of coliphage via either plaque assay or RT PCR and found that the recovery of the spiked samples varied from <1 to 52%.

A final note is the seasonality of SC and MSC in human sewage, as compared to the load of viral pathogens such as adenovirus, norovirus, and enterovirus in human sewage. It is unfortunate, but to date many studies have been conducted in the "off season" months for a particular pathogen and have not yielded any predictive or quantitative relationships. It is possible that measures of "pan viral pathogen" DNA or RNA could potentially be quantitatively related to MSC and SC concentrations, but to date, this type of assessment has not been conducted.

Skraber et al. (2004) considered the relationships between somatic coliphages and human viral pathogens in river waters of France and noted that the number of river water samples that were positive for viral pathogens increased with somatic coliphage concentration. However, little formal quantitative relationship was observed and was hampered due to the use of highly variable methods.

Issues for discussion:

It has been noted in the literature that there are strong limitations to some of the data analyses conducted, and those bear mentioning here.

1. Censored data, and data for norovirus and like enteric viruses at low concentrations is a major problem for data analyses. Many studies include an approach to turn non-detect values into a reported low value. Some of the assumptions utilized are faulty and that fully quantitative data for enteric viruses is a vital need prior to setting any future criteria for either MSC or SC.

2. Extreme events: researchers have almost no way of determined whether extreme events have played into the values that are observed during a study and incorporated into risk assessment frameworks, in beach water quality these can present themselves as wind erosion events, resuspension events, tidal events, to name a few. In the sewage treatment and disinfection literature, this can present itself in the volumes of untreated stormwater that are contributing to a system (thereby causing dilution), or volumes of water that are bypassing the system. Either way, the variability observed for these comparative studies is rarely all methodological and these factors must be considered for any future deployment.

3. Too little is known about "environmental strains" of MSC and SC. That is, many of the laboratory based studies that have been conducted and reported in the literature have repeatedly utilized the same strains. The diversity of SC and MSC in human and other warm blooded animal feces and the technology available warrants further examination of the true viral dynamics. Many of the strains used for degradation studies have been shared over the years from research laboratory to research laboratory and it bears in mind that the diversity of MSC and SC is huge. We need to include a range of different strains in a laboratory and field analyses decay setting. This was demonstrated recently by Ravva and Sarreal (2016).

4. A final note of consideration for the MSC versus SC discussion is that EPA has considered the possibility to utilize both as viral indicators. There is a dearth of literature comparing the two equally to constituents of interest. As a matter of fact, in all of the literature examined for this short review, less than 15% of the recent literature included even comparisons of both. This should serve as an indication of the fact that the effort required, even by the academic or agency researcher, to conduct full quantification of both, is prohibitive. Furthermore, it is much more difficult for a non adept technician in the laboratory to conduct MSC quantification. Somatic coliphage quantification is far easier because of ambient concentrations and necessary laboratory approaches. For molecular methods, the difficulty associated with quantification of MSC versus SC is even more magnified, because of the vital importance of any laboratory technician to be familiar with an array of RNA isolation and extraction techniques, the lability of RNA in a laboratory environment, and the issues arising from poor attention to detail for the all-important reverse transcription step that initiates any RT-PCR reaction. If a viral indicator is selected for criteria development, careful consideration and then the selection between MSC and SC should be one of the first actions in that process.

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