Investigation of Physical Characteristics Impacting Fate and Transport of Viral Surrogates in Water Systems

Doctoral Dissertation

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Investigation of Physical Characteristics Impacting Fate and Transport of Viral Surrogates in Water Systems

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Abstract

A multi-scale approach was used to investigate the occurrence and physical characteristics of viral surrogates in water systems. This approach resulted in a methodology to quantify the dynamics and physical parameters of viral surrogates, including bacteriophages and nanoparticles. Physical parameters impacting the occurrence and survival of viruses can be incorporated into models that predict the levels of viral contamination in specific types of water.

Multiple full-scale water systems (U.S., Italy and Australia) were tested including surface water, drinking water, stormwater and wastewater systems. Water quality parameters assessed included viral markers (TTV, polyomavirus, microviridae and adenovirus), bacteriophages (MS2 and ΦX-174), and coliforms (total coliforms and *E. coli*). In this study, the lack of correlations between adenovirus and that of bacterial indicators suggests that these bacterial indicators are not suitable as indicators of viral contamination. In the wastewater samples, microviridae were correlated to the adenovirus, polyomavirus, and TTV. While TTV may have some qualities which are consistent with an indicator such as physical similarity to enteric viruses and occurrence in populations worldwide, the use of TTV as an indicator may be limited as a result of the detection occurrence. The limitations of TTV may impede further analysis and other markers such as coliphages, and microviridae may be easier to study in the near future.

Batch scale adsorption tests were conducted. Protein-coated latex nanospheres were used to model bacteriophages (MS2 and ΦX-174) and includes a comparison of the zeta potentials in lab water, and two artificial groundwaters with monovalent and divalent electrolytes. This research shows that protein-coated particles have higher average $\log_{10}$ removals than uncoated particles. Although, the method of fluorescently labeling nanoparticles may not provide consistent data at the nanoscale.

The results show both that research on viruses at any scale can be difficult and that new methodologies are needed to analyze virus characteristics in water systems. A new dynamic light scattering methodology, area recorded generalized optical scattering (ARGOS) method, was developed for observing the dynamics of nanoparticles, including bacteriophages MS2 and ΦX-174. This method should be further utilized to predict virus fate and transport in environmental systems and through treatment processes. While the concentration of MS2 is higher than ΦX-174, as demonstrated by relative total intensity, the analysis of the variations of intensity over time shows that the dynamics are greater and have more variation in ΦX-174 than MS2 and this may be a result of the hydrophobic nature of ΦX-174. Relationships such as these should be further explored, and may reflect relationships such as particle bonds or hydrophobicity.

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H	Solvent Viscosity
τ	Diffusive Time Scale
Θ	Scattering Angle
Λ	Laser Wavelength
AGW	Artificial Groundwater
ANOVA	Analysis Of Variance
a.u	Arbitrary Unit
AWWA	American Water Works Association
BDL	Below Detection Limit
BGMK	Buffalo Green Monkey Kidney
BSA	Bovine Serum Albumin
CCL	U.S. EPA Drinking Water Contaminant Candidate List
CDC	Centers for Disease Control and Prevention
C_e	Equilibrium Concentration
CEC	Council Of European Communities
CFU	Colony Forming Units
C_o	Initial Phage Concentration
D	Hydrodynamic Dispersion Coefficient
DAL	Double Agar Layer
DLS	Dynamic Light Scattering
E. coli	*Escherichia coli*
E	Dielectric Constant
f	Fractal Dimension
F+	Male-Specific
FCV	Feline Calicivirus
FISH	Fluorescence *In Situ* Hybridization
FRhMK	Fetal Rhesus Monkey Kidney
fSWNTs	Functionalized Single Walled Nanotubes
g(Δt)	Autocorrelation
GAC	Granular Activated Carbon

HAdV	Human Adenoviruses
HFUF	Hollow Fiber Ultrafiltration
HPC	Heterotrophic Plate Count
HPyV	Human Polyomaviruses
<I>	Relative Total Intensity
IEP	Isoelectric Point
k_{att}	Attachment Rate Coefficients
k_{det}	Detachment Rate Coefficients
kB	Boltzmann Constant
K*f*	Freundlich Coefficient
k_i	Initial/Incident beam vector
k_s	Scattered Beam Vector
KL	Langmuir Rate Constant
LC-MS	Liquid Chromatography–Mass Spectrometry
LS	Light Scattering
MATH	Microbial Adhesion To Hydrocarbons
MBR	Membrane Bioreactor
MF	Membrane Filtration
MNV	Murine Norovirus
MPN	Most Probable Number
MUG	4-methylumbelliferyl-β-D-glucuronide
NoV	Norovirus
ONPG	o nitrophenyl-β-D-galactopyranoside
OPR	Ongoing Precision And Recovery
PCR	Polymerase Chain Reaction
PEG	Polyethylene Glycol
PFU	Plaque Forming Units
PMMoV	Pepper Mild Mottle Virus
PV	Poliovirus
PVDF	Polyvinylidene Fluoride
q	Wave Vector
qPCR	Quantitative PCR

qRT-PCR	Real Time PCR on RNA
RD	Regulatory Determinations
R_{eff}	Effective Radius
R_H	Hydrodynamic Radius
RMSD	Root Mean Square Difference
RNA	Ribonucleic Acid
rs	Spearman Rank Correlation Coefficient
RTCR	Revised Total Coliform Rule
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
SAL	Single Agar Layer
SPSS	IBM's Statistical Package For The Social Sciences
SWTR	Surface Water Treatment Rule
t	Time
Δt	Time Lag
T	Absolute Temperature
$TCID_{50}$	50 Percent Tissue Culture Infective Dose
TMV	Tobacco Mosaic Virus
TTV	Torque Teno Virus
U.S. EPA	United States Environmental Protection Agency
U.S. PHS	United States Public Health Service
U_E	Electrophoretic Mobility
UV	Ultraviolet
WHO	World Health Organization
WWTP	Wastewater Treatment Plant
z	Zeta Potential

1.0 INTRODUCTION

A pathogen is a microorganism capable of causing a disease in a host. Pathogens are excreted in the feces of infected humans and animals, and may directly or indirectly contaminate water intended for human consumption (Figueras *et al.*, 2010). Hundreds of different enteric microorganisms are known to inflect humans, and more than 140 of them are known waterborne pathogens (Figueras *et al.*, 2010; Reynolds *et al.*, 2008). Pathogens capable of causing waterborne illness include viruses, bacteria, and protozoa. The impact of waterborne pathogens in humans is often acute gastrointestinal disease. Immunosuppressed subpopulations are more likely to be infected and experience morbidity and mortality resulting from waterborne illness (Reynolds *et al.*, 2008). While testing for all enteric pathogens in drinking waters would be ideal, it is not practical because of time and financial constraints. Therefore, indicator organisms are used to assess the potential presence of pathogenic microorganisms (Yates, 2007). Although, in the future next generation sequencing may provide options for analyzing several pathogens at once.

While bacteria are commonly used indicators of drinking water quality, disease causing pathogens in developed countries are more often not bacteria but rather viruses or protozoa (Barwick *et al.*, 2000; Blackburn *et al.*, 2004; Lee *et al.*, 2002; Levy *et al.*, 1998). In addition, outbreaks in the United States may occur more frequently than recorded, owing to the limited ability of *E. coli* to represent viral presence (Craun *et al.*, 2010). Until recently, it was both difficult and time consuming to test for viruses. In addition, literature demonstrates that viruses and bacterial indicators do not co-locate exclusively with infectious viruses, because coliforms respond differently to environmental stressors and engineered treatment processes than do protozoan and viral pathogens (Abbaszadegan *et al.*, 2008; Hijnen *et al.*, 2010; Mayer *et al.*, 2008; Nasser *et al.*, 1995). Given these limitations, alternative indicators for viral pathogen risk are necessary.

This research examined indicators of viruses in water systems and environmental samples and then identify characteristics of indicators that are critical for predicting virus behavior. This research is unique in that it includes the investigation of viruses at multiple-scales, including full-scale water systems, lab scale batch analysis, and nanoscale particle analysis in order to investigate physical characteristics impacting fate and transport of viral surrogates in water systems.

This research was used to investigate the occurrence and physical characteristics of viruses which may impact treatment and survival in drinking water treatment. The outcomes of this research expand the current methodologies of nanoscale research by providing a time specific analysis of particle behavior. Determining the dynamic behavior of individual nanoparticles expands the current knowledge of viral transport in treatment processes, which is mainly based on size exclusion.

The objective of this research was to examine indicators of viruses in waters systems and identify characteristics of indicators critical for predicting virus behavior. This research considered viruses in water systems at multiple-scales. The results show both that research on viruses at any scale can be difficult and that new methodologies are needed to analyze virus characteristics in water systems. The objectives included nanoscale analysis using time-dependent light scattering to observe phage infection of bacteria and nanoparticle dynamics.

1.1 VIRAL INDICATORS IN FULL-SCALE WATER SYSTEMS

Hypotheses:

- Torque Teno Virus (TTV) is an improved indicator of viral pathogen risk compared to coliforms and coliphages.
- TTV present in wastewater, and drinking water samples and compares to the presence and concentration of other indicators.
- TTV is correlated to viral pathogen presence in multiple matrices and in different geographical locations.

TTV is a small, unenveloped DNA virus that occurs in different serotypes between non-human animals and humans. It may exhibit similar transport characteristics to pathogenic enteric viruses (Bendinelli *et al.*, 2001). The data is used to assess the ability of the indicator systems to evaluate viral pathogen risk either singly or as a suite of indicators. The data was also expanded and used to correlate indicator presence in Pisa, Italy (University of Pisa), and Brisbane, Australia (Commonwealth Scientific and Industrial Research Organization and the University of Queensland).

Samples collected include animal feces, wastewaters, source waters and treated drinking waters in four regions of the United States (U.S.) Samples were monitored for indicators (total coliforms, *E. coli*, and coliphages), traditional water quality parameters (such as pH, turbidity, total organic carbon and dissolved organic carbon), and TTV occurrence using polymerase chain reaction (PCR) analysis. Statistical analyses were conducted using IBM's Statistical Package for the Social Sciences (SPSS). The Spearman rank correlation equation was used to identify correlations among indicators. An analysis of variance (ANOVA) was used to determine both seasonal and temporal variability and also specificity to human versus non-human sources.

The expanded data set included samples from Italy including pathogen presence in wastewater treatment plant, and environmental water samples. Their data includes total bacterial count, somatic coliphages, *E. coli* and Enterococci. In addition, data from Australia included the presence of adenovirus, polyomavirus, TTV, somatic phage, *E. coli* and Enterococci in stormwater and wastewater systems.

1.2 LAB SCALE ANALYSIS OF VIRAL SURROGATES

Hypotheses:

- Adsorption of viruses is impacted by media properties, ionic strength, and pH, and altering these factors through batch scale tests will provide insight into viral characteristics.
- A representative latex sphere with appropriate size and surface characteristics will correlate to surrogate viruses during batch scale adsorption.

In order to determine adsorption to the removals of viruses, 26 nm fluorescent nanospheres and two bacteriophages, MS2 and ΦX-174 were studied. The nanospheres used were uncoated latex

particles and latex spheres coated in casein protein. Adsorption factors of surrogate viruses and nanospheres were compared in batch scale tests using ANSI/AWWA B100 filter sand media and varying water quality. Sorption to a hydrocarbon (dodecane) was also explored in order to study hydrophobicity with the use of a microbial adhesion to hydrocarbons (MATH) test. Data collected in the lab scale analysis were used to conduct a correlation analysis to determine relationships between removal rates, nanosphere type, and media.

1.3 NANOSCALE ANALYSIS UTILIZING DYNAMIC LIGHT SCATTERING

Hypotheses:

- Using artificial groundwater to provide an aqueous environment alters zeta potentials of water treatment media, and surrogates
- Dynamic light scattering provides information about virus characteristics including particle interactions and shape
- Virus dynamics in an aqueous environment correlate to particle zeta potentials

Dynamic light scattering techniques were used to define electrostatic properties, and observe time dependent behavior of the particles including particle kinetics, shape and size. Properties of electrostatic interactions were determined by examining zeta potential. The magnitude of the zeta potential gives an indication of the potential stability of the colloidal system. Physical properties of the viruses were determined by extracting data from changes in the intensity and wave vector of the scattered light.

2.0 BACKGROUND

Indicator organisms are used to establish potential risk from fecal contamination in drinking waters. Bacterial groups, such as total coliforms, are commonly used to indicate the microbiological quality of water and their detection is a component of drinking water regulations. Total coliforms and *E. coli* are used as indicators of fecal contamination in regulations by the United States Environmental Protection Agency (EPA), the Council of European Communities (CEC), and the World Health Organization (WHO). However, waterborne viral outbreaks have occurred in treated drinking water systems where the systems were in compliance with regulations (Craun *et al.*, 2006). Thus, coliform measurement is an imperfect indicator of public health risk from viruses.

There are many different indicator organisms, and not one single indicator is appropriate for every type of water system. Factors including detection methodology and survival rates influence the validity of an indicator system. The most important attribute of an indicator is a strong quantitative relationship between indicator concentration and the degree of public health risk; therefore, a strong correlation between the indicator concentration and pathogen levels is vital (Yates, 2007). Bonde (1966) first described the ideal qualities of an indicator. These qualities include criteria for indicators of public health risk and treatment efficiency. The requirements for an appropriate indicator state that an indicator should:

- Be present whenever the pathogens are present;
- Be present only when the presence of pathogens is an imminent danger (i.e., they must not proliferate to any greater extent in the aqueous environment);
- Occur in much greater numbers than the pathogens;
- Be more resistant to disinfectants and to the aqueous environment than the pathogens;
- Grow readily on simple media;
- Yield characteristic and simple reactions enabling, as far as possible, an unambiguous identification of the group;
- Be randomly distributed in the sample to be examined; and
- Grow widely independent of other organisms present, when inoculated in artificial media (i.e., the indicator bacteria should not be seriously inhibited in their growth by the presence of other bacteria) (Bonde, 1966).

While this is one definition of indicators, several other indicator characteristics have been developed by subsequent researchers. These include requirements for indicators to correlate to health risk and infectious pathogens, be cost effective, have minimal risk to the analyst, be measured in a time- and cost-efficient manner, transport similarly to pathogens in treatment and the environment, and be specific to the source of origin (Payment *et al.*, 2003; Yates, 2007). The American Water Works Association (AWWA) recommends that the selection of appropriate indicators of fecal contamination and waterborne pathogens should be based on their survivability in water, susceptibility to water disinfectants, and capability to detect increased health risks (AWWA, 2006b).

Indicators can be utilized for many different reasons. These include detection of fecal contamination, detection of wastewater contamination, determination of potential pathogenic organism presence, treatment system efficiency, and subsurface transport. The choice of an indicator is critical for assessment of the specific situation for its use. There is no single indicator that is appropriate for all situations, and there is not one single method for analysis. Although there is no perfect indicator organism, research is ongoing to determine a rapid, relatively inexpensive and accurate method of assessing the microbial quality of water (Yates, 2007).

In the future, there may be option for testing for multiple pathogens at once. Microarray assays may allow for monitoring water quality for multiple pathogens at once on a real-time basis. Current research has found that the techniques involved need to be further developed to overcome issues such as sensitivity, contamination, interference, bias and inhibition, particularly with environmental samples (Gilbride *et al.*, 2006; Girones *et al.*, 2010). Alternatively, next generation sequencing provides a high throughout put cost effective method for identifying microorganisms. Ducey *et al*. (2013) used next generation sequencing to identify the microbial community structure of anaerobic wastewater lagoons and determined that next generation sequencing could be used as a complimentary assay in identifying members of bacterial communities not identified by other methods (Ducey *et al.*, 2013). In the future, similar methods could be used for drinking water research.

2.1 TRADITIONAL BACTERIAL INDICATORS

Various bacterial groups have been commonly used to indicate the microbiological quality of water. These indicators provide a method for identifying the potential presence of pathogens. Public health regulations provide specific requirements and methods to analyze risk. This is particularly important because the reuse of wastewater effluent as a drinking water source and agricultural irrigation is becoming more of a necessity in areas in which water is scarce. Exposure to wastewater effluent requires additional measures to ensure that there are no exposures with negative public health impacts (Carducci *et al.*, 2009).

The United States (U.S.) has been using bacteria as indicators for water quality since 1914. Currently, the U.S. Environmental Protection Agency (EPA) requires drinking water suppliers to monitor for total coliforms on a routine basis; the frequency of testing is dependent upon system size. If the total coliform results are positive, then the suppliers are required to conduct repeat samplings for total coliforms and also to test both for fecal coliforms and for *Escherichia coli* (U.S. EPA, 1989b). Using coliforms as an indicator of contamination has several benefits based on their long history of use and standardized methods for detection. The coliform group has been used as an indicator of water quality for over 100 years; this history provides a depth of research knowledge that new methods cannot provide. These data allow researchers to compare data on past water quality. In addition, total coliforms are used worldwide, allowing for detailed comparisons of water quality around the world. The long history of use also provided the standardization of detection methodologies. There have also been recent advances in field tests for coliforms, making these tests appropriate for water quality monitoring in remote locations where challenges to conducting laboratory tests may exist (ADWG, 2010).

2.1.1 Coliform Bacteria

Coliform bacteria, termed the "total coliform" group, are the most commonly used indicator organisms for assessing the microbiological quality of drinking water and treated water effluent, and are the primary standard for potable water in most of the world (Payment *et al.*, 2003). Coliform bacteria are facultative anaerobic, Gram-negative, non-spore-forming, indole-negative, rod-shaped bacteria that ferment lactose with acid production in 24 to 48 hours at 35°C (APHA *et al.*, 2012; Ashbolt *et al.*, 2001). There are 16 species of total coliforms found in soils, plants, and animal and human waste. Standard methods for the detection of the coliform group include the multiple tube fermentation technique, the membrane filter technique, and the enzymatic substrate coliform test (APHA *et al.*, 2012). In addition, there are several other microbiological methods including rapid culture based methods such as immunomagnetic separation, (Attinti *et al.*, 2010)/culture, and gene sequence based methods such as polymerase chain reaction (PCR) and fluorescence *in situ* hybridization (FISH) (Ashbolt *et al.*, 2001).

2.1.2 Fecal Coliforms

Fecal coliforms (now commonly renamed thermotolerant) are coliform bacteria which grow and ferment lactose with the production of acid at 44.5°C. Bacteria in this coliform subgroup are more tolerant than other coliforms of elevated temperature (thermotolerant) and have been found to have a positive correlation with fecal contamination of warm-blooded animals (Figueras *et al.*, 2010; Toranzos *et al.*, 1997).

Some fecal coliform bacteria, specifically those belonging to the genus *Klebsiella*, have been isolated from environmental samples in the apparent absence of fecal pollution (Figueras *et al.*, 2010). Fecal coliforms display a survival pattern similar to bacterial pathogens, and therefore are limited as indicators of protozoan and viral contamination (Ainsworth, 1990). *E. coli* is one of the six types of thermotolerant fecal coliform found in animal and human waste.

2.1.3 *Escherichia coli*

Escherichia coli (E. coli) are thermotolerant coliforms 0.5 to 2.0 μm in size, lack urease and produce β-glucuronidase (Yates, 2007). *E. coli* are often used as a fecal indicator bacteria because they are nearly always present in the digestive tract of humans and other warm blooded animals in high numbers (Brenner *et al.*, 1982). *E. coli* are also capable of multiplying in the environment. *E. coli* from sewage were shown to immediately increase by about three $\log_{10}$ in number when simple nutrients were added to natural soil and fecal coliforms increased by two $\log_{10}$ within 24 hours when a minimal amount of sewage was added to soil. This indicates that the environment can provide sufficient means to support the growth of fecal coliforms and *E. coli* (Byappanahalli *et al.*, 1998). In contrast, the presence of *E. coli* in drinking water indicates recent fecal contamination because the organism does not generally multiply in drinking water systems (Brenner *et al.*, 1982). *E. coli* can be detected in water samples using elevated temperatures and an enzyme substrate system specific to *E. coli*. The enzyme system allows for fluorogenic detection of methylumbelliferyl-D-glucuronide (MUG) with methylumbelliferyl moiety, which fluoresces after irradiation with long-wave ultraviolet radiation (APHA *et al.*, 2012).

Confusion regarding the use of *E. coli* as an indicator organism can occur, as some strains are pathogenic. Pathogenic strains of *E. coli* have been categorized into seven groups: enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAggEC), diffuse

adherent *E. coli* (DAEC), and enterohemorrhagic *E. coli* (EHEC), also known as shiga toxin-producing *E. coli* (STEC). The EHEC/STEC strains include *E. coli* O157, a rare strain of *E. coli* associated with potentially dangerous outbreaks and illness. *E. coli* O157:H7 has been responsible for illnesses in recreational swimmers and for deaths linked to food and waterborne illnesses (Ashbolt *et al.*, 2001; Olsen *et al.*, 2002). One of the largest waterborne pathogenic *E. coli* outbreaks in the United States occurred in 1975 at Crater Lake National Park in southwestern Oregon. More than 2,000 individuals experienced gastrointestinal disease. The causative organism was an ETEC strain (serotype 06) that produced both heat-labile and heat-stable toxins. This serotype was found in both water and fecal samples (AWWA, 2006b).

2.1.4 Fecal Streptococci

Fecal streptococci (Schröter *et al.*, 1999) occur in the intestinal tracts of humans and many animals and rarely multiply in the environment. FS include enterococci, streptococci bovis, and streptococci equinus. The preferred FS indicator of human fecal pollution is enterococci. The EPA recommends the use of enterocci monitoring in salt water with suitable levels defined as 35 CFU/100 mL for a 30-day mean and 104 – 501 CFU/100 mL for a single sample (U.S. EPA, 1986a). This group of organisms has some advantages over the coliform group as indicators, including that these organisms rarely multiply in environmental waters, are more resistant to treatment processes, are more resilient in environmental systems, and survive longer in the environment (Yates, 2007).

FS were once thought to be a useful indicator for fecal source tracking. In the 1950s, the fecal coliform to fecal streptococcus (FC:FS) ratio was proposed as a method for determining fecal contamination source (Croft, 1959). A ratio of 4 or greater was thought to indicate human pollution, whereas a ratio of 2 or less may indicate animal pollution (Feachem, 1975; Hai *et al.*, 1982). This method was found to be inconsistent and unable to reliably indicate contamination type for ratios between 2 and 4. It was also observed that the FC:FS ratio is variable and dependent on surrounding land use, location, and temperature. The ratio is variable because fecal coliforms and fecal streptococci are found to have environmental survival and regrowth rates that are different (Gannon *et al.*, 1989) and react differently to temperature and sediment particle size (Howell, 1977). The FC:FS ratio was removed from the 15th edition of Standard Methods for the Examination of Water and Wastewater (APHA, 1981) when it was determined that different species had significantly different die-off rates for the bacteria in water.

2.1.5 Methods of Detection

In 2004, the National Research Council (NRC) defined the following ideal qualities for the methods of detection of indicators:

- Specificity to desired target organism (independent of matrix effects);
- Broad applicability;
- Precision;
- Adequate sensitivity;
- Rapidity of results;
- Quantifiable;

- Measures viability or infectivity; and
- Logistical feasibility (such as training and personnel requirements, utility in field, cost, and volume requirements) (National Research Council, 2004).

In drinking water supply systems, monitoring for total coliforms, fecal coliforms, and *E. coli* is regulated under the Total Coliform Rule (TCR) (U.S. EPA, 1989b). There are several approved methods for coliform monitoring under the TCR. These methods include the Most Probable Number (MPN) method, the Membrane Filtration (Murphy *et al.*, 1983) technique, and the Minimal Medium ONPG-MUG (MMO-MUG) Test, which is more commonly known as the chromogenic substrate method.

Standard Methods 9221 includes the MPN method (APHA *et al.*, 2012). This method enumerates coliforms based on the production of acid and/or gas from lactose in liquid media (Ashbolt *et al.*, 2001). This method includes serial dilutions of water samples. The response of coliform presence or absence in the media is indicated for each dilution after incubation. Statistical tables then are consulted in order to estimate the most probable number of coliform, fecal coliform, or *E. coli* density in the sample (APHA *et al.*, 2012).

The MPN method detects total coliforms based on the ability of the coliform group to ferment lauryl tryptose broth or lactose broth and produce acid and gas within 24 ± 2 hours or 48 ± 3 hours at 35 ± 0.5°C. Fecal coliforms are measured by inoculating EC broth with a portion of each sample, incubating at 44.5 ± 0.2°C and assaying for gas production within 24 ± 2 hours. (APHA *et al.*, 2012).

Standard Methods 9222 includes the membrane filtration (MF) method which involves passing a water sample through a 0.45 μm (pore size) filter, which traps bacteria and other particulate matter. This filter is then transferred to a saturated pad of m-Endo or Lawrence Experimental Station (LES)-Endo broth (for total coliforms) or m-FC broth (for fecal coliforms) in a petri dish (APHA *et al.*, 2012). The petri dishes are incubated at 35 ± 0.5°C (for total coliforms) or 44.5 ± 0.2°C (for fecal coliforms). After 24 ± 2 hours on m-Endo or LES-Endo broth pads, total coliforms appear as pink or dark red colonies with a metallic green surface sheen. Colonies are counted under a dissecting microscope and reported as colony forming units (CFU) per 100 mL sample. Fecal coliforms are identified as blue colonies on m-FC broth pads and contrasted with gray or cream-colored non-fecal coliforms. *E. coli* are confirmed by subculturing fecal coliform colonies onto nutrient agar containing MUG substrate. Samples are incubated for 4 hours at 35 ± 0.5°C, during which time *E. coli* positive colonies become delineated with blue fluorescence (APHA *et al.*, 2012).

The enzyme substrate method was developed because MPN and MF have several disadvantages, including lengthy incubation times (up to 96 hours for MPN confirmation), potential interference by heterotrophic plate count (HPC) bacteria, and difficulties in interpreting results as a result of bacteria are stressed or injured. Injury may be related to a number of factors, including time and temperature of exposure, disinfection levels, strain of organism, concentration of nutrients, presence of heavy metal ions, antagonistic standard plate count bacteria, and possibly other, undefined chemical and physical parameters (McFeters *et al.*, 1982). In addition, separate testing procedures are required to detect fecal coliforms. The enzyme substrate methods for coliforms and *E. coli* were introduced in the 1990s. An advantage of enzyme-based methods is they also detect traditionally non-culturable coliforms (Ashbolt *et al.*, 2001). These methods allow for significant improvements in the recoveries and identification of indicator bacteria. The enzyme substrate methods also allow for detection by specific enzyme substrates without harsh selective agents.

The enzyme-based method allows for a less complex interpretation of results for both total coliforms and *E. coli* within 24 hours with a reported detection sensitivity of 1 CFU/100 mL (Olson *et al.*, 1991).

Standard Methods 9223 includes the Colilert® Method. Colilert® utilizes two active substrates, o-nitrophenyl-p-D-galactopyranoside (ONPG) and 4-methylumbelliferyl-p-D-glucuronide (MUG), which are combined to simultaneously detect total coliforms and *E. coli*. Total coliforms produce the enzyme B-galactosidase, which hydrolyzes ONPG and thereby releases o-nitrophenol, to produce a yellow color. *E. coli* produces the enzyme P-glucuronidase, which hydrolyzes MUG and forms a fluorescent compound that can be detected using a longwave UV light (APHA *et al.*, 2012). In a study of 261 drinking water samples and 77 bathing water samples analyzed in duplicate by Standard Methods, the Colilert® method was found to be more sensitive than multiple tube fermentation or membrane filtration in detecting coliform bacteria and of equal sensitivity in detecting *E. coli* (Eckner, 1998). Additional information on this methodology is included in the Research Methods (Section 5.0).

2.2 UNITED STATES DRINKING WATER REGULATIONS

Current drinking water standards rely on bacterial indicators such as coliforms to identify contamination. For example, the EPA Safe Drinking Water Act includes the Total Coliform Rule which is based on the premise that, "the presence of any coliforms in drinking water suggests that there may be disease-causing agents in the water" (U.S. EPA, 2010b). Specifically, the Total Coliform Rule includes a maximum contaminant level (McLennan *et al.*, 2009) of 5 percent positives among monthly samples for total coliforms in drinking.

Drinking water standards based on coliforms are used throughout the world. The United States Federal Governments formally implemented drinking water standards in the late-19th and early 20th Centuries. The United States Public Health Service (U.S. PHS) was established in 1893 by the Interstate Quarantine Act (U.S. Public Health Service, 1893) and was tasked with investigating infectious diseases and drinking water standards. The Interstate Quarantine Act was amended in 1912 with the first Federal drinking water regulation, which prohibited the use of common drinking cups on carriers of interstate commerce (U.S. Public Health Service, 1912).

In 1914, the U.S. PHS adopted, as an amendment to the 1893 Interstate Quarantine Act, the Bacteriological Standard of Purity for Drinking Water Supplied to the Public by Common Carriers in Interstate Commerce (U.S. Public Health Service, 1914). Interstate carriers (such as passenger trains) that supplied water to the public were required to meet new standards. The criteria established included a 100 organisms/mL limit for total bacterial plate count, and not more than one of five 10 organisms/mL portions of each sample examined could contain *Bacillus coli* (now called *E. coli*) (U.S. Public Health Service, 1914). In 1917 the American Water Works Association recommended that this standard be considered for all water works, not only for interstate traffic (Orchard, 1917). While the Federal standards applied only to the interstate traffic, states generally used these standards in developing their own state-level regulations for public water suppliers (Roberson, 2011), and by 1970 all 50 states accepted these standards (some with modifications) as regulations or guidelines (Oleckno, 1982). The standards were amended in 1925, 1942, 1946, and 1962, and later were used to develop comprehensive Federal standards (U.S. Public Health Service, 1962; 1946; 1943; 1925). These early guidelines were important in promoting filtration and reliable chlorine disinfection

as part of the multi-barrier concept for treatment and virtually eliminated waterborne typhoid fever by the 1940s (Roberson, 2011).

In 1970, the U.S. PHS published results of a survey of public water systems and their compliance with the standards (McCabe *et al.*, 1970; U.S. Public Health Service, 1970). The study found that 41 percent of the systems surveyed did not meet the guidelines established in 1962. The study found widespread deficiencies in drinking water quality, public health risks from waterborne diseases, poor operating procedures for drinking water treatment, and inadequate treatment facilities. The U.S. PHS concluded that there were not enough established drinking water standards to ensure public safety and recommended Federal monitoring standards (U.S. Public Health Service, 1970). The results of the study generated interest in Federal safe drinking water legislation.

In addition to the impact of the U.S. PHS study, American citizens became more aware of environmental issues during the 1960s and 1970s, spurred by events such as the publishing of Rachel Carson's *Silent Spring* (1962), the media attention from buried hazardous waste in the Love Canal neighborhood of Niagara Falls, New York, and the first Earth Day (1970). Public interests led the Federal Government to consider more comprehensive environmental regulations, which would be implemented and overseen by the newly created Environmental Protection Agency (EPA). The EPA was established for the purpose of protecting human health and the environment in 1970 by executive order Reorganization Plan No. 3 to the United States Congress, creating the EPA as a single, independent agency from a number of smaller arms of different Federal agencies (Nixon, 1970).

2.2.1 Safe Drinking Water Act

The EPA became the Federal agency for administering Federal drinking water standards with the passage of the Safe Drinking Water Act (SDWA), Title XIV of the Public Health Service Act, which was signed into law on December 16, 1974, as Public Law 93-523 (U.S. EPA, 1974). The EPA was authorized to set national drinking water regulations, conduct special studies and research, and oversee the implementation of the act. The new drinking water standards were implemented as a result of concerns for chemical and microbial contamination in drinking water. These regulations were based on the drinking water standards established by the U.S. PHS. The SDWA established a new relationship between local, state, and Federal officials in providing citizens with safe drinking water. The act required the establishment of primary drinking water regulations designed to ensure safe drinking water for the consumer (AWWA, 2006a).

The SDWA has been amended several times since 1974, including 1977, 1979, 1980, 1986, 1988, 1996, and 2002 (Tiemann, 2010). The most substantial amendments were passed in 1986 and 1996. The 1986 amendments were motivated by several issues, including monitoring reports detailing water contamination by organic chemicals and pathogens, and technical advances in analytical methods and treatment processes (Tiemann, 2010). The 1986 amendments required the regulation of 83 specific contaminants by 1989, and then required 25 additional contaminant regulations every 3 years after 1989 (U.S. EPA, 1986b).

The SDWA was again amended in 1996. The amendments require EPA to strengthen protection for microbial contaminants and disinfection byproducts. The amendments also replaced the requirement for the pace at which contaminants were regulated with a requirement to decide every 5 years whether to regulate at least five contaminants based on their occurrence and risk to public health. The amendments included specific requirements for regulating arsenic, disinfection byproducts, microbial contaminants, and

radon. The amendments also added programs for small drinking water systems; required water systems to provide annual drinking water quality reports; provided for a state revolving fund (SRF), a low interest loan program to implement drinking water infrastructure projects; and increased requirements for source water protection areas. In addition, the amendments included several requirements for the EPA, including changes to the standard setting process, and requirements for analyzing risk and costs for new standards (U.S. EPA, 1996).

The 1996 amendments also defined the standard setting process for the Contaminant Candidate List (CCL) and the resultant Regulatory Determinations (RDs). CCL is the list source of priority contaminants known or anticipated to occur in public water systems. Based on this list, research is conducted and RDs are established. The first CCL (CCL 1) of 50 chemicals and 10 microbiological contaminants was published in March 1998 and CCL 2 of 42 chemicals and 9 microbiological contaminants was published in February 2005. The CCL 3 was published October 2009 and includes 104 chemicals and 12 microbiological contaminants. In 2012, the EPA requested contaminant nominations for CCL 4 (Roberson, 2011; U.S. EPA, 2012).

The SWDA was most recently amended in 2002 with the passage of the Public Health Security and Bioterrorism Preparedness and Response Act of 2002. The amendments include requirements for community water systems serving more than 3,300 individuals to conduct vulnerability assessments and prepare emergency preparedness and response plans. There are also requirements for the EPA to conduct research on preventing and responding to terrorist or other attacks (U.S. EPA, 2002).

Overall, the SDWA and its amendments created a coordinated set of programs and requirements to ensure drinking water safety. These requirements form a multiple barrier approach that includes technical and managerial barriers in order to assist in the prevention of contamination and include specific limits for biological and chemical contaminants. The following sections focus on the microbial rules, which are most relevant to this research because drinking water standards currently rely on bacterial indicators in order to indicate potential risks from pathogens.

2.2.2 Surface Water Treatment Rules

In 1989, the EPA published regulations for Filtration, Disinfection, Turbidity, *Giardia lamblia*, Viruses, *Legionella*, and Heterotrophic Bacteria. Together these regulations constitute the Surface Water Treatment Rule (SWTR) (U.S. EPA, 1989a). The SWTR was instituted under the 1986 SDWA amendments which required the EPA to protect the public from waterborne diseases, and to require disinfection and set minimum removal efficiencies for specific pathogens (AWWA, 2006a; U.S. EPA, 1986b). It requires community water systems to disinfect all surface waters or groundwater sources under the direct influence of surface water and requires filtration for most surface water sources. The SWTR also requires water systems to use both disinfection and filtration processes as part of the multi-barrier approach. The requirements may also be met through disinfection and watershed protection if the drinking water source complies with the steps to achieve a filtration avoidance waiver (U.S. EPA, 1991; 1989a).

The SWTR imposed turbidity limits on filtration processes. The SWTR requires three $\log_{10}$ (99.9%) removal and/or inactivation for *Giardia* cysts and four $\log_{10}$ (99.99%) removal and/or inactivation of viruses. To meet these requirements, treatment plants receive $\log_{10}$ credits for filtration and for disinfection. Log_{10} credit for filtration is based on the type of filter, and the plant must meet filter effluent turbidity limits.

Log_{10} credit for disinfection is based on disinfectant concentration (C) and exposure time (T) needed to inactivate pathogens. The required C·T is dependent upon disinfectant used, pathogen, and water quality parameters. Lastly, the SWTR requires both a minimum level of residual disinfectant to be maintained in the distribution system and a maximum level of turbidity not to be exceeded (U.S. EPA, 1991).

The 1996 SDWA amendments required the EPA to enhance the SWTR. Since 1996, the SWTR has been modified several times to balance the risks between microbial pathogens and disinfection byproducts (DBPs). The enhancements were included because of concerns about waterborne disease outbreaks associated with *Cryptosporidium*, specifically the Milwaukee, Wisconsin, outbreak in 1993 that resulted in over 100 deaths and approximately 400,000 incidences of gastrointestinal illness (MacKenzie *et al.*, 1995). Modifications to the SWTR were achieved through progressive rulings for the long-term treatment of surface waters. These rules include the Interim Long Term Enhanced Surface Water Treatment Rule (IESWTR), the Long Term 1 Enhanced Surface Water Treatment Rule (LT1), and the Long Term 2 Enhanced Surface Water Treatment Rule (LT2) (Roberson, 2011).

The IESWTR was enacted in 1998 and became effective in 2002. This rule included maximum contaminant level goals (McLennan *et al.*, 2009) of zero for *Cryptosporidium*, and 2 log_{10} removal of *Cryptosporidium* for filtered systems. The IESWTR requirements applied to systems serving greater than 10,000 people. The LT1 was enacted in 2002 and became effective in 2005. The LT1 expanded the IESWTR to include public water supply systems serving 10,000 or fewer people (AWWA, 2006a; U.S. EPA, 2010a).

The LT2 promulgated in January 2006 addresses higher risk public water systems, including filtered water systems with high levels of *Cryptosporidium* in their water sources and all unfiltered water systems. Water systems initially monitored their water sources to determine specific treatment requirements. Monitoring involves 2 years of monthly sampling for *Cryptosporidium*. Small filtered water systems are allowed to monitor first for *E. coli*, which is less expensive to analyze, and then only monitor for *Cryptosporidium* if the *E. coli* results exceeded regulated concentration levels (U.S. EPA, 2010a). The systems are then classified based on the results. The classifications determine the amount of additional treatment required with the majority of systems expected to require no additional treatment. All unfiltered systems are required to provide 99 percent (2 log_{10}) or 99.9 percent (3 log_{10}) inactivation of *Cryptosporidium* based on the results. Filtered systems are required to provide 90 percent (1 log_{10}) to 99.7 percent (2.5 log_{10}) reduction in *Cryptosporidium* based on the monitoring results. Treatment and management are then accomplished with a variety of strategies, termed "a toolbox approach".

2.2.3 Groundwater Rule

EPA expanded the control of microbial pathogens to include water systems with groundwater sources by implementing the Groundwater Rule (GWR). Groundwater sources were previously regulated under the 1989 Total Coliform Rule. The primary goal of the GWR is to identify groundwater systems that are susceptible to fecal contamination and to remove or inactivate pathogens in these waters (U.S. EPA, 2006b). The GWR was promulgated in 2006, and implementation began in 2009. A reduction in groundwater outbreaks is expected similar to that seen with the implementation of the SWTR for surface water systems (Craun *et al.*, 2010).

The GWR was developed in response to the 1996 SDWA amendments that required the EPA to develop regulations that require disinfection of groundwater systems "as necessary" to protect public health (U.S.

EPA, 1996). The EPA determined that extensive groundwater source protection be included in the SWDA after several documented outbreaks of waterborne pathogens in drinking waters from groundwater sources. The EPA specifically identified over 20 cases involving outbreaks associated with onsite wastewater treatment systems, wastewater collection systems, wastewater effluent, stormwater infiltration, animal waste, and water storage and distribution systems (U.S. EPA, 2006c).

The EPA included several components in the GWR. The GWR includes periodic sanitary surveys of groundwater systems, source water monitoring for wells with positive fecal indicator results, compliance monitoring to ensure disinfection treatment, and voluntary actions including hydrogeological assessments to identify wells sensitive to fecal contamination. Source water monitoring is included for all systems. Systems with significant deficiencies or fecal contamination must eliminate the contamination source, correct the deficiency, use an alternative source of water, or provide treatment which achieves at least 99.99 percent (4 $\log_{10}$) inactivation and/or removal of viruses (U.S. EPA, 2006c).

2.2.4 Total Coliform Rule

The Total Coliform Rule (TCR) was enacted in 1989 and improved upon the 1975 EPA Total Coliform standards, which were based on total coliform density. The goal of the TCR is to deliver safe drinking water both by detecting potential issues with drinking water supplies and infrastructure, and also by ensuring the integrity of drinking water. Detection of total coliform indicates the potential for fecal contamination of, breakdowns in the integrity of, or bacterial growth in the distribution system. The TCR applies to all public water systems, including systems with surface water and groundwater sources. Total coliforms are utilized as an indicator because they are more prevalent than *E. coli* or fecal coliforms, and the detection methods are relatively simple and inexpensive (U.S. EPA, 1989b).

The TCR requires public drinking water systems to routinely monitor for total coliforms. The frequency of testing is determined by size and type of system. Systems that serve fewer than 1,000 people may test once a month or less frequently, while systems with 50,000 customers test 60 times per month and those with 2.5 million customers test at least 420 times per month. Each system is required to have a sampling location plan that monitors the water quality throughout the entire system. If total coliforms are detected, then the public water supplier (PWS) must conduct repeat sampling. If repeat sample tests are positive for total coliforms, then the positive samples tested for fecal coliforms and *E. coli*. The TCR also set the MCLG for total coliforms at zero and the MCL at no more than 5 percent of the required monthly samples testing positive (AWWA, 2006a).

The TCR includes a public notification process. If a PWS has a monthly MCL violation, they must notify the state by the end of the next business day and notify the public within 30 days. Systems with routine or repeat samples that are fecal coliform or *E. coli* positive must notify the state by the end of the day and notify the public within 24 hours (U.S. EPA, 1989b; a).

There are several issues with implementation of the TCR. The local utility typically has limited control of coliform entry pathways, and water quality can be compromised by construction, main breaks, or household plumbing. In addition, the total coliform results are sensitive to frequency of sampling and timing of the sampling. It is possible for a water supplier to take extra samples in order to avoid incurring a monthly TCR violation, termed "sampling out" (Bennear *et al.*, 2009). Results can be impacted by the day they are collected, for example, if there is temporary construction, or if there are sudden population changes. The

rule requirements are complex and include variances, follow up sampling, and the notification process. There are also laboratory issues with weekend availability, laboratory locations in relation to remote sites, sample holding time, and lack of a specific time frame for laboratories to report results (U.S. EPA, 2010c). There are also public health concerns with the policy since TCR indicators may not be suitable for some fecal contamination such as *Giardia*, *Cryptosporidium* and non-fecal pathogens such as *Legionella* (U.S. EPA, 2006a).

The EPA published the Revised Total Coliform Rule (RTCR) on February 13, 2013. The revisions incorporate recommendations from a Federal advisory committee comprised of a broad range of stakeholders and consider public comments received during a public comment period held in fall 2010. The RTCR stipulates that all of the 155,000 public water systems in the U.S. that provide drinking water to over 310 million people must take steps to prevent exposure to pathogens such as *E. coli*. Under the revised rule, public drinking water systems have to notify the public if a test exceeds the maximum contaminant level for *E. coli* in drinking water. Public water systems and the state and local agencies that oversee them must comply with the revised rule from April 1, 2016; until then, public water systems and primacy agencies must continue to comply with the 1989 version of the rule (U.S. EPA, 2013).

2.3 WATERBORNE DISEASE OUTBREAKS

For an outbreak to be defined as a waterborne disease outbreak, two or more persons must have experienced a similar illness after exposure to water and the disease agent needs to be epidemiologically linked by time and location of exposure to water. In addition, the epidemiological evidence must implicate water as the probable source of illness (Craun *et al.*, 2010). Contamination in drinking water systems can occur in systems with surface water and groundwater sources. The contamination may be a result of lack of treatment or to inadequate treatment. In addition, contamination may be traced back to the distribution system instead of the source waters.

The Centers for Disease Control and Prevention (CDC) maintains data on waterborne disease outbreaks and has data dating back to 1971. State and local public health offices are responsible for detecting and investigating outbreaks. These agencies voluntarily report these outbreaks to the CDC. Many waterborne disease outbreaks go undetected, and for those outbreaks that are detected, a causative agent is often not identified (Hrudey *et al.*, 2007). Most outbreaks go undetected on account of several basic limitations in public health monitoring. There are occasions when the detection methods are insufficiently sensitive and cannot capture a full range of pathogens; in such cases, monitoring is generally not in real time. In addition, monitoring methods cannot directly determine infectivity of most pathogens (Hrudey *et al.*, 2007).

In 2006, the National Research Council estimated that only a small portion of outbreaks were reported and that reporting does not address total possible endemic illness risk. This estimate is based on the assumption that if low levels of contaminants enter a system and affect small numbers of persons, an illness might not be recognized and investigated as an outbreak. Therefore, the possible detection of an outbreak is inversely proportional to the population of an area (National Research Council, 2006).

In the 20th Century, there have been several waterborne disease outbreaks caused by viral pathogens in drinking water systems for which coliform testing results were negative. The absence or low concentration of fecal bacteria in source waters does not necessarily correlate to the absence of enteric viruses (Bosch,

1998; Jiang *et al.*, 2007). Although bacterial presence is commonly used to evaluate drinking water quality, disease causing pathogens in developed countries are more often viruses or protozoa than bacteria. (Barwick *et al.*, 2000; Blackburn *et al.*, 2004; Lee *et al.*, 2002; Levy *et al.*, 1998). Many more outbreaks like this may occur the United States and go undetected owing to the inconsistencies of *E. coli* as a representative of viral presence (Craun *et al.*, 2010).

In the period from 1971 to 2006, the CDC confirmed 780 waterborne disease outbreaks associated with drinking water supplies in the United States. The health outcomes attributed to these outbreaks include acute gastrointestinal illness (87.8%), hepatitis (3.7%), acute respiratory illness (3.1%), and undefined or mixed illnesses (5.4%). The origin of the diseases, or etiology, was determined for 432 (55%) of the 780 outbreaks. The etiologies include parasites (18.3%), bacteria (16.6%), chemicals (11.5%), viruses (8.2%), mixed agents (0.8%) and the remaining are unknown (45%). The outbreaks caused by viral pathogens were mainly attributed to norovirus (53.1%) and Hepatitis A (45.3%) (Craun *et al.*, 2010).

The factors contributing to outbreaks included fecal or wastewater contamination, inadequate knowledge of source waters, inadequate disinfection, extreme weather events, filtration failures, distribution failures, and operation and maintenance failures (Hrudey *et al.*, 2007). During the period from 1971 to 2006, the CDC identified 801 deficiencies in 780 outbreaks. The majority of the outbreaks involved the use of contaminated groundwater with no treatment or interrupted treatment (52.7%). The other major types of deficiencies included contaminated surface water (18.5%) and contamination within the distribution or plumbing system (18%) (Craun *et al.*, 2010). Many of these events occurred during heavy precipitation. Approximately, 50% of outbreaks were associated with precipitation events above the 90th percentile, and 68 percent were associated with outbreaks above the 80th percentile (Hrudey *et al.*, 2007). This 36-year time period also featured a decrease in the annual number of drinking water outbreaks as a result of improved drinking water standards, changes in water system management, and improvements to drinking water infrastructure. The decrease in the proportion of outbreaks associated with untreated or improperly treated surface water occurred after the promulgation of the SWTR and associated amendments(Craun *et al.*, 2010).

2.3.1 Surface Water Source

Contamination of surface waters by pathogens is dependent upon the surrounding watershed. Water quality is impacted by several watershed qualities, including land uses (such as impervious surfaces), surface elevations, soils (type/slope), and populations of humans and animals. This knowledge motivated New York State to embark on comprehensive watershed management with treatment measures ranging from discouraging birds from roosting on the source waters to upgrading wastewater treatment plants within the watershed to include tertiary treatment. In addition, the occurrence of pathogens in surface water is highly variable, depending on heavy rainfall events (Reynolds *et al.*, 2008). This is often a result of combined and sanitary sewer overflows (CSOs and SSOs, respectively) during wet weather conditions, which correspond to high loads of indicator organisms and pathogens. During dry weather conditions, effluents from wastewater treatment plants represent a major source for pathogen contamination (Astrom *et al.*, 2009).

The majority of the reported outbreaks associated with surface water have been in small community systems, and the absolute numbers of outbreaks have decreased since 1982. The decrease in numbers of

waterborne disease outbreaks per year is significant and attributable to improved water treatment practices stemming from SWTR compliance (Blackburn *et al.*, 2004; National Research Council, 2006).

The largest waterborne disease outbreak documented in the United States occurred in Milwaukee, Wisconsin, in 1993. The outbreak was caused by *Cryptosporidium*, with over 400,000 cases of gastrointestinal illness (MacKenzie *et al.*, 1995) and over 100 deaths associated with cryptosporidiosis (Hoxie *et al.*, 1997). During the outbreak, the water supply met all standards for coliform bacteria. The outbreak was associated with both deterioration in the raw water quality and a simultaneous decrease in the effectiveness of the coagulation and filtration processes, which together led to an increase in the turbidity of treated water and inadequate removal of *Cryptosporidium* oocysts (MacKenzie *et al.*, 1995). The epidemiological report indicated that the discharge from the sewage treatment plant was responsible for the contamination of the southern raw water intake (Fox *et al.*, 1996). At the time, water quality standards did not include testing for *Cryptosporidium* (Hrudey, 2004). SDWA was amended to prevent future similar outbreaks, as discussed in Section 2.2.

Additionally, viral outbreaks in drinking water systems with surface water sources have occurred in developed countries. This is despite compliance with drinking water regulations that included regular testing for coliforms. A Finnish municipality, Heinavesi, experienced an outbreak in 1998 of 542 confirmed primary cases and an estimated 1,700 to 3,000 cases of acute gastroenteritis. Retrospective epidemiology was conducted to find the source of the infections. The investigation identified norovirus group II in treated, untreated, and tap water samples. The same strain of norovirus was found in fecal samples of infected individuals. The drinking water source was Lake Kermajarvi, a body of water local to Heinavesi, and the treatment processes included sand filtration and chlorination. The contamination source remains unknown, although coliform bacteria were routinely examined in an environmental laboratory and were found to meet requirements. This outbreak occurred despite the municipal water samples being continuously negative for fecal coliforms prior to the outbreak (Kukkula *et al.*, 1999).

Similarly, an example of a viral outbreak caused from a drinking water system that complied with water quality regulations occurred at a ski resort in New Zealand in 2006. There was an outbreak of gastroenteritis with 48 and 83 resort staff members absent from work with acute gastrointestinal illness on July 25 and 26, 2006, respectively. The resort complied with drinking water standards with the presence of total coliforms and *E. coli* monitored weekly. Testing produced a positive result for total coliforms on July 27; however, *E. coli* were not detected in this sample. Both the entire staff and the resort's visitors during this period were surveyed, and 218 cases of gastroenteritis were identified (115 ski resort staff and 103 visitors). Virological investigations identified norovirus group I (GI/5) in fecal specimens from affected persons and in the water supply, thereby establishing a linkage between infection and the source water, a suspected contaminated surface water (mountain stream) source. This outbreak indicates that despite compliance with regulations (including required monitoring of coliforms), norovirus was able to contaminate the resort's drinking water (Hewitt *et al.*, 2007).

2.3.2 Groundwater Source

Groundwater supplies were historically thought to be free of pathogenic microbes because the subsurface environment would naturally filter pathogens. Microbial contaminants leach into groundwater in many ways, including poor wastewater management or disposal. Sources include effluent from septic tanks,

underground storage tanks, landfills, or even animal waste from sources such as poor management of land application of manure (Reynolds *et al.*, 2008). Borchardt *et al.* (2003) studied the probably of contamination in the subsurface environment by testing household wells compliant with state well installation codes. Results showed that 8 percent of wells were virus positive; these wells tended to be in coarse textured soils and were all located in subdivisions served by septic systems (Borchardt *et al.*, 2003).

Outbreaks in groundwater systems emphasize the importance of maintaining adequate, continuous disinfection. The GWR was promulgated in 2006 to address groundwater outbreaks such as those identified in the occurrence studies identified by the EPA in the GWR data availability notice such as Pennsylvania (1978), Georgia (1980), Arizona (1989), Alaska (1995), and Wyoming (2001) (Beller *et al.*, 1997; Goodman *et al.*, 1982; Lawson *et al.*, 1991; Parshionikar *et al.*, 2003; Wilson *et al.*, 1982). The GWR focuses on identification of deficiencies, protection of wells and springs from contamination, and providing disinfection where necessary to protect against bacterial and viral agents. When fully implemented, the GWR is expected to reduce groundwater associated outbreaks, similar to the decreases observed in surface water outbreaks after enactment of the Surface Water Treatment Rule (1974) and its subsequent amendments (Brunkard *et al.*, 2011).

Outbreaks associated with viruses in drinking water systems have been identified in groundwater systems in compliance with regulations. An outbreak of Norwalk-like virus occurred in 2001 in Wyoming after contamination of the groundwater supply. The 35 guests at a snowmobile lodge, who had recently stayed, were identified with acute gastroenteritis. The outbreak was attributed to geological conditions in the area and an overloaded sewage disposal system at the lodge. The sandy, porous soil had poor adsorption qualities and permitted rapid water percolation, which decreased the soil's ability to filter pathogens (Anderson *et al.*, 2003).

Similarly, 229 patrons and employees of a new restaurant in Door Peninsula in northeastern Wisconsin were affected by acute gastroenteritis in June 2007. The restaurant had opened 3 weeks prior and had a newly constructed drinking water well and septic system in compliance with Wisconsin State Code. An epidemiological investigation identified norovirus in the source water, which resulted from contamination by construction anomalies in the septic system. Unsaturated zones are assumed to protect groundwater from contamination by pathogens, but the Door Peninsula outbreak occurred despite a 35-meter thick unsaturated zone beneath the septic system demonstrating the vulnerability of karst formations. A dye tracer test showed that the septic system did not significantly attenuate the dyes, and viruses were observed in groundwater down gradient of the septic system (Borchardt *et al.*, 2011).

2.3.3 Distribution System

Waterborne pathogens can enter into the distribution system as a result of low water pressure, backflow, cross connections, and contamination of municipal water storage tanks. Little is known about the extent of distribution system inadequacies and whether they are sporadic or continually occurring. Outbreaks have been documented following external contamination in the distribution system despite the presence or requirement of residual disinfectant (Craun *et al.*, 2006; Reynolds *et al.*, 2008). Distribution system outbreaks have been attributed to chemicals (copper, chlordane, ethylene glycol, and others) and microbial contaminants, including enteric protozoa (*Giardia, Cyclospora*), enteric bacteria (*Salmonella, Shigella, Campylobacter*, and *E. coli* O157:H7) and enteric viruses (noroviruses and Hepatitis A virus) (Craun *et al.*,

2001; National Research Council, 2006). While outbreaks attributed to distributions systems constitute a relatively small proportion of total outbreaks, there was no statistically significant change in the annual number of distribution system deficiencies in public water systems since 1971. This signals the necessity of additional efforts in order to reduce this risk (Craun *et al.*, 2010).

3.0 INDICATOR SYSTEMS FOR VIRUSES

As a result of outbreaks despite compliance with current drinking water regulations, a new indicator is necessary to better characterize water quality, particularly in terms of potential viral contamination. There are several qualities required for a new indicator system to be considered useful. An effective indicator needs to be relatively inexpensive, reliable to monitor, safe to work with in the laboratory and detected when pathogens are present. Identifying a new indicator requires the development of a standard assay, monitoring of the incidences of the indicator, and quantification of the indicator in several different environments or ecosystems. Once a standard method and quantification of the indicator is complete, a potential indicator should be evaluated for the presence in drinking water sources, and assessed throughout the unit processes of drinking water treatment. Finally, the usefulness of an indicator is dependent on the occurrence of the indicator when pathogens are present (ADWG, 2010; Griffin *et al.*, 2008; Yates, 2007).

3.1 INDICATOR AND PATHOGEN ALTERNATIVES

Several researchers have suggested either coliphages as potential indicators of viral risk or direct pathogen monitoring using adenovirus or norovirus (Abbaszadegan *et al.*, 2008; Jiang *et al.*, 2001; Kopecka *et al.*, 1993; Metcalf *et al.*, 1995). Viruses have been shown to correlate more directly with other enteric viruses than fecal bacteria do in the environment and through treatment processes (Carducci *et al.*, 2008; Jiang *et al.*, 2004). Coliphages resemble many enteric viruses in their physical structure and morphology, and can be detected by plaque assay (Ashbolt *et al.*, 2001). Some groups are rarely found in individual human feces, and some can replicate in the environment (Leclerc *et al.*, 2000; Long *et al.*, 2005; Muniesa *et al.*, 2004). Coliphages are considered potential indicators because of their high population counts in wastewaters and their relatively high resistance to chlorination (Nasser *et al.*, 1993). Male-specific coliphages are present in much lower concentrations, can vary by species, and are capable of distinguishing between fecal pollution of human and animal origin (Leclerc *et al.*, 2000). Noroviruses have also been proposed as markers of fecal pollution on account of their persistence through chlorine disinfection (Shin *et al.*, 2008); however, noroviruses can exhibit seasonal fluctuations and epidemic spikes (Park *et al.*, 2011). Some investigators have proposed human adenovirus (HAdV) as a marker of fecal pollution as a result of its culturability, resistance characteristics, and lack of seasonal variability (Choi *et al.*, 2005; Grabow, 2007; Jiang *et al.*, 2007). However, in a study of urban waters, Jiang *et al.* (2002) found that adenovirus did not correlate with Hepatitis A virus or enterovirus.

Although viruses have been shown to correlate more directly with other enteric viruses as compared to fecal bacteria (Carducci *et al.*, 2008; Jiang *et al.*, 2004), it was until recently both difficult and time consuming to test for viral infectivity. In addition, there are more than one hundred fecal viruses that either cannot be detected with conventional cell culture methods, or else they exhibit poor detection efficiency (Hamza *et al.*, 2011a; Metcalf *et al.*, 1995). However, compared with the case of utilizing traditional indicators such as coliforms, research demonstrates less experience using fecal viruses in monitoring and interpreting results (Ashbolt *et al.*, 2001). Cell culture (also termed tissue culture) is a commonly used method of detection for enteric viruses. Several primate and human cell lines are used to detect the presence of enteric viruses in water samples. Commonly used cell lines are buffalo green monkey kidney (BGMK) and fetal rhesus monkey kidney (FRhMK). Cytopathic assays are initially used to qualitatively indicate the presence of enteric viruses. A plaque assay procedure is then used to quantify the viruses (Jin *et al.*, 2002).

The options for the application of molecular techniques have increased (Girones *et al.*, 2010). Molecular techniques, specifically polymerase chain reaction (PCR) methods, provide sensitive and rapid analytical tools with which to study such pathogens (Metcalf *et al.*, 1995). In some cases, they facilitate the identification, genotyping, enumeration, viability, and source tracking of human and animal contamination. Additionally, recent improvements in detection technologies have allowed the simultaneous detection of multiple targets in a single assay. However, the molecular techniques available today and those under development require further refinement in order to be standardized and applicable to a diversity of matrices (Girones *et al.*, 2010).

PCR has many advantages such as reduced detection time, high sensitivity and the ability to detect enteric viruses that do not replicate in cell culture. PCR also has some disadvantages such as possible interference of inhibitory substances; moreover, PCR does not distinguish between infectious and non-infectious viruses. PCR may be used in conjunction with cell culture to determine virus adsorption versus virus inactivation in transport studies (Jin *et al.*, 2002). PCR procedures may also be used to analyze samples quantitatively. PCR semi-quantitates the level of a specific DNA. RT-PCR is reverse transcriptase PCR and is used to semi-quantitate the levels of a specific mRNA (such as a viral RNA) in an RNA mixture (such as a cellular lysate). The total RNA is isolated from a sample and cDNA is synthesized from the RNA mixture using reverse transcriptase and either random primers or oligo-dT primers. Then regular PCR is performed on the cDNA using message-specific primers, such as those for a specific virus. Quantitative PCR (qPCR), also known as real-time PCR (real time PCR on DNA it is qPCR and real time PCR on RNA (cDNA), it is called qRT-PCR), is a technique that uses fluorescently labeled primers to conduct PCR in real time. QPCR is more quantitative than RT-PCR because non-fluorescently labeled primers can have amplicon endpoints that are identical even when the templates are different in concentration (Albinana-Gimenez *et al.*, 2009; Haramoto *et al.*, 2010; Ryu *et al.*, 2010).

Methods vary widely between molecular methods and traditional culture-based methods. Culture-based methods often take several days to complete, whereas molecular methods take hours or less. However, hybrid approaches employing brief culture periods (to ensure the culturability or infectivity of the microbe) coupled with rapid molecular detection, have the potential to rapidly detect and quantify culturable microbes in environmental samples. This has been particularly useful in decreasing the time for virus detection in cell culture (Girones *et al.*, 2010; Reynolds *et al.*, 2008).

3.1.1 Bacteriophages

Bacteriophages have been studied as possible indicators of viral pathogens since the 1970s (Kott *et al.*, 1974; Yeager *et al.*, 1979; 1977). Bacteriophages, specifically coliphages (viruses that infect coliforms), have potential as indicators of human enteric viruses because they resemble viruses in their physical structure and morphology. The detection of coliphages is achieved by a simple plaque assay, and coliphages are more easily and rapidly detected than enteric viruses (Gerba *et al.*, 1985; Yates *et al.*, 1985). In addition, they are found in higher numbers than enteric viruses in wastewater and other environments are (Yates *et al.*, 1985). Groups of bacteriophages that have been proposed as indicators include somatic coliphages, male-specific coliphages, and phages of *Bacteroides fragilis* (Leclerc *et al.*, 2000), based on their similarities to human enteric viruses in morphology, nucleic acid composition, sources, and occurrence in contaminated water.

There are several drawbacks to using coliphages as indicators. Coliphages can replicate in the environment, have a small percentage of carriers in a given host population, and according some research feature low to no correlation to viruses (Ashbolt *et al.*, 2001; Borrego *et al.*, 1990; Long *et al.*, 2005; Vaughn *et al.*, 1975). While viruses are excreted only by infected individuals for a short period, coliphages are excreted by a certain percentage of humans and animals more consistently. For example, in a study of four French rivers, Hot *et al.*, (2003) found that there was no statistical correlation between somatic coliphages and enteroviruses, human adenovirus, or Norwalk I or II viruses (Hot *et al.*, 2003).

The U.S. EPA standardized the single-layer methods (Methods 1602). The methods can be used to detect somatic coliphages (host, *E. coli* CN-13) and male-specific coliphages (host, *E. coli* F-amp) in an aquatic environment. The method includes the use of the host, $MgCl_2$, and appropriate antibiotic, and a host bacterial lawn. In Method 1602, a 100 mL water sample is supplemented with $MgCl_2$, host bacteria, and double strength agar. The mixture is poured onto petri dishes, and the plaques are counted after overnight incubation (U.S. EPA, 2001c; b). Method 1602 is further explored in the Section 5.0 Research Methods of this report. Bacterial phages can also be detected by a reverse transcriptase-polymerase chain reaction (RT-PCR) technique as shown for male-specific coliphage in fecal contaminated marine waters (Friedman *et al.*, 2009; Rose *et al.*, 1997).

Havelaar and Hogeboom (1983) studied the enumeration of coliphages utilizing multiple biotypes of *E. coli*. The enumeration of coliphages was found to be impacted by host biotype and strain, plating method, agar composition, and the addition of an antibiotic. The methodology for enumerating coliphages in river water, sewage, and secondary effluent (15 total samples) was studied because *E. coli* does not reflect the possibility of contracting viral disease from water. While coliphages are found in low numbers in individual fecal samples, they are prevalent in sewage and considered to be a possible "sewage" indicator. A statistical analysis was carried out on the decimal logarithms of the number of plaque forming units per mL. The data was compared using a two tailed t-test (P = 5%). The numbers compared were the relative efficiency of plating (E.O.P). E.O.P. was defined as the antilogarithm of the mean $\log_{10}$ difference between two methods and was expressed as the percentage of the highest mean plaque count. The strains of *E. coli* studied were B (ATCC 11303), C (ATCC 13706), CN (Mutant of C), W3110N, C-600, SC181, N205, HB101 and WG21 (ATCC 23631). Each of these strains are F negative (F-), except WG21. The strains are of rough or semi-rough laboratory biotypes because wild type biotypes of *E. coli* were found to be poor hosts of naturally occurring coliphages. Wild type *E. coli* normally have an O-antigen that can mask the majority of phage receptors. The Single Agar Layer method (SAL) and Double Agar Layer (DAL) method were compared using various media. The media studies include modified Scholten's agar (MSA), Nutrient Agar (NA), Phage Assay Agar (PAA), Modified Nutrient Agar (MNA), Escherichia Agar (EA) and Tryptone yeast extract glucose agar (TYGA). In the first experiment using DAL and an overnight culture of host strains, the *E. coli* C biotype produced 5 to 6 times more plaques than other strains. In the second experiment, which employed SAL, log phase cultures of the host strain identified 8 times more plaques on the C and CN strains when compared to the B strain. There was no significant difference in the plaque counts on the C strain and on the nalidixic acid resistant strain, CN. This indicates the possibility of utilizing the CN strain with nalidixic acid to suppress bacterial flora in the samples (Havelaar *et al.*, 1983).

The study did not identify one best method for enumerating coliphages, but instead found that a particular group of phages may be observed preferentially based on the method. It also suggests that the right method

depends on the objective of the research. Instead, the research identified a maximum production of plaques for detecting sewage pollution. A two-fold rise in the number of plaques were found using the SAL method, and the MSA and PAA medium composition performed significantly better than the other media tested, in this case MSA exhibited larger plaques with more distinct margins. The SAL was found to be the more efficient method because the plate used allowed a greater distance between plaques, the thin layer improved visibility, and a more limited amount of nutrients in the single layer may reduce the growth rate of the host strain (Havelaar *et al.*, 1983).

3.1.1.1 Somatic Coliphages

Somatic coliphages are DNA viruses of *E. coli* and perhaps related bacteria. They infect via a lipopolysaccharide on the *E. coli* cell outer surface and have been examined as fecal indicators in wastewater and drinking water treatment studies. There are four taxonomic families in the somatic coliphage group: Podoviridae, Microviridae, Myoviridae, and Siphoviridae. Somatic coliphages can be distinguished in standard plaque assays by their ability to infect the cell wall of F-minus *E. coli* hosts such as strains C and CN-13, which lack the ability to form pili. However, somatic coliphages are a heterogeneous group because they belong to different taxonomic groups with different morphologies and other characteristics. This diversity might account for the lack of the correlation of somatic coliphage and enteric virus occurrence in environmental waters (Lee *et al.*, 2011; Payment *et al.*, 2011).

Somatic coliphages have been used as indictors in previous research including indicators of fecal pollution in water systems, such as fresh surface waters, groundwater, and salt waters (Astrom *et al.*, 2009; Franke *et al.*, 2009; Locas *et al.*, 2010; Marti *et al.*, 2011). They have also been used as biotracers (microorganisms used to model fluvial and microbiological characteristics of a target population) to identify pollution sources in surface waters and aquifers (Borrego *et al.*, 1990). In addition, they may also serve as indicators for assessing viral removal efficiency during the treatment of water and wastewater (Bitton, 1987). Somatic coliphages are further discussed in Section 3.3 Relationships between Indicators and Pathogens.

3.1.1.2 Male-Specific Coliphages

Male-specific coliphages are a group of icosahedral phages that are morphologically similar to several human enteric virus groups; on account of this similarity they have been proposed as enteric virus models (Havelaar, 1991). Male-specific coliphages infect *E. coli* that contains the male-specific plasmid, which codes for the sex pilus for the phage to attach. They are also known as F, F+, and FRNA coliphages; MS2 is a type of male-specific coliphage. Male-specific coliphages resemble human viruses, in morphology, including their inability to multiply in water environments (Ballester *et al.*, 2005; Ryu *et al.*, 2010). Male-specific coliphages are considered indicators because of their high population counts in wastewaters and their relatively high resistance to chlorination (Nasser *et al.*, 1993). Male-specific coliphages are further discussed in Section 3.3 Relationships between Indicators and Pathogens.

3.1.1.3 Phages of Bacteroides Fragilis

Bacteroides fragilis is a Gram-negative bacillus bacterium species with coliphages that specifically infect this species; therefore, *Bacteroides fragilis* phages have a narrow host range. *Bacteroides fragilis* is an anaerobe found in high concentrations in the human intestinal tract, and there are more than 100 strains of

Bacteroides Phages. The group of phages that infect *B. fragilis* strain HSP40 have been proposed as a potential model for the study of viral contamination of fecal origin (Gantzer *et al.*, 1998; Jofre *et al.*, 1995; Payan *et al.*, 2005) *Bacteroides* phages have potential as fecal indicators because when compared to *E. coli*, *Bacteroides* are more abundant in fecal matter, provide higher host specificity, and are anaerobic and thus less likely to reproduce in aquatic environments than *E. coli* (Gantzer *et al.*, 2002)hey are considered an index of human fecal pollution because this strain of coliphages is human specific and are not isolated from the feces of other warm-blooded animals. They are consistently isolated from sewage, fecal polluted waters, and their sediments, but not from unpolluted samples. In addition, the levels of phages is related to the degree of pollution, the phages are found in higher numbers than human enteric viruses are, and there is no replication of these phages under simulated environmental conditions (Lucena *et al.*, 2004; Lucena *et al.*, 2003). They are more resistant to water treatment processes than bacterial indicators and other phages (somatic and male-specific) (Hot *et al.*, 2003). They are also more resistant to natural inactivation in freshwater environments than fecal coliforms and other phages (Duran *et al.*, 2002).

The low prevalence of these phages in waters with low and moderate levels of fecal pollution and the complex methodology for their recovery are the main drawbacks for the general use of these viruses as an indicator group (Lucena *et al.*, 2003). *Bacteroides fragilis* phages are much more persistent than somatic coliphages and approximately as resistant as male-specific coliphages. However, *Bacteroides fragilis* phages are detected in lower concentrations than other phages in fresh water; thus, it is more difficult to correlate the levels of *Bacteroides fragilis* phages with the levels of human enteric viruses (Jofre *et al.*, 1995). In a study of two wastewater treatment plant effluent lines, Gantzer, *et al.* found that *Bacteroides fragilis* phages were statistically significant indicators of infectious enterovirus and the enterovirus genome, although the *Bacteroides fragilis* phage concentrations lead to fluctuations in the infectious enterovirus concentration (Gantzer *et al.*, 1998).

3.1.2 Direct Monitoring of Viral Markers

Direct pathogen monitoring of specific human viruses has been proposed as an alternative to indicators for the control of drinking water quality. Until recently it was challenging to test for viruses in water samples, but technology has improved to make detection possible and more cost effective. Molecular techniques, specifically polymerase chain reaction (PCR) methods, provide sensitive, rapid, and quantitative analytical tools with which to study such pathogens, including new or emerging strains. PCR methods can provide identification, genotyping, enumeration, viability assessment, and source tracking of human and animal contamination. Additionally, recent improvements in detection technologies have allowed the simultaneous detection of multiple targets in a single assay (Girones *et al.*, 2010). Pathogens suggested for direct monitoring include human adenovirus, enterovirus, and norovirus.

3.1.2.1 Adenovirus

Adenoviruses belong to the Adenoviridae family, which includes human (49 serotypes), simian (27 serotypes), bovine (10 serotypes), equine (1 serotype), porcine (4 serotypes), ovine (1 serotype), and canine (3 serotypes) viruses (AWWA, 2006b). Adenovirus is a medium-sized, double-stranded DNA virus in a non-enveloped icosahedral capsid, and is approximately 70 to 100 nm in diameter (Grabow, 2007). All adenoviruses with human or mammalian hosts are classified under genus Mastadenovirus (Ishibashi and

Yasue, 1984). At least 51 adenovirus serotypes (Ad1–51) in six subgenera (A–F) have been described in humans. Most human adenoviruses are readily detectable by cell culture propagation (Grabow, 2007).

Human adenoviruses (HAdV) are non-enveloped icosahedral viruses containing double-stranded DNA (Jiang, 2006). Human adenoviruses can cause a variety of human diseases including gastroenteritis, acute respiratory disease, pneumonia, epidemic keratoconjunctivitis, meningoencephalitis, myocarditis, and acute febrile pharyngitis. The viruses are shed for extended periods in feces, urine, and respiratory secretions of infected persons. (Crabtree *et al.*, 1997). Investigators have proposed that human adenovirus (HAdV) can serve as an indicator of fecal pollution from human sources as a result of its culturability, resistance characteristics and lack of seasonal variability (Choi *et al.*, 2005; Grabow, 2007; Jiang *et al.*, 2007).

Adenoviruses have been found to be significantly more stable than fecal indicator bacteria and other enteric viruses during UV treatment (Ko *et al.*, 2005; Nwachuku *et al.*, 2005). Carducci *et al.* (2008) details that HAdV samples demonstrated the most appropriate relationship to other enteric viruses. However, in a study of urban waters, Jiang *et al.* (2002) found that adenovirus did not correlate with Hepatitis A virus or enterovirus.

3.1.2.2 Norovirus

Norovirus (NoV) belongs to the family Caliciviridae and the genus Norovirus. Caliciviruses are small (27-30 nm in diameter), non-enveloped, icosahedral viruses containing a single stranded, positive-sense ribonucleic acid (RNA) genome, approximately 7,000-8,000 nucleotides in length (Atmar, 2010). Viruses in the Caliciviridae family are divided into four genera: norovirus (NoV), sapovirus (SoV), lagovirus, and vesivirus. They are genetically and antigenically a diverse group and comprise five genogroups (GGI-GGV); of these, genogroups I, II, and IV infect humans (Glass *et al.*, 2009; Wang *et al.*, 2013).

NoVs are the most common cause of acute nonbacterial gastroenteritis worldwide, infecting all age groups. Infections occur around the globe and throughout the year but may be more common in winter. The emergence and detection of new strains often coincide with the increase in NoV outbreaks (Parashar *et al.*, 2012). Human NoVs have been difficult to characterize because they do not grow in cell culture and lack a suitable animal infectivity model (Atmar, 2010). The development of RT-PCR has increased the accuracy of disease surveillance for NoVs to the point that the majority of gastroenteritis cases that otherwise might have been considered to be of "unknown etiology" are now attributable to NoVs, with an estimated 23 million cases per year in the United States (AWWA, 2006b).

3.1.2.3 Polyomavirus

Polyomaviruses is the sole genus in the family Polyomaviridae. Polyomavirus genera identified in humans include JCV and BKV human polyomaviruses (HPyV), both of which have similarly structured genomes (Sáenz-Robles *et al.*, 2001); KI polyomavirus, WU polyomavirus (Gaynor *et al.*, 2007) and MC polyomavirus (Feng *et al.*, 2003). These viruses have a double-stranded DNA genome surrounded by an 40-50 nm icosahedral capsid that consists of three capsid proteins, and a double-stranded, covalently closed circular DNA genome (Beller *et al.*, 1997; Sáenz-Robles *et al.*, 2001). Viral persistence occurs in the kidney and can show remarkably high levels of virus release (Dörries, 2002). Polyomavirus infection is primarily asymptomatic, with latent renal infections in immunocompromised individuals (McQuaig *et al.*,

2011). In the late 1990s, researchers began to document the presence of the human polyomavirus (HPyV) species BKV and JCV in urban raw sewage (Bofill-Mas *et al.*, 2000).

3.1.2.4 TTV

TTV is a small, non-enveloped DNA virus that was discovered in 1997 (Nishizawa *et al.*, 1997). Torque Teno Virus (TTV), family *anelloviridae*, is an unenveloped, single-stranded, DNA virus 3.4 to 3.9 kb in length and circular, with a negative polarity diameter of 30-32 nm (Desai *et al.*, 1999; Itoh *et al.*, 2000; Okamoto, 2010). TTV's genome is a circular, single-stranded piece of DNA of a negative polarity, approximately 3.8 kb in length; it is a non-enveloped virus with a virion of about 40 nm in diameter.

TTV is classified in the genus and family *anellovirus circoviridae* (Diniz-Mendes *et al.*, 2004). The virus has considerable genetic diversity with at least forty genotypes, classified in at least five groups that could theoretically have different levels of virulence (Maggi *et al.*, 2009). Human TTV is classified in 29 genetically distinct species, which cluster in five branches in phylogenetic trees (Maggi *et al.*, 2011). TTV was first discovered in a search for potential causative agents of non-A to G hepatitis (Nishizawa *et al.*, 1997).

3.1.3 Abiotic Particle Monitoring

Abiotic spheres are very small beads that are typically made from latex, such as polystyrene with divinyl benzene crosslinking. They are spherical in shape and are available in a variety of sizes from several suppliers such as Bang Laboratories, Interfacial Dynamics Corp., and Polysciences. The beads can be prepared with a variety of functional groups on the outer surface, for example, carboxylated microspheres have a functional –COOH group conjugated to their surface (Pang *et al.*, 2009). Latex spheres are used in a variety of filtration experiments, from bench scale to full-scale water systems. The potential use of latex spheres as surrogates for viruses in treatment studies is desirable given the hazards and difficulties in preforming challenge tests with human viral pathogens (Pontius *et al.*, 2009).

Pang *et al.* (2009) studied the potential of latex spheres coated in casein protein to exhibit zeta potentials similar to *E. coli* and bacteriophage, MS2. Zeta potentials were determined using laser Doppler microelectrophoresis for *E. coli*, MS2, casein (purified and non-purified) and the microspheres, 20 nm and 1 μm, (uncoated and protein-coated). The zeta potentials were measured in a 10^{-3} mol/L NaCl background electrolyte at various pH values adjusted with 10^{-2} mol/L NaOH or HCl. Over a range of pH values from 2 to 8, the charge characteristics of the uncoated microspheres did not correlate to MS2 or *E. coli*. The uncoated microspheres had greater negative charges and the charges were more constant over the pH range when compared to the microorganisms. The zeta potentials of MS2 ranged from 10 mV at a pH of approximately 3 to a zeta potential of -30 mV at a pH of approximately 7.5, while the zeta potential of the uncoated latex spheres remained at a -40 mV (+/- 10 mV) throughout a pH range of 2.51 to 11.33). The zeta potentials of the microspheres covalently coated with purified casein were correlated to those of the microorganisms. The zeta potentials of the 20 nm coated latex spheres ranged from 30 mV at a pH of 2 to a -30 mV zeta potential at a pH of 9 with a negative slope similar to the MS2 phage with a zeta potential of 10 mV at a pH of 3 and a -25 mV zeta potential at a pH of 7.5. This study shows that the surface charge of a microorganism can be closely mimicked by microspheres that are covalently coated with a protein having a zeta potential similar to that of the microorganisms (Pang *et al.*, 2009). Abiotic particles are further

described in Section 3.3 Relationships between Indicators and Pathogens, and Section 4.3 Particle Interactions.

3.2 TTV PRESENCE AND PERSISTENCE

This research focuses on the possibility of the TTV as a viral marker that exhibits similar transport characteristics to pathogenic enteric viruses. In order to be considered a good indicator of water quality, the virus needs to be present in the human population. Importantly, TTV infections have been identified throughout the world, with 70 to 90 percent of the general population is infected, although highest infection rates have been identified in countries with poor sanitation (Maggi *et al.*, 2011).

TTV was first isolated in Japan and was later detected in blood samples from patients in several other countries, including the United States, France, Italy, and Brazil (Bassit *et al.*, 2002; Bendinelli *et al.*, 2001; Biagini *et al.*, 2000; Devalle *et al.*, 2005; Diniz-Mendes *et al.*, 2004; Leary *et al.*, 1999; Maggi *et al.*, 2001). Research suggests that TTV may cause chronic, possibly lifelong viremias in most people regardless of age, health status, and other variants. TTV appears to be present ubiquitously in humans, elicits seemingly innocuous infections, and does not appear to exhibit seasonal fluctuations or epidemic spikes. TTV in humans can be found throughout the body, including in blood and feces, and replicates actively in most tissues and organs (Maggi *et al.*, 2009; Okamoto, 2009a). In addition, TTV appears to exhibit similar physical characteristics and transport mechanisms as pathogenic viruses (Abe *et al.*, 1999; Bendinelli *et al.*, 2001).

Vasilyev *et al.* (2009) researched TTV distribution in a healthy population of Russian Olympic Athletes in Moscow, Russia. Out of a population of 512 healthy individuals, 485 (94%) had a TTV viral load of more than 1,000 copies per 1 mL of blood. There were no significant differences between men and women or between age groups (Vasilyev *et al.*, 2009). A separate study conducted in Japan identified the presence of TTV in approximately 50 percent of patients with acute or chronic hepatitis and 12 percent of blood donors (Nishizawa *et al.*, 1997; Okamoto *et al.*, 2000).

Alavi *et al.* (2011) researched clinical outcomes of torque teno virus-infected thalassemic patients with and without hepatitis C virus infection in Tehran, Iran. The research identified that 50.5 percent of the thalassemic patients and 27.1 percent of controls were TTV infected. Although, the research indicated that Thalassemic patients had a greater chance of TTV infection (Alavi *et al.*, 2011).

Ergunay *et al.* (2008) researched the detection of TTV (TTV) by three PCR methods targeting different regions of viral genome in children with cryptogenic hepatitis, chronic B hepatitis, and asymptomatic hepatitis carriers in Ankara, Turkey. TTV occurred in the patients at a rate of 64.7 percent for those infected with cryptogenic hepatitis, 47.1 percent for those infected with asymptomatic hepatitis B, 55.6 percent for those infected with chronic hepatitis B virus, and 47.5 percent for the control group. Differences in TTV DNA detection were not statistically significant between the study groups with all methods. No significant correlation was detected between presence of TTV DNA and liver enzyme levels. TTV detection rate increased with age, suggesting a non-parenteral, environmental exposure to the virus among the study population (Ergunay *et al.*, 2008).

Pinho-Nascimento *et al.* (2011) studied TTV in fecal samples of patients with gastroenteritis. In this study, three PCR methods, including two conventional and one real time assays were used to investigate the

presence of TTV DNA in fecal samples from 135 Brazilian patients with gastroenteritis ages 0 to 90 years old. The three PCR methods included single-round conventional PCR, nested conventional PCR and TaqMan real-time PCR. Of the samples, 123 (91.1%) were positive with at least one method, and 37 (27.4%), 27 (20.0%), 57 (42.2%), 29 (21.5%), and 33 (24.4%) fecal samples contained TTV isolates belonging to genogroups 1–5, respectively. Fifty-two samples (38.5%) contained than one TTV genogroup. Viral loads ranged from 2.6 to 6.5 $\log_{10}$ genome equivalents per gram of feces (Pinho-Nascimento *et al.*, 2011). This study indicates both a high prevalence and a diversity of TTV isolates in feces of patients with gastroenteritis.

Infection with TTV is not restricted to humans. It has also been detected in certain animal species, including non-human primates (Cong *et al.*, 2000; Okamoto *et al.*, 2000; Verschoor *et al.*, 1999), farm animals (pigs, chickens, cows, and sheep) (Brassard *et al.*, 2010; Devalle *et al.*, 2005; Lang *et al.*, 2011; Leary *et al.*, 1999; Liu *et al.*, 2011a; Martinez Guino *et al.*, 2010; Sibila *et al.*, 2009), companion animals (dogs and cats) (Biagini *et al.*, 2007; Okamoto, 2009b; Zhu *et al.*, 2011), and wild animals (wild boar and sea lions) (Martinez *et al.*, 2006; Ng, 2009). These studies identify animal TTV strains with the similar genomic structure, although there is also great variability within these strains.

In China, a study of 158 fecal samples collected from dogs younger than 1 year old with diarrhea in a pet clinic, established 20 s TTV presence in the environment. Hamza *et al.* (2011) evaluated pepper mild mottle virus, human picobirnavirus, and TTV as indicators of fecal contamination in river water in North Rhine Region, Germany. They found that TTV was not a suitable indicator of fecal contamination in water as a result of low detection rate. In a similar study, Vecchia (2009) quantified TTV and fecal pollution in the Arroio Dilúvio, a waterstream that crosses the city of Porto Alegre, RS, Brazil. TTV was present in approximately 10.7 percent (3/28) of the samples (Vecchia, 2009). Occurrence of TTV was sporadic in the environment and not considered to be a consistent viral marker when compared to other viral agents.

Lan *et al.* (2011) found that 20 out of 158 specimens (13%) were positive for Torque Teno canis virus DNA using detection with PCR. Zhu, *et al.* (2011) undertook a molecular detection and sequence analysis of feline TTV in Pet Clinic, Shanghai, China. Two isolates (SH-F1 and SH-F2) of Torque teno felis virus (feline TTV) were detected in 2 of 16 (12.5%) serum samples. Full-length genomes were cloned and sequenced. Phylogenetic analysis showed that they were clustered with the strain of Japan (Fc-TTV4, AB076003) and the strain of France (PRA4, EF538878) (Zhu *et al.*, 2011).

Sibila *et al.* (2009) researched TTV infection in sows and suckling piglets in Madrid, Spain, to identify the role of the sow in transmitting TTV to piglets. A study was conducted of TTV infection in 44 sows and 215 piglets. The study included the infection dynamics of two swine TTV genogroups (TTV1 and TTV2). TTV1 was detected in higher percentages than TTV2 in sows (75% positive TTV1 and 43% TTV2) and piglets (at 3 weeks of age, 32% positive TTV1 and 12% TTV2). TTV1 and TTV2 co-infections were observed in higher percentages in sows (34%) than in piglets (at 3 weeks of age, 4%). These results suggest that while there may be some transmission from sow to piglet, infections also occur through additional transmission routes (Sibila *et al.*, 2009).

Martínez-Guinó *et al.* (2010) also investigated swine TTV in aborted and slaughterhouse collected fetuses in Girona, Spain. The researchers collected 98 abortion cases and fetuses from 55 pregnant sows at a slaughterhouse. The prevalence in aborted swine fetuses was 17.0 percent for TTV1 and 29.6 percent for

TTV2. Fetuses were also 10.9 percent TTV1 PCR positive and 40.0 percent were positive for TTV2. There were no statistically significant differences when comparing prevalence of swine (Martinez Guino *et al.*, 2010).

TTV presence in the environment has also been established. Haramoto, *et al.* (2010) studied real-time PCR detection of adenoviruses, polyomaviruses, and TTVs in river water in Tamagawa River, Japan. The research included 18 samples with only one positive for TTV (1/18, 5.6%). TTVs were detected in one sample at a concentration of 1.58 10^3 copies/l, suggesting that an increased volume of filtrate is needed for successful detection of TTVs in river water samples (Haramoto *et al.*, 2010). In a similar study, Vecchia *et al.* (2009) quantified TTV and fecal pollution in the southern region of Brazil. TTV was present in approximately 10.7 percent (3/28) of the samples. Occurrence of TTV is only sporadic in water considered to be contaminated compared to other viral agents, such as adenovirus and enterovirus (Vecchia, 2009).

Although it is the case that TTV presence has been established in the environment, researchers have found that it may not be present frequently enough to use as a marker in the environment. Hamza *et al.* (2011) evaluated pepper mild mottle virus, human picobirnavirus, and TTV as indicators of fecal contamination in river water in North Rhine Region, Germany. They found that TTV was not a suitable indicator of fecal contamination in water because of its low detection rate. In a similar study, Vecchia (2009) quantified TTV and fecal pollution in the Arroio Dilúvio, a water stream that crosses the City of Porto Alegre, RS, Brazil. TTV was present in approximately 10.7 percent (3/28) of the samples (Vecchia, 2009). Occurrence of TTV was sporadic in the environment and not considered to be a consistent viral marker when compared to other viral agents.

3.3 RELATIONSHIPS BETWEEN INDICATORS AND PATHOGENS

While bacterial indicators are important factors in drinking water quality standards, bacteria and viruses have many differences, and often epidemiological studies fail to show a relationship between viral pathogens and bacterial indicators in both environmental systems and treatment processes (Ashbolt *et al.*, 2001). The susceptibility of indicator organisms to removal or inactivation in treatment processes is an important consideration in the suitability of an indicator for assessing public health risk in drinking waters. Numerous studies have investigated removal of indicators and pathogens in bench, pilot, and full-scale drinking water treatment facilities, and assessed the relationships between pathogens and indicators. Individual treatment processes provide varied removal rates for viruses and bacteria. In addition, viral removal rates are compared to virus inactivation.

3.3.1 Environmental Systems

The survival of microorganisms is a complex function of exposure to light, temperature, moisture content, soil type, and the specific organisms. Scandura *et al.*, (1997) found that higher groundwater pH was associated with greater virus frequency (Scandura *et al.*, 1997). Azadpour–Keeley and Keeley (2003) conducted a literature search and determined viruses were transported more easily through natural aquifer systems when compared to coliforms (Azadpour-Keeley *et al.*, 2003). Overall, virus persistence and mobility generally exceed that of bacteria in environmental systems. Bacterial and viral indicators respond to sunlight in different ways. Enteric viruses (including poliovirus, echovirus, coxsackievirus) and coliphages are considerably more resistant to sunlight than *E. coli and E. faecalis* (Fujioka *et al.*, 2002).

Viruses survive aeration and sunlight during spray irrigation as well as percolation through 1.5m of soil (Gerba *et al.*, 2005). *E. coli* is not well suited as an indicator of human enteric viruses in recreational water because of the rate of inactivation in sunlight (Fujioka *et al.*, 1985).

Temperature and seasonality are another important factors for pathogens. At the lower temperatures characteristic of the groundwater in most areas of the United States, both poliovirus and echovirus persist in very long period, up to 28.8 days before one $\log_{10}$ reduction (Yates *et al.*, 1985). In surface waters in Finland, no correlation was found between season and norovirus, while bacterial indicators experience seasonal variation (Horman *et al.*, 2004). Sampling of Beaverdam Creek Tributary in Beltsville, Maryland, identified an increase of fine particles, and organic carbon led to slower inactivation of *E. coli* and an average inactivation of 1.98 for 24°C compared to 14°C and a 4.68 ratio of inactivation for 14°C compared to 4°C indicating a lower inactivation rate at lower temperatures (Garzio-Hadzick *et al.*, 2010). In addition, lower densities of *E. coli* in a watershed in Ontario, Canada, were found during the winter and early spring months (Dorner *et al.*, 2007).

Schijven *et al.* (2000) found in solutions of high pH with sandy soils, MS2 was found to be a conservative tracer virus; however, in the presence of multivalent cations, ΦX-174 attached less than MS2. These results demonstrate that in soils near neutral pH and with total high concentrations of multivalent cations, bacteriophage ΦX-174 may be the better choice for a relatively conservative tracer virus in field and column studies (Schijven *et al.*, 2000b).

Bacterial and viral indicators correlate differently to water turbidity. Cizek *et al.* (2008) researched the behavior of two pathogens (*Cryptosporidium*, *Giardia*) and several common indicator organisms (fecal coliform, *E. coli*, Enterococci, and coliphage) in natural waters under both dry and wet weather conditions. Samples were collected in the winter and spring of 2006-2007 and collected in the summer of 2007 from 5 different locations from the Kensico Reservoir. A statistical analysis of the pathogen and indicator correlations was conducted using the Spearman Rank correlations. Coliphages exhibited association with settable particles, in a reservoir, especially during wet weather events, while bacterial indicators (fecal coliforms, *E. coli*, and Enterococci) were consistent with amount of settable particles under both wet and dry weather conditions. A weak correlation of bacterial indicators to *Giardia* and *Cryptosporidium* was identified in reservoir samples during dry weather and wet weather events with correlations between total concentrations of fecal coliform and *Giardia* and *Cryptosporidium* of (R of 0.167 and 0.231 with p-values of 0.35 and 0.19, respectively, indicating confidence levels around 65 and 81%) (Cizek *et al.*, 2008). This was confirmed by another study of the Grand River watershed in Canada, where *E. coli* correlated to turbidity, but pathogenic viruses did not correlate with a Spearman rank correlation analysis over the duration of wet and dry weather (Dorner *et al.*, 2007).

Viruses have also been found to survive over greater distances within fresh water river systems (Astrom *et al.*, 2009). For example, poliovirus persists at 26°C in well water for 3 to 5 days before one $\log_{10}$ reduction (Yates *et al.*, 1985). Coliphages also have longer survival times when compared to bacterial indicators in natural aquatic environments (Borrego *et al.*, 1990). Borrego, *et al.* (1990) concluded that fecal coliforms as a result of their low survival in natural river systems could not be considered good indicators of fecal pollutants.

Somatic coliphages have been reported to replicate in *E. coli* under environmental conditions (Borrego *et al.*, 1990). Therefore, one of the potential drawbacks of somatic coliphages is their potential replication outside the gut, which does not adequately reflect the viral contamination in aquatic environment. Payment *et al.* (2011) found that non-fecal indicators, total coliforms, and aerobic endospores were found more frequently than coliphages in virus positive samples. Somatic and male-specific coliphages were not found to be a good predictor of virus presence or absence because they were present only in low numbers and less frequently than bacterial indicators (Payment *et al.*, 2011).

Male-specific coliphages have also been identified as an indicator for human enteric viruses; however, this research suggests the somatic coliphages are just as valuable. The use of the *E. coli* strain C (ATCC 13706) allows for the use of a nalidixic acid resistant clone to detect indigenous coliphages. This clone allowed for the incorporation of the nalidixic acid to control overgrowth of bacteria in the concentrated samples, particularly the river water samples. In addition, the enumeration of male-specific coliphages utilized the WG49 *Salmonella* strain. This strain is genetically engineered with a plasmid, which codes for F-pili production and removes the potential interference from somatic coliphages counts. However, strict quality control is required for the production of the WG39 strain because this plasmid can be lost during production. This production variability can be controlled by instead using somatic bacteriophage enumeration and the parent *Salmonella* strain, WG45 (Payment *et al.*, 1993).

Yates *et al.* (1985) found no significant differences in the decay rates of poliovirus, echovirus, and MS2 coliphages in contaminated groundwater, and infer from this that MS2 coliphages may be a model for animal virus survival. In addition, inactivation rates of MS2 were equal or slower than those of the animal viruses (Yates *et al.*, 1985). In another study, several groundwater samples where no bacterial indicators were identified, but were positive for human adenovirus and male-specific coliphages (Ogorzaly *et al.*, 2010). Grabow (2001) also found that detection of male-specific coliphages by plaque assays is not as easy and simple as in the case of somatic coliphages. The male-specific fimbriae are produced only by host bacteria in the logarithmic growth phase; thus, cultures for plaque assays have to be timed carefully (Grabow, 2001).

Okoh *et al.* (2010) studied inadequately treated wastewater as a source of human enteric viruses in river water downstream of wastewater treatment facilities in the Eastern Cape Province of South Africa. Human adenoviruses were detected in about 22 percent of river water samples and about 6 percent of treated water samples in South Africa (Okoh *et al.*, 2010). In a study of a two groundwater aquifers in France, raw samples were spiked into groundwater microcosms and after 90 days no bacterial indicators were identified, while several (7/60) samples were positive for human adenovirus (Ogorzaly *et al.*, 2010). Groundwater samples with no bacterial indicators were identified, while several samples were positive for human adenovirus (Haramoto *et al.*, 2007).

Pathogen retention in soils and groundwater is dependent on several variable including physical, chemical and bacterial (Bradford *et al.*, 2008). For example, soils high in sand or a sand gravel mix do not achieve high rates of virus removal, and fissured limestone aquifers under shallow soils allow virus transport over great distances (Metcalf *et al.*, 1995). Saturated column experiments with packed field soil, demonstrated much greater transport potential for somatic coliphage than bacterial indicators (Bradford *et al.*, 2009). Gerba, *et al.* (1987) also found that following applications of a chlorinated secondary effluent by an irrigation system to a sandy soil, poliovirus and echovirus were detected in drains well below the surface.

According to Gerba *et al*. (1981), MS2 behavior is similar to the adsorptive behavior on soil of animal viruses, such as coxsachkievirus B4 and echovirus 1. The survival times of adenovirus in groundwater were found to be longer than that of bacterial indicators (Yates *et al.*, 1987).

Ogorazaly *et al.* (2010) studied viral loads in surface water samples and compared them to groundwater samples. They found that 11.7 percent of the groundwater samples and 75 percent of the surface water samples were adenovirus positive. The hydrogeological characteristics of the groundwater studied explain the difference because a low permeability layer overlies confined aquifers, commonly limiting the migration of bacterial contaminants into the aquifer. Survival times of adenoviruses in groundwater may be attributable to detection of their genomes in the field and may not be a result of recent contamination (Ogorzaly *et al.*, 2010). While, confined aquifers were once thought to protect the water quality of groundwater sources, viruses found in a confined aquifer in Wisconsin were able to penetrate the overlying aquitard; there were no fecal coliform bacteria detected in the virus positive water samples (Borchardt *et al.*, 2004).

3.3.2 Wastewater Treatment

In treatment systems, bacteria and viruses have different removal and inactivation patterns (Nasser *et al.*, 1995). In a wastewater treatment facility with activated sludge and final chlorination, no significant correlation was found between bacterial indicators and the presence of viruses or their abatement (Carducci *et al.*, 2008). In treated drinking water samples from seven full-scale plants, no correlation was found between presence of viruses and indicator bacteria (Payment *et al.*, 1985). Physical removal of viruses through membrane systems is more challenging than removal of bacteria. For example, ultrafiltration and microfiltration can provide an absolute barrier to bacteria and protozoa; however, virus removal through membranes is dependent on the specific membrane characteristics (Jacangelo *et al.*, 2008; U.S. EPA, 2001a). Inactivation kinetics also differ, as many enteric viruses are more resistant than bacteria to ozone, chlorine, and ultraviolet radiation (Blatchley *et al.*, 2007; Lee *et al.*, 2011; Mamane *et al.*, 2007; Shin *et al.*, 2008; 2003; Thurston-Enriquez *et al.*, 2005; Thurston-Enriquez *et al.*, 2003).

Scandura *et al.* (1997) analyzed onsite wastewater treatment systems and viral transports. They found that the removal rate for enterovirus decreased between 5 and 6 $\log_{10}$ units in 3 to 5 days in a seeded septic tank (similar to a constantly stirred tank reactor), and that viruses were discharge with septic effluent into the system drain field. They also found relatively weak correlations between the levels of viruses and fecal coliforms in the tested waters (Scandura *et al.*, 1997).

Boudard *et al.* (2012) studied the enhancement of conventional treatment with ultrafiltration membranes with spiked river water during a pilot scale test. The object of the study was to assess the removal rates for male-specific bacteriophages, represented by MS2, Qβ, and GA by a conventional pretreatment process (coagulation-flocculation-settling-sand filtration) followed or not by an ultrafiltration membrane. MS2 was used because it is often used as an indicator of viruses in the United States; Qβ was used for the same purposes in Japan. Both are similar in characteristics and thought to be a "worst case scenario" in terms of virus removal. GA was selected as a potential indicator because it has not often been studied and the physical characteristics may make it a model, which is most difficult to remove in drinking water processes. The bacteriophages presence was measured with the detection of infectious phages (PFU) by double agar layer method and reverse transcription polymerase chain reaction (RT-PCR) method to detect phage nucleic

acids. RT-PCR detects bacteriophages regardless of their infectivity. The water was pretreated at a pilot scale by sequential coagulation, flocculation, laminar settling, and sand filtration (filtration velocity 2.3 m/h, sand height 80 cm, column inner diameter 10 cm, and sand volume, 6.3L). The pretreated water progressed to 4 mm inner diameter hexa-canal tube ultrafiltration membranes at a flow rate of 0.84 L/h and 0.3 bars pressure. The three analysis campaigns were each completed with triplicates per phage; assays were conducted in triplicate. The raw water samples of 100 L were each spiked with bacteriophages to a concentration of 1×10^6 PFU/mL. Samples were taken from the raw water tanks after both clarification and the filtration process. The process was again challenged in the same manner for the ultrafiltration with samples from the raw water tanks and after the ultrafiltration modules. Three different membrane modules were tested. Membrane A was external-internal polyvinylidene fluoride membrane with 100 cm^2 active surface area, 0.03μm cut off pore size, and 300 L per hour-m^2-bar permeability. Membrane B was external-internal polyvinylidene fluoride membrane with 100 cm^2 active surface area, 200 KDa cut off pore size, and 300 L per hour-m^2-bar permeability. Membrane C was internal-external polyethersulfone membrane with 100 cm^2 active surface area, 100 KDa cut off pore size, and 600 L per hour-m^2-bar permeability. Chlorine disinfection assays were also conducted at a bench scale. Demineralized water and treated water samples were subjected to a target residuals level of 0.3 mg/L for chlorine after 30 minutes contact time. The chlorine assays were also completed in triplicate. While the concentrations were not the same, the pattern of removal behavior was similar between infectious and total bacteriophages throughout the processes (Boudaud *et al.*, 2012).

Hot *et al*. (2003) conducted an analysis of 68 surface water samples obtained from four rivers located in the north of France (Nord-pas de Calais, Cote d'Opale, France) and were sampled monthly or semimonthly between February 1999 and January 2000. For each river, the sampling site was located before the point where treated wastewater was discharged. They found that there was no statistical correlation between somatic coliphages and enteroviruses, human adenovirus, or Norwalk (I and II) virus (Hot *et al.*, 2003). On the other hand, in a study of two wastewater treatment plant effluent lines, Gantzer *et al.* (1998) found that somatic coliphages were statistically significant indicators of infectious enterovirus and the enterovirus genome.

These removal rates were similar in order of magnitude to a study conducted by Simmons *et al.* (2011). They analyzed the removal rates of enterovirus, norovirus, and adenovirus throughout the treatment process of the Traverse City, Michigan, Wastewater Treatment Plant (WWTP). Eight sampling events were conducted approximately monthly (between January and August 2008). The treatment process included a membrane bioreactor (MBR) combined with biological nutrient removal. There were four sampling locations: primary settling (influent and effluent), and membrane bioreactor (influent effluent). There were 32 total samples collected with (32/32) samples testing positive for enterovirus and adenovirus and (20/32) samples testing positive for norovirus. The virus concentrations were similar from the influent to the primary effluent. Adenovirus and enterovirus concentrations increased from influent to membrane influent, while norovirus concentrations reduced. The $\log_{10}$ removal rates for removal through the MBR were 4.1-6.3 for adenovirus, 4.1-6.3 for enterovirus, and 3.5-4.8 for norovirus, while the removal rates throughout the entire process were 3.1 for adenovirus, 3.6 for enterovirus, and 4.7 for norovirus. The virus concentrations were associated more with the settled sludge as compared with the filter supernatant during secondary biological treatment. Norovirus and adenovirus removals did not have seasonal correlations, but enterovirus removal was found to vary by season with higher concentration during the winter months. This

may indicate that during the winter months enterovirus attachment to the floc could be reduced more easily than warmer months (Simmons *et al.*, 2001).

Kuo *et al.* (2010) researched a full-scale MBR system (also used in Simmons *et al.* 2001) by using the previously described samples to focus on adenovirus. They found that viral particles could be removed by MBR systems via direct capture by microfilter membranes, biofilm growth on the membrane, or by absorption into the biomass, and then indirectly removed by the membrane. Adenovirus concentrations for the raw sewage and primary sedimentation effluent were relatively stable at approximately 10^6 viral particles per liter. The adenovirus concentrations for the membrane influent were significantly higher (10^8-10^9) than those of the raw sewage and primary effluents. The membrane influents samples were allowed to settle and the adenovirus concentrations of the settled activated sludge were always much higher than those for the supernatant portion (1.07×10^9 compared to 2.5×10^6 virus particles per liter) with the viruses mostly associated with the solids. The removal rates for the MBR was 5.0 $\log_{10}$ removal and 2.9 $\log_{10}$ removal throughout the wastewater treatment plant (Kuo *et al.*, 2010).

Katayama *et al.* (2008) studied six wastewater treatment plants in Japan but did not observe removal rates comparable to those observed in the studies mentioned above. These treatment plants were sampled year round and were from different prefectures of Japan. Each plant was sampled once a month for 72 sampling events. Samples were collected at the plant influent, after secondary treatment, before chlorination, and effluent after chlorination. Triplicate assays were done on a series of three dilutions and in some cases where positive results were obtained among the most dilute series, further decimal dilution was done until all three were virus negative. Samples were tested for fecal coliforms, norovirus 1 and 2, enterovirus, and adenovirus. Each of the four tested viruses was detected in all of the influent samples, with one exception: WWTP-A in October was negative for norovirus 1. The researchers found a 3.04 $\log_{10}$ reduction in fecal coliforms through secondary treatment, while there was not a significant decrease in the presence of viruses in the samples from secondary treatment to final effluent. Norovirus 1 was identified in 94 percent of the secondary effluent samples and 92 percent of the final effluent samples; norovirus 2 was identified in 92 percent of the secondary effluent samples and 89 percent of the final effluent samples; enterovirus was identified in 65 percent of the secondary effluent samples and 57 percent of the final effluent samples; and adenovirus was identified in 99 percent of the secondary effluent samples and 100 percent of the final effluent samples. The mean concentrations of fecal coliforms reduced from 7.7×10^1 CFU/mL after secondary treatment to 7.9×10^0 CFU/mL after chlorination, while the concentration of viruses after secondary treatment and after chlorination was not significantly different. They found that while chlorination was effective in removing fecal coliforms, viral concentrations were not impacted. In addition, noroviruses (group I and II) had similar seasonal profiles and were more abundant in the winter (approximately 100 times more). Enteroviruses were constant year round, and adenoviruses were statistically affected by month, although they did not exhibit a clear seasonal profile. The ratio of concentrations in the influents for each of the viruses to that in the effluent was mostly stable throughout the year for all viruses, which showed no seasonal effect on the removal of viruses in the wastewater treatment. The adenovirus had the highest concentration throughout the year (a preferable feature of a potential indicator) (Katayama *et al.*, 2008).

Carducci *et al.*, also studied virus removal rates in wastewater treatment in two separate studies (2008 and 2009) of the Pisa, Italy wastewater treatment plant. The first study included sampling from April to

June 2007, with twenty sampling events with samples collected at the influent and effluent for a total of 40 samples. The process is activated sludge followed by chlorination. All samples were run in triplicate with positive and negative controls. Each sample was processed for adenovirus, torque TTV, and hepatitis A virus. The adenovirus was identified in all 40 samples with no seasonal profile demonstrating 2.0 $\log_{10}$ reduction through the treatment plant. The torque TTV was identified in 90 percent (36/40) of the samples with a 1.58 $\log_{10}$ reduction through the treatment plant. Adenovirus and torque TTV indicated a resistance to chlorine disinfection with exit concentrations of 2.4×10^3 gc/mL and 6.71×10^3 gc/mL, respectively. There was no correlation identified between the viruses and bacterial indicators (Carducci *et al.*, 2008).

The second study was conducted between March 2007 to June 2007 and November 2007 to April 2008, with 29 sampling events at the influent and effluent for a total of 58 samples. The samples were analyzed for adenovirus, torque TTV, hepatitis A virus, rotavirus, enterovirus, norovirus (groups I and II), somatic coliphages, *E. coli,* and Enterococci. The bacterial indicators and coliphages did not significantly correlate to any of the pathogens, although when considering removal rates between adenovirus and somatic coliphages, the somatic coliphages may be an indicator of treatment efficiency. Adenovirus was shown to have a high resistance to chlorine disinfection, and while the TTV also showed resistance and high correlations, attributable to the great variability in DNA copy counts, reduction rates, and virus unculturability, it precludes the torque TTV as an ideal indicator (Carducci *et al.*, 2009).

TTV seems to exhibit several qualities that would make it a good indicator of fecal and wastewater contamination. The prevalence in humans is high, and the serotypes are different in humans and animals, which would be a component of the identification of the contamination source. It is also potentially harmless to humans, so it may be safer to use on a laboratory setting compared to using a pathogenic agent such as adenovirus (Sidhu *et al.*, 2010; Vaidya *et al.*, 2002). Carducci, *et al.* (2009) studied viral removal by wastewater treatment by monitoring indicators and pathogens at the wastewater treatment facility in Pisa. No significant correlations were found between bacterial indicators and the viruses considered, confirming their inadequacy for virological risk. TTV DNA was detected in 72 percent (21/29) of the raw sewage and in 62 percent (18/29) of the exit samples, with a mean removal of 1.6 $\log_{10}$ with a standard deviation of 1.16 (Carducci *et al.*, 2009).

In 2008, Carducci *et al.* also researched the use of TTV as an indicator in wastewater treatment facilities. They determined that there are issues with viral culturability, which prohibits the valuation of actual survival rates. Thus, TTV should be subjected to additional analysis, and additional research is required to assess the qPCR of TTV as a viral indicator. There were also several issues with the use of qPCR to define TTV, including variability in DNA copy count, their reduction rates, and the virus' unculturability, which precluded the evaluation of actual survival rates In addition, further analysis should include a comparison of TTV to bacterial indicators in order to demonstrate potential resistance of TTV to treatment effects and to determine additional relationships of TTV to other enteric viruses and indicators (Carducci *et al.*, 2008).

TTV seems to exhibit several qualities which would make it a good indicator of fecal and wastewater contamination (Haramoto *et al.*, 2008). Samples were collected from a wastewater treatment plant in Japan, and TTV DNA was detected in all 12 influent samples tested, with a geometric mean concentration of 1.7×10^4 genomic copies/liter. The concentration of TTV DNA in the influent samples showed no clear seasonal pattern, suggesting that TTV infections occur constantly throughout the year. The high prevalence of TTV in wastewater suggests that TTV may be an appropriate indicator of fecal contamination.

3.3.3 Drinking Water Treatment

In 1995, Jofre, *et al.* compared viruses and bacteriophages in raw waters and identified bacteriophages in treated water samples of full-scale conventional treatment facilities. The study included bacteriophages infecting *B. fragilis*, somatic coliphages, and male-specific coliphages as potential indicators of enteroviruses in full-scale conventional treatment. Three drinking water treatment facilities in Barcelona, Spain, were used: two plants (A and B) drew water from the Llobregat River (with known heavy pollution of human origin) and the third plant (C) drew water from the Ter River. Each plant supplied drinking water that met water quality criteria based on bacterial indicators. The three plants all had prechlorination, coagulation-sedimentation, and filtration (plant A had both sand and activated carbon filtration, Plant B had sand filtration, and Plant C had activated carbon filtration). All three of the plants had postchlorination as a final step, and Plant A had an ozonation step between sand filtration and activated carbon filtration. The study compared phage counts identified by the double agar layer (PFU) method to the infectivity of enteroviruses in buffalo green monkey (BGM) cells. Bacteriophages were identified in 100 percent (24/24) of the 10 mL raw water samples for each of the Plants (A and B) drawing from the Llobregat River. Bacteriophages were identified in 31.8 percent to 36.4 percent of the 20 raw 10 mL water samples from the plant drawing from the Ter river (Plant C). Enteroviruses were identified in 55 percent of the samples from Plant A, 50 percent of the samples from Plant B, and none of the samples from Plant C. The number of enteroviruses detected in the raw waters of Plant A ranged from less than 20 to 630/1,000 L, and Plant B ranged from less than 20 to 158/1,000 L. In addition, larger samples of 100 mL were analyzed for bacteriophages throughout the treatment processes utilizing a presence-absence test (% positive per 100 mL). The 68 samples of finished waters resulted in 11.7 percent were positive for phages infecting *B. fragilis*, 2.8 percent for somatic coliphages, and 1.4 percent for male-specific coliphages. The removal of enterovirus could not be calculated as a result of the minimal presence in the raw water samples and the absence in treated water. In the conventional treatment, phages infecting *B. fragilis* were more resistant to treatment than somatic or male-specific coliphages (Jofre *et al.*, 1995). This study found that bacteriophages were present post treatment while infectious enteroviruses were unable to be detected, demonstrating bacteriophages can be a more conservative representation of infectious viruses.

Payment *et al.* (1985) did not find a direct correlation between the indicator bacteria and the viruses measure, although the study did identify benefits for the use of bacteriophages as surrogates. The study included the removal of viruses and indicator bacteria during the treatment process of seven full-scale treatment facilities. The facilities were sampled twice a month for 12 months. The study allowed for the comparison of traditional bacterial analysis to the infectivity and cytopathic effects of viruses. Plants 1 and 2 treated water with pre-chlorination, coagulation and sedimentation, filtration, ozonation, and post-chlorination. Plant 3 had similar treatment, with the pre-chlorintaion being only seasonal. Plants 4 and 5 had similar treatment to Plants 1 and 2 without ozonation. Plant 6 treated water with filtration and post-chlorination, and Plant 7 treated water with only chlorination. The plant capacities ranged from 0.9 to 100×10^4 m^3/day. Samples were obtained through treatment including raw water, post-chlorination, post-sedimentation, post- filtration, post-ozonation, and finished (tap) water (Payment *et al.*, 1985).

The raw water quality of the Payment (1985) study was generally poor with total coliforms exceeding 10^5 CFU/L and the average total virus count of 3.3 MPNCU/L (with several samples of over 100 MPNCU/L). Total plate counts were also evaluated as a measure of total bacterial populations, and the

finished water had lower counts results ranging from averages of 2.6 to 3.8 $\log_{10}$ CFU/L. Viruses were detected in 11 of 155 (7%) of the finished water samples (1,000 L) with an average density of 0.0006 MPNCU/L (with the highest density measured at 0.02 MPNCU/L). The average cumulative virus reduction was 95.15 percent after sedimentation and 99.97 percent after filtration, but remained consistent after ozonation or final chlorination. The overall observed virus reduction was approximately 4 logs. The viruses, which were isolated from the samples, were all enteroviruses including poliovirus Type 1, 2, 3; coxsackievirus Types B3, B4, and B5; echovirus Type 7 and untyped picornavirus. The presence of viruses in raw water was weakly correlated with some of the parameters, including total coliforms and fecal coliforms with r-values in the 0.5 to 0.7 range. The treated water samples did not have any correlations between the presence of viruses and the measured parameters. While coliphages were not found to correlate directly with enteric viruses, multiple regression analysis showed that somatic coliphages were an explanatory variable for viruses in settled waters. While somatic coliphages are not normally in high numbers, it is easier to maintain their host cells than it is to maintain the host cells of male-specific coliphages (Payment *et al.*, 1985).

In a later study, Payment and Franco (1993) evaluated large volume samples (100 to 2000 liters, with up to 20,000 liters for selected finished water samples) in three full-scale conventional water treatment facilities in Montreal. All three plants used river water sources that were contaminated with sewage, thus increasing the likelihood that indicators and pathogens would be present in the raw waters. All had conventional treatment, with one plant also having biological filtration and one plant also having ozonation. Human enteric viruses were found in all raw waters, and *Giardia* cysts were found, depending upon treatment plant, in 80 to 100 percent of samples. *Cryptosporidium* oocysts were more variable, ranging from 0 to 100 percent of samples depending on plant. Regarding indicators, somatic and male-specific coliphages were detected in all raw samples, with concentrations ranging from tens of thousands to hundreds of thousands per 100 L. Indicator and pathogen levels were reduced through treatment, with levels consistently lower from raw to settle to filtered to finished waters. Overall, removal of both indicators and pathogens was on the order of 5 to 7 $\log_{10}$, with calculated values dependent on raw water values and detection limits (Payment *et al.*, 1993).

In a limited study by Gerba *et al.* (2003), removal of *E. coli*, *Encephalitozoon intestinalis*, feline calicivirus, and coliphages (fr, MS2, and PRD-1) was evaluated in a conventional pilot plant. The intent of the study was to provide additional information about organisms (microsporidia as *E. intestinalis* , and calicivirus) on the U.S. EPA Drinking Water Contaminant Candidate List (CCL) (EPA, 1995) Three challenge studies were conducted in which microorganisms were seeded into raw water at concentrations ranging from 10^3 to 10^8 per mL. Overall removals for the indicators and pathogens were 1.85 to 3.21 $\log_{10}$, with filtration accounting for the majority of the removal for *E. coli*, *E. intestinalis*, and PRD-1, and sedimentation being more important for calicivirus, MS2, and fr. The bacteria and phages were detected using traditional agar methods, and the calicivirus was propagated in feline kidney cells and assayed by the $TCID_{50}$ method. A detailed statistical analysis of the data was not conducted (Gerba *et al.*, 2003). This study again identifies MS2 and PRD-1 as suitable representations of virus removals, with PRD-1 exhibiting the least incidence of removal of any microorganism and serving as a worst case example prior to disinfection.

Xagoraraki *et al.* (2004) also researched MS2 through filtration. The purpose of the study was to identify removals of emerging pathogens and pathogen indicators in conventional treatment by pilot scale

coagulation, flocculation, sedimentation, and granular media filtration. Seven challenge tests were performed to investigate the effects of water quality and unit process condition on pathogen removals. The pathogens were spiked at concentrations of *C. parvum* (1×10^5 oocysts/L), *E. intestinalis* 2×10^5 spores/L, *E. coli O157:H7* (6×10^7 CFU/L), *A. hydrophila* (2×10^7 CFU/L) and bacteriophage MS2 (5×10^7 PFU/L). The pilot plant received raw water from Lake Mendota in Madison, Wisconsin, and included two parallel treatment trains. A detailed statistical analysis was completed and Tukey's test was used to compare mean removals and correlate turbidity (Xagoraraki *et al.*, 2004). The results did not show any strong correlation between the indicators (MS2 and turbidity) and the removal of pathogens.

Mayer *et al.* 2008 studied enhanced coagulation in jar tests with human enteric pathogens and bacteriophages. The optimization tests demonstrated both that bacteriophage removal increased as coagulant dose increased and also that bacteriophage removal improved slightly with reductions in pH. The enhanced coagulation conditions were optimized at 40 mg/L ferric chloride and a pH of between 5 and 6.5. The enteroviruses were each tested twice with replicate assays, and the bacteriophages were tested twice with triplicate assays. At a coagulant dose of 40 mg/L ferric chloride and a pH below 6.5 the maximum, removals were achieved with $\log_{10}$ removals of coxsackie B virus (3.0 $\log_{10}$), echovirus (1.75 $\log_{10}$), poliovirus (2.5 $\log_{10}$), fr (1.8 $\log_{10}$), ΦMS2X-174 (1.3 $\log_{10}$), MS2 (0.36 $\log_{10}$) and PRD-1 (0.29 $\log_{10}$) (Mayer *et al.*, 2008).

Abbaszadegan *et al.* (2008) studied the removal of adenovirus, calicivirus, and bacteriophages through bench and pilot scale testing of conventional treatment. Though the objective of this study was to evaluate removal rates of microorganisms from the second EPA CCL (U.S. EPA, 2005), the study needed to provide sufficient data on virus and bacteriophage removal rate correlations, in order to only use bacteriophages in the later stages of the research. Adenovirus Type 4 and feline calicivirus (surrogates for enteric adenovirus Type 40 and 41, and human caliciviruses, respectively) were evaluated along several bacteriophages, including MS2, PRD-1, ΦX-174, and fr. Abbaszadegan *et al.* identified bacteriophage as an indicator because the double layer agar assay used to quantify the microorganisms was less complex, and less expensive, than the standard in vitro cell culture methods used for animal viruses; in addition, not all viruses had an available in vitro cell culture technique (Abbaszadegan *et al.*, 2008).

The source water for the Abbaszadegan *et al.* (2008) study was raw water from the Chandler Water Treatment Plant in Chandler, Arizona. This raw water had an average water turbidity of less than 0.2 NTU, a pH of approximately 8.0, and alkalinity ranging from 200 to 220 mg/L as $CaCO_3$. Ferric chloride was dosed from 0 to 50 mg/L. The raw water was seeded with 1×10^6 PFU for each bacteriophage and 1×10^6 50 percent tissue culture infective dose ($TCID_{50}$) per mL for each virus. This seeding methodology allowed for the comparison of bacteriophages in the double layer agar method to the infectivity and cytopathic effects of viruses. The coagulant was tested in increments of 10 mg/L in doses ranging from 0 to 50 mg/L. The jar tests to identify the concentration thresholds were conducted with 5 experimental runs and duplicate assays. Concentrations of 20 and 40 mg/L ferric chloride were selected as the low and high thresholds, respectively. The jar tests to identify average $\log_{10}$ removals were conducted with three experimental runs and duplicate assays. MS2 was reduced by 1.62 $\log_{10}$ removal at 20 mg/L ferric chloride and 2.13 $\log_{10}$ removal at 40 mg/L ferric chloride. The MS2 removals at 20 mg/L were followed by fr (1.45), PRD-1 (0.45) and ΦX-174 (0.40) (estimated from Figure 3 of the study), with the same trend for removals at 40 mg/L, supporting studies that find male-specific bacteriophages (MS2 and fr) are removed to a greater extent

through coagulation and settling when compared to somatic bacteriophages (PRD-1 and ΦX-174) (Gerba *et al.*, 2003; Jofre *et al.*, 1995). At 40 mg/L, removals for adenovirus and FCV were 1.4 $\log_{10}$ and 1.5, respectively with removals for PRD-1 and ΦX-174 at 1.0 and 0.65 $\log_{10}$, respectively (Abbaszadegan *et al.*, 2008). This study suggests that PRD-1 and ΦX-174 may be appropriate surrogates (process indicators) for both adenovirus and FCV in removal studies.

Abbaszadegan *et al.* (2007) conducted a bench scale analysis to evaluate enhanced coagulation and settling. Conventional drinking water treatment includes coagulation, which focuses on turbidity removal, while enhanced coagulation focuses on the removal both of turbidity and of natural organic matter. The focus of the study was to determine the balance between the risks of bacterial and chemical contamination. DOC was measured as a potential precursor to DBPs as described in the ESWTR and D/DBP rule. While the purpose of the study was to determine optimization of enhanced coagulation for the reduction of natural organic matter effects as indicated by the DOC level and the removal of emerging pathogens, the study also included removals for adenovirus Type 4, feline calicivirus, MS2, PRD-1, ΦX-174 and fr (Abbaszadegan *et al.*, 2007).

In order to optimize conditions for the enhanced coagulation, Abbaszadegan *et al.* (2007) conducted jar tests in order to adjust ferric chloride dose and pH with respect to DOC removal. The turbidity of the raw water ranged from 3 to 20 NTU, a pH from 8 to 8.5, and alkalinity ranging from 125 to 200 mg/L as $CaCO_3$. Ferric chloride was used in as a coagulant because it is more efficient than alum in the removal of natural organic matter. The raw water was seeded with 1×10^6 PFU for each bacteriophage and 1×10^6 50 percent tissue culture infective dose ($TCID_{50}$) per mL for each virus. Separate jar tests were conducted for the bacteriophages, surrogate viruses, and DOC because it was found that the nutrient media for the stock cultures distorted the DOC concentrations. The bacteriophages were studied with one jar test and triplicate assays, and the two jar test experiments were completed for the surrogate viruses with duplicate assays. Coagulant doses ranging from 0 to 120 mg/L ferric chloride were tested in increments of 20 mg/L, and the pH values ranged from 5.5 to 7.5 and were tested in increments of 0.5. The bench scale testing demonstrated that the optimal conditions were 40 mg/L $FeCl_3$ and a pH between 5 and 6. These conditions resulted in maximum removals of 2.58 $\log_{10}$ of adenovirus type 4, 2.50 $\log_{10}$ of feline calicivirus, 2.49 $\log_{10}$ of fr, 2.32 $\log_{10}$ MS2, 1.75 $\log_{10}$ of PRD-1, 1.52 $\log_{10}$ of ΦX-174, and 56 percent of DOC (Abbaszadegan *et al.*, 2007). These removals indicate that bacteriophages are a conservative representation of viruses with enhanced coagulation.

Nasser (1995) used jar tests to optimize flocculation doses and bench scale high rate filtration columns (100 cm) to observe virus removals. In the first part of the study, enteric pathogenic viruses, represented by hepatitis A and poliovirus, were compared to male-specific bacteriophage in coagulation and flocculation tests. The MS2 was enumerated by double agar layer method, while poliovirus and hepatitis A (HAV) were enumerated by the plaque assay method. Tests were conducted to find to optimum doses of coagulant for use in the filtration test. HAV, poliovirus, and MS2 coliphage were seeded to concentrations of 10^4-10^5 PFU/mL. The initial removals were 49 percent (MS2), 88 percent (HAV), and 47 percent (poliovirus), while optimized removals were 99 percent (MS2), 93 percent (HAV), and 91 percent (poliovirus). Throughout the testing, the removals of MS2 were similar to poliovirus and were often less than the removals of Hepatitis A, making MS2 a, appropriate indicator of viruses (Nasser *et al.*, 1995)

While studying enhanced coagulation, Mayer *et al.* (2008) identified removal rates for multiple viruses and bacteriophages in a bench scale analysis. Removals were identified DOC, coxsackievirus, echovirus, poliovirus, and bacteriophages as potential indicators including MS2, PRD-1, ΦX-174, and fr. The series of jar tests was conducted in a similar fashion to Abbaszadegan *et al.* (2007). The source water was untreated central Arizona surface water with turbidities of 9-22 NTU, pH of 7-8, and alkalinity of 140-260 mg/L $CaCO_3$. The samples were seeded with final concentrations of 1×10^6 PFU/mL for the bacteriophages or $10^{3.5}$ 50 percent $TCID_{50}$ per mL for the viruses. The analysis resulted in removals of 3.0 $\log_{10}$ coxsackievirus B6, 1.75 $\log_{10}$ echovirus 12, 2.5 $\log_{10}$ poliovirus 1, 1.8 $\log_{10}$ fr, 1.3 $\log_{10}$ ΦX-174, 0.36 $\log_{10}$ MS2, 0.29 $\log_{10}$ PRD-1 and 41 percent DOC. Coxsackievirus B6 was removed more efficiently than the bacteriophages, indicating these may be suitable indicators, with fr and ΦX-174 being more representative of the viruses and MS2 and PRD-1 being more conservative than fr and ΦX-174. Echovirus had the lowest removal rates of the viruses and was most suitably indicated by MS2 and PRD-1 (Mayer *et al.*, 2008). In addition to also identifying bacteriophages as surrogates, this study suggests that different viruses may be represented by different bacteriophages, indicating that one bacteriophage may not able to represent all viruses, but instead multiple bacteriophages may be more well suited to indicating enteric viruses.

Abbaszadegan *et al.* (2007), as previously discussed, continued with a pilot study using the optimized coagulant dose and pH from the jar tests. These tests were conducted using only the bacteriophages, since the use of animal viruses were not permitted at the facility. The pilot plant processes include coagulation, flocculation, sedimentation, and filtration, as compared to the jar tests, which did not include filtration. The pilot test was performed at a fast flow rate of 0.5 gpm and a slow flow rate of 0.25 gpm. The filters were multimedia including gravel, sand, and GAC. Samples were collected from three separate treatment locations: the influent, after sedimentation and filtered water. The pilot plant was challenged with seeded samples with a final concentration of 1×10^6 PFU/mL for each of the bacteriophages. There was a statistically significant difference between the removals of the bacteriophages at the high and low flow rates, except for PRD-1. At the low flow rate of 0.25 gpm, the greatest removal was for PRD-1 (6.67 $\log_{10}$ removal), followed by MS2 (4.60 $\log_{10}$ removal), fr (4.54 $\log_{10}$ removal) and ΦX-174 (3.94 $\log_{10}$ removal). At the high flow rate of 0.5 gpm, the greatest removal was for fr with 7.87 $\log_{10}$ removal, followed by PRD-1 with 6.87 $\log_{10}$ removal, MS2 with 6.87 $\log_{10}$ removal, and ΦX-174 with 4.47 $\log_{10}$ removal (Abbaszadegan *et al.*, 2007).

Abbaszadegan *et al.* (2008) found similar results expanding on Abbaszadegan *et al.* (2007) identifying removal mechanisms. The pilot testing included MS2, PRD-1, ΦX-174, and fr, and were conducted at 20 and 40 mg/L ferric chloride. The pilot treatment included coagulation, flocculation, sedimentation, and filtration. Three runs were conducted, and duplicates were completed for each test. The virus removal rates trended as (MS2 and fr greater than PRD-1 and ΦX-174) for both the jar and pilot tests, although the removal rates for the jar tests were more conservative than the pilot testing. The removal rates were generally higher at the 0.5 gpm flow rate, but only the MS2 removal was impacted by the coagulant dose with the higher removals at the 40 mg/L ferric chloride. The bacteriophages had maximum removal rates of 5.1 $\log_{10}$ for MS2, 4.9 $\log_{10}$ for fr, 3.5 for PRD-1 and 1.3 for ΦX-174. MS2 and fr were removed with the greatest removals during filtration and PRD-1 and ΦX-174 showed the greatest removal during sedimentation. The mechanism for virus removal during physical and chemical separation processes was found to be adsorption and charge neutralization followed by gravitational separation; this was found despite the fact that individual factors of adsorption, such as hydrophobicity, surface charge, and isoelectric

point, cannot fully explain the adsorption process. These mechanisms may not be enough to meet the SWTR minimum 4 $\log_{10}$ reduction in viruses. While the MS2 and fr met the criteria of removal credits without disinfection, PRD-1 and ΦX-174 did not, meaning that full-scale treatment processes may not be efficient at removing some viruses (Abbaszadegan *et al.*, 2008). The type and dosing of coagulant typically affects the extent of pathogen removal and the coagulant type may affect removal of bacteriophage by subsequent filtration. While filtration is an important step in the treatment process, this study indicates that the steps prior to filtration impact removal rates.

Nasser *et al.* (1995), as described previously, studied high rate filtration, by determining the removal efficiency of enteric pathogenic viruses. Pilot tests were conducted for high rate filtration using a 100 cm sand bed. The alum was added in line at the optimized concentration of 20 ppm, and kaolin and humic acid were added for turbidity. The viruses and phage were added in-line to a final concentration of 10^4-10^5 PFU/mL. The removal of turbidity and MS2 coliphages were minimal by high rate filtration without the addition of the alum. The alum enhancement increased the removal of viruses and turbidity, and the addition of the alum and the 1 mg/L of a cationic polyelectrolyte further increased the removal of viruses and turbidity. The removals observed were 99.5 percent for turbidity, 99 percent for MS2, greater than 93 percent for hepatitis A and 91 percent for poliovirus. This study indicated that turbidity removal was greater than the removal of viruses and is therefore not a suitable indicator for viruses (Nasser *et al.*, 1995).

Noroviruses have also been proposed as markers of fecal pollution; however, noroviruses can exhibit seasonal fluctuations and epidemic spikes. From June 2003 to June 2004, Haramoto *et al.* (2006) researched noroviruses, total coliforms, *E. coli*, and male phages in raw sewage, secondary treated sewage, and final effluent at a wastewater treatment plant in Tokyo, Japan. The concentration of human noroviruses, determined by qPCR, in raw sewage varied from 0.17 to 260 copies/mL for Genotype 1 and from 2.4 to 1,900 copies/mL for Genotype 2, showing much higher values in winter, while the concentration of total coliforms, *E. coli*, or male phages in raw sewage was almost constant throughout the year. Human noroviruses of Genotype 2 were removed most effectively with an average removal of 3.69 $\log_{10}$, followed by *E. coli* with an average removal of 3.37 $\log_{10}$, total coliforms with an average removal of 3.05 $\log_{10}$, Male phages with an average removal of 2.81 $\log_{10}$, and human noroviruses of genotype 1 with an average removal of 2.27 $\log_{10}$. No correlations were identified between the tested bacteria and human noroviruses in final effluent (Haramoto *et al.*, 2006).

Shin and Sobsey (2008) studied the inactivation of norovirus (NV) in drinking water by chlorine disinfection. The objective of the study was to determine the inactivation of purified and dispersed norovirus by bench scale free chlorine disinfection in typical water treatment condition using RT PCR viral assays. The inactivation of poliovirus (PV1) and MS2 coliphage were also included in the study to compare the rates of the enteric viruses and to determine the relationship between virus inactivation based on infectivity assays and based on RT PCR assays. A virus mixture of PV1 (1.3×10^4-1.6×10^4 PFU/mL), MS2 (1.2×10^5-9.2×10^6 PFU/mL), and norovirus (10^4-10^5 polymerase chain reaction units per mL) were added to the bench scale batch system. Values were the average of duplicate cultures or assays. The CT values were calculated for 2-4 $\log_{10}$ inactivation. The inactivation of MS2 based on infectivity assays was very rapid and reached the detection limit within 20s contact time. The inactivation of PV1 based on infectivity assay was also rapid and reached the detection limit within 10 minutes contact time. Meanwhile, the inactivation of norovirus was 2 $\log_{10}$ at 3 minutes contact time, which was slower than MS2 but faster

than PV1 (Shin *et al.*, 2008). This demonstrates the persistence of norovirus and poliovirus, and suggests that MS2 may not be a suitable surrogate for human infectious viruses.

Norovirus was further studied by Park *et al.* (2011) for the effects of ultraviolet (UV) irradiation in spiked samples. The study determined the inactivation profiles of three surrogates of norovirus, and MS2 coliphages using bench scale tests. The infectivity of murine norovirus (MNV), feline calicivirus (FCV), and echovirus 12 was determined by cell culture infectivity, and MS2 infectivity was determined by plaque assay. All data was averaged from at least four replicate of two independent experiments. The viruses were tested at tires of 10^8, $10^{8.5}$, and $10^{8.9}$ for MNV, FCV, and echovirus 12, respectively. An interpolation of linear regression for first-order inactivation identified UV doses to achieve 4 log inactivation of 29, 25, 30, and 70 (mJ cm^{-2}) for MNV, FCV, echovirus, and MS2, respectively. The reaction profiles for MNV and echovirus were statistically similar, while the inactivation rates of FCV with echovirus and FCV and MNV were statistically different. A UV dose of 30 mJ cm^{-2} was able to achieve 4 log_{10} reduction of the three mammalian norovirus surrogates, and therefore it is likely that human norovirus is effectively controlled by UV disinfection recommendation for viruses of 40 mJ cm^2 (Park *et al.*, 2011). While norovirus was more persistent than MS2 in the Shin and Sobsey (2008) study, norovirus was found by Park *et al.* 2011 to be suitably inactivated by UV.

Hijnen *et al.* (2010) utilized a pilot plant with granular activated carbon (GAC) adsorption filters, and calculated removals for indicators MS2, *E. coli*, and spores of *C. bifermentans*; and pathogenic oocysts of *C. parvum*, and *G. lamblia*. The plant had with two parallel GAC filters--one with fresh GAC and the other with loaded GAC--and each was backwashed prior to the test to create proper hydraulic conditions (mimicking full-scale beds). The influent for the pilot plant was water from River Meuse (The Netherlands) after the impounded reservoirs, coagulation, and rapid sand filtration. The influent to the pilot plant was inoculated with MS2, resulting in influent concentrations of approximately 1.2×10^9 PFU/L for the fresh GAC and approximately 7.2×10^6 PFU/L in the loaded GAC columns. The influent inoculations for *E. coli* resulted in average concentrations of 1.1×10^6 CFU/L; spores of *C. bifermentans* of 2.0×10^6 CFU/L; oocysts of *C. parvum* of 1.6×10^5; and *G. lamblia* 9.8×10^4 cysts/L . The sampling was conducted over 4 consecutive days, and effluent samples were collected at 0, 10, 30, and 60 minutes. The removal rates for *E. coli* removal ranged from 0.1 to 1.1 log_{10} removals, with dosed *E. coli* through the fresh GAC being the lowest and removal of indigenous *E. coli* through the loaded GAC. The removal of the indicator bacteria was attributed to attachment. The removal for MS2 phage was below the level of detection, and the removals for *E. coli* and anaerobic spores were limited ranging from less than 0.1-1.1 log. Conversely, the removals were significant for oocysts of *C. parvum* with 1.3 log_{10} removal and *G. lamblia* with 2.7 log_{10} removal (Hijnen *et al.*, 2010). This study showed that GAC adsorption filters as a separate unit process are not an adequate barrier for viruses, as represented by presence of MS2 bacteriophages and limited capacity to eliminate pathogenic bacteria, as represented by presence of *E. coli* (Hijnen *et al.*, 2010).

Hijnen, *et al.* (2010) continued the GAC analysis in full-scale facilities with MS2 phages (representing viruses), *E. coli*, and *G. lamblia* in granular activated carbon (GAC) adsorption filtration. The study investigated the performance of eight water treatment plants during different seasons and was able to show that "fresh" (i.e. recently backwashed GAC) GAC filtration media, was less effective at removing bacteria than "loaded" or late-cycle GAC. Their study found that while *G. lamblia* was significantly removed, the phage and *E. coli* removal was limited (Hijnen *et al.*, 2010). The results highlighted the variability in

performance of granular media filtration to remove bacteria and viruses, and that removals may be dependent on several operational and design parameters. Additional performance considerations for utility operators include the effects of filter shut-down (i.e. stop-start events), ripening and increasing flow rate during high demand periods (Hijnen *et al.*, 2010).

Several filtration experiments have been conducted to compare the transport of microspheres to *C. parvum.* Dai *et al.* (2002) used microspheres as a surrogate for *C. parvum* oocysts in filtration experiments. A bench scale filtration system was used to compare fresh media removals of microsphere to the oocysts. The column was packed with 0.55 mm spherical glass beads to a depth of 25 cm and a porosity of 40. The research included comparing the zeta potential, hydrophobicity, and filterability of 5 μm carboxylated latex microsphere and oocysts for a variety of solution conditions. *C. parvum* oocysts had a slightly negative zeta potential (-1.5 to -12.5 mV) at a pH 6.7 over a wide range of calcium concentrations (10^{-6} to 10^{-1} M) while the microspheres were more negatively charges (-7.4 to -50.2 mV). The results suggested that when groundwater is hard or high ionic strength (greater than 10^{-3} M Ca^{+}) microspheres mimic the transport of oocysts. The microspheres also consistently had lower removals that the oocysts and were a conservative estimate of oocyst removal in filters containing hydrophilic negatively charged filter media (Dai *et al.*, 2002).

Emelko *et al*. (2004) conducted pilot scale filtration studies with microspheres and *C. parvum*. The comparisons between the microspheres and oocysts varied during operating conditions and process challenges. In-line filtration experiments were conducted at two pilot facilities, the city of Ottawa and University of Waterloo; the loadings rates were 2.7 and 4.3 gpm/ft^2, respectively. The filters were 28 inches deep, and the filter material was varying heights of anthracite, sand, and garnet. Seeded samples were added at the filter influent for 1-hour periods, with a minimum of 10 replicates at concentrations of: 1×10^6 oocysts per L and 2,500 oocysts per microsphere; 1,000 oocysts per L and 500 oocysts per microsphere; and 100 oocysts per L and 50 oocysts per microsphere. *C. parvum* removals ranged from 0.2 log during no coagulation experiments to 5.7 logs during stable operations, and microsphere removals ranged from 0.2 to 5.1 logs. Oocysts and microsphere removals were generally similar thought the operating conditions. A statistical analysis was conducted and the linear coefficients of determination ranged from 0.74 to 0.96 (Emelko *et al.*, 2004).

While size and shape of microorganisms and microspheres can be similar the surface characteristics of the microspheres limit their ability to represent microorganisms, and Harvey *et al.* (2008) observed through a limestone column the differences in the mobility and recovery of *Cryptosporidium parvum* oocysts and carboxylated polystyrene microspheres of similar sizes, even though their diameters, aspect ratio of length to width, and buoyant densities were similar. Microspheres were more negatively charged ($\zeta = -18.7$ at pH = 7.0, and $\zeta = -21.0$ mV at pH = 7.8) compared with the oocysts ($\zeta = -3.6$ at pH = 7.0, and $\zeta = -6.7$ mV at pH = 7.8). Harvey *et al.* (2008) determined that the surface charge of particles had the greatest impact to transport velocity. The researchers also determined that these observed transport differences were attributable to the difference in surface charge, and thus appropriate to identify a surrogate with surface characteristics more similar to microorganisms (Harvey *et al.*, 2004).

Latex spheres have also been used as a potential viral surrogate in low-pressure membrane studies. Pontius *et al.* (2009) conducted a challenge study of microfiltration and ultrafiltration membranes using two

bacteriophages (MS2 and PRD1) and two fluorescent latex spheres of representative sizes compared to the bacteriophages (26 and 67 nm). The study evaluated the suitability of fluorescent microspheres as viral surrogates, taking into account both size and surface charge. The microspheres were carboxylated with a point of zero charge of less than 2, while the isoelectric points of MS2 and PRD1 were 3.9 and 4.2, respectively. Rejection experiments were conducted using 0.22 μm hydrophilic and hydrophobic polyvinylidene fluoride (PVDF) MF membranes, a 100 kD hydrophilic regenerated cellulose (RC) ultrafiltration membrane, and a 100 kD hydrophobic polyethersulfone (PES) ultrafiltration membrane. Surface characteristics were studied, such as zeta potential with a ZetaPlus analyzer, contact angle with a goniometer, and membrane thickness and roughness with scanning electron microscope and atomic force microscopy (Pontius *et al.*, 2009). Challenge studies were conducted at two different pH levels (6.5 and 8.5) for the four different membranes, with initial phage concentrations of 10^6 PFU/mL and microsphere concentrations of 10^{10}-10^{11} per mL. The phage concentrations were assayed with the Adams soft agar overlay method, and the microsphere concentrations were determined by florescence spectroscopy with a fluorescence spectrophotometer. This study found that fluorescent microspheres were an inconsistent surrogate when compared to phages in membrane studies and found that further study on the impact of surrogate surface characteristics was necessary to predict rejection (Pontius *et al.*, 2009).

3.3.4 Bacterial and Viral Association

Bacteria and viruses have many differences, and often epidemiological studies fail to show a relationship between viral pathogens and bacterial indicators in both environmental systems and treatment processes (Ashbolt *et al.*, 2001; Hamza *et al.*, 2011a; McQuaig *et al.*, 2011). The differences in the fate of bacteria and viruses may be expected because of differences in structure and transport mechanisms. Viruses are 10 to 100 times smaller than bacteria and require a host for replication, while bacteria replicate through cell division. Viruses and bacteria also vary in genome size(for example *E. coli* is 4.2 Mb and Hepatitis B is 0.0032 Mb), and the bacterial genome is made of DNA while the viral genome can be made of DNA, RNA, or both (Upadhyay *et al.*, 2010). Another factor is that bacteria are self-contained and viruses cannot survive without a host; in other words, bacteria can self-replicate, while viruses need host cells in order to replicate their genomes(Yates *et al.*, 1985).

The size and structure of the microorganisms are important factors in fate. Viruses consist of a protein coat that holds a coiled string of nucleic acid. Viruses are tiny geometric structures that can only reproduce inside a living cell. They range in size from 20 to 250 nanometers. Bacteria, however, are prokaryotic singled celled organisms and do not have a cell nucleus which consist of ribosomes, nucleoid (DNA), a cell wall, cytoplasm, and a flagella. The average bacterium is 1,000 nanometers long (Upadhyay *et al.*, 2010).

A single virus particle (virion) is primarily made up of nucleic acids and protein capsomeres, which surround the genetic code. The external surface is the capsid (or the coat protein) and may have the morphology of helical, icosahedral or complex. There is also a distinction between enveloped and non-enveloped viruses. The surface properties of a virion is determined by the characteristics of the capsid. Enveloped viruses have an additional external component derived from the host cell and consists of proteins and lipids, and determines the surface properties. An important factor in the terms of quality as an indicator is the ability to move multiplying in the environment. In terms of motility, viruses do not possess structures that enable them to move purposefully through the environment. Bacteria, on the other hand, are able to

move through the environment using a structure known as the flagellum. (Giddings, 1998). Viruses are intracellular parasites which enter host cells, use the host cell machineries to replicate their genome, and synthesize their proteins to produce additional viruses that are then released.

Viruses bind to the membrane of a host and can bind to a specific receptor, or multiple receptors. Although viruses can initially bind to non-specific sites which are not capable of direct virus entry and these sites can provide initial adhesion sites. This binding allows for viruses to accumulate at the capsid based on electrostatic attractions. It is possible for these accumulations to allow for the virons to interact with specific receptors (Villanueva *et al.*, 2005). Bacteriophages are referred to by the receptor types that initiate bacterial entry to host cells. Somatic phages are capable of binding to components of the cell wall. In contrast, male-specific phages attach to filamentous appendages (sex-pili) attached to bacterial cell walls (Leclerc *et al.*, 2000).

Coliform bacteria are insufficient indicators of viral pathogens because these microorganisms cannot designate viral pathogen risk in the environment (Haramoto *et al.*, 2007). Although total coliforms are normally present in the waste of warm blooded animals, they tend to be poor indicator microorganisms through treatment processes because of their short survival and susceptibility to water treatments (Moe, 2004). Also, pathogenic viruses have been found in waters in which the number of coliforms had not exceeded water quality standards (Fong *et al.*, 2005). Moreover, these bacterial indicators are not specific to feces and have the ability to grow in natural waters (Ashbolt *et al.*, 2001; Fong *et al.*, 2005; Toranzos *et al.*, 2007).

Most bacteria are removed in the soil surface by filtration, sedimentation, and adsorption while viruses are removed primarily through adsorption (Gilbert *et al.*, 1976). However, salt concentrations, pH, organic matter, soil composition, infiltration rates, and climatic conditions may affect the degree of retention of bacteria and viruses by soil. Furthermore, the survival and movement of the retained bacteria and viruses are influenced by soil moisture, temperature, pH, and nutrient availability. The ability of some coliforms to grow in the environment, lack of correlation between coliforms and pathogenic microorganism concentrations, and detection of atypical strains make coliforms unsuitable as indicators of pathogens (Figueras *et al.*, 2010).

Male-specific coliphages are present in much lower concentrations, can vary by species, and are capable of distinguishing between fecal pollution of human origin and fecal pollution of animal origin (Leclerc *et al.*, 2000). It has been found, however, that somatic coliphages have higher concentrations than male-specific phages in wastewater and raw water sources (Grabow, 2001).

Long *et al.* (2005) studied the use of male-specific coliphages as potential indicators of fecal contamination and their use in fecal source tracking. While coliforms are utilized as indicator organisms, they lack source specificity. Water suppliers utilize a multi-barrier approach to providing drinking water with source water protection as one of the barriers. In order to protect those sources, a method to identify potential sources of fecal contamination is required. Male-specific coliphages may provide this information not only to assess relative risk but also to provide information that can develop appropriate corrective actions. This study does not focus on the use of coliphages as a surrogate for human enteric viruses, but instead proposes the use of coliphages as their own indication of fecal contamination. The study identified DNA versus RNA genotypes of coliphages and compared the genotypes and the potential use of specific genotypes as source indicators.

The densities were representative of previously published studies of similar indicators. Male-specific DNA coliphages were identified in statistically significant numbers of samples supporting their potential as fecal indicators. In addition, male-specific DNA coliphages were more prevalent in the wastewater influent and effluent samples when compared to animal waste lagoons. Liquid composite waste samples such as wastewater samples and waste lagoons indicated higher levels of male-specific RNA coliphages when compared to individual sources, such as septic tanks and grazing animal fecal samples. This may occur because the host cells in wastewater allow the coliphage from individual fecal sources to multiply in wastewater liquids. The male-specific RNA coliphages also demonstrate more statistical significance in identifying differences in coliphage isolates. The majority of groups I and IV male-specific RNA coliphages were associated with animal sources, while groups II and III were more often associated with human sources. These results indicate that coliphages may provide additional information concerning fecal source tracking, and should be considered as a tool in drinking water quality assessment (Long *et al.*, 2005).

Havelaar, et *al.* (1990) studied the occurrence of somatic coliphages, male-specific bacteriophages, and *E. coli* strains sensitive to infection by male-specific phages. The study included samples from humans (n = 33), pigs (n = 36), cattle (n = 31), and chickens (n = 28). *E. coli* was identified in all of the untreated samples of human feces and not often detected when antibiotics were used. Pig feces samples were usually positive for *E. coli*, cattle sample *E. coli* counts were usually low, and *E. coli* was found in high numbers (3.9×10^3 to 8.0×10^3 CFU/g) in chicken feces. Somatic coliphages were detected in high numbers (greater than, 10^3 PFU/mL) in all feces types. However; male-specific phages were rarely detected in the feces of humans (6%) and cattle (10%) and were more frequent in pigs (47%) and chickens (64%). Phages were detected in very low numbers (13 -22 PFU/g) in only two of the 33 samples of human feces. Despite this, male-specific phages are often detected in domestic wastewater samples, identifying male-specific phages as a possible indicator of wastewater pollution but not of fecal pollution (Havelaar *et al.*, 1990).

However, research provides a sufficient amount of data to allow for the use of coliphages as representatives of viruses in unit process studies without the use of human viruses. This is important, because while coliphages are similar to viruses in size and isoelectric points, they are less expensive and easier to assay. These studies confirm the use of bacteriophages as surrogates for viruses. In most instances, the bacteriophages have lower removals than viruses, meaning they could be considered a conservative indication of virus removal through drinking water treatment.

4.0 PARTICLE CHARACTERISTICS

As described in Section 3.0, viruses and traditional indictors have different fates in treatment processes and environmental systems. Often, viruses are more persistent. Traditional physical treatment methods using gravity and size exclusion are unreliable in regards to viral pathogens. Treatment and removals at the nanoscale are instead dependent on the interaction of forces impacting physicochemical attachment.

4.1 LIGHT SCATTERING

Light scattering (LS) is an optical method of analyzing particle characteristics and dynamics. LS can also be used to study the stability of an aqueous solution. LS methods include a light source (often a laser), through a sample and an analysis of changes in the intensity of the scattered light. Experimentally, LS intensity fluctuations are a function of size of the particle, the shape of surface structures, particle concentration, and the type of ions in solution (Berne *et al.*, 2000; Brar *et al.*, 2011; Kaszuba *et al.*, 2008).

Particles undergoing Brownian motion produce fluctuations in scattered light intensity, I, which LS can quantify. The light source at wavelength, λ, hits particles, the light scatters in all directions. When the laser, incident beam k_i, hits a particle in the sample, the beam is scattered and sampled at a scattering angle, θ, with a detector. The measurements can be analyzed for specific wave vectors. The wave vector is the difference in the incident beam vector and the scattered beam vector, k_s. A wave vector is a function of the wave length, viscosity and scattering angle, as shown in Equation 4-1.

$$|\vec{q}| = \frac{4\pi n_d \sin\left(\frac{\theta}{2}\right)}{\lambda}$$

(Equation 4-1)

Where $|\vec{q}|$ is wave vector, λ wavelength, n_d refractive index (for aqueous samples, viscosity), and θ scattering angle. The wave vector $|\vec{q}|$ is the difference between the incident beam vector (k_i) and the scattered beam vector (k_s). These variables are outlined in Table 4-1.

Table 4-1: Light Scattering Definitions

Light Scattering Variable	Definition
<I>	Quantity Of Scattered Light Intensity
Λ	Laser Wavelength
k_i	Initial/Incident Beam Vector
Θ	Scattered Beam Angle
k_s	Scattered Beam Vector
Q	Wave Vector
<I>(q, t)	Relative Total Intensity

Light Scattering Variable	Definition
F	Fractal Dimension (Models Shape)
R_{eff}	Effective Radius (Models Size)
RMSD	Root Mean Square Difference

The set up for a generic light scattering experiment is shown in Figure 4-1.

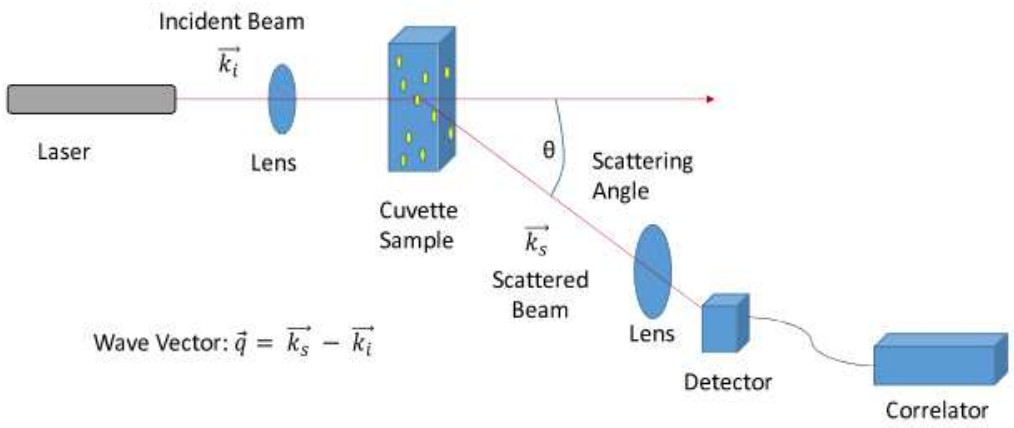


Figure 4-1: Light Scattering Components

Dynamic light scattering (DLS) is also referred as Photon Correlation Spectroscopy or Quasi-Elastic Light Scattering. DLS provides data based on the particle scattering in a system on a time scale ranging from tens of nanoseconds to seconds. The measurement of the intensity correlation function allows evaluation of the diffusion coefficient, D, of the scattered particles. The DLS measurements can demonstrate the ability of the particles to aggregate over time by determine time impacts or increases of the hydrodynamic radius.

For example, Pasquini *et al.* (2012) used static and dynamic light scattering to determine the structural morphology and aggregation state of functionalized single walled nanotubes (fSWNTs). Static light scattering (SLS, ALVGmbH, Germany) was used to obtain the fractal dimension, Df, of the particles. Measurements were taken every 1° using eight detectors and collected every 20 seconds time over the range of $0.00516 < q < 0.03397$ nm^{-1}, corresponding to θ of 17−153°.

To compare the extent of dispersion of the fSWNTs, dynamic light scattering (DLS) measurements were collected. DLS measured g(Δt), the autocorrelation of the scattered light intensity at θ. Diffusion of particles in the sample causes fluctuations in the scattered light intensity, and g(Δt) decays exponentially as described in Equation 4-2.

$$g(\Delta t) = e^{\frac{-\Delta t}{\tau}}$$

(Equation 4-2)

Where Δt is the time lag and τ is the diffusive time scale in the system. The diffusion coefficient, D, is determined from the measured diffusion time, τ, through Equation 4-3.

$$\tau = \frac{1}{2q^2 D}$$

(Equation 4-3)

The hydrodynamic radius (R_H) can be calculated from the diffusion coefficient utilizing the Stokes−Einstein equation, as shown in Equation 4-4.

$$R_H = \frac{k_B T}{6\pi n_d D}$$

(Equation 4-4)

Where k_B is the Boltzmann constant, T is the absolute temperature, and η is the solvent viscosity. Although DLS is typically used to determine size of spherical particles, in this experiment, it was used to determine the relative size of rod-shaped materials. By using DLS the experiment was able to analyze the aspect ratio and the diffusion coefficient of the particles (Pasquini *et al.*, 2012).

As demonstrated, an analysis of intensity fluctuations provided a quantification of the velocity of the Brownian motion and then the particle size using the Stokes-Einstein relationship. Brownian motion was quantitatively characterized by the diffusion coefficient, D. The diffusion coefficient depends on particle size and shape, as well as on the ambient temperature and solution viscosity. By converting a diffusion coefficient into a hydrodynamic radius (the radius of a hard sphere with the same diffusion coefficient as the scatterer), temperature and viscosity are factored out (Berne *et al.*, 2000; Brar *et al.*, 2011; Holz *et al.*, 1978). While this demonstrates physical characteristics of the particle, water quality research, particularly research focused on adhesion, primarily utilizes DLS to measure electrostatic characteristics.

4.2 ELECTROSTATIC CHARACTERISTICS

Electrophoresis is the movement of charged particles, such that when an electrical field is applied to an aqueous environment, the charged field induces a double layer around each particle. The thickness of the double layer depends upon the concentration of ions in solution the available valence electrons. Negatively charged colloids move toward the positive charge, and the speed of the particles, electrophoretic mobility, is calculated based on the applied voltage gradient, the charge on the particle, and the viscosity of water. (Hendricks, 2010).

Zeta potential (ζ) is the energy potential of the particles, and is a typical measurement of electrostatic interactions of colloids. Zeta potential measures the colloidal charge at the point of the slipping plane. It indicates the degree of repulsion between similarly charged particles and the magnitude quantifies the potential stability of the system (da Silva *et al.*, 2010). The zeta potential of a virus can be measured to predict electrostatic interactions impact the behavior of a virus in a particular environment and what which water quality parameters alter the virus (Malvern Instruments, 2012; Tanneru *et al.*, 2012).

The zeta potential is calculated by converting the electrophoretic mobility using the Helmholtz-Smoluchowski or the Henry Equation.

4.2.1 Isoelectric Points

The pH at which the net surface charge is neutral is the isoelectric point (IEP) and is a characteristic of the particle in equilibrium. This charge determines particle mobility and contributes to the sorption process. Biotic nanoparticles, such as viruses and bacteriophages, have a pH dependent surface charge in polar media and in the case of non-enveloped viruses, the functional groups of the coat protein determine the net surface charge. IEPs of viruses are found in pH range from 1.9 to 8.4 and are most frequently in the range of 3.5 to 7. Michen *et al*. (2010) fitted virus IEPs to a Gaussian function with a mean value of 5.0 and a standard deviation of 1.3. Unfortunately, the literature indicates that a range of IEPs is attributed to each virus dependent on the research methodology.

The Michen *et al.* (2010) literature review included ten citations for the IEP of MS2 bacteriophage with a mean IEP value of 3.5, a standard deviation of 0.6, and a difference between highest and lowest IEP (ΔIEP) of 1.8. Values shifted from 3.1 to 3.9 by increasing the concentration of $NaNO_3$ from 1 to 100 $mmol^{-1}$. The study determined a representative value of MS2 by excluding measurements with undefined purity, different strains, and those conducted at high ionic strengths to focus the IEP towards the point of zero charge. The resultant value was an average value of 3.4, a standard deviation of 0.8, and a ΔIEP of 0.8. In the case of ΦX-174 bacteriophage, a representative value was determined with a mean IEP of pH 6.6, a standard deviation of 0.05, and a ΔIEP of 0.1 (Michen *et al.*, 2010).

Michen *et al.* (2010) also found that isoelectric points varied by host, species, and strain. Strains within a single species may vary because of differences in the coat proteins because the coat can define the exchange of amino acids with other peptides. The variation among a single species can also be caused by lab handling including the purification of virus stock is inconsistent. The review determined that IEP values reported in literature should only be used in estimations of sorption in waters of comparable chemistry.

Langlet *et al.* (2008) studied the alteration of IEPs by changing the water chemistry of solutions. The research included bench scale analysis of the electrokinetic properties and size variations for four male-specific bacteriophages including MS2, GA, Qβ, and SP with diameters ranging from 21-30 nm over a range of pH values 1.5 to 7.5 and $NaNO_3$ electrolyte concentration (1-100 mM). The research identified significant aggregation of MS2 phages in systems with pH ≤ pI. The pI measured at 1 mM ionic strength were MS2 3.1 ± 0.1, GA 2.1 ± 0.1, Qβ 2.7 ± 0.1, and SP2.1 ± 0.3 and at 100 mM ionic strength were MS2 3.9 ± 0.3, GA 2.3 ± 0.1, Qβ 1.9 ± 0.3, and SP 2.6 ± 0.1. The size analysis identified trends in aggregation. For MS2, aggregation was not dependent on ionic strength. This may be a result of MS2 phage being one of the most hydrophobic phages (Lytle *et al.*, 1995). MS2 aggregation did vary with pH with a mean hydrodynamic radius (R_H) of 9 $\mu m^2/s$ for neutral pHs and at lower pHs near the pI the R_H was 0.3 $\mu m^2/s$, indicating aggregation at the high pHs. This data suggests a potential bias for PFU assays based on water quality caused by PFU counts less than the sum of its constituent particles (Langlet *et al.*, 2008).

While electrostatic forces have been shown to impact adsorption, they the only driving force in adsorption. Chattophadhyay *et al.* (1999) conducted experiments in order to examine the adsorption of bacteriophages (T2, MS2 and ΦX-174) on four different clays in 0.01 M NaCl at pH 7. The different phage types were observed to adsorb to the clays in decreasing removals T2, MS2, and ΦX-174. This research showed that hydrophobic interactions drove the adsorption process rather than electrostatic forces. (Chattopadhyay *et al.*, 1999).

An IEP is able to provide a measurement of a particle characteristic. Yet IEP is highly dependent upon the specifics of the particle and the environment in which it is measured. While specific IEPs were not attributed to each phage, when particle interactions are researched, IEPs and zeta potentials are frequently utilized to define a particle and its potential to interact with other particles.

4.3 PARTICLE INTERACTIONS

Derjaguin, Landau, Verwey, and Overbeek (DLVO) theory accounts for van der Waals and electrostatic interactions between colloidal particles and is used to describe sorption behavior (Hendricks, 2010). DLVO theory is based on the stability of a particle in solution being dependent on total potential energy (Derjaguin *et al.*, 1941; Ryan *et al.*, 1996; Verwey *et al.*, 1948). For a system to be considered stable, the electrostatic (double layer) repulsive forces must be greater than the van der Waals attractive forces (Malvern Instruments, 2012).

DLVO theory provides a method to quantify molecular interaction profiles, by summing electrostatic and van der Waals interactions (Hendricks, 2010). Particle surfaces become charged when placed into an electrolyte solution. For example, if the particles come into contact with oxygen molecules, then hydrogen atoms in the water will be attracted to the charged molecules. Oxygen molecules will interact with the hydrogen molecules, leaving a net negative charge on the particle. The net negative charge attracts positively charged ions in the solution that would normally have been dissociated (Ryan *et al.*, 1996).

While DLVO theory is used to describe colloidal interactions, it is not able to consistently describe all particle relationships. Non-DLVO interactions, such as hydrophobic interactions, are included in some models that are known as extended DLVO theories (XDLVO). Many non-DLVO interactions are not completely understood, and a comprehensive theory has not been uniformly applied (Bradford *et al.*, 2008). Interfacial potential energy modeled with XDLVO is calculated by summing the forces contributed by van der Waals (ΦvdW), electrostatic double layer (ΦEdI), and hydrophobic interactions (Φhydrophobic). Wong *et al.* (2012), includes steric interactions as shown in Equation 4-5 (Wong *et al.*, 2012).

$$\Phi XDLVO + steric = \Phi vdW + \Phi EdI + \Phi hydrophobic + \Phi steric$$

(Equation 4-5)

Hydrophobicity is a result of the interaction of the aqueous solution with the particle. Current research includes the measurement of interactions between hydrophobic surfaces, such as biological molecules exposing hydrocarbon groups, and hydrophobic surfaces are attracted to one another. Hydrophobic interactions have been suggested in the adhesion and aggregation of particles including proteins and collagen (Li, 2009).

Hydrophobic interactions can be described theoretically (Van Oss, 1995) or empirically (Yoon *et al.*, 1996). Contact angle is used to empirically determine hydrophobicity of an aqueous solution by measuring the water contact angle (θ) on a solid surface (θ refers to hydrophobic character). The solutions are described by their relationship to the surfaces as wetting ($\theta < 90°$) and non-wetting ($\theta > 90°$) surfaces. Hydrophobic interactions are defined as $\theta > 65°$, and hydrophilic surfaces have $\theta < 65°$ (Vogler, 1998). The impacts of hydrophobic interactions on colloid transport remain an open area of research (Liang *et al.*, 2006).

Batch, column, and field studies of colloid transport provide information about the applicability of DLVO and XDLVO theories for particle interactions. In addition to DLVO, studies of environmental and groundwater transport have found that many factors affect the subsurface transport of biotic and abiotic particles including soil properties such as mineralogy, grain size, surface roughness, and organic content; water properties such as pH, ionic strength; and colloid properties such as isoelectric point, surface charge, surface morphology, and size.

Knappett *et al.* (2008) utilized replicate column experiments to quantify the impacts of ionic strength and grain size on the transport of colloids. Column diameters met the minimum d_{col}/d_{10} ratio (the ratio of 50 that is recommended to minimize wall effects. Bacteriophage, MS2, and 0.025 μm carboxylated microspheres were used as surrogates for bacteria and viruses saturated columns of crushed silica. MS2 was quantified using the double layer agar method in duplicate or triplicate with 30-300 PFU per mL. Microspheres were Flouresbrite™ carboxylated polystyrene with a mean diameter of 1.5 μm and a particle density of 1.055 gram per cm and enumerated with fluoresce. Microspheres were used because of the ease of enumeration, absence of possible inactivation, and potential growth associated with microbial surrogate. Grain size of the media, angular silica sand of 99.7 percent quartz, was varied from medium sand (d_{50}=0.70 mm) to fine sand (d_{50}=0.34 mm). In column experiments, bromide concentration was analyzed with an ion chromatograph and used to estimate hydrodynamic dispersion coefficient (D). Surrogates were suspended in artificial groundwater (AGW) containing a ionic strength (5nM) of 1mM Ca^{2+}, 2 nM Na^{+}, and 4 nM Cl^{-} and a high ionic strength (34 nM) of 4.8 nM Ca^{2+}, 19.5 nM Na^{+}, and 29.1 nM Cl^{-}. Increasing the Ca^{2+} concentration from 1 to 4.8 mM resulted in complete attenuation. The microspheres were found not to accurately model MS2 as evidenced by the greater than 6 log reduction of MS2 in C/C_o while only a one log reduction in the concentration of microspheres (Knappett *et al.*, 2008).

The results from Knappett *et al.* (2006) found that at high overall ionic strength (34 nM) MS2 had no breakthrough in columns with either grain size, indicating total removal of the virus colloids from the free liquid. While at the low ionic strength (5 nM), MS2 exhibited a complete breakthrough in the medium sand and a 5 log reduction in concentration in the fine sand. Effluent concentrations were decreased through the fine sand for the MS2 with a 5 log decrease and microspheres with a 2.5 log decrease. Changes in ionic strength or grain size influenced virus removal in saturated porous media. This model was compared to a literature review of research of natural systems and was found to under predicted colloid retention by angular sand over distances as short as 20 cm (Knappett *et al.*, 2008).

Knappett *et al.* found an increase in attachment consistent with DLVO theory as represented by the increase in ionic strength consistent with a compression of the double layer of ions for the particles, media and colloids. The compression reduced the double layer repulsion and allowed the colloid to approach the like charged grain. The researchers found that when the particles were proximate, the colloid was held by van der Waals forces in a low net potential energy state (Knappett *et al.*, 2008). The increase in retention was found to be a result of attachment efficiency, and colloid retention in porous media increases when the ionic strength is increased.

The transport of bacteriophage PRD1 in groundwater through a natural sand aquifer was studied by Ryan *et al.* (1999). Zeta potentials were measured for the phage and soil types throughout the aquifer. The soils with heterogeneous surfaces were negative and soils with ferric oxyhydroxides and clay mineral edges had positive charges. Attachment of phage, were PRD1 was identified in positive charged sites in the aquifer.

When NaOH was injected to reverse the charge on the aquifer, the phages were released, indicating that electrostatic forces dominated the attachment of PRD1 to aquifer soils (Ryan *et al.*, 1999).

You *et al.* (2005) used iron particles to study the removal MS2 and ϕX-174 in batch and column experiments. MS2 had a higher adsorption rate compared to ϕX-174 in the batch experiment. While the phages were found to have similar removals in column experiments with groundwater at pH 7.5, the recovery of both adsorbed viruses was 0.1 percent for MS2 and ϕX-174, indicating that the phages were either irreversibly adsorbed or rendered non-infectious. In columns filled with a mix of sand and iron, particles both phages were retained at 4 $\log_{10}$ removal. Sterile artificial groundwater was continuously pumped through the filtration bed for 10 days, and then column removal of the phages improved to 5 $\log_{10}$ removal. The increase in removal was potentially from the formation of iron oxides during the continuous wash cycle. (You *et al.*, 2005). The improved removals also identifies the dominance of electrostatic forces in attachment.

Dowd *et al.* (1998) performed transport experiments in 73 cm (5 cm inner diameter) columns injecting five different bacteriophages (MS2, PRD1, Q, ΦX-174, and PM2). Their purpose was to test the correlation between viral transport and isoelectric points. The porous material was from a sandy aquifer (95% sand, 7% silt, and 2% clay). The experiment included two experimental set ups: one using a conventional batch, flow through column and the other was a continuous flow through column in which the column effluent was re-injected into the influent end. This was done to simulate longer distances of travel through an aquifer (Dowd *et al.*, 1998).

For the column experiments, Dowd *et al.* (1998) injected 2.1 pore volumes of virus seeded groundwater into the influent and measured the effluent virus concentration over 10 pore volumes of flushing with water free of bacteriophage. The phages were injected separately into the column (except MS2 and ΦX-174, which were introduced concurrently). Influent bacteriophage concentrations (C_o) ranged from 10^4 to 10^9 PFU/mL. With the large diameter (approximately 60 nm), bacteriophage PRD1 (69% adsorbed with a pI of 4.2) has a higher removal than PM2 (30% adsorbed with a pI of 7.3). Similarly with the phages (approximately 24 nm), MS2 (46%, 3.9) has a higher removal than ΦX-174 (2.5% adsorbed with a pI of 6.6). An exception to this trend was Q (53% adsorbed with a pI of 5.5) (Dowd *et al.*, 1998). The result was that the IEP of a virus determined efficiency of viral adsorption within aquifers.

Porubcan and Xu (2011) researched colloids within natural heterogeneous porous media. They conducted column experiments to investigate the transport of latex particles of 0.46 µm, 2.94 µm, 5.1 µm, and 606 µm diameter latex particles through mixtures of 0.78 mm, 0.46 mm, and 0.23 mm diameter quartz sands. Glass chromatography columns of 2.5 cm diameter and 15 cm in length were used with an acrylic end fitting the featured a 0.051 mm stainless steel membrane. The columns were set up vertically and sand was layered using wetted sand mixtures. Colloid attachment was minimized by suspending the microspheres in Nanopure water to maximize the electrostatic repulsion between the colloids and the sands. The smallest (0.46 µm) particles traveled through the media. The results showed minimal physicochemical filtration or straining attributable to the repulsive charges between the latex particles and cleaned sands. The straining of the larger particles was modeled and the particle straining was found to correlate the ratio of the colloid diameter to the average grain size and empirically derived straining capacity term (λ) (Porubcan *et al.*, 2011).

Xu *et al.* (2006) examined the straining kinetics of colloidal particles in saturated porous media. Column experiments were conducted with 0.5, 1.1, 3.1 and 5.1 µm latex particles with carboxyl surface functional groups. The columns were filled with high purity quartz sand in five class sizes: 0.71-0.85 mm, 0.3-0.355 mm, 0.125-0.15 mm, 0.106-0.125 mm and 0.09-0.106 mm. The sand was prepared by boiling the for 24 hours in concentrated nitric acid, washing with 0.1 M NaOH on a shaker table for 12 hours, and rinsed with deionized water. The sand was then dried in an oven at 80°C and stored in Pyrex beakers until used in experiments. The research identified a threshold value of dp/dg less than 0.008 for the transport of colloids as a result of straining. Above this limit the straining rate coefficient increased linearly with the ratio of colloid diameter and the average diameter of sand grains and below this value straining are assumed negligible. Xu continued this research (2008) and extended the relationship to non-spherical particles with impact from straining determined by the minor sizes attributable to their orientation along the flow direction. Therefore, with a particle diameter of 26 nm and a mean grain diameter of 0.45 mm the dp/dg is 0.00006 and straining was assumed to be negligible.

Johanson *et al.* (2012) conducted saturated quartz sand column experiments to study the impact of Entrococcal surface protein (esp) on the transport of *E. faecium*. The sand used in the experiments was cleaned in concentrated nitric acid to remove metal hydroxides, soaked in dilute NaOH to remove clay particles, and boiled a second time on the nitric acid to remove any metal residues. The zeta potentials (ζ) of the sand were analyzed in ionic solutions of pH 7.2 with strengths of 1, 2.5, 5, 20 and 50 mM. The solutions were buffered with 0.2 mM $NaHCO_3$ and the total ionic strength was adjusted using NaCl. The zeta potentials were measured with a ZetaPals (Brookhaven Instruments) analyzer. Sand ζ identified an increase as ionic strength increased likely a result of the compression of the electrostatic double layer (Johanson *et al.*, 2012).

Research demonstrates that removal of colloids (colloidal size is 1 nm to 100 nm and biotic colloids include protozoa, bacteria, and viruses) consisted of an interaction of several forces resulting in physicochemical attachment. Fate and transport of biotic and abiotic microparticles and colloids in saturated porous media were affected by heterogeneity of media, solutions chemistry, and colloid surface properties. Particle properties such as zeta potentials and electrostatic relationships were complicit in nanoparticle sorption.

4.4 EQUILIBRIUM ANALYSIS

An isotherm is a model of adsorption at equilibrium. It can be generated from data obtained during an adsorption process run at constant temperature until equilibrium is achieved. Experimental data for equilibrium are often model by the Langmuir isotherm and the Freundlich isotherm (LeVan *et al.*, 1981; Schijven *et al.*, 2000a; Yates *et al.*, 1987).

The Freundlich model represents the isothermal variation of adsorption of a quantity adsorbed by unit mass of solid adsorbent. The Freundlich model equation is as shown in Equation 4-6.

$$q_e = K_f C_e^{\frac{1}{n}}$$

(Equation 4-6)

Where q_e is the uptake of contaminant adsorbed per unit adsorbent (mg/g), Ce is the equilibrium concentration (mg/L), K_f is the Freundlich Coefficient, and n is an empirical coefficient.

The Langmuir model assumes a uniform surface, a single layer of adsorbed material, and constant temperature. In addition, the rate of attachment to the surface as proportional to a momentum force times an area. The momentum force is the concentration in the fluid, and the area is the amount of available surface (Bungay, 2000). The Langmuir model depends upon both the rate at which molecules cover the surface of the adsorbent and also the rate at which other molecules leave the surface. At steady state, both rates equal such that the rate of molecules attaching to the surface equals the rate of molecules detaching from the surface.

At equilibrium, the Langmuir model is described by Equation 4-7.

$$\mathrm{K_L C_e}(1-\theta) = \mathrm{K_L}\,\theta$$

(Equation 4-7)

Where: K_L is the Langmuir rate constant, Ce is the equilibrium concentration (mg/L), and Θ is a ratio of amount of adsorption in units of moles adsorbate per mass adsorbant, and the maximum adsorption. When K_L is much less than 1, the Langmuir equation may be linearized (Schijven *et al.*, 2000a). While Langmuir isotherm is derived directly from the equilibrium equation, the empirically derived Freundlich isotherm is often used in practice because it may be applied in cases of limited data (Hendricks, 2010).

4.4.1 Batch Experiments

In batch experiments, a solute such as water containing a known number of viruses is mixed with media such as soil, and the change in concentration of viruses remaining in solution particles is measured. Removals are measured as virus concentrations decline with time, and after a period of time equilibrium is achieved between the solid and liquid phase. Batch experiments can provide estimates for adsorption parameters.

Batch experiments provide attachment rate coefficients, detachment rate coefficients, and a distribution coefficient for equilibrium adsorption, assuming inactivation of viruses is neglected. Since the scale of time for a virus batch system study is often a few hours, the inactivation of viruses is assumed to be negligible. In batch experiments, the concentration of the solution is assumed spatially uniform.

Virus concentrations in batch experiments are described by Equation 4-8 (Schijven *et al.*, 2000b).

$$C/C_o = \frac{k_{det} + k_{att}\, exp[-(k_{att} + k_{det})t]}{k_{att} + k_{det}}$$

(Equation 4-8)

Where C is the number of viruses per unit volume in the aqueous phase (L^{-3}), and k_{att} and k_{det} are the attachment and detachment rate coefficients for the kinetic sites, respectively, (t^{-1}). The rate coefficient of k_{att} may be evaluated from early measurements of a batch experiments, and k_{det} may be determined as a function of k_{att} and the concentration at equilibrium. The kinetic behavior in batch experiments prior to equilibrium is used to calculate virus attachment, and virus detachment. Batch analysis provides initial parameters for a lab scale analysis but the attachment rates can be much higher in batch analysis because constant stirring provides access to additional accessible sites for adsorption.

Schijven, *et al.* (2000) modeled adsorption of MS2, PRD1, ΦX-174, Qβ, and PM2 in batch and column experiments. Batch experiments were performed using 50 mL centrifuge tubes containing 5 grams of aquifer material, 9 mL of groundwater with counts of 10^6-10^{10} plaque forming units (PFU) of bacteriophages. One mL of the groundwater diluted stock was then added to a time zero tube, which was vortexed, serial diluted, and the virus enumerated to provide an initial phage concentration (C_o). The remaining tubes containing groundwater and sediment were inoculated and placed in a shaking incubator at 21°C. One tube at a time was removed at 10, 20, 40, and 90 minutes and centrifuged at 1000 × g for 2 minutes in order to sediment the soil. The supernatant was then sampled and assayed to determine remaining virus concentration. Batch attachment decreased in the order of C/Co 0.0015 MS2, 0.015 PRD1, 0.19 Qβ, 0.2 ΦX-174 and 0.21 PM2 (estimated from graphs). Higher attachment of negatively charged viruses may be found in the presence of positively charged sites. The results indicated that under conditions of high pH in sandy soils, MS2 is a conservative tracer, while in the presence of multivalent cations, bacteriophage ΦX-174 may be more conservative. The research determined that for soils near neutral pH, with high concentrations of multivalent cations, bacteriophage ΦX-174 may be the better choice for a relatively conservative tracer virus in field and column studies than MS2 (Schijven *et al.*, 2000a).

Goyal and Gerba studied the adsorption of viruses to nine different soil types with varied percentages of sand, clay and silt. Several viruses and phages were studied including echovirus Types 1 to 8, 11 to 13, 22, 24 to 28, 29, and 31; poliovirus Types 1 to 3; and coxsackievirus Types BI to B6. In addition, several enteroviruses isolated from groundwater beneath a wastewater land disposal site were studies including five strains of echovirus Type 1; two strains of coxsackievirus type B4; three strains of poliovirus Types 2; and 4 strains of poliovirus Type 3. Other viruses used were isolated from estuarine water included echovirus Type 1 and poliovirus Type 2. Finally, to compare characteristics several phages were also included in the study MS2, ΦX-174, T2, T4 and f2.

In the batch tests test tubes were used with 2 grams of test soil, 2 mL of test solution and counts of 10^6 to 10^7 PFUs of test virus. The test tube was stoppered, hand shaken, and then placed on a rotary shaker at 200 rpm for 30 minutes. The soil was then removed from suspension by centrifugation for 5 minutes at 2,500 x g, and the supernatant was assayed. Most of the viruses adsorbed very well to the sandy loam (test

soil FM), with more than 90 percent of the added virus adsorbing to the soil. Although, there were a few viruses with lower attachments to sandy loam including echovirus 1, 12, and 29 and rotavirus (SA-11), of which only 55.0, 78.0, 14.0, and 51.6 percent adsorbed. These results indicate that sorption of virus to a given soil is very strain dependent. In batch tests to compare results in varied ionic strengths adsorption of all of the viruses appeared to be enhanced in 0.01 M $CaCl_2$ but not in 0.001 M $CaCl_2$. These results indicated there was not one specific single phage or virus which modeled adsorptive behavior of enteric viruses to soils (Goyal *et al.*, 1979).

Syngouna *et al*. (2010) studied batch sorption of bacteriophages on to clay particles, kaolinite and bentonite. The effect of temperature was investigated at 4 and 25°C. Interaction energies between viruses and clays were calculated using DLVO theory. The bacteriophages used were MS2 (hydrophobic protein coat), and ΦX-174 (hydrophilic protein coat). Test tubes without soil were used to represent virus inactivation.

The batch tests were conducted in 50 mL glass centrifuge tubes containing 0.5 grams of the clay at a concentration of 10 mg clay per mL of PBS solution with virus concentrations of 10^3 to 10^9 PFU/mL. Batch tests were conducted both under static conditions and under dynamic conditions at which samples were agitated with a small benchtop tube rotator. Samples were enumerated every 24 hours for 7 days. Prior to sampling, tubes were centrifuged at 2000g for 30 minutes. For each deposition kinetics experiment, one equilibrium adsorption value was obtained. The equilibrium adsorption data where fitted to a linear isotherm with Kd (mL/mg sorbent) as the distribution coefficient. Distribution coefficients for the batch tests are included in Table 4-2 (interpreted from graphs).

Table 4-2: Equilibrium Adsorption Data for MS2 and ΦX-174

Phage	Clay	Temperature (ºC)	Initial Concentration (PFU/mg)	K_d (mL/mg)	
				Static	Dynamic
ΦX-174	Kaolinite	25	4×10^3	0.078	0.068
		4	8×10^6	0.019	0.021
	Bentonite	25	9×10^7	0.016	0.038
		4	9×10^7	0.021	0.024
MS2	Kaolinite	25	10×10^6	0.041	0.076
		4	15×10^7	0.046	0.054
	Bentonite	25	15×10^6	0.084	0.095
		4	10×10^7	0.050	0.057

Kd values were higher for the dynamic than static experiments (except ΦX-174 on kaolinite at 25ºC) owing to agitation; this is because the number of accessible sites for attachment is much higher in dynamic than static experiments. Adsorption was higher onto clays at 25ºC when compared to 4ºC and Kd values increased with temperature (Syngouna *et al.*, 2010).

Zhang *et al*. (2007) studied the impact of the air-water interface influences on estimated adsorption removals. Batch sorption experiments were conducted with sorption of MS2 on to sandy fluvic soil, red

loam soil, and red clay soil. Glass vials of 50 mL of with 10, 25 or 50 mL of lab water seeded at 10^1-10^9 PFU/mL were used with 10 g of each soil type. The samples were placed on a 300 r/min shaker table at 4°C for 3 hours. Samples were analyzed with non-sterilized and sterilized soils. Virus recovery efficiency in a blank experiment (no soil) was also evaluated for inactivation. The presence of air-water interface altered the results of virus adsorption in different soils because of different soil properties associated with virus inactivation, and the presence of air-water interface significantly decreased virus recovery efficiency (Zhang *et al.*, 2007).

4.4.2 Microbial Adhesion to Hydrocarbon

Microbial adhesion to surfaces interactions and roles that microorganisms play in the natural and synthetic environments are associated with bacteria adhesion and cell surface hydrophobicity (CSH). There are several techniques utilized to measure bacteria hydrophobicity, including the Microbial Adhesion to Hydrocarbons (MATH) test and the contact angle measurement (CAM) test. The MATH test utilizes partitioning of aqueous and hydrocarbon phases. Often, the method consists of vortexing microorganisms with a hydrocarbon (n-Dodecane) in 4:1 ratio, allowing for phase separation and measuring the absorbance of aqueous phase.

Absorbance is then compared with the initial absorbance of the bacterial suspension and the difference is used as the measure of bacteria suspended in the hydrocarbon phase. MATH test result is usually expressed as % cell surface hydrophobicity, where A control is the absorbance of a control culture not subjected to MATH test and A MATH is the absorbance of aqueous phase of cell culture subjected to MATH test and is determined by Equation 4-9.

$$\% \text{ hydrophobicity} = 100*(A_{CONTROL}-A_{MATH})/ (A_{CONTROL})$$

(Equation 4-9)

Hori *et al.* (2008) researched adsorption of bacterium *Acinetobacter sp.* strain to a hydrocarbon surface. Bacterial cells were harvested at the stationary growth phase by centrifugation, washed with sterile water, and resuspended to an optical density at 660 nm in a basal salt medium (pH 7.0) or fresh ultrapure water (pH 6.1). Aliquots (1 ml) of the cell suspension were transferred to test tubes and 10 to 1,000 µl of hexadecane was added. After vigorous vortex mixing for an interval between 5 and 60 seconds, the optical density of the aqueous layer was measured. For the detachment test, the aqueous layer was removed after the MATH test, leaving the emulsion layer containing hexadecane droplets. The same volume of pure water as that removed was carefully delivered along the inner wall of the test tube. After vortex mixing for an interval between 5 and 60 seconds, the two phases were allowed to separate, and the OD660 of the aqueous phase was measured again to determine the concentration of the cells detached from the hexadecane surface. Results of adsorption were confirmed by scanning electron microscope and analyzed. The research indicated that adsorption of bacterial cells to hydrocarbon surfaces can be described by the Langmuir adsorption isotherm. (Hori *et al.*, 2008).

Saini *et al.* (2011) measured bacterial hydrophobicity with the Microbial Adhesion to Hydrocarbons (MATH) test. Glass culture tubes had 4 ml of cell culture suspended in 10 mM KCl and vortexed 1 ml of dodecane for 2 minutes. The phases were then allowed to separate at room temperature for 15 minutes. An

aliquot was carefully removed from the aqueous phase using a disposable pipette, and its absorbance was measured at 600 nm using a UV–VIS spectrophotometer. The control cultures represent the cells washed and suspended in the aqueous phase and were not subjected to the MATH assay (hydrocarbon addition). The MATH samples represent the cells that were subjected to the MATH assay and were used to determine the hydrophobicity of bacterial cultures as well as the change in cell size attributable to hydrocarbon exposure during the MATH test. Bacterial cultures were shown to be weakly hydrophobic, particularly the *E. coli* strains (Saini *et al.*, 2011).

Gargiulo *et al.* (2008) studied the role of water quality and bacteria surface hydrophobicity in bacterial transport and deposition under unsaturated flow conditions using column experiments. Hydrophobicity of *D. radiodurans* and *R. rhodochrous* was quantified using the microbial adhesion to hydrocarbon (MATH) approach. The microbial cultures were collected at different growth stages and centrifuged at 7100 xg for 10 minutes at 25°) and resuspended in a 10^{-1} M NaCl solution. A glass test tube was filled with 3 mL of the bacteria suspension, and the optical density of the bacteria solution was measured at 600 nm in a spectrophotometer (DU800, Beckman Coulter, Fullerton, CA). A sample of 300 µL of n-hexadecane was added to the suspension, and the glass tube was vortexed for 2 minutes. The mixture was allowed to separate, and a sample of the aqueous phase was analyzed for optical density at 600 nm. The relative hydrophobicity *H*r was then calculated from where OD_i denotes the optical density of the original suspension and OD_f is the optical density of equilibrated aqueous phase after partitioning, as shown in Equation 4-10.

$$Hr = \left(1 - \frac{ODf}{ODi}\right) \times 100\%$$

(Equation 4-10)

The research included researching removals through column experiments in addition to the MATH tests and found that removals for hydrophobic bacteria in the sand increased with decreasing water saturation. (Gargiulo *et al.*, 2008).

5.0 RESEARCH METHODS

The objective of this research was to examine indicators of viruses in waters systems and identify characteristics of indicators critical for predicting virus behavior. This research is unique in that it includes the investigation of viruses at multiple-scales, including full-scale water systems, lab scale batch testing, and nanoscale particle investigation. The full-scale water systems included fecal, wastewater, stormwater, surface water, groundwater, and distribution systems. The lab scale analysis included batch adsorption tests for comparisons of water quality on the efficiency of virus removal as modeled by abiotic nanospheres. The nanoscale analysis included time-dependent light scattering using the ARGOS method to observe phage infection of bacteria and particle dynamics. The research methods are described briefly in the following and the standard operating procedures are included in Appendix A.

5.1 SAMPLE COLLECTION FULL-SCALE WATER SYSTEMS

The project included an analysis of water quality parameters in various water systems, including drinking water, surface water, groundwater, and wastewater systems. The purpose of this analysis was to analyze specific indicators of water quality. This project includes a multiple-scale analysis of water quality indicator systems including traditional water quality parameters, traditional indicators (total coliforms and *E. coli*), and alternative indicators (abiotic particles, somatic and male-specific coliphages).

Indicators and viral markers were evaluated by collecting and analyzing samples from animal feces, wastewater, stormwater, surface water (fresh and salt), groundwater, and drinking water distribution samples. Samples were collected and analyzed by Worcester Polytechnic Institute (Worcester, MA, U.S.), the University of Wisconsin-Madison (Madison, WI, U.S.), the University of Pisa (Pisa, Italy), and the Commonwealth Scientific and Industrial Research Organization (CSIRO) (Brisbane, Australia). Appropriate positive and negative controls were analyzed for all tests. All samples were collected aseptically to prevent cross contamination. Samples were diluted or concentrated as appropriate to achieve acceptable detection limits. The samples are summarized in Table 5-1.

Table 5-1: Summary of Full System Samples, United States, Italy and Australia

Source	Location	Dates (MM/YY)	Total Samples	Individual Samples	Sample Type
Fecal	United States	06/10 to 04/11	75	10	Chicken
				15	Dog
				22	Equine
				3	Rabbit
				25	Ruminant
Wastewater	United States	06/10 to 04/11	25	13	Influent
				12	Effluent
	Italy	04/04 to 03/05	24	12	Influent
				12	Effluent
		03/07 to 04/08	58	29	Influent
				29	Effluent
	Australia	01/10 to 06/10	44	22	Influent
				22	Effluent
Stormwater	Australia	01/12 to 03/12	40	16	Markerston Catchment
				24	Fitzgibbon Catchment
Surface Water	United States	05/11 to 03/12	15	15	Fresh Surface Water
	Italy	05/04 to 04/05	12	12	River Water
	Italy	05/04 to 04/05	12	12	Sea Water
Groundwater	United States	05/11 to 03/12	4	4	Raw Groundwater
Drinking Water	United States	05/11 to 03/12	20	20	Distribution System

In the United States, fecal, wastewater, and drinking water samples were collected from four different regions (Northeast, South, Midwest, and West) throughout multiple seasons. Samples were collected year round from geographically diverse areas of the United States to assess spatial and temporal variability. Fresh fecal samples (n = 75) were collected from private farms and included five animal groups: chicken, dog, equine (horse and donkey), rabbit, and ruminant (cow, sheep, goat and llama). Animals were monitored by the sampler, and feces were collected in sterile containers immediately after defecation (Figure 5-1).

Figure 5-1: Animal Feces Samples Collected in Sterile Containers

Wastewater and drinking water samples were collected from municipal sources (Figure 5-2). Wastewater samples (1 L) included influent and effluent samples (n = 12) prior to disinfection. Drinking water samples (20 L) included ground and surface sources and water from distribution systems (Plummer *et al.*, 2014). Drinking water samples were concentrated with hollow fiber ultrafiltration (HFUF) for primary and polyethylene glycol (PEG) precipitation for secondary concentrations by a factor of up to 25 times prior to enumerations as seen in Figure 5-2.

Figure 5-2: Drinking Water Samples Collected from Municipal Sources

In Italy, wastewater and surface water samples were collected by collaborators at the University of Pisa from the greater Pisa area, localized in the Tuscany region during two separate sampling periods. Samples (n = 24, first sampling; n = 58, second sampling) were collected from the city of Pisa activated sludge wastewater treatment plant (1L influent and 10 L effluent). Surface water samples (10 L) were also collected from the river Fiume Morto (n = 12) downstream from the city of Pisa treatment plant and from a seawater outfall (n = 12). (Carducci *et al.*, 2006; Verani *et al.*, 2006).

In Australia, stormwater and wastewater samples were collected by collaborators from CSIRO from the greater Brisbane area. Stormwater samples (n = 40) were collected from two sites (Fitzgibbon and Markerston catchment areas) in Brisbane. Multiple samples were collected during three storm events. Samples were collected using automated sampling infrastructure (ISCO 6700 or equivalent) triggered by automated flow measurement (Doppler flowmeter or weir). Wastewater samples, influent (1 L) and (20 L) effluent, were collected from the Luggage Point, Oxley Creek and Bundamba wastewater treatment facilities (n = 44) (Sidhu *et al.*, 2013; Sidhu *et al.*, 2010).

5.2 COLIFORM ENUMERATION

Data were collected on three bacterial indicators: total coliforms, *E. coli*, and *Enterococcus*. In the United States, total coliforms and *E. coli* were enumerated using Standard Methods 9223 with Colilert® (IDEXX, Westbrook, ME) in the multiple well format (Quanti-Tray®, IDEXX, Westbrook, ME) (as shown in Figure 5-3) and equated to a Most Probable Number (MPN) of the target organisms per 100 mL. Duplicate tests were conducted and results were averaged. In the United States, dilutions of fecal and wastewater samples were performed via 10 or 100 fold serial dilutions in appropriate buffer water. Fecal samples were resuspended in buffer water and serially diluted using Standard Method 9050c.1a (APHA *et al.*, 2012).

IDEXX Quanitrays® and Colilert® utilize two active substrates, o-nitrophenyl-p-D-galactopyranoside (ONPG) and 4-methylumbelliferyl-p-D-glucuronide (MUG), which are combined to simultaneously detect total coliforms and *E. coli*. Total coliforms produce the enzyme β-galactosidase, which hydrolyzes ONPG and thereby releases o-nitrophenol, which produces a yellow color. *E. coli* produce the enzyme β-glucuronidase, which hydrolyzes MUG to form a fluorescent compound (APHA *et al.*, 2012). Detailed procedures are included in Appendix A, Standard Operating Procedures.

Figure 5-3: Coliform Enumeration Quantitrays

In Italy, *E. coli* (EC) and intestinal *Enterococci* levels present in the samples were determined by Bio-Rad miniaturized methods (Bio-Rad Laboratories, Milan, Italy): MUG/EC Microplates *E. coli* and MUD/SF Microplates *Enterococcus* based on culture in liquid media (Most Probable Number) for the detection and enumeration of these parameters according to ISO 9308-3 (ISO, 1998b) and ISO 7899-1 (ISO, 1998a) respectively (Bofill-Mas *et al.*, 2010).

In Australia, fecal bacteria (*E. coli* and *Enterococcus*) were quantified using the membrane filtration technique. Samples (1 and 10 mL) were filtered with 0.45 µm nitrocellulose (Millipore, Billerica, MA) filters and placed on respective selective agar plates in triplicate. *E. coli* was enumerated on Chromocult™ coliform agar (Merck, Munchen, Germany) and *Enterococcus* spp. on Chromocult™ Enterococci agar (Merck, Munchen, Germany). The plates were incubated overnight at 37°C and then typical colonies were counted (Sidhu *et al.*, 2012).

5.3 COLIPHAGE ENUMERATION

The United States samples were analyzed for somatic and male-specific (F+) coliphages by EPA Method 1602, the single layer agar method. *E. coli* CN-13 (ATCC 700609; resistant to nalidixic acid) and *E. coli* FHS (pFamp) R (*E. coli* F-amp; ATCC 700891; resistant to streptomycin and ampicillin) were used as hosts for somatic and male-specific coliphages, respectively (U.S. EPA, 2001c). Samples were supplemented with magnesium chloride, log phase host bacteria, and tryptic soy agar. Plates were incubated overnight at 36 °C and examined for plaque forming units (PFU)/100 mL, as demonstrated in Figure 5-4. A blank, an ongoing precision and recovery (OPR), and a matrix spike were used to evaluate performance of this method (U.S. EPA, 2001c). Detailed procedures are included in Appendix A-Standard Operating Procedures.

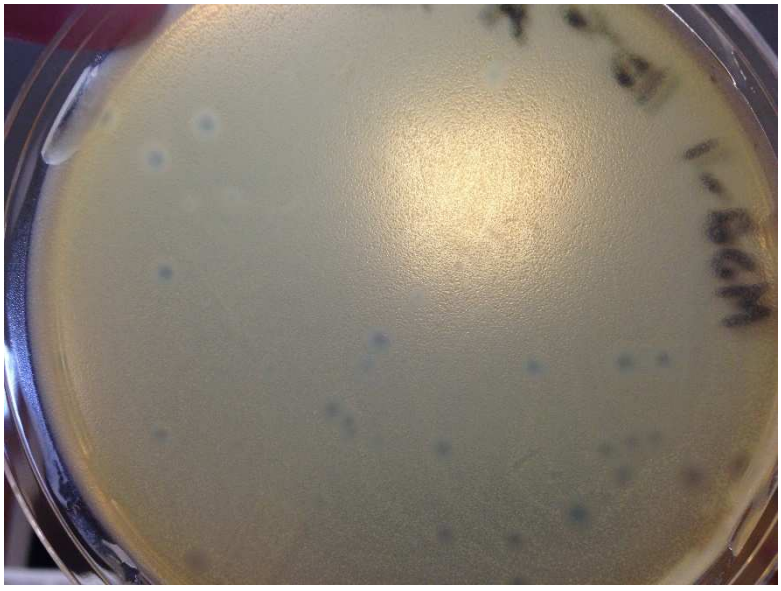


Figure 5-4: Coliphage Plaques

In Italy, somatic coliphages were enumerated by collaborators at the University of Pisa using the ISO double agar layer plaque assay method using *E. coli* C, ATTC 13706 as host strain (ISO, 1999). The sample, host and top layer agar were mixed and added to a plate with a hard layer of agar. The plates were incubated overnight at 36°C and counted for PFU/100 mL.

In Australia, somatic coliphages (Microviridae family) were enumerated by collaborators from CSIRO using quantitative PCR (qPCR) with Bio-Rad iQ5 (Bio-Rad Laboratories, California, U.S.) using iQ Supermix (Bio-Rad) real-time PCR kit. Each 25 μL PCR reaction mixture contained 12.5 μL of SuperMix, 300 nM of each primer, 200-250 nM corresponding TapMan probes and 3 μL of template DNA. Bovine serum albumin (BSA) was added to each reaction mixture to a final concentration of 0.2 μg μL-1 to prevent PCR inhibition.

5.4 VIRAL MARKERS

This research is part of a larger project to evaluate various indicator systems for viruses, including testing a potentially new viral indicator, Torque Teno virus (TTV), and proposed viral targets. While viral markers were not enumerated as a part of this portion of the project at Worcester Polytechnic Institute, Worcester, MA, the viral presence and concentration were statistically compared to factors tested as a part of this report. The methods for the viral markers used by the partner universities are included in Appendix A5. In addition, these methods are further discussed in current literature published by these partners (Carducci *et al.*, 2009; Liu *et al.*, 2011b; Long *et al.*, 2010; Verani *et al.*, 2006).

5.5 BIOTIC VIRAL SURROGATES

This research project investigated the physical characteristics of viruses by utilizing bacteriophages. MS2 and ΦX-174 were used because their structure resembles many human enteric viruses; they have in fact been previously studied as surrogates in several research applications (Attinti *et al.*, 2010; Havelaar, 1991; Yates, 1988). They are also less expensive and easier to handle during laboratory procedures than an enteric virus. The surrogates are each described in Table 5-2.

Table 5-2: Biotic Viral Surrogates

Bacteriophage	Description	Size	Isoelectric Point
MS2	Icosahedral, single stranded RNA	27 nm	3.5
ΦX-174	Icosahedral, single stranded DNA	23 nm	6.7

MS2 is an icosahedral phage with a diameter of 27 nm and a low isoelectric point of 3.5 (Schijven *et al.*, 2000b), conversely ΦX-174 icosahedral, single-stranded DNA bacteriophage, is less hydrophobic than MS2 and has an isoelectric point of about 6.7 and a size of 23 nm (Dowd *et al.*, 1998).

MS2 (ATCC 15597-B1) and ΦX-174 (ATCC 13706-B1) were purchased from the American Type Culture Collection (ATCC, Manassas, VA). MS2 was enumerated and the concentration counts were 1.0×10^{10} and 9.1×10^{9}. ΦX-174 was enumerated and concentrations were 8.33×10^{5} and 2.670×10^{5}. Coliphages were prepared according to instruction from ATCC. The concentrations of the phages were increased by using centrifugal filter devices (Centricon Plus-70, Millipore, Billerica, MA, U.S.). Detailed procedures are included in Appendix A-Standard Operating Procedures.

5.6 ABIOTIC VIRAL SURROGATES

Uncoated and coated latex nanospheres were used as a biotic model of viruses. The nanospheres, manufactured and coated by Bang Laboratories, consisted of both uncoated glacial blue fluorescent dyed 26 nm carboxyl-modified polystyrene spheres, and protein-coated flash red fluorescent dyed 26 nm carboxyl-modified polystyrene spheres. Each are described in Table 5-3.

Table 5-3: Abiotic Viral Surrogates

Nanosphere	Description	Size	Fluorescent Dye
Uncoated	Carboxyl-Modified Polystyrene Spheres	26 nm	Glacial Blue
Coated – Casein Protein	Carboxyl-Modified Polystyrene Spheres	23 nm	Flash Red

Casein coating was used in order to compare results to Pang *et al.* 2009 because casin coating provided a simple IEP to MS2. The characteristics of the microspheres are 15 mL at 1 percent solids (approximately 10 mg beads/mL), and a density of approximately 1.06 g/cm^3. Concentrations for both nanospheres were 1.0260×10^{15} microspheres per mL.

Fluorescent microspheres were prepared with a dye that fluoresces at a specific wavelength, allowing them to be directly counted under a fluorescent microscope. The concentrations were measured using a fluorescence spectrophotometer (LS 55, Fluorescence Spectrophotometer, Perkin Elmer, Waltham, MA U.S.), as shown in Figure 5-5. In order to determine the fluorescence spectrum of a sample, concentration curve was used to align the measured readings to particle concentrations. This spectrum was then analyzed to provide or confirm identification of the sample's composition. Several runs of various serial dilutions were conducted in order to identify both the appropriate range of sensitivity for the instrument and also the best fit for excitation and emission wavelengths. Concentrations of 10^{6} to 10^{-2} were found to be detected consistently by the instrument, and initial concentrations of 10^{5} were used. Detailed procedures and product specifications are included in Appendix A-Standard Operating Procedures.

Figure 5-5: Fluorescence Spectrophotometer

5.7 PARTICLE ANALYSIS

Adsorption and efficiency of removals were modeled by bacteriophages and microspheres during equilibrium batch analysis.

5.7.1 Water

Two types of water were used for particle analysis: lab water and Sigma water. The lab water exceeded ASTM Type I, ISO 3696 and CLSI-CLRW Type I standards. Lab water was treated with a four-stage deionization process combined with a UV lamp, an ultrafilter, and a 0.2 micron filter. The Sigma water was Grade A water LC-MS CHROMASOLVR from Sigma Aldrich. This water was prepared for high purity applications including liquid chromatography–mass spectrometry (LC-MS). Sigma water quality was $\leq$ 0.0001 percent non-volatile impurities, $\leq$ 1 ppb fluorescence (254 nm), transmittance 210 nm, and filtered through 2μm filter.

The solutions used for analyzing the impacts of ionic strength were prepared using lab water and research grade salts, including NaCl and $CaCl_2$. Two types of artificial groundwater were used: AGW1 at 5 nM (1mM Ca^{2+}, 2 nM Na^{+}, and 4 nM Cl^{-}) and AGW2 at 34 nM (4.8 mM Ca^{2+}, 19.5 nM Na^{+}, and 29.1 nM Cl^{-}) (Knappett *et al.*, 2008). The solutions were analyzed at approximately 2.5, 4, 6, 8 and 10 pH. The solution pHs were adjusted using solutions of 1 N solutions of HCl and NaOH immediately before experiments are carried out.

5.7.2 Media

The sand used was ANSI/AWWA B100 Filter Sand, 150# with mean grain diameter of 0.45-0.55 mm with less than 1.50 UC and 0 percent silt and clay from Unifilt Corporation, Wilkes-Barre Twp, Pennsylvania. The sand was washed with a 10 percent soap solution of MPbio ES7X phosphate free soap, and rinsed thoroughly with distilled water (Thompson *et al.*, 1998). A portion of the washed sand was treated with a series of acid/base washes in order to remove all organics and surface metals, such as iron. The treated sand was boiled for 24 hours in 70 percent nitric acid, rinsed with lab water, rinsed with 0.1 M NaOH for 12 hours, and boiled dried and rinsed with lab water until the pH of the water was the same as the lab water (Figure 5-6) (Porubcan *et al.*, 2011; Xu *et al.*, 2006; Xu *et al.*, 2008). The cleaned sand was autoclaved in lab water, oven dried at 105^{o}C overnight, and stored in autoclaved beakers (Thompson *et al.*, 1998).

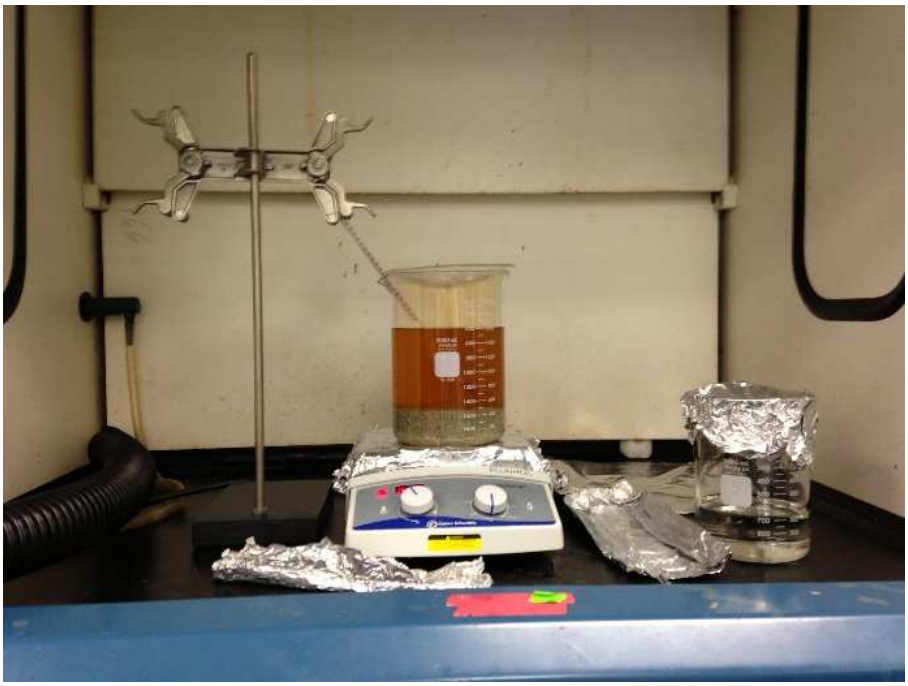


Figure 5-6: Sand Media Prepared in Concentrated Acid

Then, 10g of sand was pulverized into small particles with an agate mortar and added to an autoclaved 50 mL beaker with 20 mL of solution (Liu *et al.*, 2009). The particles were suspended for a minimum of 1 hour. Detailed procedures are included in Appendix A-Standard Operating Procedures.

5.7.3 Microbial Adhesion to Hydrocarbons

The hydrophobicity of MS2 was analyzed using microbial adhesion to hydrocarbons. MS2 was tested in lab water, AGW1, and AGW2. The literature review used optical density to define initial and final conditions for bacteria; this procedure was revised to evaluate initial and final conditions using the coliphage enumeration SOP. Samples were prepared with 1 mL of the phage sample to 9 mL ionic solution at pHs of 4, 6, and 8 and vortexed. A 4 mL sample of the colloidal suspension was transferred to a glass rounded-bottom test tube, and a sample of 1 mL of dodecane was then added to the sample. The solution was vortexed for 5 minutes. The solution was then left undisturbed for 15 minutes at room temperature

allowing a phase separation with an emulsion layer on top and an aqueous layer on the bottom. A 1mL sample was extracted from the aqueous layer with a pipette, and coliphages were enumerated using the procedure detailed previously. The plates were then counted and % hydrophobicity was calculated using Equation 5-1,

$$\% \text{ hydrophobicity} = 100*(A_{CONTROL}-A_{MATH})/ (A_{CONTROL})$$

(Equation 5-1)

Where, hydrophobicity is expressed as % cell surface hydrophobicity, where A control is the absorbance of a control culture not subjected to MATH test and A MATH is the absorbance of aqueous phase of cell culture subjected to MATH test. Detailed procedures are included in Appendix A - Standard Operating Procedures.

5.7.4 Batch Analysis

Batch tests were used to assess adhesion to filtration media. Adhesion is considered to be the main removal mechanism for colloids (Lytle *et al.*, 1995). The tests were conducted with viral surrogates (protein-coated 26 nm nanosphere and uncoated 26 nm nanosphere) over a period of two hours. This research includes colloid removals in artificial groundwater across several pHs, The removal of viruses was quantified using Equation 5-2,

$$\text{Log}_{10} \text{ removal} = - \log\frac{N}{N_o}$$

(Equation 5-2)

Where N is the number of viruses (or surrogates) in the filtrate, and N_o is the number of viruses (or surrogates) in the challenge solution (Pontius *et al.*, 2009). The removals were analyzed with initial concentrations of 10^5 particles per mL at pHs of 4, 6, and 8 for LW, AGW1, and AGW for sand and acid-washed sand. The concentrations were calibrated and the original methodology was altered to reflect the range of detection (10^6 -10^{-2} particles per mL), while Pang *et al.* found a detection limit of 10^8 particle per mL.

The batch tests were conducted in 40 mL glass bottles that are wash in 20 percent sulfuric acid solution, autoclaved, and oven dried at 121°C for 4 hours (Thompson *et al.*, 1998). Glass tubes were used to minimize virus inactivation (Thompson *et al.*, 1998).

The glass tubes received 9.9 mL of the appropriate ionic solution (AGW1 and AGW2) and 0.01 mL solutions of nanospheres combined to final concentration of approximately 10^5 particles per mL. A 1:1 ratio of sand to solution was used, and 10g of sand (washed and acid/base washed) was added. The inoculated samples were capped and immediately placed on a shaker table at 100 rpm in an incubator at 20°C.

Duplicate tubes were removed at 10, 20, 40, 90, and 120 minutes. Additionally each trial was run with control samples without media. The supernatant was then sampled and assayed to determine remaining virus concentration (Schijven *et al.*, 2000b). Samples were vortexed, serially diluted, and enumerated. Samples at time zero were used to provide an initial phage concentration (C_o). Glass vials without sand were also sampled to observe potential impacts from inactivation.

5.8 LIGHT SCATTERING

5.8.1 Zeta Potentials

The objective of measuring the zeta potential is to predict how the surrogate will behave through electrostatic interactions. The magnitude of the zeta potential gives an indication of the potential stability of the colloidal system. If all the particles in suspension have a large negative or positive zeta potential, they will tend to repel each other.

The electrophoretic mobility of the bacteriophages, microspheres, and filter media were determined by Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK). The detection angle of the Zetasizer is 90°. The electrophoretic mobilities were converted to ζ using the Smouluchowski equation (Ryan *et al.*, 1999). Duplicate samples were conducted with 5 runs, each featuring a minimum of 10 trials. The Zetasizer Nano ZS utilizes a conversion of the Henry Equations with a Smoluchowski approximation in which the Henry function is assigned 1 for particle sizes less than 0.2 μm and 1.5 for colloids greater than 0.2 μm. The Henry equation 5-3 is:

$$U_E = \frac{2\varepsilon\zeta f(ka)}{3\eta}$$

(Equation 5-3)

Where U_E is the electrophoretic mobility (charge on ion/frictional coefficient), ε is the dielectric constant, ζ is zeta potential (mV), , η is the viscosity (Pa*s), and f(ka) is Henry's function (Malvern Instruments, 2004). Detailed procedures are included in Appendix A-Standard Operating Procedures.

5.8.2 Area Recorded Generalized Optical Scattering

A new method for analyzing time-dependent light scattering was developed by the physics department at, WPI, Worcester, Massachusetts. While this method was used initially to calculate shape and size of latex spheres, this research provided an opportunity to use this system to observe system dynamics over time. The area recorded generalized optical scattering (ARGOS) approach to light scattering employs large image capture array. This method can be used for both static and dynamic measurements of a wide variety of sample environments. This process is fully described in the dissertation by Saad Algarni (2014), Physics, WPI, Worcester, Massachusetts (Algarni, 2014).

The system allows for a well-defined geometry in which images may be manipulated to extract structure with intensity at a specific wave vector (I(q)) and dynamics with intensity at a specific wave vector over time (I (q,t)) for a wide range of sample types. The components of the ARGOS method, as shown in Figure 5-7, are vary from traditional light scattering methods, owing to the use of the screen detector, the beam attenuator, and a camera to save images over time. This allowed for the measurement of total intensity and intensity at specific wave vectors.

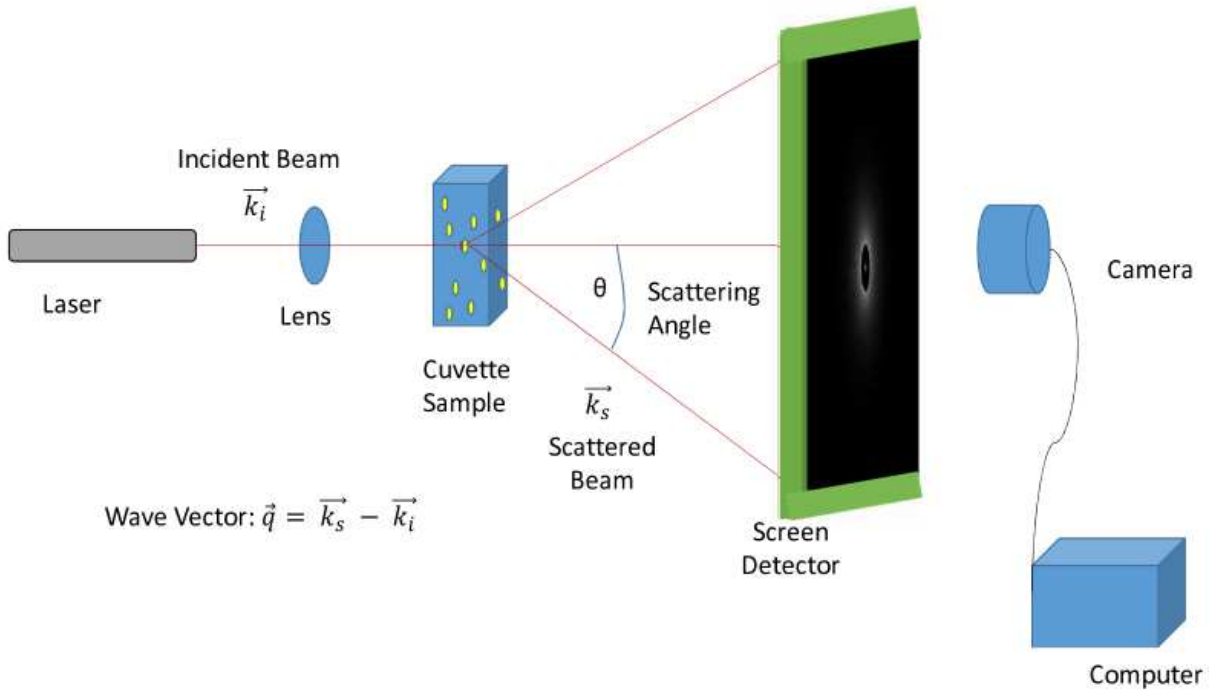


Figure 5-7: Components of the ARGOS Method

The method employs a translucent screen upon which the scattered light is converted to diffuse light and is then imaged by a camera as a function of time. The placement and size of the screen determine the range of the wavevector to be measured, while the camera sensitivity, resolution, and speed determine the intensity of the scattered light. The ARGOS setup is shown in Figures 5-8 and 5-9.

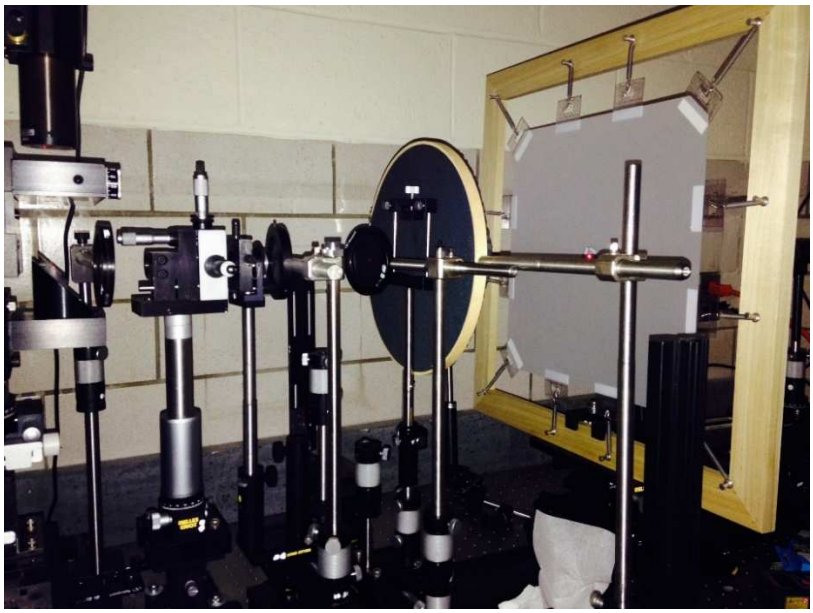


Figure 5-8: ARGOS Set-Up Portraying the Laser, Lens, Filters, Sample Holder and Front of Screen

Figure 5-9: ARGOS Set-Up Portraying Camera, Beam Attenuator and Camera

The laser (JDSU HeNe Model (1125), Edmund Optics, Barrington, NJ, U.S.), was used at 5 mW at 632.8 nm, with random polarization. The CCD camera (Model EO-0813M 1/3, Edmund Optics, Barrington, New Jersey, U.S.) was an 8-bit monochromatic with a resolution of (1024 pix × 670 pix), and the shutter speed was 1/30 second. The lens also purchased from Model 16 MM EO MEGAPIXEL FIXED FL Edmund Optics, Barrington, New Jersey, U.S.) had a 16mm focal length, C-Mount, and working distance minimum 100 mm. The screen was semi translucent (400 mm × 400 mm) comprised of optical paper (DuPont, Wilmington, DE, U.S.). The beam attenuator was made of multiple layers of ND filters; the number of the layers was selected according to the strength of the central beam intensity. The programming and all codes for this method were developed based on LabView 2009 64bit, Assistance Vision program (AVP) version. Samples were prepared in aseptic conditions. Samples were measured in Spectrosil® Quartz cuvettes, 12.5 mm (width), 12.5 mm (length), and 45 mm (height) (Vernier Software & Technology, Beaverton, OR). The room temperature was maintained at 23°C, unless otherwise noted. Detailed procedures are included in Appendix A-Standard Operating Procedures.

The light from the laser is scattered onto the screen. The digital camera captured pictures of the data in images similar to Figure 5-10.

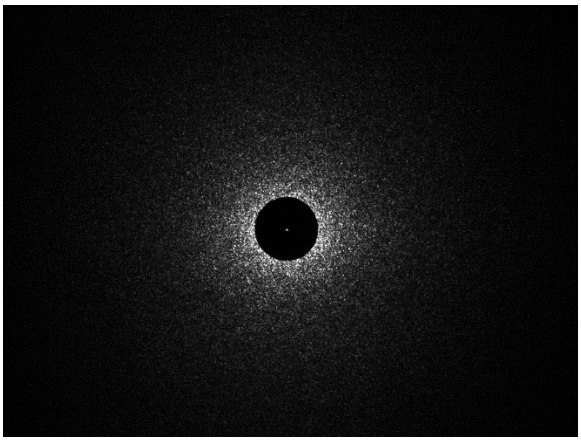


Figure 5-10: ARGOS Light Scattering Data

Photographs of the screen were obtained at time specific intervals and the light intensities. The total light intensity and the change in light intensity was determined for each time interval over the course of the experiment. Each experiment was run several times and reproduced a minimum of three times. The description of the light intensity analysis is included in the dissertation of Saad Algarni, WPI Physics Department, November, 2014 (Algarni, 2014).

5.9 STATISTICAL ANALYSES

Statistical analyses were conducted on the data from each phase of this research. The statistical analyses were conducted using analytical software developed for research including SPSS and LabView.

5.9.1 SPSS Analysis

Statistical analyses were completed to review the quantitative, binary, and categorical data to find both correlations among and variations between the population sets and various sample sets. These statistical analyses were conducted utilizing IBM's Statistical Package for the Social Sciences (SPSS) product, Version 17.0. A review of the outliers was completed both to determine the strength of inference and also to investigate their impact upon average and median values.

5.9.1.1 Analysis of Variance

Analysis of variance (ANOVA) evaluation was conducted on the quantitative data sets. ANOVA is a statistical technique for dividing the total variation in a population or subset into a number of components attributable to different variables. The mean for each parameter was calculated and compared to the mean of the population or sub group, as shown in Equation 5-4. The null and alternative hypotheses are included where $\mu_1,\mu_2,\ldots\mu_k$ are sample set means.

$$H_o: \mu_1=\mu_2=\ldots=\mu_k \qquad \text{(Equation 5-4)}$$

H_a: H_o is not true and the means differ from one another.

5.9.1.2 Spearman Rank Correlation

The Spearman rank correlation was calculated because it is nonparametric and does not rely on data belonging to any particular distribution equivalent, as opposed to the Pearson correlation which assumes normal distribution. The Spearman rank correlation coefficient can be used to describe the relationship between nonlinear data and can be used for data at the ordinal level. The Spearman rank correlation was run for the quantitative data sets for the fecal, wastewater, and drinking water samples, and defines relationships within the data. The Spearman rank correlation coefficient (r_s) is a measure of the strength of a relationship between two variables. It is calculated using the ranks of paired sample data entries (n = number of paired data entries; d = difference between the ranks of a paired data entry) (Larson and Farber, 2003). The function for the Spearman rank correlation is included as (Equation 5-5):

$$r_s = 1 - \frac{6\Sigma d^2}{n(n^2 - 1)}$$

(Equation 5-5)

5.9.2 LabView Analysis

A variety of image processing algorithms were developed to correct for dead pixels, camera response, and intensity normalization. Programing and all codes that were used were developed with Lab- View 2009 64bit, Assistance Vision program (AVP) version(9). Of particular use was the development of the root mean square difference image to probe dynamic (Algarni, 2014).

5.9.2.1 Root Mean Square

RMSD was used to calculate the difference in the intensity of the initial condition (in this research, the first photograph in a series) from that of each subsequent condition. These calculations provided a method of observing dynamic changes in the kinetics of a particle and were particularly useful in observing dynamics when structural or concentration changes were not observed.

The root mean square difference (RMSD) is an average value of the difference for a time varying function. RMSD is a statistical measure of magnitude of the difference of a varying quantity. RMSD used to define the difference between values predicted by a model and the values actually observed, as shown in Equation 5-6.

$$RMSD = \sqrt{\frac{\sum_{i=1}^{n} (X_{obs,i} - X_{model,i})^2}{n}}$$

(Equation 5-6)

.

6.0 RESULTS

Viruses were researched at multiple-scales using viral markers (TTV and adenovirus), biotic surrogates (male-specific and somatic coliphages), and abiotic surrogates (latex nanospheres). The following sections summaries the results of the research conducted. The detailed data is included in Appendix B – Data.

6.1 FULL-SCALE WATER SYSTEMS

Given the limitations of coliforms, this study analyzed a new potential viral indicator for describing water quality. The data collected were used to analyze the use of TTV as an indicator of wastewater and fecal contamination in water systems (data as accepted by the Journal of Water and Health, January 2015). In addition, traditional water quality parameters, traditional indicators (total coliforms and *E. coli*), and alternative indicators (somatic coliphages, male-specific coliphages and microviridae) were reviewed for correlations to viral markers (TTV, polyomavirus and adenovirus). Combing data sets from United States (U.S.), Italy, and Australia provided a unique opportunity to expand the original data sets and compare results from three independent locations.

6.1.1 Bacterial Indicator Results

Bacterial indicator data is summarized in Tables 6-1 to 6-3. For the fecal samples with detectable levels of bacterial indicators, coliforms and *E. coli* ranged from below detection limits to 6.1×10^8 MPN per gram in chicken samples. The median total coliforms ranged from 3.7×10^3 MPN per gram in rabbit samples to 3.0×10^6 MPN per gram in chicken and dog samples. The median *E. coli* ranged from 155 MPN per gram in rabbit samples to 3.0×10^6 MPN per gram in dog samples.

Table 6-1: Enterococci Data

Country	Source	Sample Type	N	Enterococci (CFU/100 mL)		
				Min	Median	Max
U.S.	Fecal	Chicken	10	NT	NT	NT
U.S.		Dog	15	NT	NT	NT
U.S.		Equine	22	NT	NT	NT
U.S.		Rabbit	3	NT	NT	NT
U.S.		Ruminant	25	NT	NT	NT
U.S.	Wastewater	Raw	13	NT	NT	NT
U.S.		Final	12	NT	NT	NT
Italy		Raw	29-41	7.90E+03	1.00E+06	4.00E+06
Italy		Final	29-41	400	1.80E+04	9.10E+04
Australia	Stormwater	Stormwater	40	180	5.80E+03	3.90E+04
Italy	Surface Water	Sea	12	NT	NT	NT
Italy		River	12	NT	NT	NT
U.S.		Fresh	15	NT	NT	NT
U.S.	Groundwater	Raw	4	NT	NT	NT
U.S.	Drinking Water	Distribution	20	NT	NT	NT

NT-Not Tested, BDL - Below Detection Limit

Table 6-2: Total Coliform Data

Country	Source	Sample Type	N	Total coliforms (MPN/100 mL or g)		
				Min	Median	Max
U.S.	Fecal	Chicken	10	BDL	3.00E+06	6.10E+08
U.S.		Dog	15	5.40E+04	3.00E+06	1.00E+08
U.S.		Equine	22	50	9.80E+03	4.90E+07
U.S.		Rabbit	3	BDL	3.80E+03	2.60E+05
U.S.		Ruminant	25	BDL	7.40E+05	2.60E+07
U.S.	Wastewater	Raw	13	6.60E+05	1.60E+07	1.00E+08
U.S.		Final	12	900	5.50E+04	9.80E+05
Italy		Raw	29-41	NT	NT	NT
Italy		Final	29-41	NT	NT	NT
Australia	Stormwater	Stormwater	40	NT	NT	NT
Italy	Surface Water	Sea	12	NT	NT	NT
Italy		River	12	NT	NT	NT
U.S.		Fresh	15	5.4	190	1.60E+03
U.S.	Groundwater	Raw	4	BDL	BDL	BDL
U.S.	Drinking Water	Distribution	20	BDL	BDL	6.3

NT-Not Tested, BDL - Below Detection Limit

Table 6-3: *E. coli* Data

Country	Source	Sample Type	N	*E. coli* (U.S.-MPN/100 mL or g, IT/AUS CFU/100 mL)		
				Min	Median	Max
U.S.	Fecal	Chicken	10	BDL	2.30E+06	3.40E+08
U.S.		Dog	15	6.60E+04	3.00E+06	1.00E+08
U.S.		Equine	22	BDL	7.00E+03	4.70E+07
U.S.		Rabbit	3	BDL	160	2.60E+05
U.S.		Ruminant	25	BDL	4.60E+05	1.10E+07
U.S.	Wastewater	Raw	13	3.50E+04	2.10E+06	7.30E+06
U.S.		Final	12	120	4.90E+03	8.90E+04
Italy		Raw	29-41	3.60E+04	5.60E+06	4.50E+07
Italy		Final	29-41	520	7.90E+04	2.00E+06
Australia	Stormwater	Stormwater	40	42	530	4.50E+03
Italy	Surface Water	Sea	12	0.1	0.1	15
Italy		River	12	1.90E+05	7.40E+05	1.70E+06
U.S.		Fresh	15	BDL	0.67	35
U.S.	Groundwater	Raw	4	BDL	BDL	BDL
U.S.	Drinking Water	Distribution	20	BDL	BDL	BDL

NT-Not Tested, BDL - Below Detection Limit

Bacterial indicators in the U.S. wastewater samples (n = 25) had a maximum concentration of 1.0×10^8 total coliforms MPN per 100 mL and $7.3x10^6$ *E. coli* MPN per 100 mL in raw influent, but decreased by between 2 and 3 orders of magnitude through treatment. The levels also reached as low as 900 total coliforms and 120 *E. coli* MPN per 100 mL in final wastewater samples. The bacterial indicators (*E. coli* and Enterococci) in wastewater samples (n=58) from Italy had a maximum concentration of 4.5×10^7 *E. coli* MPN per 100 mL in raw wastewaters, and decreased by an order of magnitude through treatment. The levels in Italy reached as low as 520 MPN per 100 mL in final wastewater. The stormwater samples (n=40) from Australia had similar ranges for *E. coli* and Enterococci with a minimums of 42 and 100 CFU/100 mL, and maximums of 4.5×10^3 and 3.9×10^4 CFU/100 mL, respectively.

The sea water (n=12) samples (10 L) had a maximum value of 15 CFU/100 mL for *E. coli*, and a median of 0.1 CFU/100 mL (detection limit). Similarly, the U.S. surface water (n=15) samples (20 L) had minimums below detection limits for bacterial indicators. The maximum values for total coliforms and *E. coli* were 1.6×10^3 MPN/100 mL. The surface water samples (n=12) from Italy (10 L) had much higher *E. coli* concentrations with a median concentration of 7.4×10^5 CFU/100 mL and a maximum of 1.7×10^6 CFU/100 mL.

Groundwater samples (n=4) were collected from one location in the U.S. Midwest. The groundwater samples were negative for total coliforms and *E. coli*. In addition, U.S. drinking water distributions system samples (n=20) were collected from systems using both ground and surface water sources. They were negative for *E. coli*, while one sample was positive for total coliforms at 6.3 MPN/100 mL.

6.1.2 Coliphage Indicator Results

Coliphage indicators are summarized in Tables 6-4 to 6-5. Male-specific and somatic coliphages were detected in approximately half of the fecal samples. Most of the samples that tested positive were below detection limits: 41 of 75 samples (54.7%) below detection limits for male-specific coliphages and 32 of 75 (42.7%) below detection limits for somatic coliphages. For fecal samples with detectable levels of coliphage, the maximum male-specific coliphage concentration was 2.0×10^6 PFU/100 mL in chickens (median below the level of detection) and maximum somatic coliphage concentration was 2.5×10^7 PFU/100 mL in chickens (median 2.0×10^4 PFU/100 mL).

Table 6-4: Male Specific Coliphage Indicator Data

Country	Source	Sample Type	N	Male-Specific Coliphage (PFU/g or 100 mL)		
				Min	Median	Max
U.S.	Fecal	Chicken	10	BDL	BDL	2.00E+06
U.S.		Dog	15	BDL	BDL	170
U.S.		Equine	22	BDL	9.2	2.90E+04
U.S.		Rabbit	3	BDL	370	4.90E+04
U.S.		Ruminant	25	BDL	BDL	5.20E+04
U.S.	Wastewater	Raw	13	2.20E+03	9.00E+04	3.00E+05
U.S.		Final	12	BDL	120	760

Country	Source	Sample Type	N	Male-Specific Coliphage (PFU/g or 100 mL)		
				Min	Median	Max
Italy		Raw	41	NT	NT	NT
Italy		Final	41	NT	NT	NT
Australia	Stormwater	Stormwater	40	NT	NT	NT
Italy		Sea	12	NT	NT	NT
Italy	Surface Water	River	12	NT	NT	NT
U.S.		Fresh	15	BDL	BDL	1.8
U.S.	Groundwater	Raw	4	BDL	BDL	1
U.S.	Drinking Water	Distribution	20	BDL	BDL	190

Table 6-5: Somatic Coliphage Indicator Data

Country	Source	Sample Type	n	Somatic Coliphage (PFU/g or 100 mL)		
				Min	Median	Max
U.S.	Fecal	Chicken	10	BDL	2.00E+04	2.50E+07
U.S.		Dog	15	BDL	6.1	1.80E+04
U.S.		Equine	22	BDL	BDL	1.00E+05
U.S.		Rabbit	3	BDL	BDL	3.00E+05
U.S.		Ruminant	25	BDL	180	8.40E+04
U.S.	Wastewater	Raw	13	733	4.00E+04	1.60E+05
U.S.		Final	12	170	1.40E+03	5.10E+05
Italy		Raw	41	4.00E+05	2.40E+06	1.00E+07
Italy		Final	41	1.00E+03	1.90E+04	2.00E+06
Australia	Stormwater	Stormwater	40	1	91	870
Italy	Surface Water	Sea	12	0.1	250	700
Italy		River	12	4.60E+04	1.70E+05	4.60E+05
U.S.		Fresh	15	BDL	BDL	5.8
U.S.	Groundwater	Raw	4	BDL	BDL	0.34
U.S.	Drinking Water	Distribution	20	BDL	BDL	0.52

The U.S. raw wastewater samples had maximum concentrations of 3.0×10^5 and 1.6×10^5 PFU/100 mL, with medians of 9.0×10^4 and 4.0×10^4 PFU/100 mL for male-specific and somatic coliphages, respectively. The coliphage reduction through treatment varied significantly with average reductions of 10^4 for male-specific coliphages and no significant reduction for somatic coliphages. The wastewater samples

from Italy contained somatic coliphages in the raw samples had a maximum concentration of 1.0×10^7 PFU/100 mL, with a median of 2.4×10^6 PFU/100 mL with a median reduction of 2 orders of magnitude through treatment. Stormwater samples had a median of 90 PFU/100 mL and a maximum of 870 PFU/100 mL for somatic coliphages.

Coliphage concentrations in surface waters from the U.S. were much lower than in samples from Italy. Somatic coliphage in the sea water (10 L) samples (n=12) had a maximum of 700 PFU/100 mL and a median of 250 CFU/100 mL. The median for U.S. surface water samples was below the level of detection and the median for the Italy surface water samples was 10^5 PFU/100 mL. U.S. surface water samples (20 L) were below detection limits for most samples. For the U.S. surface water samples maximum concentrations of male-specific coliphage were 1.8 PFU/100 mL and maximum concentrations of somatic coliphage were 5.8 PFU/100 mL. Somatic coliphage concentrations for U.S. and Italy waters varied by several orders of magnitude.

The percentage of samples below the level of detection for coliphages was high in groundwater and distribution system samples for male-specific and somatic coliphages. The groundwater samples had median coliphage concentrations of non-detectable and maximum concentrations of 0.99 PFU/100 mL male-specific coliphage and 0.34 PFU/100 mL somatic coliphage. Similarly, the distribution system samples had medians of non-detectable for both coliphage groups and a maximum of 193 PFU/100 mL for male-specific coliphage.

6.1.3 Viral Marker Quantitative Results

TTV was found in 36 of 58 wastewater samples in Italy (collected by collaborators at the University of Pisa) had maximum concentrations of 3.6×10^5 genomic copies per mL in raw wastewaters and a median reduction of an order of magnitude through treatment. The stormwater and wastewater samples from Australia were quantified for genomic copies per mL of adenovirus, TTV, polyomavirus, and microviridae. In the wastewater samples from Australia (n=44), adenovirus had a maximum concentration of 9.1×10^3 genomic copies per mL, with 2 $\log_{10}$ removals through treatment; TTV had a maximum concentration of 2.4×10^3 genomic copies per mL, with reductions 2 $\log_{10}$ removal through treatment. These results are included in Table 6-6.

Table 6-6: TTV and Adenovirus

Country	Source	Sample Type	N	TTV (genomic copy per mL)			Adenovirus (genomic copy per mL)		
				Min	Median	Max	Min	Median	Max
Italy	Wastewater	Raw	29	BDL	697	3.60E+05	NT	NT	NT
Italy		Final	29	BDL	17	2.40E+04	NT	NT	NT
Australia		Raw	11-22	130	250	2.40E+03	110	510	9.10E+03
Australia		Final	11-22	0.19	0.9	3.9	0.18	0.83	6
Australia	Stormwater	Stormwater	24-40	0.01	2.2	13	0.004	0.22	9.1

The Australian wastewater samples (collected by collaborators from CSIRO) were enumerated for polyomavirus and microviridae (including somatic phages), with maximum concentrations of 2.2×10^3 and

5.5×10^3 genomic copies per mL with two $\log_{10}$ removal through treatment. Stormwater samples tested for viral markers were primarily in the one and tens of genomic copies per mL, with maximum concentrations of 9.10, 32.5, and 12.5 genomic copies per mL for adenovirus, polyomavirus, and TTV, respectively. These results are included in Table 6-7.

Table 6-7: Additional Viral Markers Tested in Australia

Country	Source	Sample Type	n	Polyomavirus (genomic copy per mL)			Microviridae (genomic copy per mL)		
				Min	Median	Max	Min	Median	Max
Australia	Wastewater	Raw	11-22	410	1.00E+03	2.20E+03	1.20E+03	2.20E+03	5.50E+03
Australia		Final	11-22	0.077	0.26	1.2	0.09	0.43	3.8
Australia	Stormwater	Stormwater	24-40	0.01	0.01	33	NT	NT	NT

6.1.4 Viral Markers Presence/Absence

In addition to the quantitative virus data, presence/absence testing was conducted. The surface water, groundwater, and distribution system samples in the U.S. were tested for TTV and adenovirus with traditional PCR; results were analyzed as either positive or negative. The surface water samples from Italy (collected and analyzed by collaborators at the University of Pisa) were negative for adenovirus and had a majority of negative TTV results (1/12). The groundwater samples from the U.S. were negative for adenovirus (0/4) and one sample was positive for TTV (1/4). There were four positive (4/20) samples of TTV in distribution system samples. Eleven of the U.S. distribution system samples were tested for adenovirus (including the four TTV positive samples); all were negative. These results are included in Tables 6-8 and Tables 6-9.

TTV was present in 3 of 76 fecal samples (4.0%). In wastewaters, TTV was present in 38 to 49 percent of samples, depending on sample type (raw versus final) and location (country). Surface water detection was rare, with 3 of 12 river waters in Italy positive for TTV; however, no sea water samples in Italy and no surface water samples in the U.S. had TTV. One of four groundwater samples and 4 of 20 drinking water samples tested positive for TTV in the U.S. Adenovirus was not found in any fecal samples, surface waters, groundwaters, or drinking waters in the U.S., but was detected in the majority of wastewater samples (100% of raw samples and 67% of treated samples).

Table 6-8 TTV Presence/Absence

	Source	Sample Type	N	TTV Positive	
				Number	Percentage
U.S.	Feces	Chicken	10	1	10%
U.S.		Dog	15	2	13%
U.S.		Equine	22	0	0%
U.S.		Rabbit	3	0	0%
U.S.		Ruminant	25	0	0%
U.S.	Wastewater	Raw	13	5	38%
U.S.		Final	12	5	42%
Italy		Raw	41	20	49%
Italy		Final	41	16	39%
Italy	Surface water	Sea	12	0	0%
Italy		River	12	3	25%
U.S.		Fresh	15	0	0%
U.S.	Groundwater	Raw	4	1	25%
U.S.	Drinking Water	Distribution	20	4	20%

Table 6-9 Adenovirus Presence/Absence

	Source	Sample Type	n	Adenovirus Positive	
				Number	Percentage
U.S.	Wastewater	Raw	13	12 (of 12)	100%
U.S.		Final	12	8	67%
Italy		Raw	41	NT	NT
Italy		Final	41	NT	NT
Italy	Surface water	Sea	12	NT	NT
Italy		River	12	NT	NT
U.S.		Fresh	15	0	0%
U.S.	Groundwater	Raw	4	0	0%
U.S.	Drinking Water	Distribution	20	0 (of 11)	0%

6.1.5 Analysis of Variance

Table 6-10 includes the results for the one-way analysis of variance (ANOVA) used to determine whether there are any significant differences between the means of two or more independent groups at a 95 percent (<0.05) confidence level. Seasonal variances were calculated for drinking water, surface water, stormwater, wastewater, and fecal samples. No seasonal variances were identified. An ANOVA was also calculated between countries for the wastewater samples. Results indicate that somatic coliphage concentrations varied by country.

Table 6-10: Results for the One-Way Analysis of Variance (ANOVA)

		Location ANOVA	**Seasonal ANOVA**				
Indicators		**Wastewater Country (AUS, IT, U.S.)**	**Drinking Water (U.S.)**	**Surface Water (IT, U.S.)**	**Stormwater (AUS)**	**Wastewater (AUS, IT U.S.)**	**Fecal Samples (U.S.)**
Coliform (CFU per 100 mL or g)	Varies	ID	N	N	ID	N	N
	Coefficient	-	0.634	0.677	-	0.969	0.776
	N	-	20	15	-	25	75
E. coli (CFU per 100 mL or g)	Varies	N	N	N	N	N	N
	Coefficient	0.104	0.623	0.742	0.854	0.665	0.572
	N	107	20	27	40	107	75
Enterococci (CFU per 100 mL)	Varies	ID	ID	ID	N	N	ID
	Coefficient	-	-	-	0.179	0.239	-
	N	-	-	-	40	58	-

Indicators		Wastewater Country (AUS, IT, U.S.)	Drinking Water (U.S.)	Surface Water (IT, U.S.)	Stormwater (AUS)	Wastewater (AUS, IT U.S.)	Fecal Samples (U.S.)
Male-Specific Coliphage (PFU per 100 mL or g)	Varies	ID	N	N	ID	N	N
	Coefficient	-	0.120	0.577	-	0.338	0.295
	N	-	20	15	-	25	75
Somatic Coliphage (PFU per 100 mL or g)	Varies	Y	N	N	N	N	N
	Coefficient	0.0005	0.147	0.359	0.235	0.110	0.357
	N	107	20	15	40	87	75
Microviradae (genomic copies per mL)	Varies	ID	ID	ID	ID	N	ID
	Coefficient	-	-	-	-	0.207	-
	N	-	-	-	-	22	-
TTV (genomic copies per mL)	Varies	N	ID	ID	ID	N	ID
	Coefficient	0.106	-	-	-	0.071	-
	N	80	-	-	-	80	-
Adenovirus (genomic copies per mL)	Varies	N	ID	ID	ID	N	ID
	Coefficient	0.126	-	-	0.564	0.345	-
	N	61	-	-	40	61	-
Polyomavirus (genomic copies per mL)	Varies	ID	ID	ID	N	N	ID
	Coefficient	-	-	-	0.645	0.889	-
	N	-	-	-	40	44	-

ID - Insufficient Data (There are fewer than two groups for dependent variable. No statistics are computed.)

6.1.6 Correlation Analysis

The Spearman Rank correlation analysis was conducted for indicators and viral markers in drinking water, surface water, stormwater, wastewater, and fecal samples. The following tables include a summary of the parameter correlations. Detailed calculations are included in Appendix C – SPSS Correlation Data. The tables summarize correlations with a Y (with number of cases) for a statistically significant correlation at the 95 percent confidence level, N for no significant correlation and ID for insufficient data. These results demonstrate correlations between indicators is specific to the water system.

Table 6-11 includes the results for drinking water quality parameters. For these results, coliforms, *E. coli*, male-specific coliphage, and somatic coliphage correlate to one another. These results indicate a relationship between bacteria and coliphages, but may be impacted by the sample size (n = 20).

Table 6-11: Drinking Water Quality Parameter Correlation Analysis

	Coliform (CFU per 100 mL or g)	***E. coli* (CFU per 100 mL or g)**	**Male-Specific Coliphage (PFU per 100 mL or g)**	**Somatic Coliphage (PFU per 100 mL or g)**
Coliform (CFU per 100 mL or g)	1			
E. coli (CFU per 100 mL or g)	Y n = 20	1		
Male-Specific Coliphage (PFU per 100 mL or g)	Y n = 20	Y n = 20	1	
Somatic Coliphage (PFU per 100 mL or g)	Y n = 20	Y n = 20	Y n = 20	1

Table 6-12 includes the results for surface water quality parameters. The results demonstrate that of the parameters tested (coliforms, *E. coli*, male-specific coliphages, and somatic coliphages) *E. coli* and somatic coliphage correlated. These results show that while *E. coli* is correlated to somatic coliphages, they are not correlated to male-specific coliphages.

Table 6-12: Surface Water Quality Parameter Correlation Analysis

	Coliform (CFU per 100 mL or g)	***E. coli* (CFU per 100 mL or g)**	**Male-Specific Coliphage (PFU per 100 mL or g)**	**Somatic Coliphage (PFU per 100 mL or g)**
Coliform (CFU per 100 mL or g)	1			
E. coli (CFU per 100 mL or g)	N	1		
Male-Specific Coliphage (PFU per 100 mL or g)	N	N	1	
Somatic Coliphage (PFU per 100 mL or g)	N	Y n = 27	N	1

Table 6-13 includes the results for stormwater quality parameters. The results demonstrate that of the parameters tested (*E. coli*, Enterococci, somatic coliphages, TTV, adenovirus, and polyomavirus) *E. coli* and Enterococci correlated. These results show that while two of the bacterial indicators correlated, the bacterial indicators did not correlate to the viral markers.

Table 6-13: Stormwater Quality Parameter Correlation Analysis

	E. coli **(CFU per 100 mL or g)**	**Enterococci (CFU per 100 mL)**	**Somatic Coliphage (PFU per 100 mL or g)**	**TTV (genomic copies per mL)**	**Adenovirus (genomic copies per mL)**	**Polyomavirus (genomic copies per mL)**
E. coli (CFU per 100 mL or g)	1					
Enterococci (CFU per 100 mL)	Y n=40	1				
Somatic Coliphage (PFU per 100 mL or g)	N	N	1			
TTV (genomic copies per mL)	N	N	N	1		
Adenovirus (genomic copies per mL)	N	N	N	N	1	
Polyomavirus (genomic copies per mL)	N	N	N	N	N	1

Table 6-14 includes the results for the wastewater quality parameter correlation analysis. The parameters in this analysis include coliforms, *E. coli*, Enterococci, male-specific coliphage, somatic coliphage, microviridae, TTV, adenovirus and polyomavirus. There are several significant correlations between wastewater quality parameters. These results show that while bacteria and coliphage indicators tended to correlate to one another, and viral markers tended to correlate to one another, there were limited correlations between the groups. The correlations did identify a relationship between TTV and Enterococci.

Table 6-14: Wastewater Quality Parameter Correlation Analysis

	Coliform (CFU per 100 mL or g)	***E. coli* (CFU per 100 mL or g)**	Enterococci **(CFU per 100 mL)**	**Male-Specific Coliphage (PFU per 100 mL or g)**	**Somatic Coliphage (PFU per 100 mL or g)**	**Microviradae (genomic copies per mL)**	**TTV (genomic copies per mL)**	**Adenovirus (genomic copies per mL)**	**Polyomavirus (genomic copies per mL)**
Coliform (CFU per 100 mL or g)	1								
E. coli (CFU per 100 mL or g)	Y n = 25	1							
Enterococci (CFU per 100 mL)	ID	Y n = 58	1						
Male-Specific Coliphage (PFU per 100 mL or g)	Y n = 25	Y n = 25	ID	1					
Somatic Coliphage (PFU per 100 mL or g)	Y n = 25	Y n = 87	Y n = 43	Y n = 25	1				
Microviradae (genomic copies per mL)	ID	ID	ID	ID	ID	1			
TTV (genomic copies per mL)	ID	Y n = 58	Y n = 58	ID	N	Y n = 22	1		
Adenovirus (genomic copies per mL)	N	N	ID	N	N	Y n = 22	Y n = 22	1	
Polyomavirus (genomic copies per mL)	ID	ID	ID	ID	ID	Y n = 22	Y n = 22	Y n = 44	1

Table 6-15 includes the results for the fecal sample parameter correlation analysis. The results were that coliforms and *E. coli* correlate and somatic coliphage correlated to coliforms, *E. coli* and male-specific coliphage. These results demonstrate that while the somatic coliphages correlate to the bacterial indicators, that the male-specific coliphages did not correlate to the bacterial indictors. These results are similar to the surface water correlations where somatic coliphages correlated to *E. coli*, but male-specific coliphages did not correlate to either bacterial indicator.

Table 6-15: Fecal Sample Parameter Correlation Analysis

	Coliform (CFU per 100 mL or g)	***E. coli* (CFU per 100 mL or g)**	**Male-Specific Coliphage (PFU per 100 mL or g)**	**Somatic Coliphage (PFU per 100 mL or g)**
Coliform (CFU per 100 mL or g)	1			
E. coli (CFU per 100 mL or g)	Y n = 75	1		
Male-Specific Coliphage (PFU per 100 mL or g)	N	N	1	
Somatic Coliphage (PFU per 100 mL or g)	Y n = 75	Y n = 75	Y n = 75	1

6.2 ADSORPTION ANALYSIS

Adsorption of particles was tested through microbial adhesion to hydrocarbons (MATH) and batch tests with drinking water filter media. These results demonstrate the limited ability of current testing methodologies to identify characteristics of nanoparticles.

6.2.1 MATH

MATH tests were initially planned for all of the particles, but only performed on MS2. The initial methodology was to test MATH for all variations of surrogate, water and pH. After several tests, there were no obvious changes in the results, and tests were terminated. Current literature also suggested that while MATH may be an appropriate way to test for hydrophobicity with microorganisms, this method may not be sensitive enough for nanoparticles (Rosenberg, 2006). The results are summarized in Table 6-16, and the detailed results are included in Appendix B – Data.

Table 6-16: MATH Results

	Log Removals		
	4	**6**	**8**
L	0.061	0.038	0.061
H	0.098	-	-

6.2.2 Batch Nanoparticles

Batch tests were conducted to evaluate the concentrations of abiotic nanoparticles when mixed with artificial groundwater and filter media. The $\log_{10}$ removals for the coated or uncoated nanoparticles with sand or acid-washed sand in lab water, low ionic strength water or high ionic strength water of pH 4, 6, or 8 are summarized in the following figures and the detailed data is included in Appendix B - Data. Each

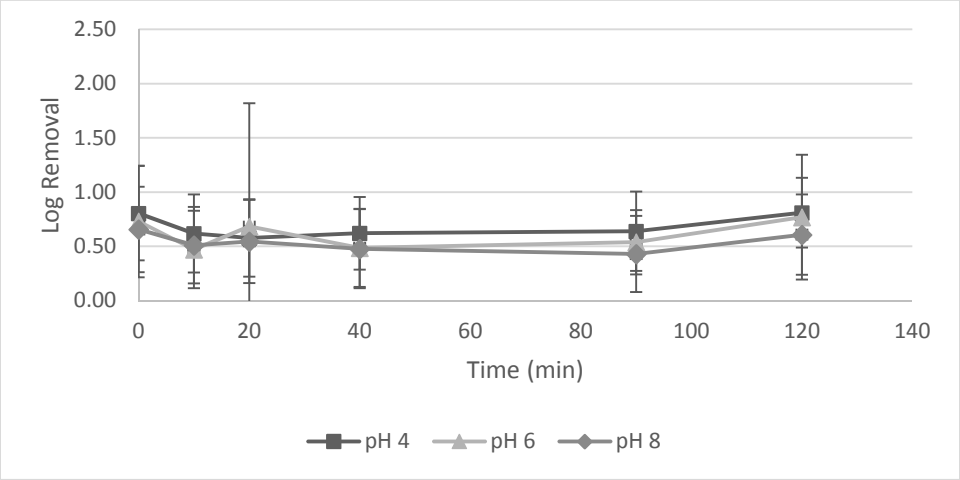
particle type in each media type is detailed in the following Figures identifying log_{10} removals in the specific water type and pH. The figures also include error bars for each data point representing standard deviation. Table 6-17 and corresponding figures summarize the minimum and maximum log_{10} removals for uncoated and protein-coated nanoparticles. The log_{10} removals are greater for particles with the acid-washed sand when compared to particles with sand media, with the exception of protein-coated nanoparticles in low ionic strength water. For the particles with sand media, removals were greatest in low ionic strength water. For uncoated particles with acid-washed sand, removals were greatest in high ionic strength water. For protein-coated nanoparticles, removals were greatest in lab water.

Table 6-17: Log Removals for Nanoparticles with Media

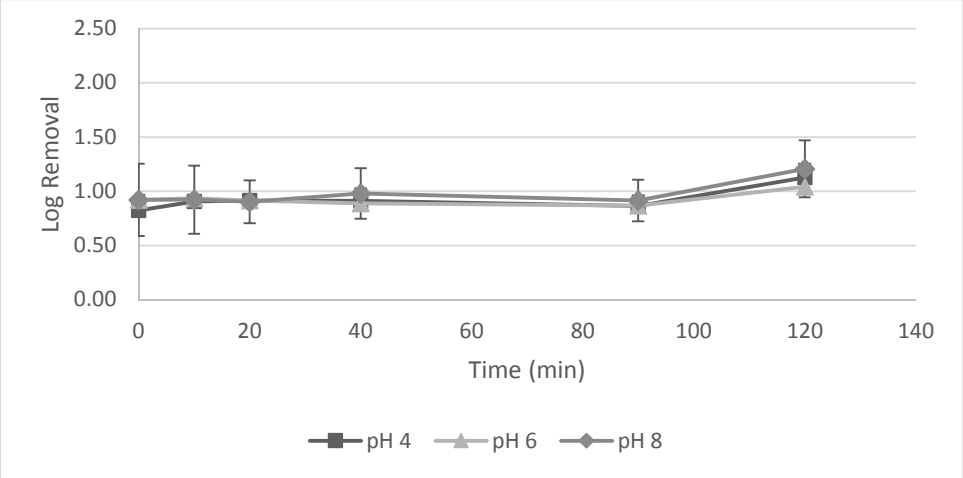
		Media - Sand		**Media Acid-Washed Sand**	
Nanoparticles	**Water**	**Minimum Log Removal**	**Maximum Log Removal**	**Minimum Log Removal**	**Maximum Log Removal**
Uncoated	LW	0.61 pH 8	0.81 pH 4	1.16 pH 6	1.42 pH 8
	L	1.04 pH 6	1.21 pH 8	1.36 pH 4	1.48 pH 8
	H	0.46 pH 8	0.64 pH 6	1.52 pH 4	1.74 pH 8
Protein-Coated	LW	1.20 pH 4	1.25 pH 8	1.43 pH 4	1.78 pH 8
	L	1.33 pH 4	2.22 pH 8	1.27 pH 4	1.66 pH 8
	H	1.28 pH 6	1.43 pH 4	1.30 pH 4	1.72 pH 8

The log_{10} removals for uncoated nanoparticles with sand media are included in Figure 6-1. The overall log_{10} removals ranged from 0.46 log_{10} in lab water at pH 8 to 1.21 log_{10} in low ionic strength water at pH 8. The log_{10} removals for uncoated nanoparticles with acid washed sand media are included in Figure 6-2. The overall log_{10} removals ranged from 1.16 in lab water at pH 6 to 1.74 in high ionic strength water at pH 8. The log_{10} removals for protein coated nanoparticles with sand media are included in Figure 6-3. The overall log_{10} removals ranged from 1.20 in lab water at pH 4 to 2.2 in low ionic strength water at pH 8. The log_{10} removals for protein coated nanoparticles with acid washed sand media are included in Figure 6-4. The log_{10} removals ranged from 1.27 in low ionic strength water at pH 4 to 1.78 in lab water at pH 8. These Figure are further detailed in the Section 7.0 Discussion.

(a)



(b)



(c)

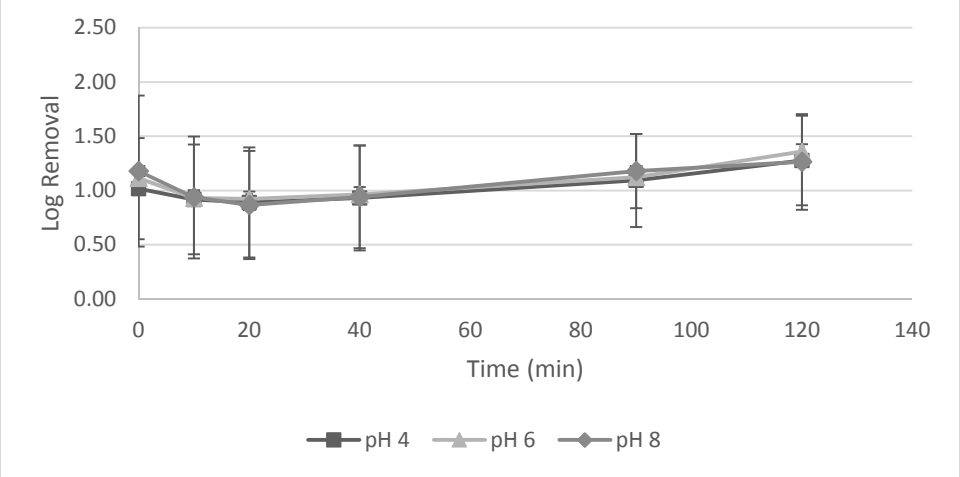
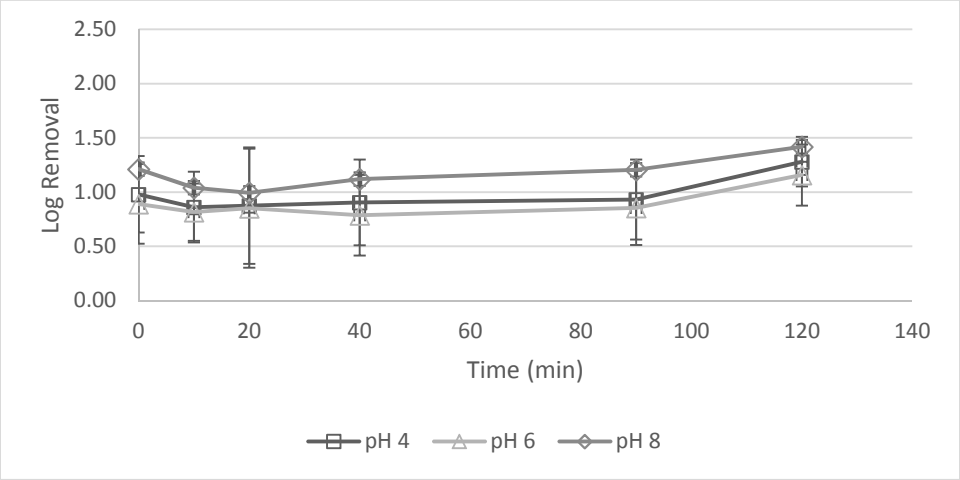
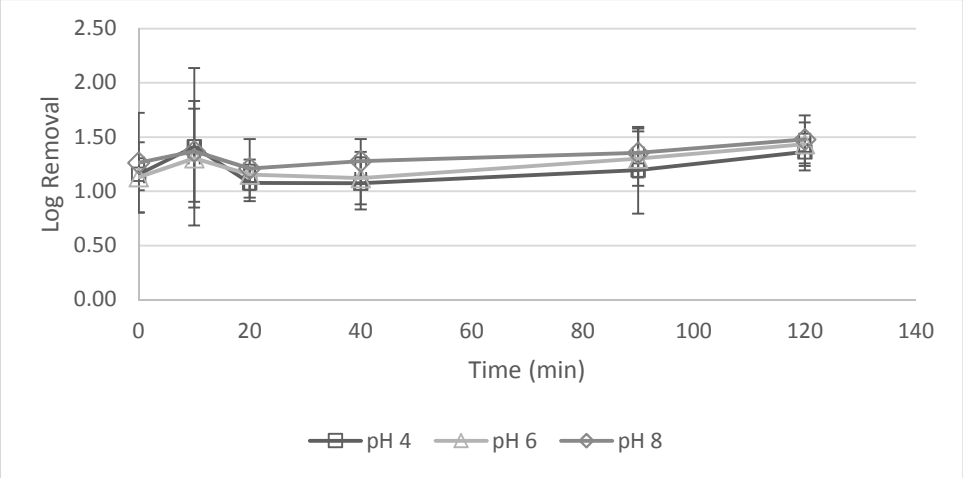


Figure 6-1: Log Removals in pH 4, 6, 8 for Uncoated Particles with Sand Media (a) Lab water (b) Low Ionic Strength Water (c) High Ionic Strength Water (error bars represent standard deviation, n=8)

(a)



(b)



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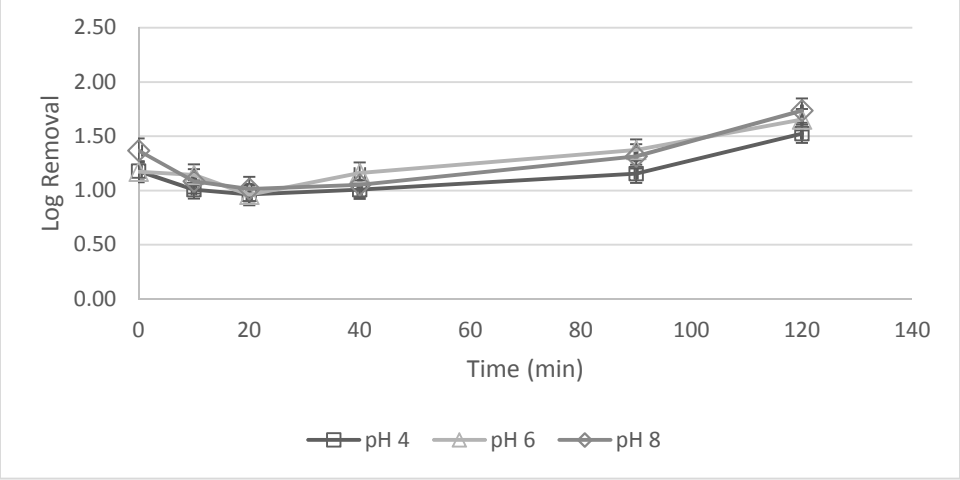
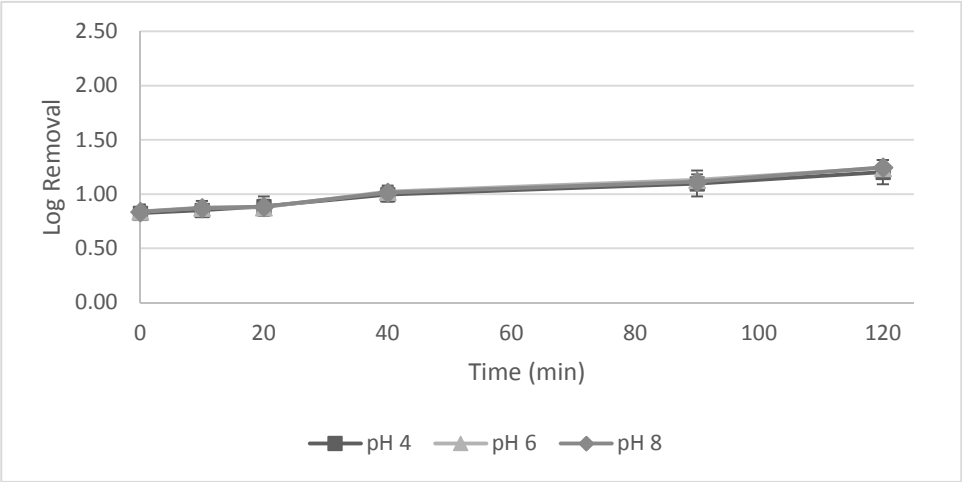
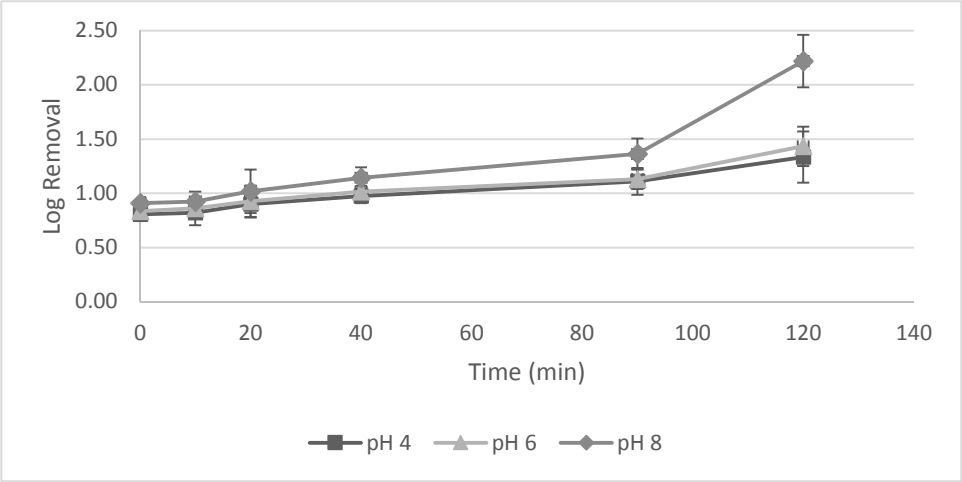


Figure 6-2: Log Removals in pH 4, 6, 8 for Uncoated Particles with Acid Washed Sand Media (a) Lab Water (b) Low Ionic Strength Water (c) High Ionic Strength Water (error bars represent standard deviation, n=8)

(a)



(b)



(c)

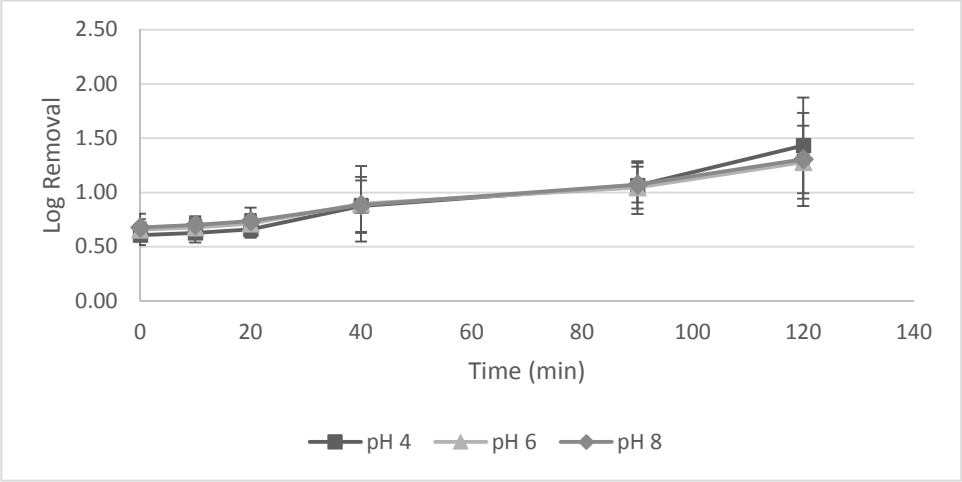
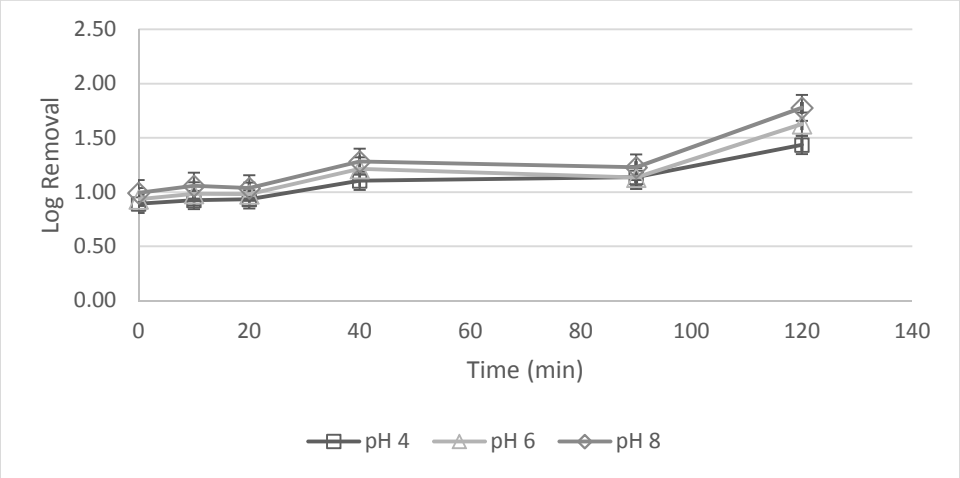
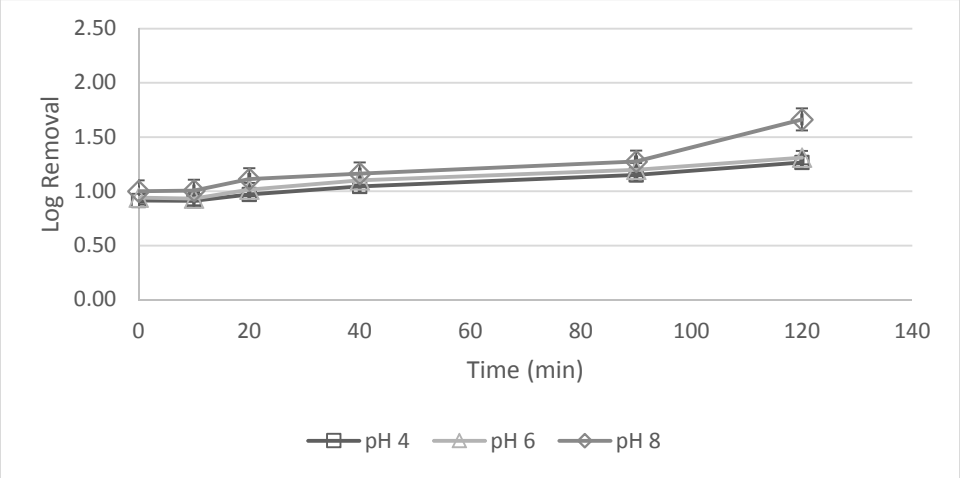


Figure 6-3: Log Removals in pH 4, 6, 8 for Protein Coated Nanoparticles with Sand Media (a) Lab Water (b) Low Ionic Strength Water (c) High Ionic Strength Water (error bars represent standard deviation, n=8)

(a)



(b)



(c)

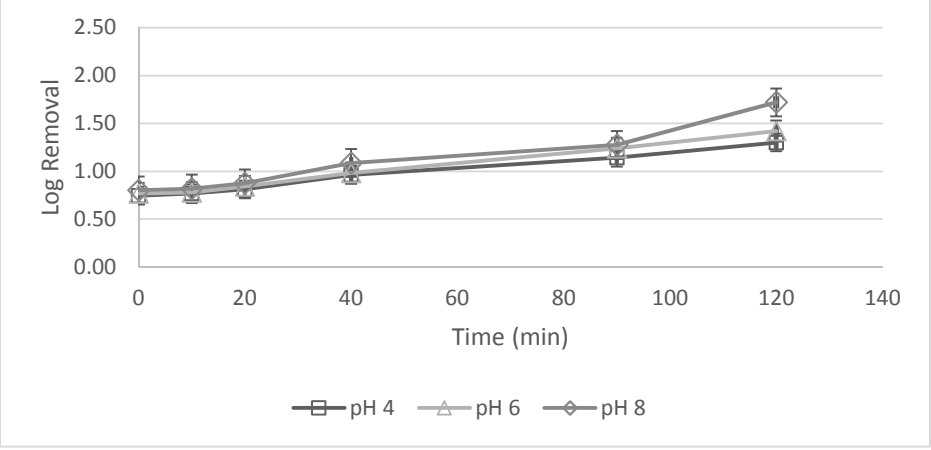
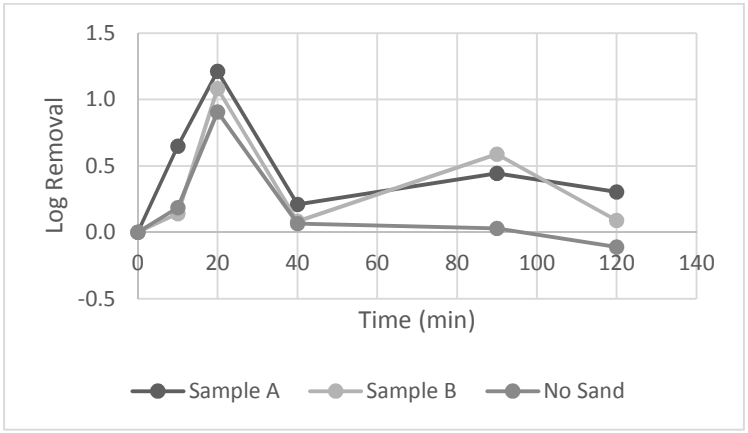


Figure 6-4: Log Removals in pH 4, 6, 8 for Protein Coated Nanoparticles with Sand Media (a) Lab Water (b) Low Ionic Strength Water (c) High Ionic Strength Water (error bars represent standard deviation, n=8)

6.2.3 Batch MS2

Batch tests were conducted to evaluate the concentrations over time of biotic nanoparticles when mixed with artificial groundwater and filter media. The original intent was to conduct this experiment for MS2 and ΦX-174 phages with sand or acid-washed sand in lab water, low ionic strength water or high ionic strength water with pH 4, 6, or 8. The results of the first few experiments were inconsistent and current literature suggested that viruses had a low affinity for silicon dioxide based materials (Michen *et al.*, 2010). These tests were suspended and nanoparticle characteristics were further explored with time-dependent light scattering experiments. The $\log_{10}$ removals are summarized in Figure 6-5 and the detailed data is included in Appendix B - Data. In each of the control cases without media, the concentration of MS2 increased with time. The final average $\log_{10}$ removals for MS2 with sand in low ionic strength water were 0.198 and 0.545, for pH 6 and 8, respectively.

(a)



(b)

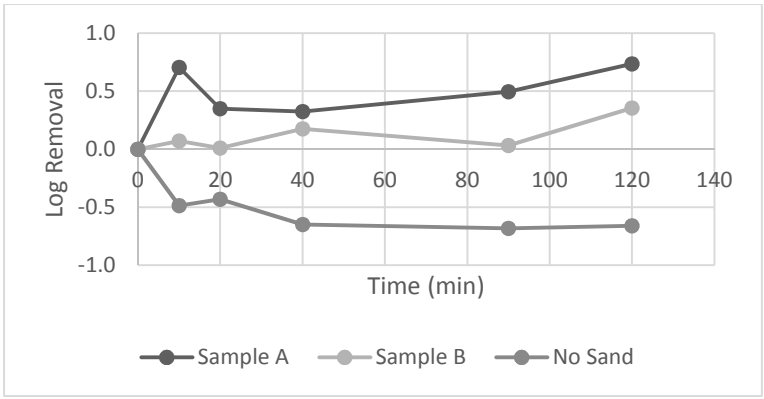


Figure 6-5: Log Removals of MS2 with Sand Media in Low Ionic Strength Water at (a) pH 6 and (b) pH 8

6.3 ZETA POTENTIALS

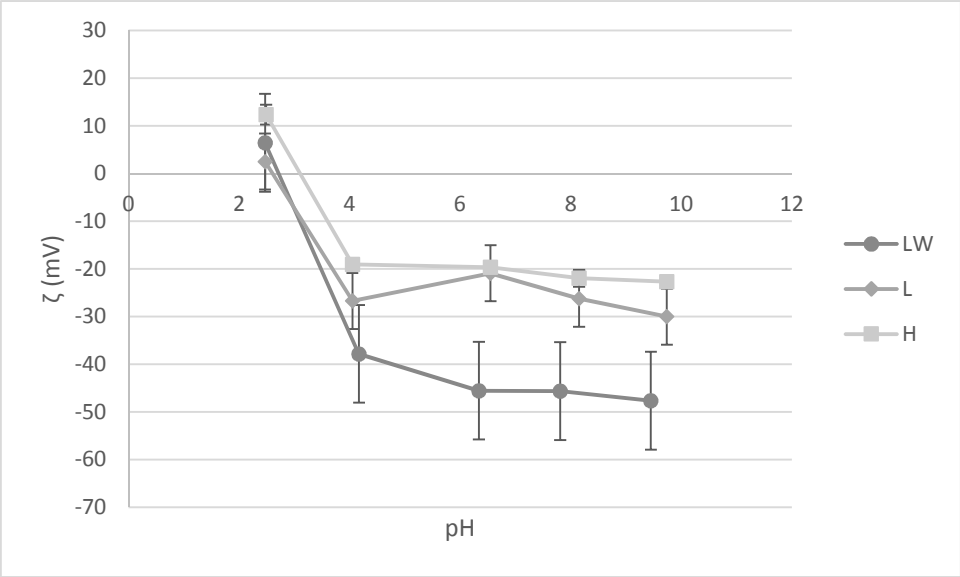
Zeta potentials were identified for each particle type in solution with pH ranging from 2.5 to 10. The solutions used to suspend the particles were lab water, low ionic strength water and high ionic strength water. The following figures summarize the data and the detailed data tables are included in Appendix B, Data. Zeta potential is a component in the dispersion and aggregation processes of a particle. The greater the absolute value of the zeta potential, the more stable the system will be because the charged particles repel one another reducing the probability of aggregation. Stable particles are less impacted by additions of electrolytes and tend towards lower viscosities.

The zeta potentials trended lower as pH increased and zeta potentials trended higher as ionic strength increased. Figure 6-6 includes the average zeta potentials of sand and acid-washed sand in solution. The zeta potentials had the greatest change from pH 2.5 to 4 with more gradually change after pH of 4. The maximum values were identified in high ionic strength water at pHs of about 2.5 with zeta potentials of 9.41mV for sand in pH 2.49 and 6.05 mV for acid-washed sand at pH 2.5. The minimum values were identified in lab water at pHs of approximately 10 with zeta potentials of -47.68 mV for sand in pH 9.45 and -53.65 mV for acid-washed sand in pH 9.87.

The zeta potentials of the uncoated nanoparticles remained relatively constant values, while the zeta potentials of the protein- coated nanoparticles gradually trended lower as pH decreased. Figure 6-7 includes the average zeta potentials of uncoated and protein-coated nanoparticles in solution. The uncoated particles in low and high ionic strength water had similar zeta potentials while the zeta potentials were lower in the lab water. The uncoated nanoparticle had maximum and minimum zeta potentials of -19.35 mV in high ionic strength water at pH 8.65 and -53.38 mV in lab water at pH 6.08, respectively. The protein-coated nanoparticles had zeta potentials which increased as ionic strength of the water increased. The maximum and minimum zeta potentials were -13.20 mV in high ionic strength water at pH 2.43 and -47.88 mV in lab water at pH 4.02, respectively.

The zeta potentials trended lower as pH increased and zeta potentials trended higher as ionic strength increased. Figure 6-8 includes the average zeta potentials of phages, MS2 and ΦX-174 in solution. The maximum and minimum zeta potentials for MS2 were -9.07 mV in high ionic strength solution at pH 2.43 and -25.98 mV in lab water at pH 10.90, respectively. The zeta potentials of ΦX-174 increased slightly after approximately pH 8. The maximum and minimum zeta potentials for ΦX-174 were 2.70 mV in low ionic strength solution and -29.30 mV in lab water at pH 8.87, respectively.

(a)



(b)

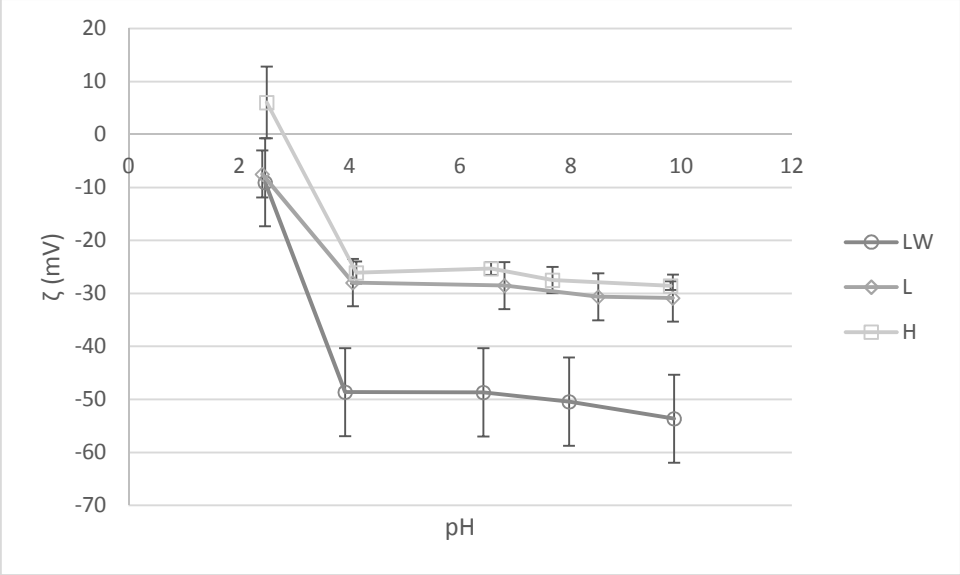
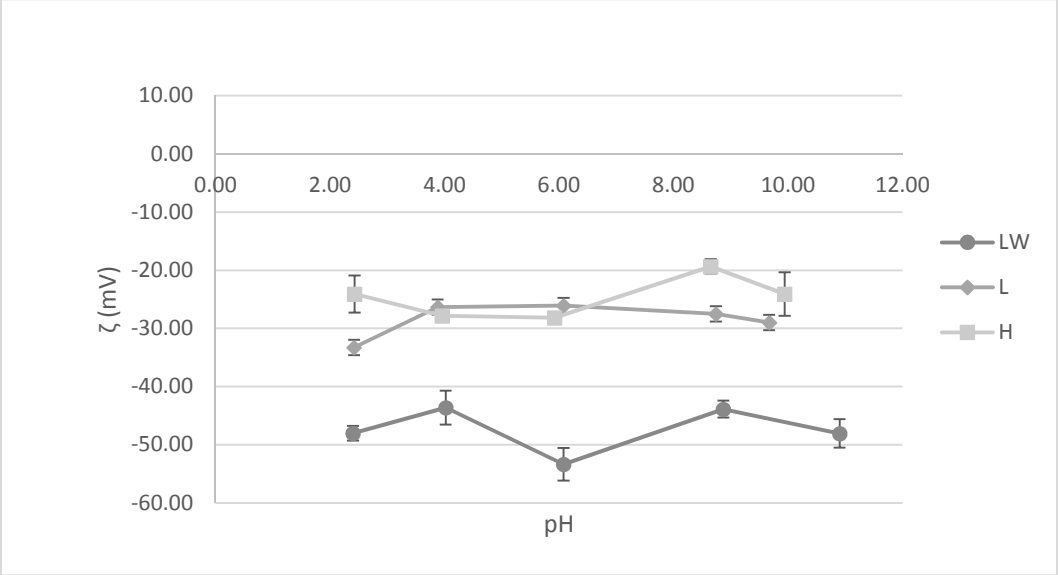


Figure 6-6: Zeta Potentials of Filter Media in Solutions of Varying Ionic Strengths (a) Sand and (b) Acid-Washed Sand (error bars represent standard deviation, n=4)

(a)



(b)

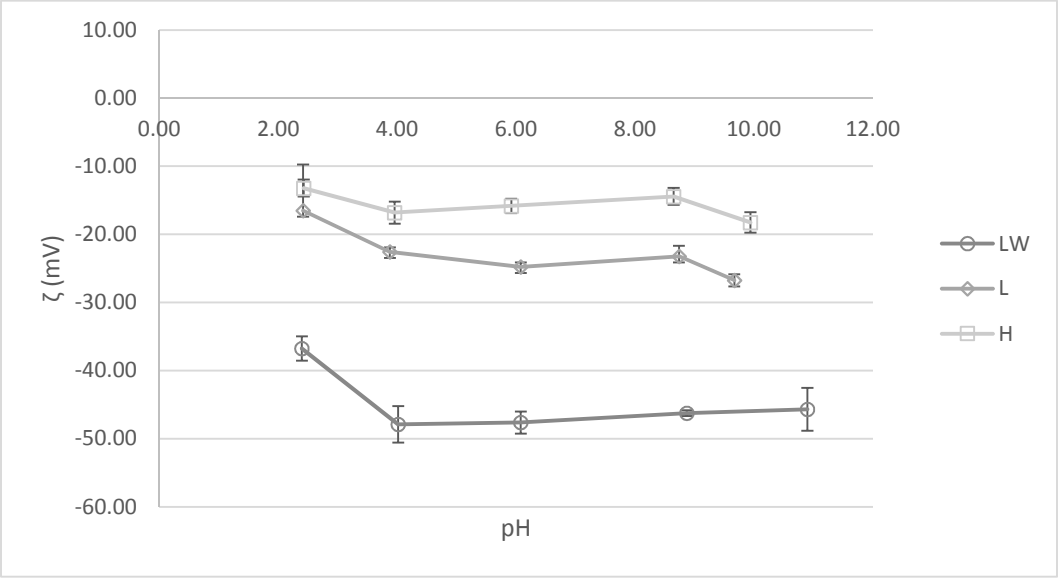
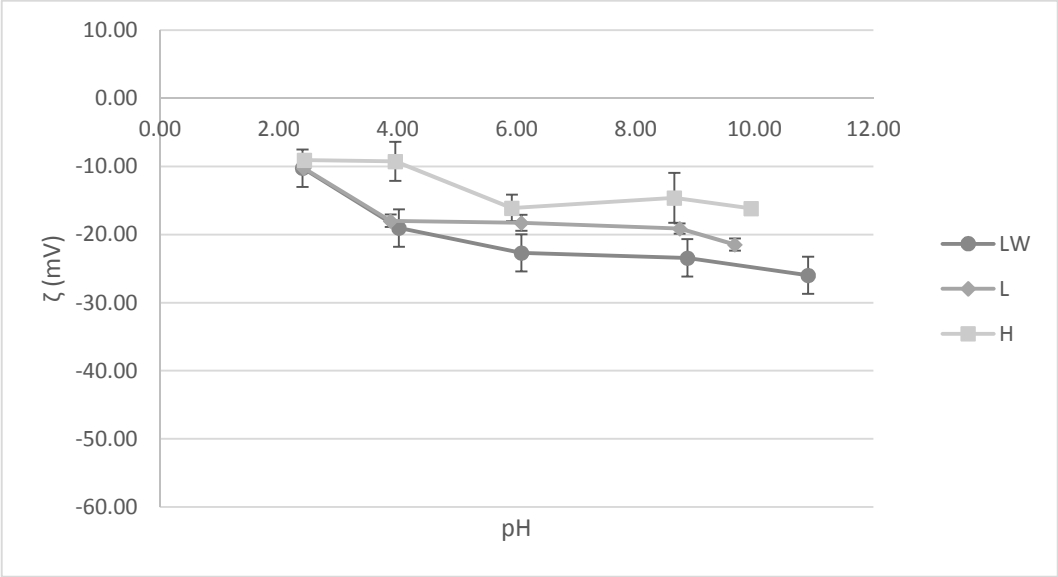


Figure 6-7: Zeta Potentials of Abiotic Particles in Solutions of Varying Ionic Strengths (a) Uncoated and (b) Protein-Coated Nanoparticles (error bars represent standard deviation, n=4)

(a)



(b)

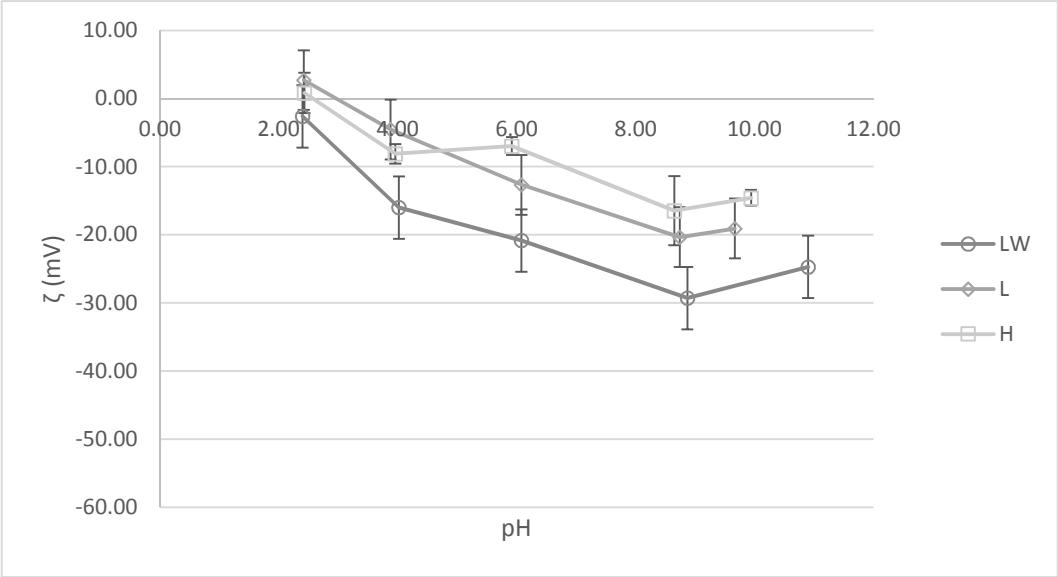


Figure 6-8: Zeta Potentials of Biotic Particles in Solutions of Varying Ionic Strengths: (a) MS2 and (b) ΦX 174 Bacteriophages (error bars represent standard deviation, n=4)

6.4 BACTERIA DYNAMICS

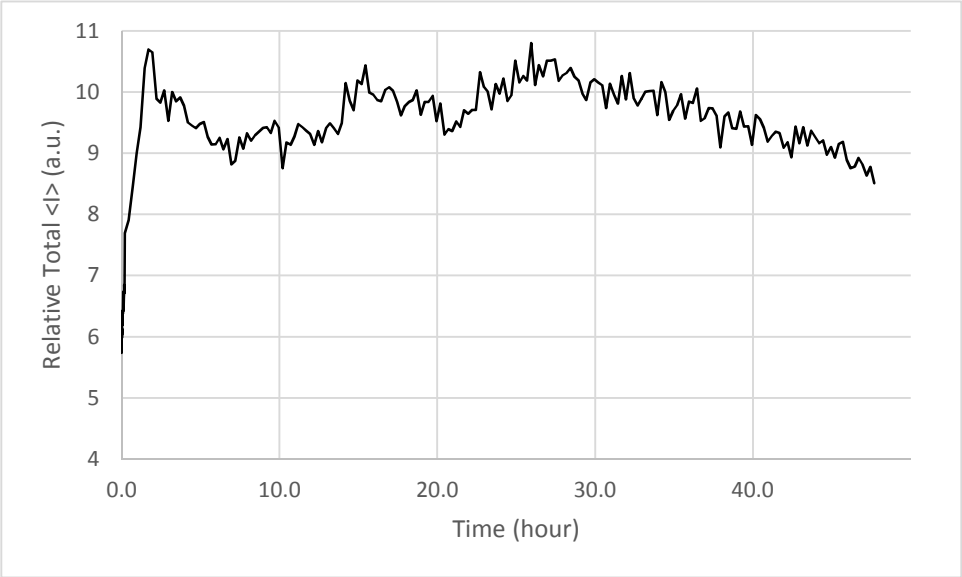
Dynamics of a particle help to define the transport characteristics and several models deriving from the dynamic properties provide particle characteristics, such as shape and size. The dynamics of bacteria were observed with the ARGOS method using time dependent light scattering. These tests were conducted several times over the course of a several months (2013-2014) in order to establish a procedure for data collection. The following data are from the final observations for the bacteria. The data was collected from a series of photographs which are available upon request as a part of the electronic appendix for Appendix B - Data. The photographs were then analyzed using a program created in LabView software package. The details of the software and data extraction are included in the WPI Doctoral Dissertation of Saad Algarni, November 2014 (Algarni, 2014).

Dynamic light scattering was used to observe *E. coli* F-amp (bacteria) in solution. The bacteria samples were prepared according to Section 5.0 Research Methods of this report and observed for a 48-hour period. The initial intent of the experiment was to get a baseline of the bacteria prior to conducting infectivity evaluations. The research allowed for several findings on bacterial behavior based on an extended time-dependent study of bacteria.

Figure 6-9 summarizes the data for relative total intensity over the 48-hour observation period. The x-axis is shown both for linear and log scale time measurements (which allows for observations about the initial relative intensity changes). The bacteria were observed for relative total intensity which provided information about total system dynamics. The relative total intensity increases rapidly to an initial peak at 1.7 hours. The relative total intensity fluctuates with a second and third peak at 15 hours and 25 hours, and then steadily decreases.

The root mean square difference (RMSD) was also calculated for specific wave vectors. This analysis allowed for observations about particle dynamics at a regime specific to the particle which provided information about individual particle dynamics. A wave vector of 7500 was selected because this vector corresponds to the regime which would specify the dynamics of particles on the microscale. This analysis RMSD was calculated thus: an image was taken at time zero, and the average difference over a specified wave vector (q) across all succeeding images was plotted as a function of time quantifying particle dynamics over time. Figure 6-10 summarizes the data for relative total intensity RMSD over the 48-hour observation period. The x-axis is shown both for linear and log scale time measurements.

(a)



(b)

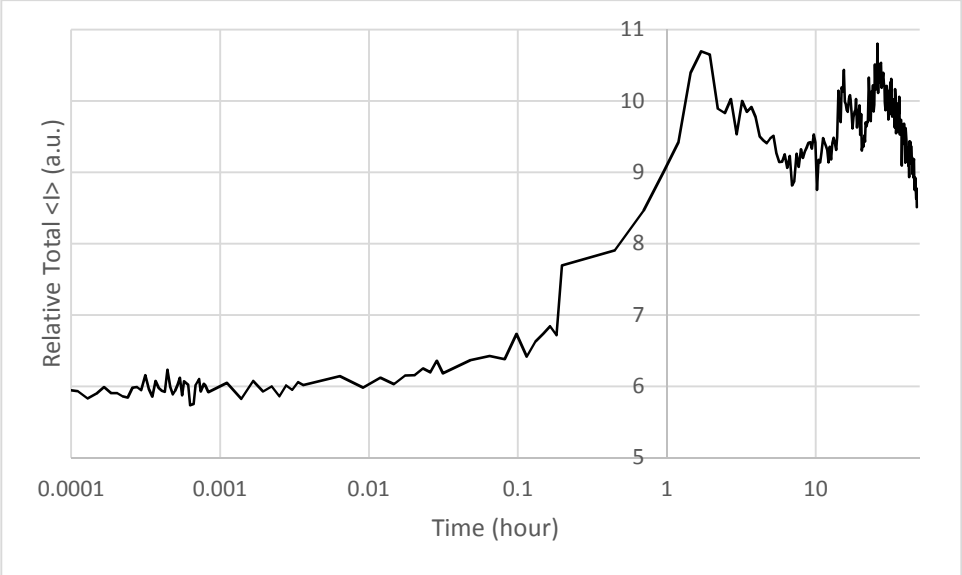
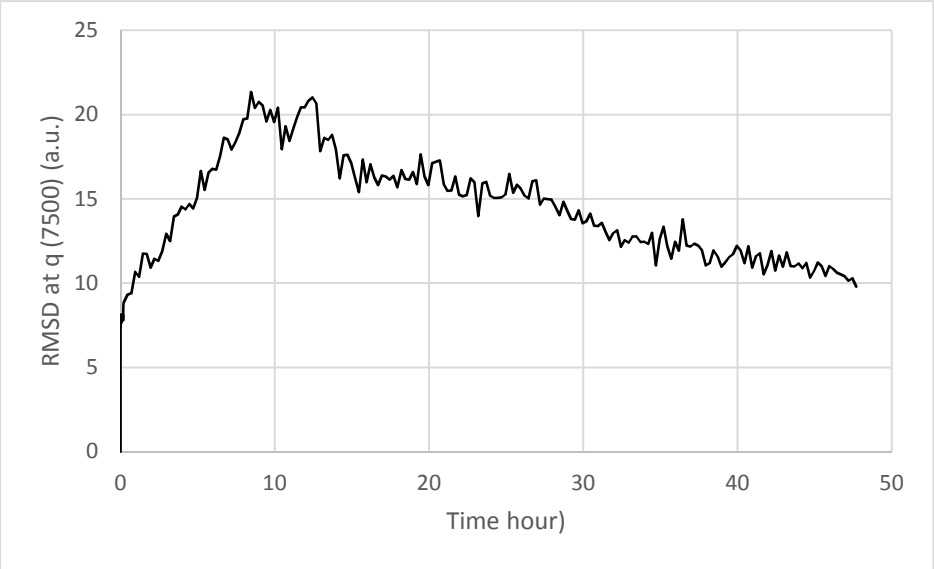


Figure 6-9: Relative Total Intensity for F-amp *E. coli* over a 48 hour period (a) x- axis linear scale, and (b) x-axis log scale

(a)



(b)

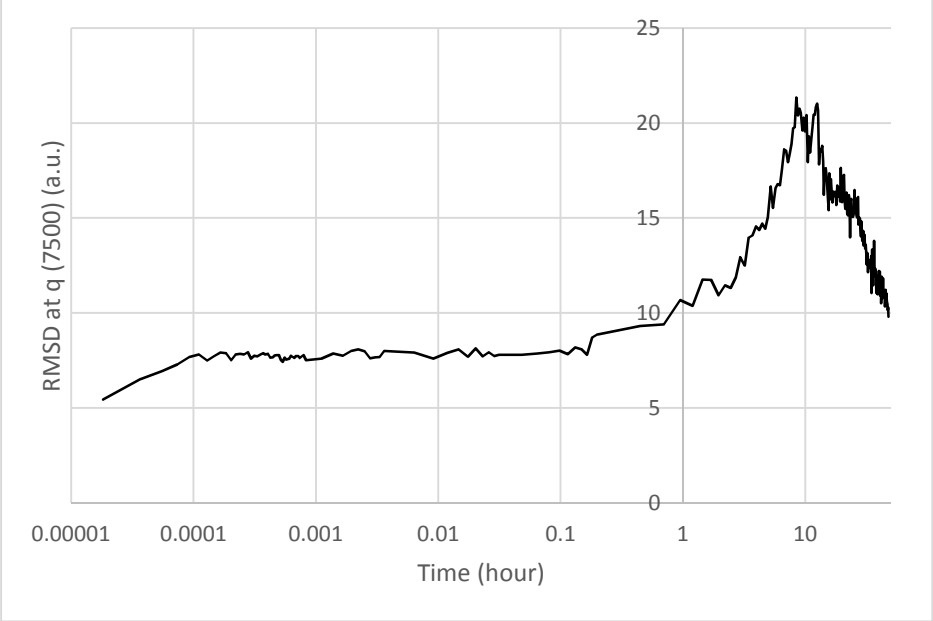


Figure 6-10: Root Mean Square Difference for F-amp *E. coli* over a 48 hour period (a) x- axis linear scale, and (b) x-axis log scale

The shape of a particle can be analyzed using the data from the relative total intensity <I> and wave vectors (q) owing to the integration of intensities and angular distributions. The shape can be observed for specific times with the model log <I> vs log q (Berne *et al.*, 2000). Relative total intensity <I> changes was observed at times corresponding to changes in the dynamics (1) 0.3 hour, the beginning of the analysis; (2) 1.4 hours, prior to the peak; (3) 9 hours, prior to the peak; (4) 13 hours, post peak; and 39 hours, end of the analysis. Changes in the particle shape are modeled by Figure 6-11 graphing log <I> vs. log q for specific times.

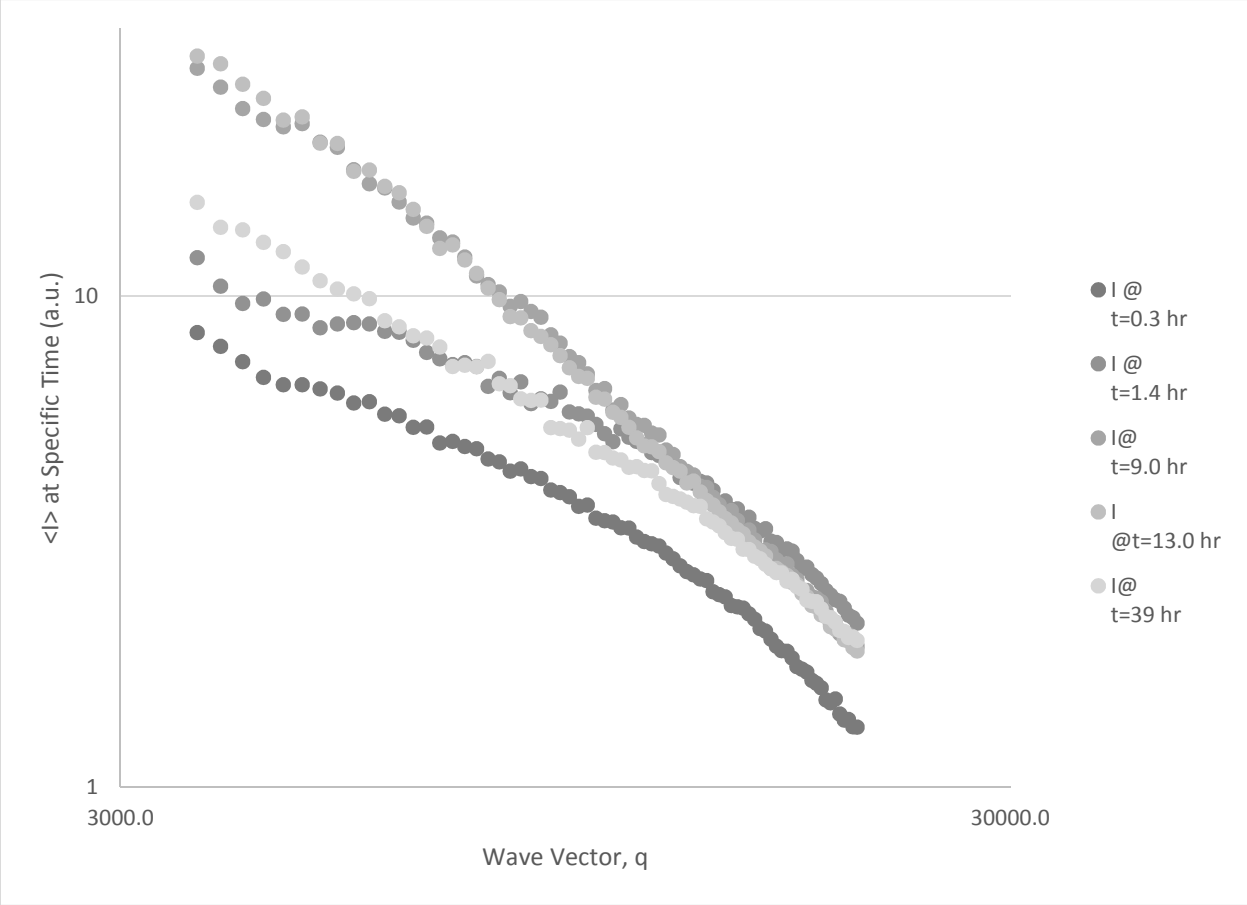


Figure 6-11: <I> v q at Specific Times

Changes in shape can also be observed by calculating the fractal dimensions at specific times. These calculations are included in Figure 6-10. The fractal dimension (f) was modelled using Equation 6-1 where an elongated rod models, f=1; flat disk, f=2 and sphere, f=3, (Berne *et al.*, 2000).

$$f = \frac{\log < I >}{\log q}$$

(Equation 6-1)

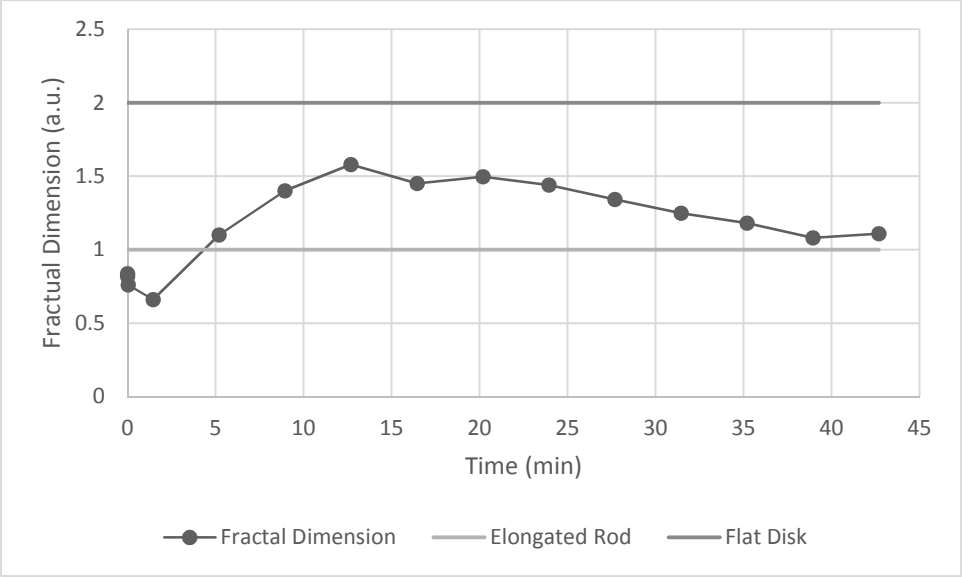


Figure 6-12: Fractal Dimension of Famp *E. coli* over the 48 Hour Period

The fractal dimension of the F-amp *E. coli* particles early in the analysis is modelled by elongated rods, progresses to a shape change modelled by a flat disk, and then returns to a model more similar to an elongated rod, as shown in Figure 6-12. Similarly, the effective radius was modeled using the intensity and wave vector. The effective radius can be modelled using Equation 6-2 (Berne *et al.*, 2000).

$$R_{eff} = \frac{\ln < I >}{q^2}$$

(Equation 6-2)

The results for the effective radius over the 48-hour period are included in Figure 6-13. The effective radius ranges from a minimum of 1.56 µm at 20 hours to a maximum of 1.75 µm at 9 hours.

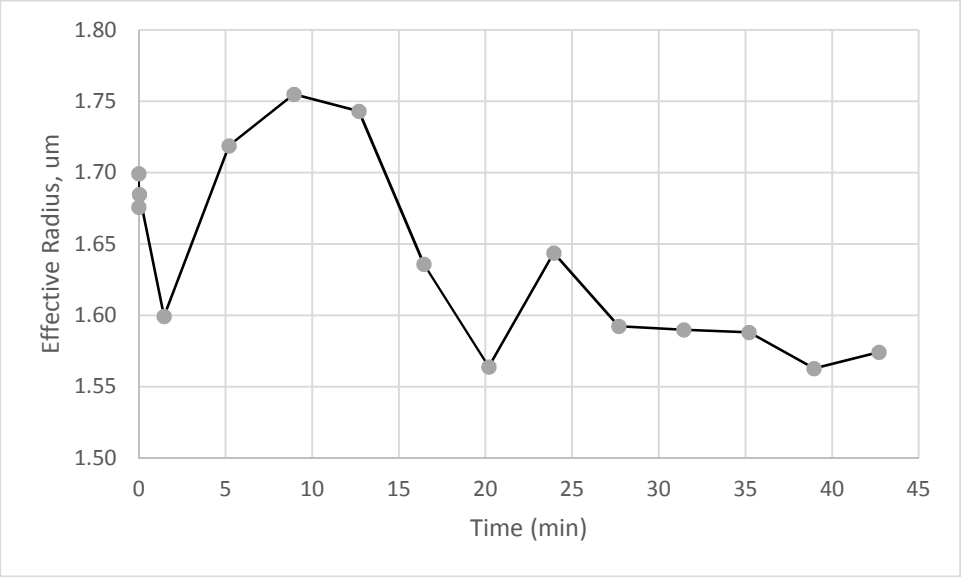


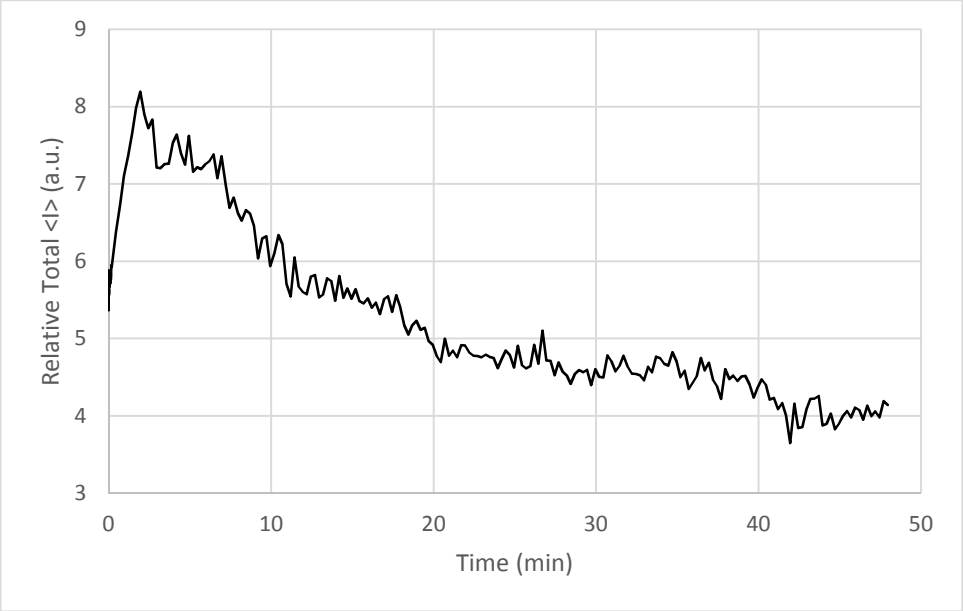
Figure 6-13: Effective Radius Over Time

6.5 BACTERIA WITH PHAGE DYNAMICS

The bacteria (F-amp), was then studied during infection by bacteriophage (MS2). The bacteria was inoculated with a bacteriophage virus and observed over a 48-hour period. Figure 6-14 details the relative total intensity over the 48-hour period with (a) linear x-axis and (b) log scale x-axis. The relative total intensity allows for observations of the entire system dynamics. The relative total intensity of the system increased rapidly to a peak at 1.95 hours and decreased steadily over the remaining study period. The dynamics analyzed were those of the entire system. Potential factors impacting the system dynamics such as host lysis or pili removal were not specifically assessed. Future research should include a parallel analysis with microscopy to account for all physical events observed.

The characteristics of the bacteria were evaluated by calculating RMSD, fractal dimension and effective radius over the 48-hour period. These results are shown in Figure 6-15. The RMSD reflects the dynamics of the particles and increased rapidly to a peak at 6.20 hours and decreased steadily over the remaining study period. The RMSD was calculated over the study period for a wave vector, q (7500), which is specific to the micro-scale of the bacteria.

(a)



(b)

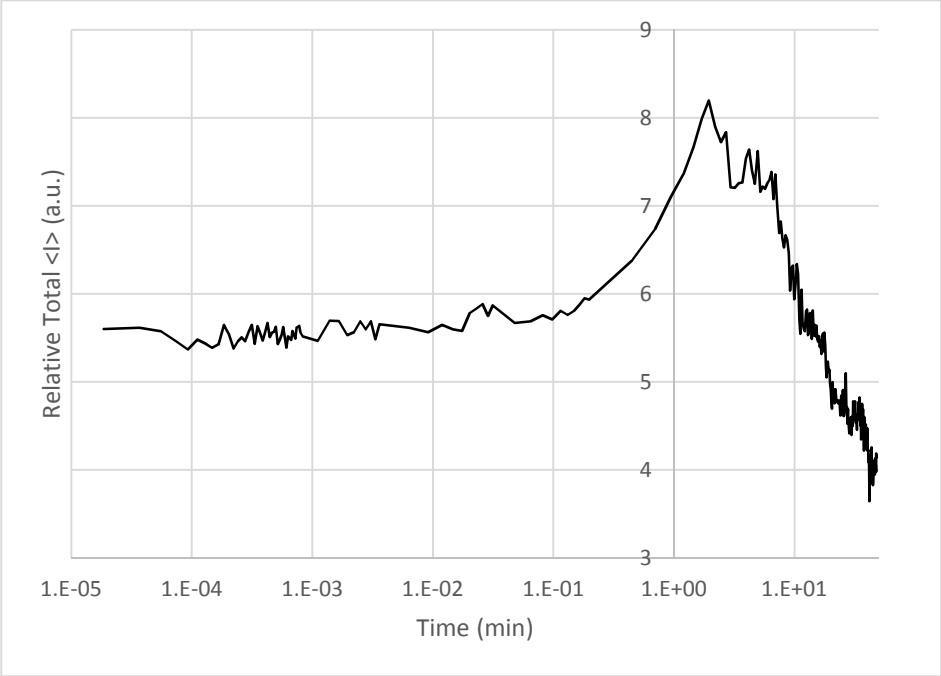
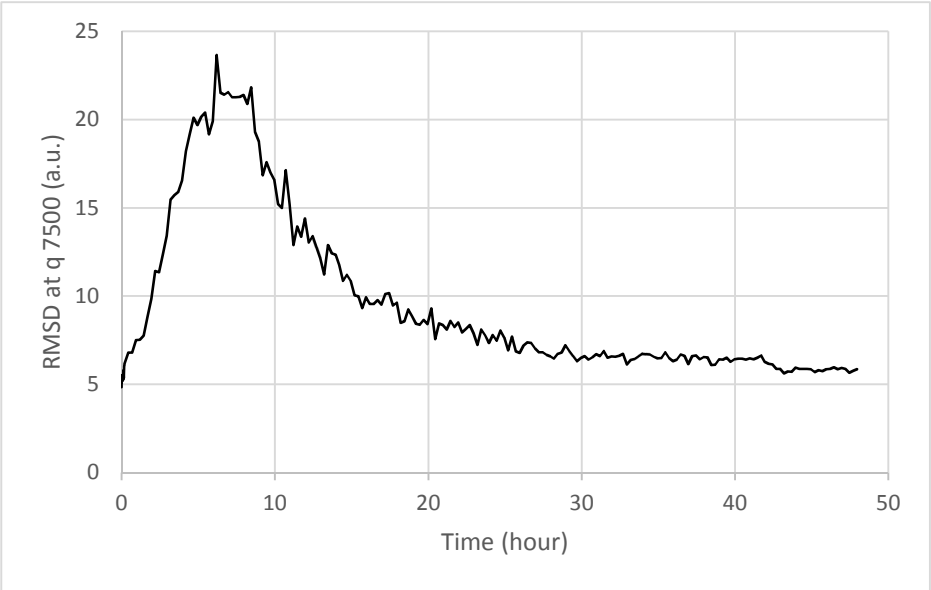


Figure 6-14: Relative Total Intensity for F-amp *E. coli* with MS2 over a 48-Hour Period (a) x-axis Linear Scale, and (b) x-axis Log Scale

(a)



(b)

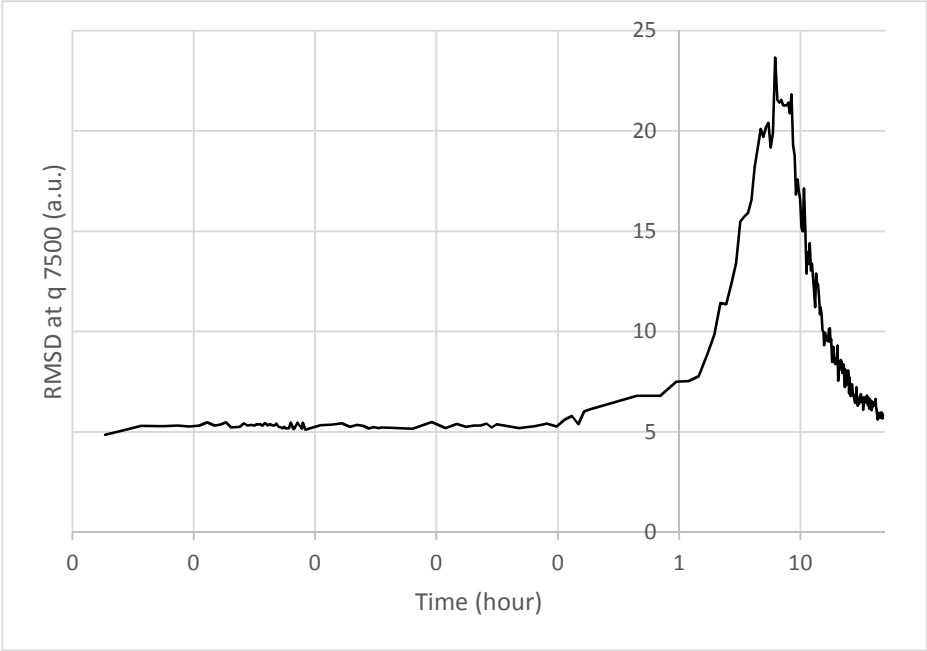
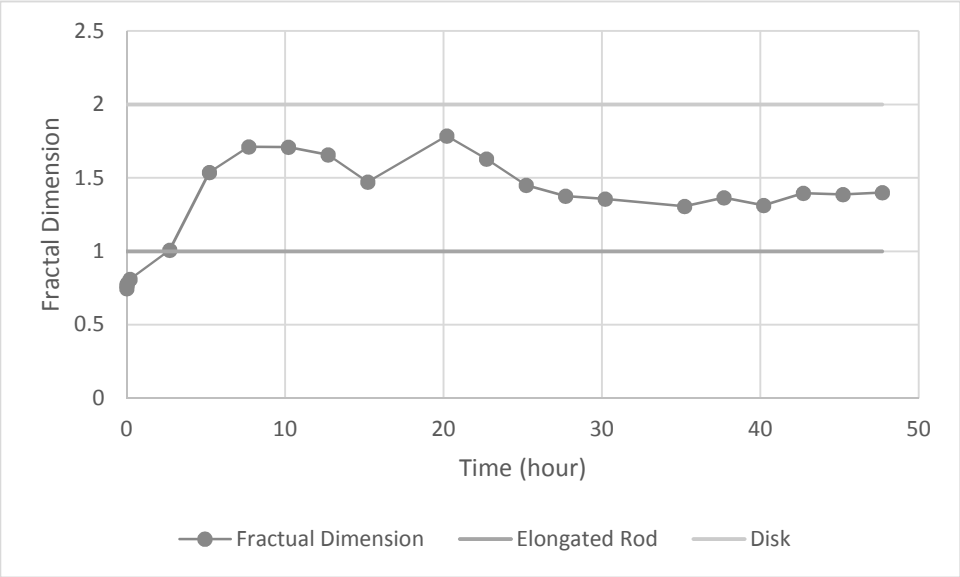


Figure 6-15: Root Mean Square Difference for F-amp *E. coli* with MS2 Over a 48 hour Period (a) x-axis Linear Scale, and (b) x-axis Log Scale

The fractal dimension of the bacteria particles was calculated for the 48-hour period. The initial shape model for the bacteria was an elongated rod. For the period between 5.0 and 25.0 hours, the bacteria more closely fit the model of a flat disk. After 25 hours, the particles trended back toward a model for the elongated rod. The effective radius ranged from a minimum of 2.51 µm at 35 hours to a maximum of 2.99 µm at 8 hours. The morphology of the bacteria over the 48-hour period is shown in Figure 6-16.

(a)



(b)

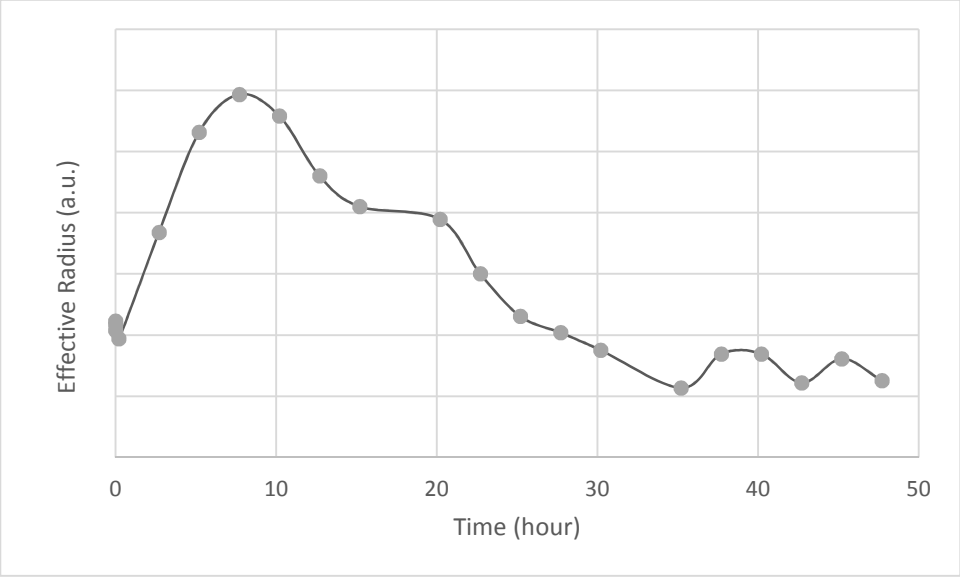


Figure 6-16 – F-amp *E. coli* Characteristics during Infection

6.6 NANOPARTICLE DYNAMICS

The ARGOS time-dependent light-scattering method was used to find the total intensity of the particle behavior over time and the root mean square difference was calculated at specific wave vectors (q) to identify changes in particle behavior. The intensity of the laser was held constant so that comparisons could be made between the total intensities of the particles. The study was originally calibrated to observe particles at the micro-scale. Over the period of a few months, the methodology was re-calibrated to observe the dynamics of nanoparticles. The method was used to characterize MS2, ΦX-174, uncoated particles and protein coated particles. The use of abiotic particle may serve to focus the description of the particles on specific properties, such as hydrophobicity or type of capsid.

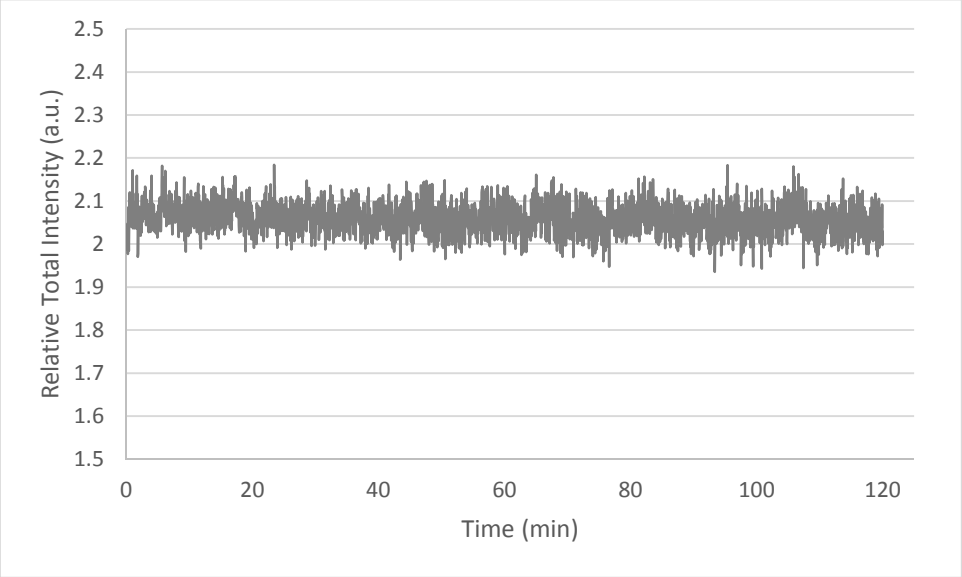
Table 6-13 summaries the data for the nanoparticle dynamics. The complete data set is included in Appendix B – Data. The greatest intensities were for the uncoated nanoparticles with a range of 6.005 to 9.541 relative total intensities. The lowest intensities were for ΦX-174 with a range of 0.739 to 2.104 relative total intensities.

Table 6-18: Statistics for Nanoparticle Dynamics

Relative Total <I>	MS2	ΦX-174	Uncoated Nanoparticles	Protein-Coated Nanoparticles
Mean	2.059	0.968	6.945	2.637
Median	2.059	0.921	6.848	2.626
Mode	2.067	0.907	6.775	2.550
Standard Deviation	0.034	0.191	0.480	0.103
Range	0.248	1.365	3.537	0.890
Minimum	1.936	0.739	6.005	2.369
Maximum	2.184	2.104	9.541	3.260

The particle dynamics for each particle type is included in the following figures which include (a) relative total intensity, <I> and (b) RMSD calculated for 3 wave vectors. The data is included in Figure 6-17 for MS2, Figure 6-18 for ΦX-174, Figure 6-19 for uncoated nanoparticles and Figure 6-20 for protein-coated nanoparticles. This data is further analyzed in Section 7.0 Discussion.

(a)



(b)

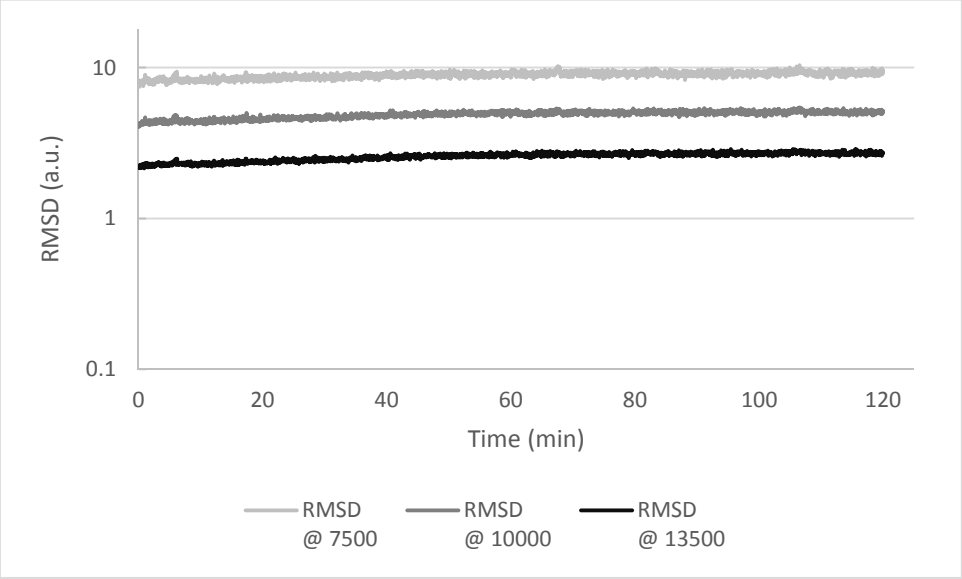
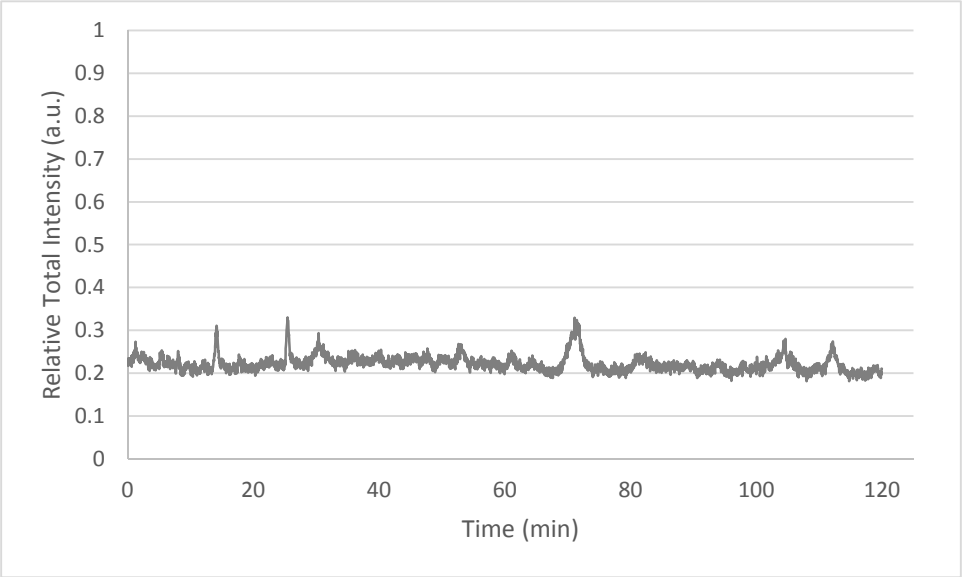


Figure 6-17: MS2 Dynamics (a) Relative Total Intensity and (b) RMSD

(a)



(b)

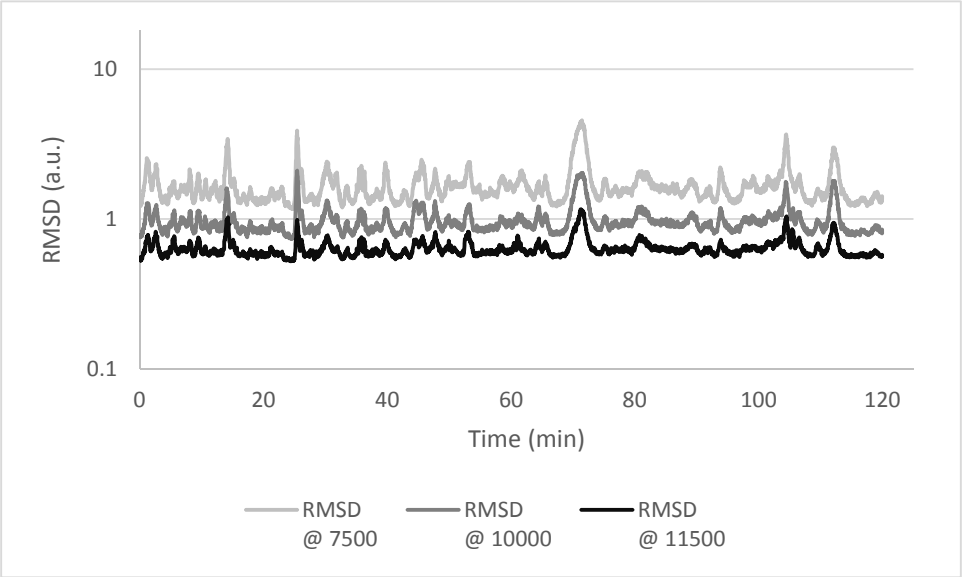
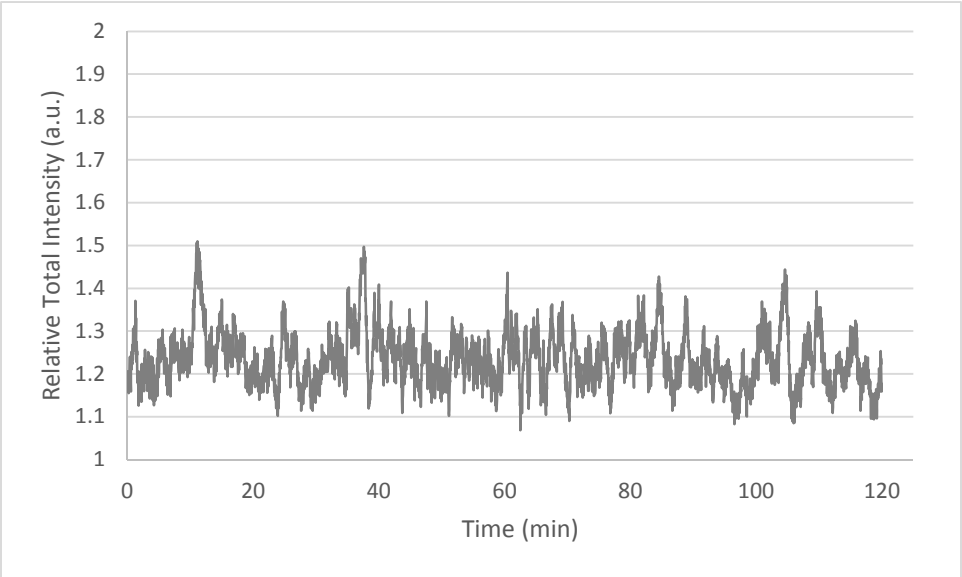


Figure 6-18: ΦX-174 Dynamics (a) Relative Total Intensity and (b) RMSD

(a)



(b)

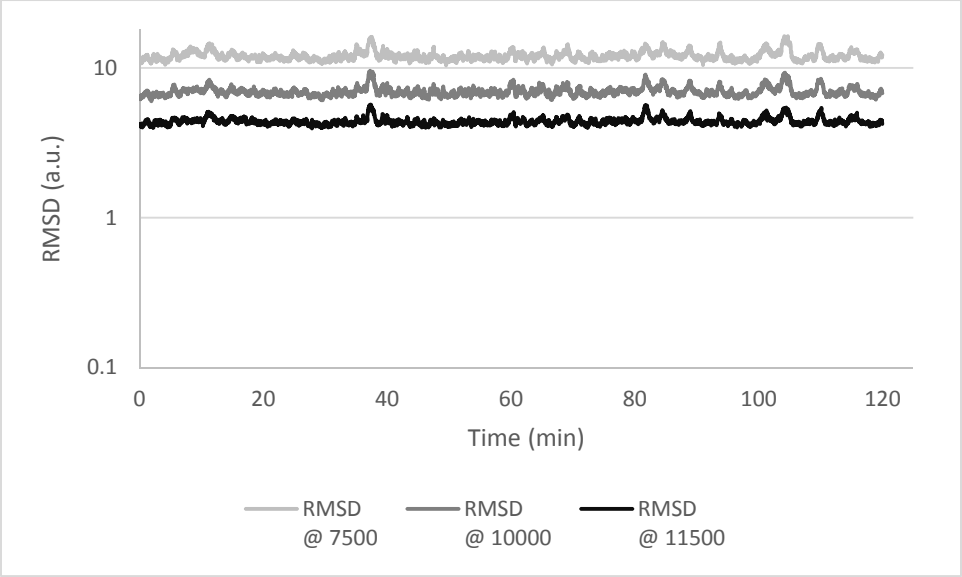
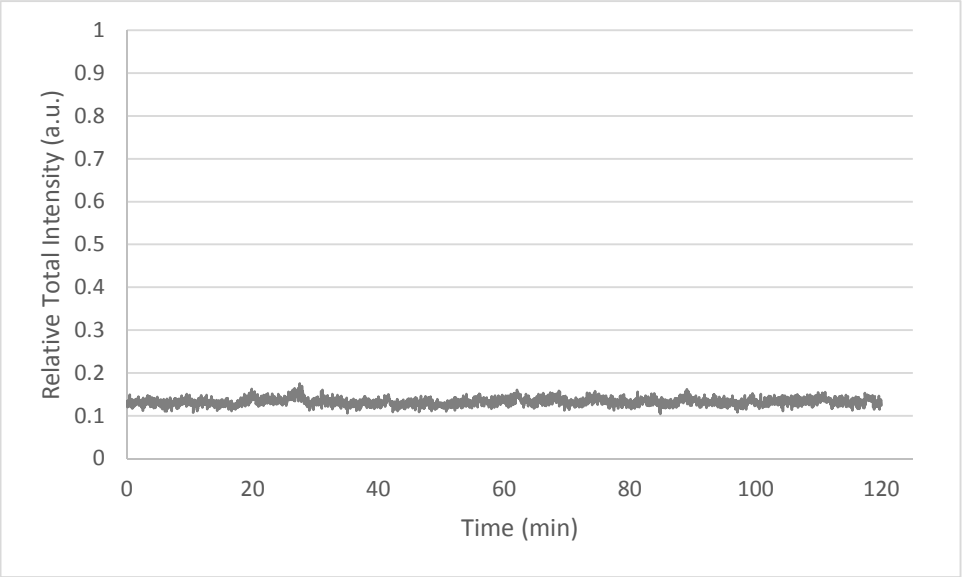


Figure 6-19: Uncoated Nanoparticle Dynamics (a) Relative Total Intensity and (b) RMSD

(a)



(b)

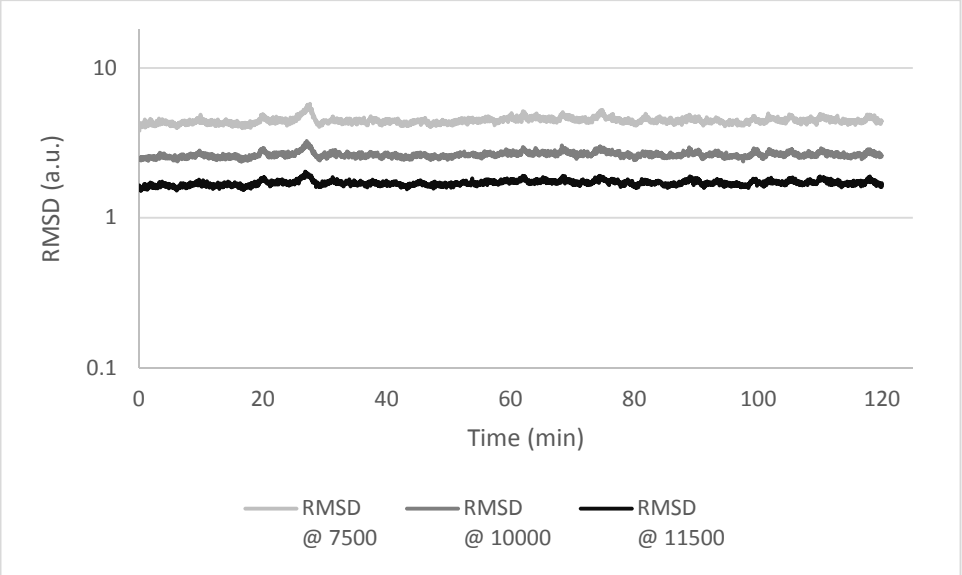


Figure 6-20: Protein Coated Nanoparticle Dynamics (a) Relative Total Intensity and (b) RMSD

7.0 DISCUSSION

The objective of this research was to examine indicators of viruses in waters systems and identify characteristics of indicators critical for predicting virus behavior. This research is unique in that it includes the investigation of viruses at multiple-scales, including full-scale water systems, lab scale batch testing, and nanoscale particle investigation. The full-scale water systems included fecal, wastewater, stormwater, surface water, groundwater, and distribution systems. The lab scale analysis included batch adsorption tests for comparisons of water quality on the efficiency of virus removal as modeled by abiotic nanospheres. The nanoscale analysis included time-dependent light scattering using the ARGOS method to observe phage infection of bacteria and particle dynamics.

Multiple-scale analyses are important because systems can exhibit different behaviors at different scales. The analysis is summarized in Figure 7-1. Researching water quality indicators at the multiple-scale level provided an opportunity to observe unique particle characteristics.

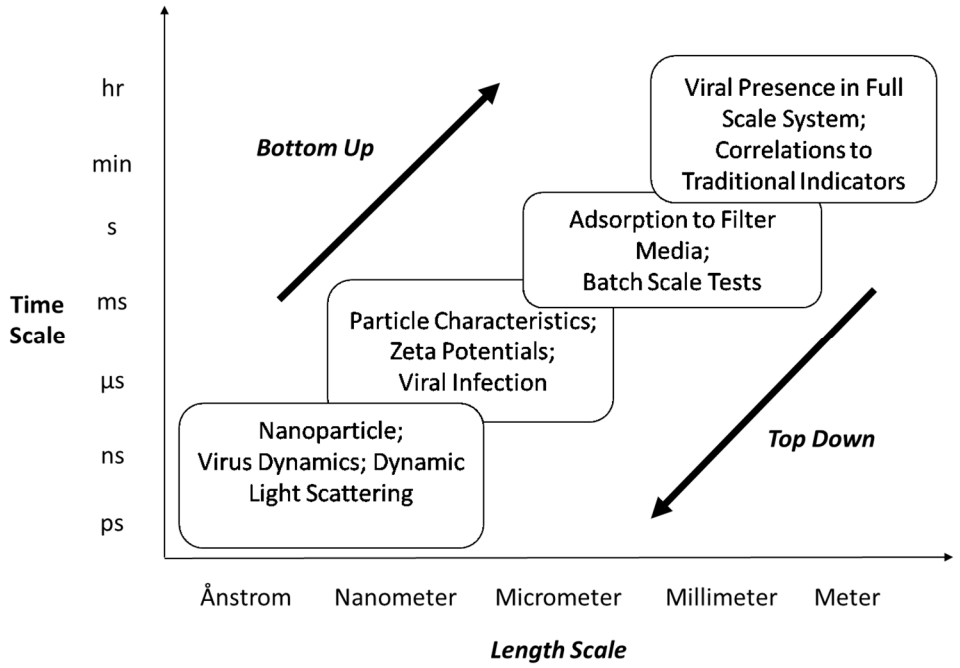


Figure 7-1: Multi-Scale Analysis

7.1 FULL-SCALE WATER SYSTEMS

Water quality results were compared for the data collected in U.S., Italy, and Australia. Combing data sets provided a unique opportunity to expand the original data sets and compare results from three independent locations. While combining the data provided larger sets, it is important to note that the collection and identification methods were not identical. Each method is described briefly in Section 5.0 Research Methods and more fully in Appendix A. While the results were comparable to current literature, it is important to note that different methods, serotypes and primers have different detection efficiencies.

The full-scale water system measurements were assessed using IBM's Statistical Package for the Social Sciences (SPSS) product, Version 17.0. Analysis of Variance (ANOVA) was conducted on the quantitative data sets to evaluate differences based on season and country. Correlations analyses were conducted to determine correlations among the bacterial indicators, coliphages and viruses. The Spearman Rank correlation analysis was used for quantitative data sets. Statistical analyses were conducted for data sets with over 20 results. Analyses were conducted at the 95 percent confidence level (α=0.05). Since correlations and ANOVA were calculated for data sets with over 20 results, subsequently, these are not calculated for distribution system, groundwater and salt water data.

7.1.1 Analysis of Variance

Seasonal variances were calculated for fecal, wastewater, fresh surface water, and stormwater samples, as presented in Section 6.1.5. In this current research study, no seasonal variances were identified for the indicators. The lack of seasonal variability is an important quality for a water quality indicator in order for the parameter to be useful year round. In addition for this study, an ANOVA was also calculated between countries for the wastewater samples. Somatic coliphages varied by country (U.S. and Italy).

The lack of seasonal variability for the viruses is one of the factors in proposing enteric viruses as water quality markers. For example, adenovirus was proposed as an indicator of fecal pollution from human sources based on its culturability, resistance characteristics and lack of seasonal variability (Choi *et al.*, 2005; Grabow, 2007; Jiang *et al.*, 2001; Jiang *et al.*, 2007; Simmons *et al.*, 2011). Similarly, TTV seems to exhibit several qualities which would make it a good indicator of fecal and wastewater contamination (Haramoto *et al.*, 2008). In 2007, Haramoto *et al.* collected samples from a wastewater treatment plant in Japan, and TTV was detected in all 12 influent samples tested, with a geometric mean concentration of 1.7×10^4 genomic copies per liter. The concentration of TTV in the influent samples showed no clear seasonal pattern, suggesting that TTV infections occur constantly throughout the year.

Long *et al.* found the lack of seasonal variability in coliphages was consistent with a study of male-specific coliphage for source tracking (Long *et al.*, 2005). The study included fecal samples, agricultural and wastewater samples from different geographical locations in different seasons. No seasonal variations were identified for male-specific coliphage in wastewater. In the studies where seasonal variations were not identified for fecal samples or wastewater samples, there tended to be had statistically high populations of bacteria.

While other studies of treated wastewater and surface waters found seasonal variations. These results are in contrast to a study of the fate of waterborne bacteria and viruses in treated wastewater found that coliphages exhibited seasonal effects with concentration higher in the summer than those observed in the winter (Blatchley *et al.*, 2007). Similarly in a study of male-specific coliphages in surface waters, male-specific coliphages were found to vary by season and found higher inactivation rates of male-specific coliphage in warmer months (Cole *et al.*, 2003).

While the lack of seasonal variability in bacteriophage for this research project may be an indication of survival characteristics, the presence of bacteriophage is also a function of bacteria populations. The bacteria populations in this project also were not found to vary by season. In particular, fecal samples and wastewater, in general, are known to be high in fecal bacteria, and since bacteriophage need a host, the presence and characteristics of the coliphage are significantly impacted by the seasonal variations of the

hosts. In studies of wastewater, coliforms would be consistently present and temperature is fairly constant, and therefore, allow for hosts and bacteriophage to be present consistently through the years. While in studies of surface water such as Cole *et al.* 2003 the coliforms would be impacted by colder weather, and would, as a results, limit hosts and bacteriophage presence. Similarly, host presence would be impacted in disinfected samples such as Blatchley *et al.* 2007.

7.1.2 Correlation Analysis

The Spearman Rank correlations were calculated between the indicators with quantitative data sets. In the fecal and surface water samples, correlations were calculated for the bacterial indicators and coliphages. There were correlations between the bacterial indicators and somatic phages, but there were no correlations to male-specific coliphage. In fecal samples, somatic coliphages correlated to both coliforms and *E. coli* and for the surface water samples somatic coliphages correlated to *E. coli*. Correlations to bacteria may have been impacted by high variability in concentrations and potential sampling error (Knappett *et al.*, 2011; Oliver, 2014; Rasmussen *et al.*, 2014).

The surface water results may have been impacted by the number of samples (n=27) and the inconsistent testing. The surface water samples were drawn from the U.S. and Italy. The samples from Italy did not include results for total coliforms or male-specific coliphages. The small sample set for the male-specific coliphages (n=15) alters the validity of the analysis (Coats *et al.*, 2014; Green, 2011). The correlations were also impacted by the difference in sampling concentrations between U.S. and Italy. For example the maximum results for the U.S. samples were 35 MPN per 100 mL for *E. coli*; comparatively the samples from Italy had much high much higher *E. coli* concentrations with maximum concentration of 1.7×10^6 CFU per 100 mL. These results may be a result of land uses in the area of the surface waters. The samples from the U.S. came from drinking water reservoirs, while the samples from Italy were sampled downstream of a wastewater treatment plant outfall. In a study of land uses patterns and the occurrence of coliphages in surface water, the results demonstrated differences in concentrations of somatic coliphages between areas of different land use (Franke *et al.*, 2009).

The results for the fecal samples may have been effected by levels in some fecal samples that were below the detection level of the methodology. In the results, one cow fecal sample and one chicken fecal sample had coliforms and *E. coli* below detection limits. There were also inconsistent results in another chicken sample where coliforms and *E. coli* concentrations were 1×10^2 CFU per g. These are in contrast to the samples excluding these results which had concentrations in the ranged from 5.7×10^4 to 5.0×10^8 CFU per g in chicken feces for coliforms and *E. coli*. It is unusual to not detect coliforms and *E. coli* in fecal matter from chickens and cows. Previous studies have shown consistent levels of *E. coli* and coliforms in the gastrointestinal tract of domestic and farm animals (Grauke *et al.*, 2002; Havelaar *et al.*, 1986). This suggests both that these samples may have been compromised and that the concentration results attributed to errors in the sampling and transporting of samples.

The quantity of samples below the level of detection for coliphages may have influenced correlations between coliphages and other indicators. For fecal samples, total coliforms, *E. coli* and male-specific coliphages were correlated; however, somatic coliphages were not correlated to other indicators. While bacterial indicators were detected in almost all samples, coliphages were not detected in approximately 50

percent of the samples; for male-specific coliphages 42 of 76 samples were below detection limits and for somatic coliphages 33 of 76 were below detection limits.

Somatic coliphages were analyzed in stormwater and did not correlate to other parameters. This was in contrast to the distribution system samples, in which each of the parameters tested correlated to one another (including total coliform, *E. coli*, somatic coliphages and male-specific coliphages). Both of these analyses may have been influenced by the small sample sizes (n=20, n=27, respectively). It is recommended that further studies include larger sample sets.

In the correlations calculated for the fresh surface water, a relationship was identified between *E. coli* and somatic coliphage. The bacterial indicators were not found to relate to male-specific coliphage. In some studies, however, somatic coliphages were found to be present in greater numbers than male-specific coliphages; this result was assumed to be on account of environmental persistence (Brion *et al.*, 2002; Lee *et al.*, 2011).

This study found the expected correlations in wastewater samples between bacterial indicators (coliforms and *E. coli* and *E. coli* and Enterococci*)* and between viral markers (polyomavirus, adenovirus, and TTV). In a similar study of sewage treatment, Vaidya *et al*. (2002) also found TTV correlated to enteric viruses in raw sewage featuring a prevalence of TTV that was statistically similar to the prevalence of hepatitis E virus RNA and hepatitis A virus RNA (Vaidya *et al.*, 2002). This study also included TTV correlations to *E. coli*, microviridae, and Enterococci; coliforms and *E. coli* correlated with both male-specific coliphage and somatic phage. Enterococci correlated to *E. coli*, somatic phage and TTV; and microviridae correlated to adenovirus, polyomavirus, and TTV. Microviridae (a family of bacteriophage which includes somatic phage ΦX-174) may more widely represent viral markers as an indicator, as is demonstrated through these correlations. These results are similar to the relationships identified with the Pisa, Italy, wastewater treatment facility, where no significant correlation between bacterial indicators and the presence of adenovirus was found (Carducci *et al.*, 2008).

Removal rates of TTV through wastewater treatment in this study were similar to those featured in a study conducted by Hamza *et al.* (2011). In a study of wastewater removals through a wastewater treatment plant discharging into the Ruhr River in Germany, similar removal patterns were identified, with approximately 1.7 to 2.3 and 2.6 to 3.5 $\log_{10}$ removals for adenovirus and TTV, respectively, although influent concentrations were found to be higher at 1.7×10^8 and 1.3×10^3 for adenovirus and TTV, respectively. The influent concentrations of TTV in the wastewaters in this study correspond to concentrations detected in Japan by Haramoto *et al.* (2008) in a study of a wastewater treatment plant in Japan, where samples were samples were collected monthly (2005-2006). This study identified TTV in all of the samples (12 of 12), with mean concentration of 1.7×10^4 and maximum concentration of 4.8×10^4 genomic copies per liter.

Statistically, the use of TTV as an indicator may be limited to the detection occurrence. Out of the total number of samples tested for TTV (324) in this study, only 33 percent (108) were positive for TTV. In a 2009 study of the Ruhr and Rhine Rivers in Germany, the surface water results showed that most samples (108 of 111) were positive for adenovirus and about half the samples (56 of 108) were positive for TTV (Hamza *et al.*, 2011a). A study of the Tamagawa River in Japan had similar results to this study and identified TTV in 5 percent of samples (500 mL volume) (Haramoto *et al.*, 2005). In a similar study,

Vecchia, *et al.* (2009) quantified TTV and fecal pollution in the southern region of Brazil and TTV was present in approximately 10.7 percent (3 of 28) of the samples (Vecchia, 2009).

The stormwater results did not show correlations between viral markers, including TTV and bacterial and coliphage indicators. The results were influenced by the methodology used for TTV testing. The samples were tested for human TTV but not for animal TTV. Stormwater runoff includes a potential for contamination from human sources such as failed septic systems, but is often contaminated with animal waste (Arnone *et al.*, 2007; Cizek *et al.*, 2008). TTV has been identified in a wide range of fecal samples and is not restricted to human feces. It has also been detected in certain animal species, including non-human primates (Cong *et al.*, 2000; Okamoto *et al.*, 2000; Verschoor *et al.*, 1999), farm animals (pigs, chickens, cows, and sheep) (Brassard *et al.*, 2010; Devalle *et al.*, 2005; Lang *et al.*, 2011; Leary *et al.*, 1999; Liu *et al.*, 2011a; Martinez Guino *et al.*, 2010; Sibila *et al.*, 2009), companion animals (dogs and cats) (Biagini *et al.*, 2007; Okamoto, 2009b; Zhu *et al.*, 2011), and wild animals (wild boar and sea lions) (Martinez *et al.*, 2006; Ng, 2009). For example, in China, a study of 158 fecal samples collected from dogs younger than 1 year old with diarrhea in a pet clinic, 20 specimens (20 of 158, 13%) were positive for Torque Teno canis virus DNA using detection with PCR (Lan *et al.*, 2011). While TTV has been identified in a variety of animal fecal samples, this study only had a 4.0 percent detection in the fecal samples. Although a human TTV sequence was utilized, and therefore presence of this sequence would not be expected in animal fecal samples. Any positive detection of TTV may have been a result of cross-contamination with humans because these animals were domestic pets.

7.2 LAB SCALE ANALYSIS

Lab scale systems were examined for uncoated and protein-coated nanospheres with drinking water filter media (sand and acid-washed sand). The concentrations of the nanospheres were observed for 2-hour periods across multiple pHs with waters of varying ionic strength. Appendix D – Batch Analysis includes summaries of for $\log_{10}$ removals for each pHs 4, 6, and 8. Uncoated abiotic particles and viruses differ in their surface characteristics--the main difference is that uncoated, unmodified abiotic particles lack a protein capsid. These differences have an important influence on particle retention and transport in porous media (Bales *et al.*, 1997).

Some researchers have used uncoated fluorescent latex as surrogates for transport studies in porous media. While abiotic particles are chemically stable and easy to detect by methods such as spectrofluorometry or flow cytometry, the results have generally been unsatisfactory (Harvey *et al.*, 2011). Additional methods such as DNA labels, epifluorescence microscopy or radioactive labels have provided more consistent results, but have their own drawbacks, such as time-consuming assays or restricted applications (Boualam *et al.*, 2002; Bradley *et al.*, 2007; Pang *et al.*, 2012).

While size and shape of microorganisms and microspheres can be similar, the surface characteristics of uncoated latex spheres limit their ability to represent microorganisms (Harvey *et al.*, 2008; Harvey *et al.*, 2004). Altering the surface of the particle may provide for a particle that is a better representative of viruses. This is a factor because Harvey *et al.* (2008) determined that the surface charge of particles had the greatest impact to transport velocity. Researchers have also determined that these observed transport differences were attributable to the difference in surface charge, and thus appropriate to identify a surrogate with surface characteristics more similar to microorganisms.

Researchers have also used bacteriophages as surrogates, and research has demonstrated that they are conservative predictions of virus removal in many conditions. In 1995, Jofre, *et al.* compared viruses and bacteriophages in raw waters and identified bacteriophages in treated water samples of full-scale conventional treatment facilities. The researchers found that bacteriophages were present post-treatment while infectious enteroviruses were unable to be detected, demonstrating bacteriophages phages infecting *B. fragilis* were a more conservative representation of enteroviruses. Abbaszadegan *et al.* (2007) conducted a bench-scale analysis to evaluate enhanced coagulation and settling, and found that removals indicated that bacteriophages were a conservative representation of viruses with enhanced coagulation. Transport studies have also shown that MS2 does not represent rotavirus and adenovirus accurately. In a laboratory study using hematite coated glass fiber, the adsorption capacity of rotavirus differed from that of MS2 by four to five orders of magnitude (Gutierrez *et al.*, 2009). A more appropriate virus surrogate, such as protein-coated nanospheres, will reduce uncertainty in risk assessments

Similarly, Schijven, *et al.* (2000) modeled adsorption of MS2, PRD1, ΦX-174, Qβ, and PM2 in batch and column experiments. The results indicated that under conditions of high pH in sandy soils, MS2 is a conservative tracer, while in the presence of multivalent cations, bacteriophage ΦX-174 may be more conservative. Latex spheres have also been used as a potential viral surrogate in low-pressure membrane studies. This study found that fluorescent microspheres were an inconsistent surrogate when compared to phages in membrane studies and found that further study on the impact of surrogate surface characteristics was necessary to predict rejection (Pontius *et al.*, 2009).

Additionally, more than one virus surrogate in a system may be necessary. Mayer *et al.* (2008) studied enhanced coagulation identified removal rates for multiple viruses and bacteriophages and found that different viruses may be represented by different bacteriophages, indicating that one bacteriophage may not able to represent all viruses. The researchers found instead that multiple bacteriophages may be better suited to indicating enteric viruses. Protein-coated nanospheres may also be an efficient way to provide multiple models of enteric viruses within a single treatment study.

This present research included varying ionic strengths to mimic groundwater. The research showed that for uncoated nanoparticles, removals increased as ionic strength increased, as demonstrated in Figure 7-2a. This is consistent with the theory that increased ionic strength will reduce the magnitude of the repulsive energy barrier between the negatively charged sand and particles. The results from this present study are similar to those found in a study of the adsorption and aggregation of norovirus-like particle attachment where attachment increased with increasing ionic strength (da Silva *et al.*, 2010).

(a)

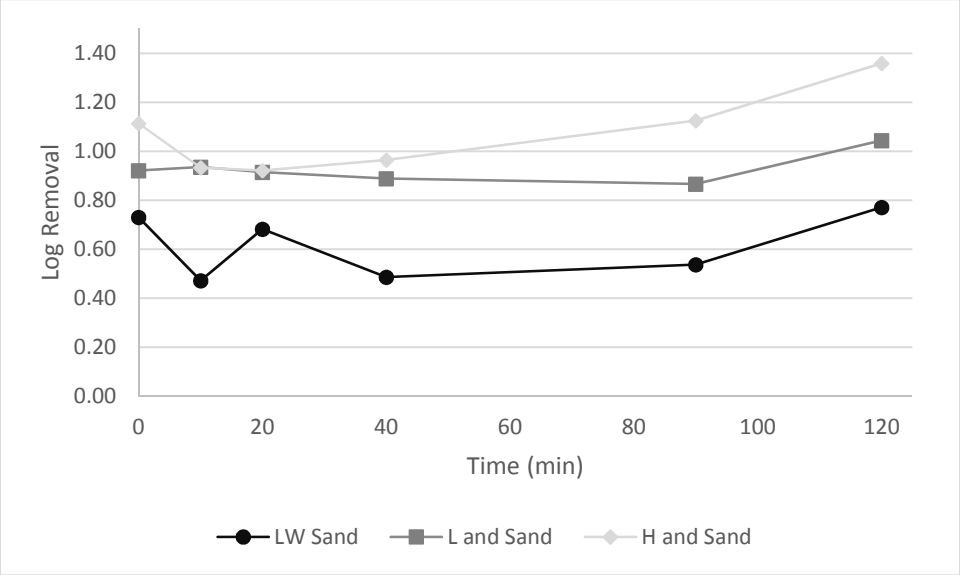


Figure 7-2a: Average Log Removals with Sand and Varied Ionic Strength for Uncoated Nanoparticles

While these results were observed for the uncoated particles, the removals of the protein-coated particles (included in Figure 7-2 b) were similar to one another in both media across the varied ionic strengths. These results indicate that the protein-coated nanospheres may behave similarly to MS2. In a study of deposition kinetics in packed beds of quartz, Penrod *et al.* (1996) identified removals for two bacteriophages, MS2 and λ. The tests were conducted at pHs similar to the isoelectric points of the bacteriophages. Initially, both bacteriophages showed a low retention at less than one $\log_{10}$ removal in the sand column at pH 5 and 0.01 M NaCl. When the NaCl concentration was increased 0.3 M NaCl, the removal of MS2 was not affected. While at 0.3 M NaCl the adsorption of λ was significantly improved to an average of five $\log_{10}$ removal. These results indicate that as ionic strength increases, adsorption also increases. The research suggests that this may have resulted from increased van der Waals interactions between λ and the sand (Penrod *et al.*, 1996).

(b)

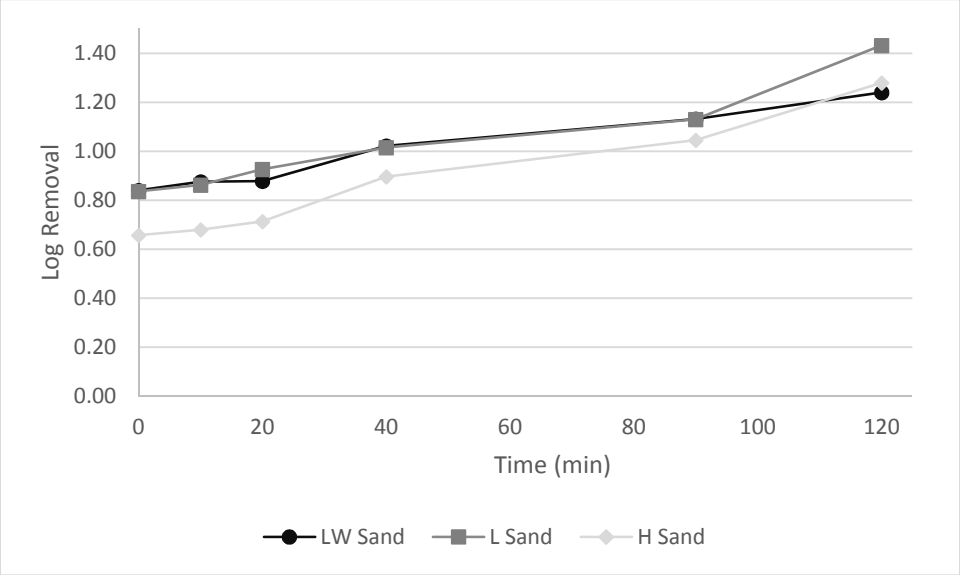


Figure 7-2b: Average Log Removals with Sand and Varied Ionic Strength for Protein-Coated Nanoparticles

Virus attachment to sand has been widely studied and found to be a function of several factors, including ionic strength and pH, as observed in this present study (Bradford *et al.*, 2008; Crist *et al.*, 2004; Torkzaban *et al.*, 2006; Wan *et al.*, 1994). While the impacts of pH and ionic strength are widely discussed in literature, the important aspects of this research are the removal rates of the uncoated nanospheres compared to the rates of the protein-coated nanospheres. The average removals of the protein-coated nanoparticles were consistently higher than those of the uncoated particles. The results for particles in lab water at pH 4 and low ionic strength water at pH 6 waters are included in Figure 7-3 which demonstrates that with varied pH and ionic strength, in both cases the protein-coated nanospheres had greater removals.

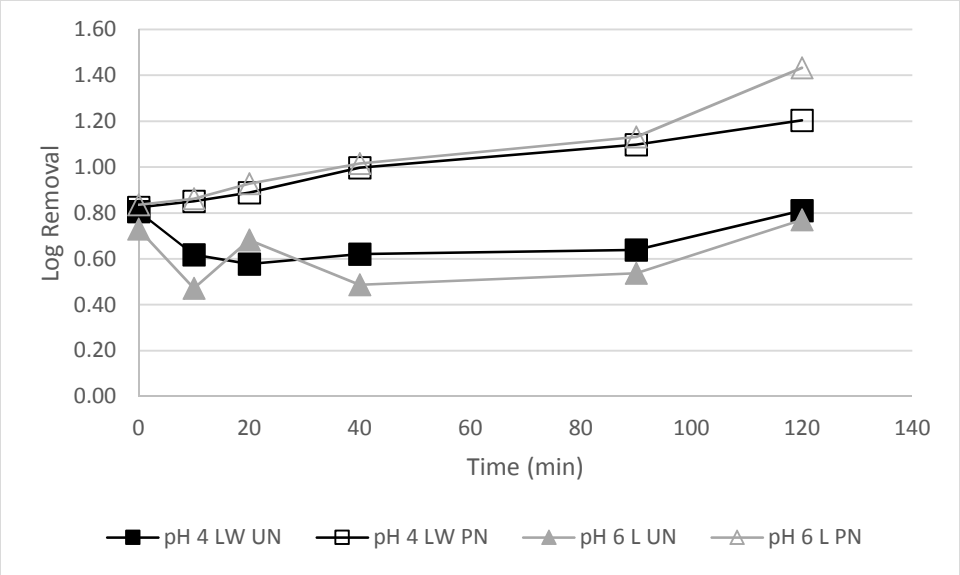
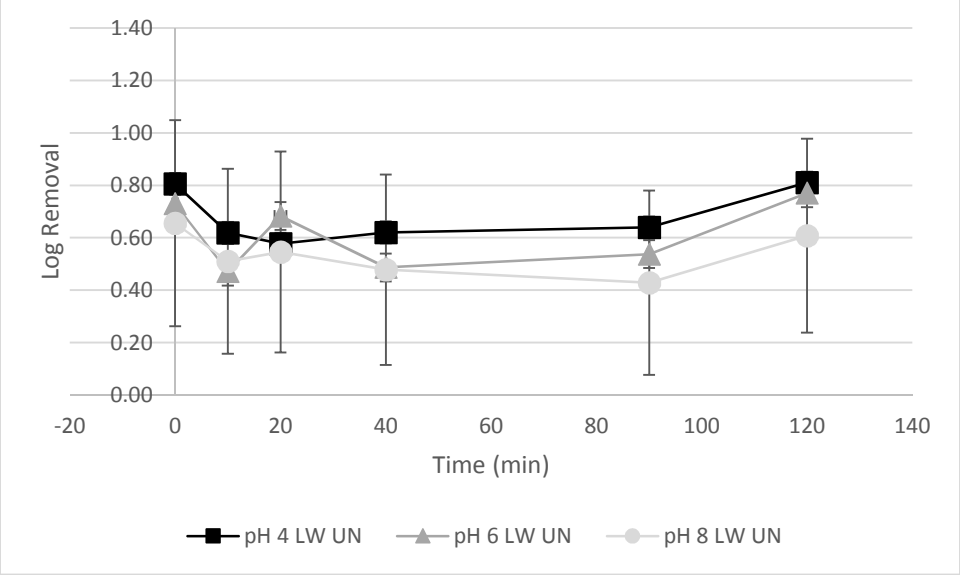


Figure 7-3: Average Log Removals of particles with sand in lab water with (1) pH 4 and (2) pH 6

The research demonstrates that the protein coating impacted attachment over time and enhanced final removals. Figure 7-4 is representative of the behaviors of uncoated particles, which show inconsistent concentrations, and of particles that seem to attach and detach. Also, the error bars which represent standard deviation indicate that the concentrations show wide variations and may not adsorb to the sand particles at all.

(a)



(b)

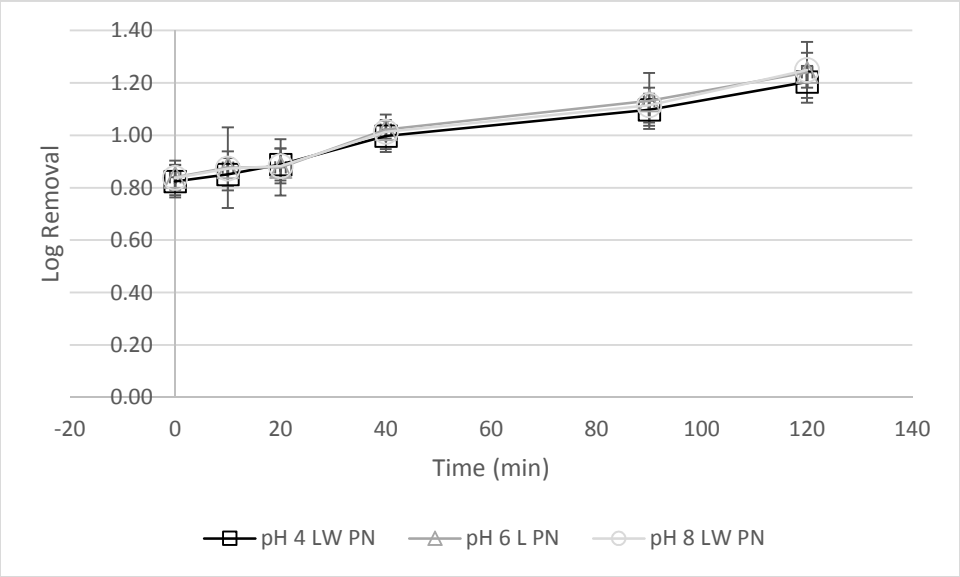


Figure 7-4: Particles in Lab Water with Sand Media (a) Uncoated and (b) Protein Coated (error bars represent standard deviation, n=8)

Lab scale bench tests were also conducted with MS2. These results were inconsistent based on duplicate testing. The data was not reproducible. This testing was not pursued further. These results may have resulted from the fact that while sand filtration is often practiced in wastewater and drinking water treatment without the addition of flocculation aids, this process is inefficient and the degree of virus removed is erratic. Viruses do not adsorb to sand, and removals are determined by factors such as flow rates, pH, ionic strength, and organic matter. Sand has an isoelectric point of less than 4 and a negative surface charge (like most viruses), and so the low attachment rate of viruses to sand can be accounted for by the repulsive electrostatic forces (Porubcan *et al.*, 2011; Truesdail *et al.*, 1998).

7.3 NANOSCALE ANALYSIS

Dynamic light scattering (DLS) can be used to determine of particle size in Brownian systems and has long been used for the measurement of Brownian motion of particles at the nanoscale, such as colloids, proteins and macromolecules. DLS collects information about intensity fluctuations, and calculations are based on the time scale of movement of the scattered particles. Current DLS techniques can make accurate measurements over a very short timescale, are non-invasive, and have a high sensitivity which provides for an investigative tool for biological cells. Particle dynamics can be expanded upon to provide calculations on the particle such as velocity distributions, the average velocities, and the fraction of non-motile cells (Holz *et al.*, 1978; Reufer *et al.*, 2012). While there are many references which detail the theory and models developed for light scattering (Berne *et al.*, 2000; Boustany *et al.*, 2002), this discussion is specific to the characteristics of the particles observed.

7.3.1 Zeta Potentials

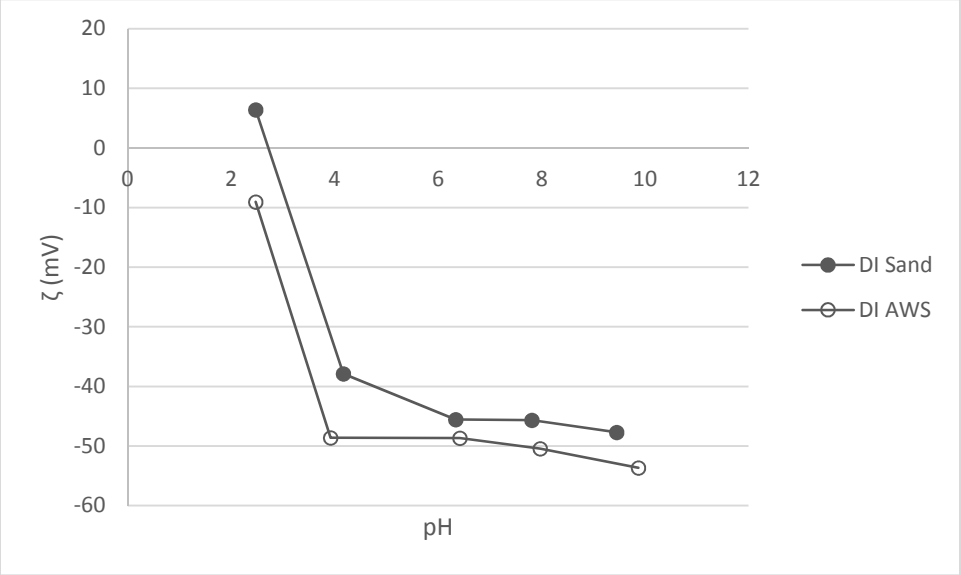
Zeta potentials were obtained using dynamic light scattering. The results include average zeta potentials for the media, abiotic particles, and biotic particles in the three tested solutions with varied ionic strengths and pHs ranging from 2.5 to 10. This research expands upon Pang *et al.* (2009) which included the study of zeta potentials of latex spheres coated in casein protein and found zeta potentials of coated particle to be similar to *E. coli* and MS2. Pang *et al*. found the surface charge of a microorganism can be closely mimicked by microspheres that are covalently coated with a protein having a zeta potential similar to that of the microorganisms low ionic strength monovalent lab water. This present research study includes lab water and high ionic strength solutions; and also expanded the findings to include an additional bacteriophage, ΦX-174.

The importance of testing the zeta potentials was borne out by the fact that changes in electrostatic layers impact viral adsorptions to solid interfaces. Ryan *et al.* 2002 studied bacteriophage adsorption to iron oxide-coated sands and found that viral transport was controlled by electrostatic interactions. Dowd *et al.* (1998) similarly studied virus adsorption and transport through sandy soils (composed of sand, silt, and clay) in groundwater. The was performed using transport experiments in 73 cm (5 cm inner diameter) columns injecting five different spherical bacteriophages (MS2, PRD1, Q, ΦX-174, and PM2). The purpose was to test the correlation between viral transport and isoelectric points. The bacteriophages were selected based on varied isoelectric points of 3.9 (MS2), 4.2 (PRD1), 5.3 (Q), 6.6 (ΦX-174) and 7.3 (PM2). The data suggested that the isoelectric point of a virus is the predetermining factor controlling viral adsorption within aquifers, underlining the major role of electrostatic forces in virus adsorption (Dowd *et al.*, 1998).

The surface concentration of the ionizable groups determines the total charge. The pH at which a virus has no net charge is the isoelectric point (IEP). The IEP varies with structure of the capsid. At lower pH, net protonation of ionizable groups produces positive charge, whereas at high pH deprotonation dominates to give a net negative charge. In this study, the zeta potentials trended lower as pH increased. This trend was also identified in a study of the transport of PRD1 in an iron oxide-coated sand aquifer (Ryan *et al.*, 1999; Ryan *et al.*, 2002). As the pH approached the virus IEP, aggregation resulted from a (neutralization) decrease in the absolute values of the electrostatic repulsive interactions.

The results demonstrated that the zeta potentials of the acid-washed acid were generally lower than for the washed sand. These results are demonstrated in Figures 7-5 where the zeta potentials of sand and acid-washed sand are shown for suspension is lab water and high ionic strength water. These results are similar to a study of the impact of mineral grain zeta potential on colloid transport in geochemically heterogeneous porous media. The mineral grain surfaces coated with iron oxide were analyzed for zeta potentials in varied pH and ionic strength changes. The results included zeta potentials of acid-wash sand which were lower than the coated sand. The study also suggested that that sand in the environment (such as heterogeneous subsurface porous media, such as iron oxide-coated sand aquifers) was the most important factor deposition kinetics of colloidal particles (Elimelech *et al.*, 2000).

(a)



(b)

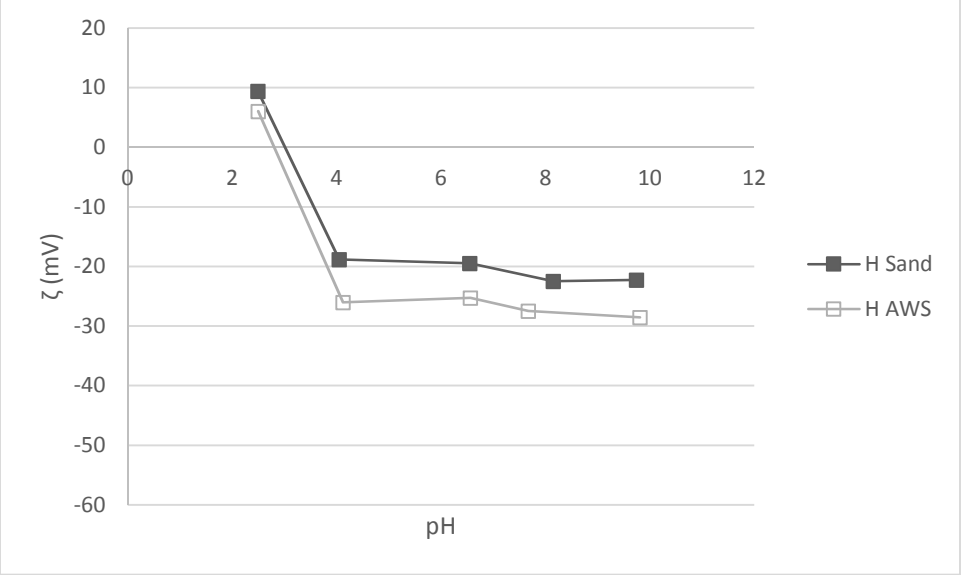
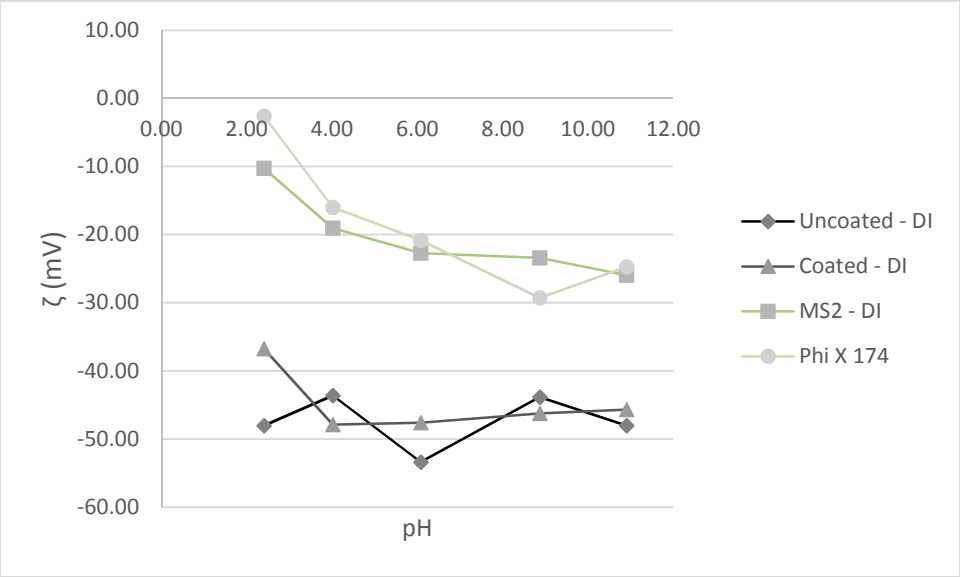


Figure 7-5: Zeta Potentials of Sand and Acid-Washed Sand in (a) Lab Water and (b) High Ionic Strength Solution

Figure 7-6 shows the average zeta potentials of the four nanoparticles in lab water and high ionic strength water. The zeta potentials were higher in the higher ionic strength solution. These results are similar to a study of electrostatically driven adsorption of silica nanoparticles on functionalized surfaces, which found the addition of NaCl reduced repulsive charges (Li et al., 2013). Also, a study of *E. coli* adhesion and transport in saturated porous media found that as concentrations of KCl increased, electrophoretic mobility decreased (Haznedaroglu et al., 2008). These observations result from the compression of the electrostatic double layer impacted by the electrolyte in solution (da Silva *et al.*, 2010).

(a)



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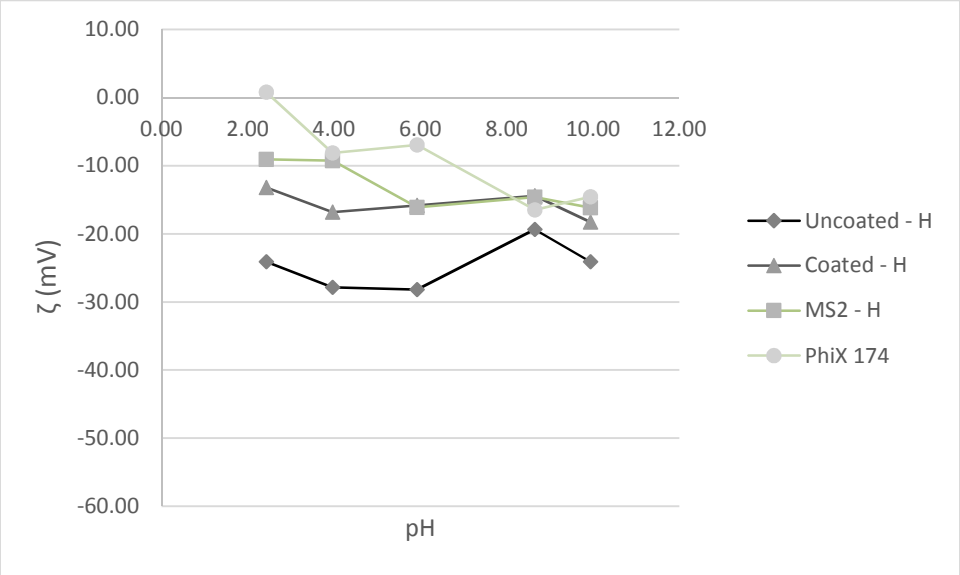

Figure 7-6: Average Zeta Potentials of Particles in Solutions for 2.5 to 10 in (a) Lab Water and (b) High Ionic Strength Water

The electrostatic layer around each particle is impacted by distribution of ions on the surface and is compressed with an increase in diffuse counter-ions provided by the higher ionic strength (Hendricks, 2010). In addition, while studies of pH usually have the particles in a low-strength monovalent electrolyte, this present study included two types of artificial groundwaters with monovalent and divalent electrolytes. Similarly in a study of deposition and aggregation kinetics, interactions with monovalent and divalent solutions changed the characteristics of rotavirus (Gutierrez *et al.*, 2010). The results of this study and this

present study indicate that the ionic strength of the water significantly impact the nature of the particle's electrostatic layer.

Surface charge is an important aspect of particle attachment to media (such as sand). Research demonstrates that MS2 a conservative virus surrogate among the phages because it is poorly adsorbed onto porous media, although this may not be true for engineered porous media. For example, Nasser *et al.* (1995) observed removals under hydrophobic conditions, in the presence of divalent cations, and at water temperatures above 10°C. The results were that filtration removals of MS2 (95%) were higher than poliovirus (38%) in a sand medium containing cationic polyelectrolyte (Nasser *et al.*, 1995). The results of this research and the results of this present study demonstrate that the relationships of particles and water quality parameters need to be further studied particularly in terms of impact on attachment to filter media.

The zeta potentials for each particle were statistically analyzed for each water type. Correlations were calculated using SPSS at the 99% confidence level. Tables 7-1 through 7-3 summarize the correlations between zeta potentials. The detailed results are included in Appendix C - Correlations.

Table 7-1: Zeta Potential Correlations in Lab Water

Particle Type	Uncoated Particles	Protein Coated Particles	MS2	ΦX-174
Uncoated Particles	1			
Protein-Coated Particles	N	1		
MS2	N	N	1	
ΦX-174	N	N	Y	1

Table 7-2: Zeta Potential Correlations in Low Ionic Strength Solution

Particle Type	Uncoated Particles	Protein Coated Particles	MS2	ΦX-174
Uncoated Particles	1			
Protein-Coated Particles	N	1		
MS2	N	Y	1	
ΦX-174	N	Y	Y	1

Table 7-3: Zeta Potential Correlations in High Ionic Strength Solution

Particle Type	Uncoated Particles	Protein Coated Particles	MS2	ΦX-174
Uncoated Particles	1			
Protein-Coated Particles	N	1		
MS2	N	N	1	
ΦX-174	N	N	N	1

While correlations were identified between the bacteriophages and protein-coated nanoparticles low ionic strength water, they were not correlated in in lab water and high ionic strength water. Changes in ionic strength have been shown to impact transport of latex spheres. The study adsorption in varied high ionic strength microspheres mimic transport of oocysts. *C. parvum* oocysts had a slightly negative zeta potential (-1.5 to -12.5 mV) at a pH 6.7 over a wide range of calcium concentrations (10^{-6} to 10^{-1} M) while the microspheres were more negatively charges (-7.4 to -50.2 mV). The results suggested that when groundwater is hard (high ionic strength, greater than 10^{-3} M Ca^{+}), microspheres mimic the transport of oocysts (Dai *et al.*, 2002).

In the low ionic strength water, zeta potentials across the pH range of 2.5 to 10 have linear, positive correlations at the 99 percent confidence level between the coated particles and both bacteriophages, while the uncoated particles do not. In the high ionic strength water, neither the coated nor the uncoated particles were correlated to MS2 or ΦX 174, in addition the two bacteriophages did not correlate to one another. The results demonstrate the importance of understanding groundwater quality and sorption media prior to estimating colloid removals. The results also show the ability of a protein-coated nanoparticle to model the electrophoretic mobility of bacteriophages (as model viruses) is dependent on ionic strength.

Pang *et al*. (2009) was able to observe the ability of protein-coated nanoparticles to model MS2. Zeta potentials were measured in 10^{-3} mol per liter NaCL background electrolyte. The results of research by Pang *et al*. also found that the zeta potentials of the protein-coated nanoparticles correlated to the zeta potentials of MS2 in low ionic strength solution. The results of this present study found similar results with protein-coated nanospheres correlating zeta potentials to MS2 in low ionic strength. Although, these results were not observed when the ionic strength was increased or when the particles were in lab water. The ability to use protein-coated nanospheres may be dependent on the ionic strength of the water.

Latex nanospheres have several benefits because they can be rapidly detected and may be custom manufactured in varying sizes, shapes, and coatings. In addition, there are also no health concerns when using nanospheres in the lab. For these reasons, it is recommended that they continue to be explored as viral surrogates. Although at this time, research methods are inconsistent and new methodology need to be

developed. In terms of this research, the ability of protein-coated nanospheres was dependent on water quality including pH and ionic strength of a sample.

7.3.2 Bacterial Dynamics

Bacterial dynamics were observed over a 48-hour period using the ARGOS method. Instead of taking traditional grab samples based on a time sensitivity of an order of magnitude of about a minute, this method allowed for the investigation of the same sample with no volume changes at a time sensitivity of 0.001 sec. This method also provided for a measurement of population dynamics that is more sensitive than CFU per mL. While methods such as bacteria staining have been used to study bacteriophage infection of bacteria, this research provides a more time-specific method in which the bacteria remains within the initial conditions.

Traditional observations of bacterial survival are made in lab conditions that provide for optimized growth. The traditional view of the bacterial life cycle prevalent in most biology textbooks, such as *Basic Molecular Biology* (Upadhyay *et al.*, 2010), is based on a four phase growth cycle. The growth cycle includes an initial lag phage, an exponential phase, a stationary phase, and a death phase, as depicted in Figure 7-7. The traditional definition assumes bacteria grown in a batch culture will inevitably reach a point when the growth rate decreases and initiates a stationary phase in which a culture both shows no further increase in the number of cells and is followed by a decrease in population (or the "death phase").

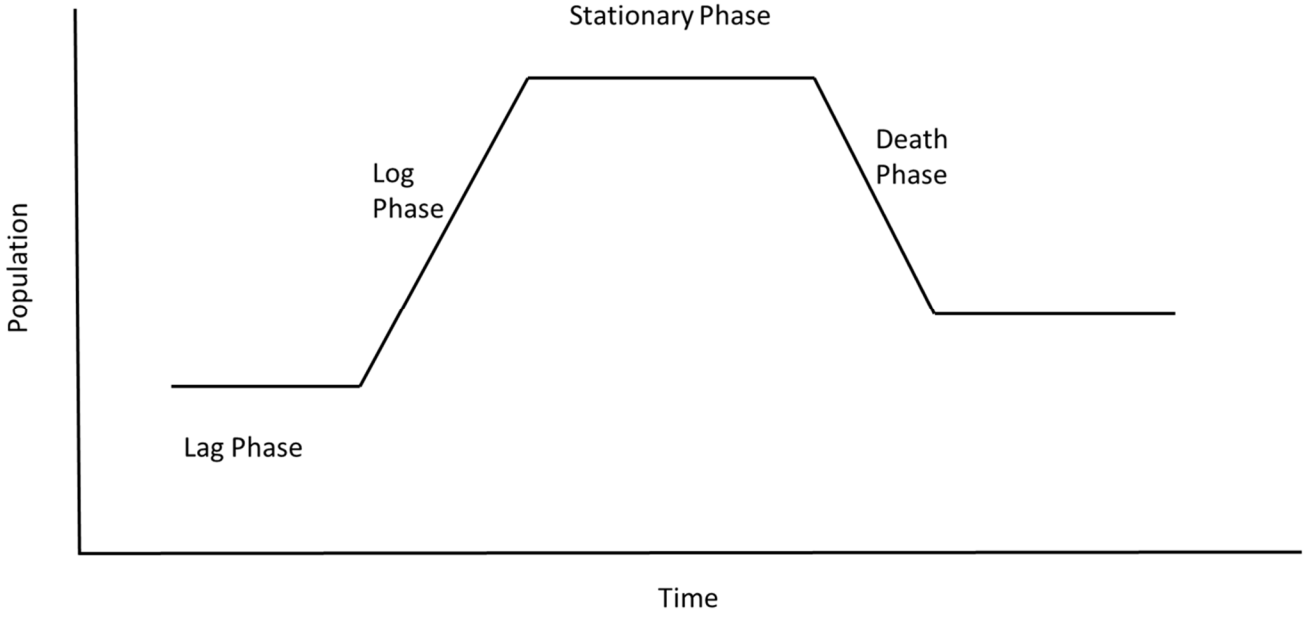

Figure 7-7: Traditional View of Bacterial Growth

Traditional growth models do not provide for conditions similar to the natural environment. Microorganisms in the environment are commonly observed in conditions of varying nutrient availability and stressed conditions including water flow, wind, temperature, light, and other organisms (Finkel, 2006). Therefore, the analysis of the population dynamics of bacterial growth is limited by a number of factors. An *in situ* method of observation may provide more accurate models of growth.

Traditional population models are not sensitive enough to observe total population dynamics. Traditionally, bacterial concentrations are enumerated with units of coliform forming units on a logarithmic scale. This method does not provide the sensitivity needed to observe the dynamic changes after the log phase. In addition, aliquots of the sample must be removed in order to complete the methodology. Removing these aliquots changes the initial volume of the sample, and also removes the bacteria from the solution in order to be enumerated. This method also does not allow for a specificity or precision of time. The method requires an estimation of time when removing aliquots of the sample and during the enumeration process, and the sensitivity of this method is approximately a minute (Camper *et al.*, 1991; Fujioka *et al.*, 2002).

The traditional growth curve includes a death phase which is often reflected as a loss of viable counts using standard plating assays. Death phase research strives to identify the triggers from the stationary phase to the death phase. The theories include the notion that the phase is triggered by a stochastic event based on resources and perhaps that the bacterial cell is programmed for altruistic suicide (Finkel, 2006). Finkel (2006) observed bacterial populations over extended periods of time and found that with only the addition of sterile water bacterial populations could maintained at densities of 10^6 CFU per mL for more than 5 years. This phase in the growth cycle was termed long-term stationary phase and was characterized by a balance of ”birth” and “death” rates. The populations during the long-term stationary phase were found to be sinusoidal with periods of days and weeks.

Finkel (2006) studied the long term population dynamics of bacterial growth and survival. Many of the properties identified as stationary phase may be important for growth under conditions of limiting or poor nutrient availability and that the stationary phase may represent a maximally slow growth rate. The study found that after the stationary phase, bacterial metabolic activity is greatly reduced. The study also found that stress response genes and metabolic pathways may determine dynamics during the death phase.

The present study found that the population dynamics of bacteria do not follow a traditional growth model and that the ARGOS method allowed for the observation of bacterial changes in terms of individual particles and population dynamics in real time. Data analysis software OriginPro 8.0 was used to analyze the data from the dynamic light scattering and Figure 7-8 includes the relative total intensity over time. The observations of relative total intensity suggest that there is no stationary phase and that the bacterial growth curve demonstrates sinusoidal system dynamics throughout the growth cycle that are not included in the traditional bacterial growth curve.

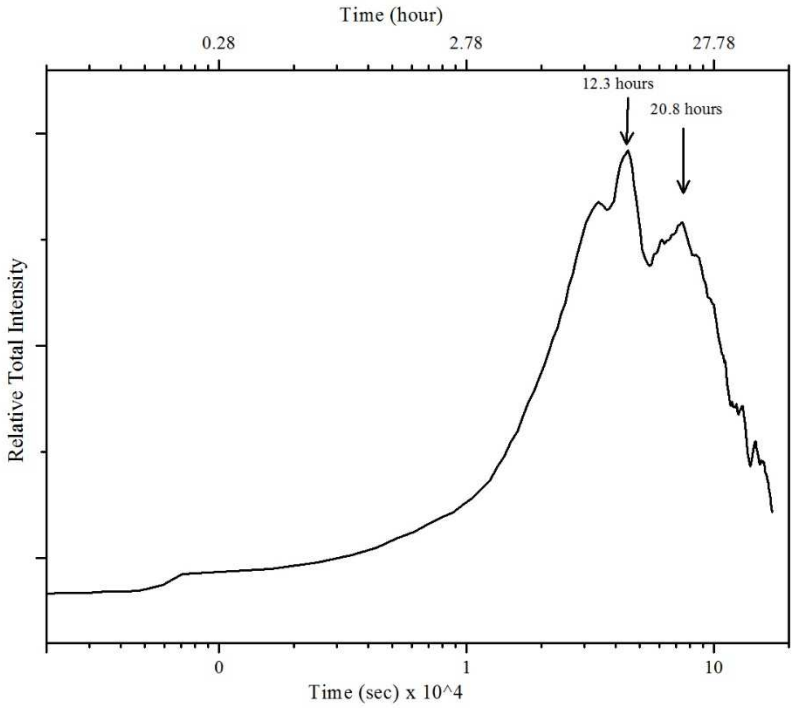

Figure 7-8: Relative Total Intensity – Multiple Peaks During Staionary Phase

The dynamics of the system include decreasing sinusoidal type patterns consistently subsequent to the log phase growth. The dynamics are those of a bacterial population trying to attain a long-term stationary phase as identified by Finkel (2006). The dynamics of the present study occur within short time intervals therefore, the standard plating assay was not able to detect this process (as detailed in Section 5.0 Research Methods). The dynamics of the long-term stationary phase in the research conducted by Finkel (2006) was able to be observed with plating methods because the periods occurred in time intervals of days and weeks.

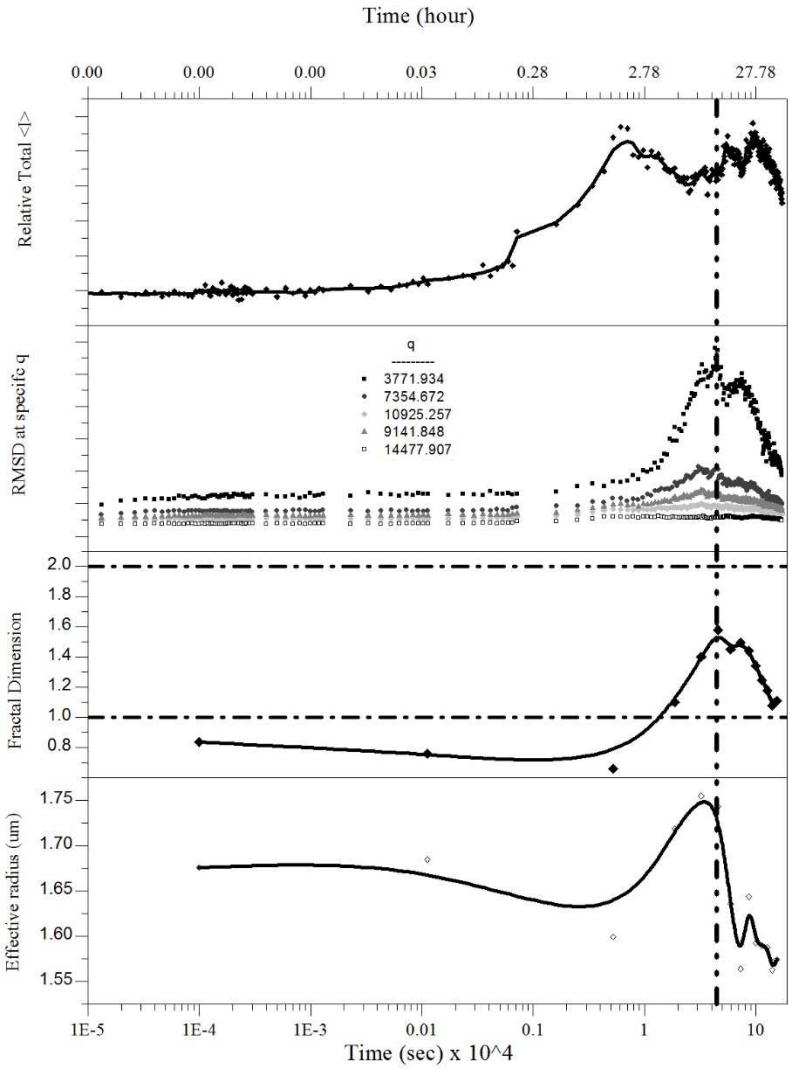

Figure 7-9: Demonstration of Morphological Changes

Population dynamics compared to cell morphological changes are compared in Figure 7-9. The relative total intensity is an indicator of total population dynamics. This is demonstrated by the observations of the effective radius twelve hours into the analysis (as marked by a vertical line on the figure). It is possible for an intensity to be impacted by an increase in the number of particles or by an increase in particle size. The observations at hour 12 confirm that while the effective radius decreases, the intensity remains consistent and then increases. This may be a result of the particles swelling at first, and then splitting; thus creating a total population increase, while effective radius decreases. The fractal dimension also shows at this moment that the shape model (the shape model is detailed in Section 6.4 Bacteria Dynamics) peaks at a coccoid shape more similar to a disc and progresses at this point back to the original shape model of an elongated rod. These results confirm the ability of relative total intensity to model population dynamics.

Morphological changes of bacteria throughout the growth cycle have been observed through both light and electron microscopic examination. The rod shape of growing *E. coli* is lost in stationary phase because cells become much smaller and almost spherical as the result of several cell divisions without an increase in cell (Ingraham *et al.*, 1983). This present study found similar results with calculations of fractal dimension and also during observations with the phase contrast microscope. This research included phase contract microscope studies in parallel to the ARGOS procedure. Images from this analysis are included in Figure 7-10.

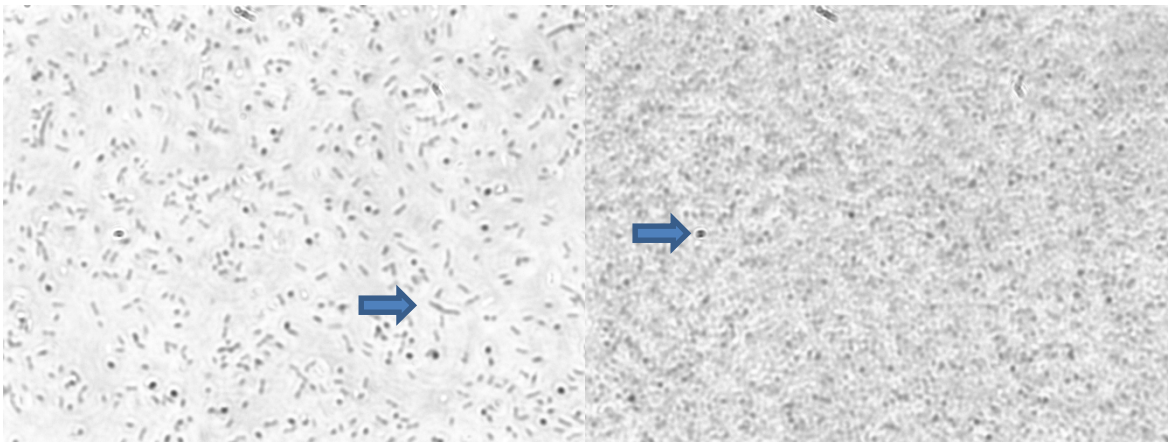

Figure 7-10: Phase Contrast Microscope – 40x– Morphological Changes in *E. coli* (a) [right hand side] at time, 40 minutes; (b) [left hand side] at time, 12 hours, 3 minutes

The observations from the phase contract microscope confirm the morphology calculated by the fractal dimension. The morphology 40 minutes into the observations clearly show particles which are elongated while the morphology at 12 hours and 3 minutes into the observation identify a higher bacterial population and bacteria shaped as cocci (discs). While similar experiments were conducted with a phase change microscope, the ARGOS methodology was able to run for longer periods of time with a high frequency of measurements and was able to collect data from a much wider range of wave vectors.

7.3.3 Infectivity Dynamics

The initial intent of the bacterial study was to provide a baseline for bacterial populations in order to study the dynamics of infectivity. While observations of nanoparticle characteristics can be difficult to observe, bacteria dynamics are often studied. One way of observing bacteriophage nanoparticle characteristics is to examine their impact upon bacteria populations.

While observations concerning phages at the nanoscale are complex, and phages have been studied at the micro-scale since the 1940s, Luria, *et al*. (1950) studied bacteriophages using a phase contract microscope. The research included observations of bacterial lysis, and changes in bacterial population with the use of chromatinic material. The research observed the effects of infection of *E. coli* with phage T2 and T2r and compared that with the effects of the *E. coli* with T2 and T2r X-ray and UV irradiation. Cytological observations confirmed the specificity of structural changes to bacteria following phage infection. The

observations confirmed identified that bacteria infected with inactive phage failed to produce active phage (Luria *et al.*, 1950).

While methods such as bacteria staining have been used to study bacteriophage infection of bacteria, this current study provides a more time-specific method in which the bacteria remained within the initial conditions. The research also included a time-specific analysis of infection with more time specificity than provided by analyzing concentrations of plaque-forming units.

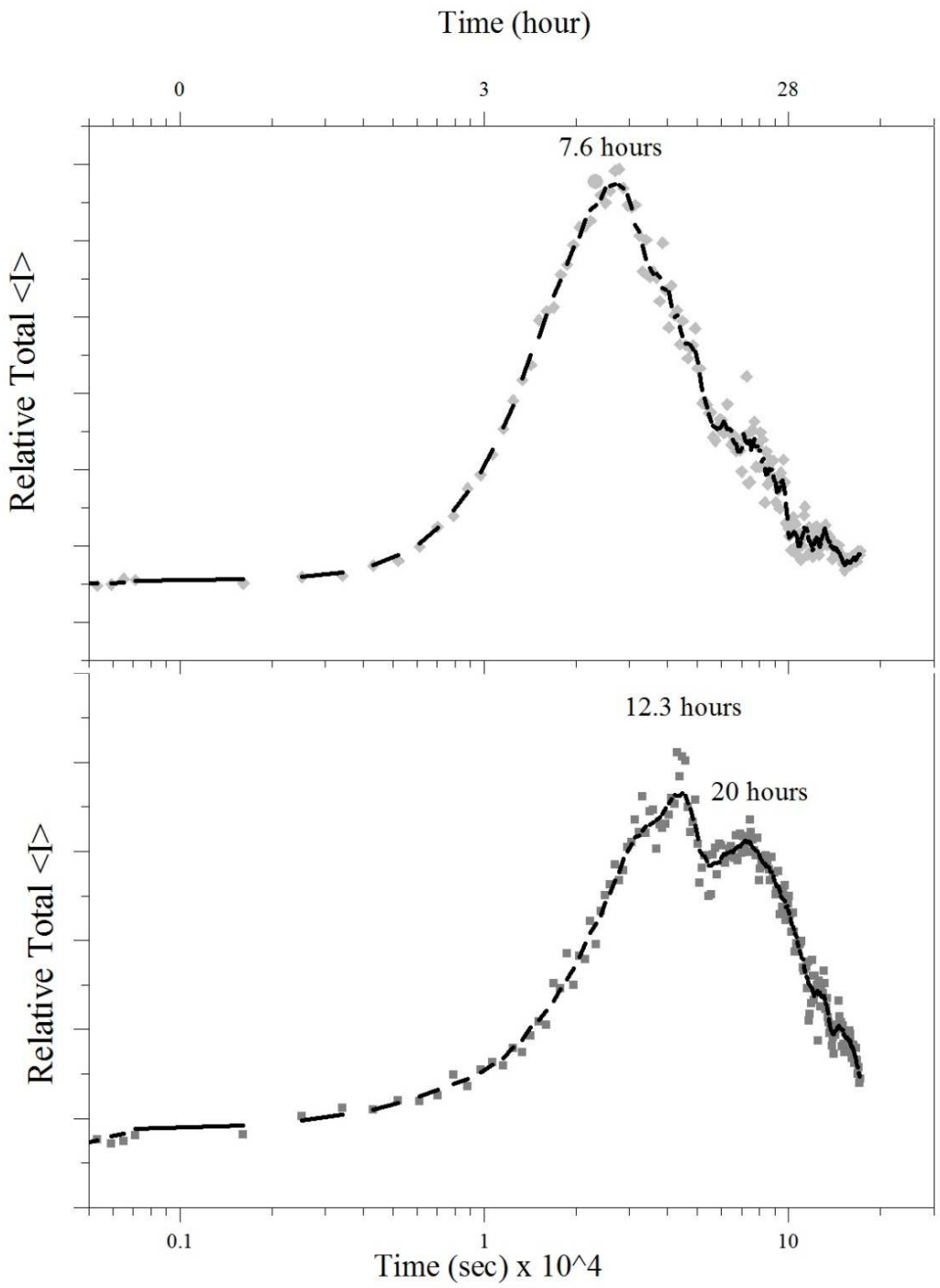

Figure 7-11 compares the growth rate of F-amp *E. coli* to the populations during infectivity of F-amp *E. coli* by MS2. Figure 7-11: Comparison of Relative Total Intensity for the Infectivity Analysis (top panel) and Relative Total Intensity for the Bacterial Analyses (bottom panel)

Figure 7-11 shows that dynamics of both the relative total intensity of F-amp *E. coli* infected with MS2 (top panel) and the relative total intensity observed in the bacteria alone (bottom panel). The relative total

intensity peak occurred earlier for F-amp *E. coli* infected with MS2 at 7.6 hours. This also shows that the infectivity analysis does not demonstrate the dual peaks observed at 12.3 hours and 20 hours for the uninfected F-amp *E. coli*. Since it is not appropriate to compare scale of relative total intensity, it is possible that the peaks were not of the same magnitude and that the exponential growth of the bacterial was interrupted and pushed into a death phase (Charles *et al.*, 2009; Costan-Longares *et al.*, 2008).

The rates of growth and death were observed for F-amp *E. coli* while uninfected and infected with MS2, as shown in Figure 7-12. The growth rate (rates are defined with the variable "m") of the infected bacteria occurred at a rate higher than the uninfected bacteria, m(1) 1.25×10^{-4} and m(3) 5×10^{-4}, respectively, as shown in Figure 7-13. Similarly, the death rates were also higher for the infected bacteria than the uninfected bacteria, m(2) 4.43×10^{-4} and m(4) 1.35×10^{-4}, respectively. The present study indicates that bacteria may react to infection by increasing the rate of population growth.

The morphological changes during infectivity demonstrate specific characteristics. These changes can be observed in Figure 7-13. The fractal dimension starts near one dimensional rod-like particles and becomes more of a disc-like particle. It is interesting to note that the populations drop off and the particles never get a chance to reduce back down to a one-dimensional rod-like structure. The effective size also drops down past the initial effective radius. In addition, in the infected bacteria the relative total intensity model population, and the final concentration is less than the initial concentration; meanwhile, the final population of the uninfected bacteria is higher than the initial populations.

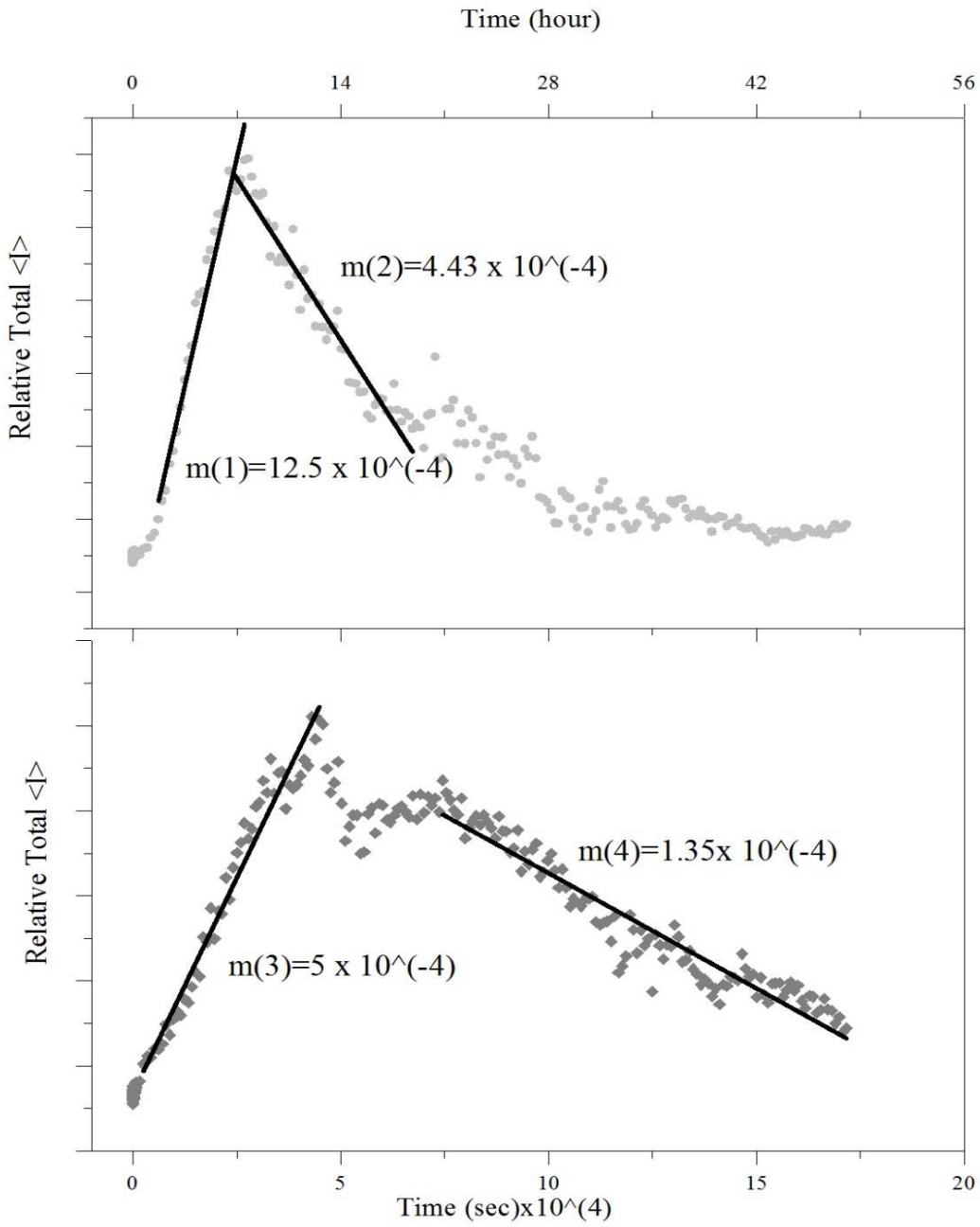

Figure 7-12: Growth and Death Rates for Infectivity and Bacterial Analyses

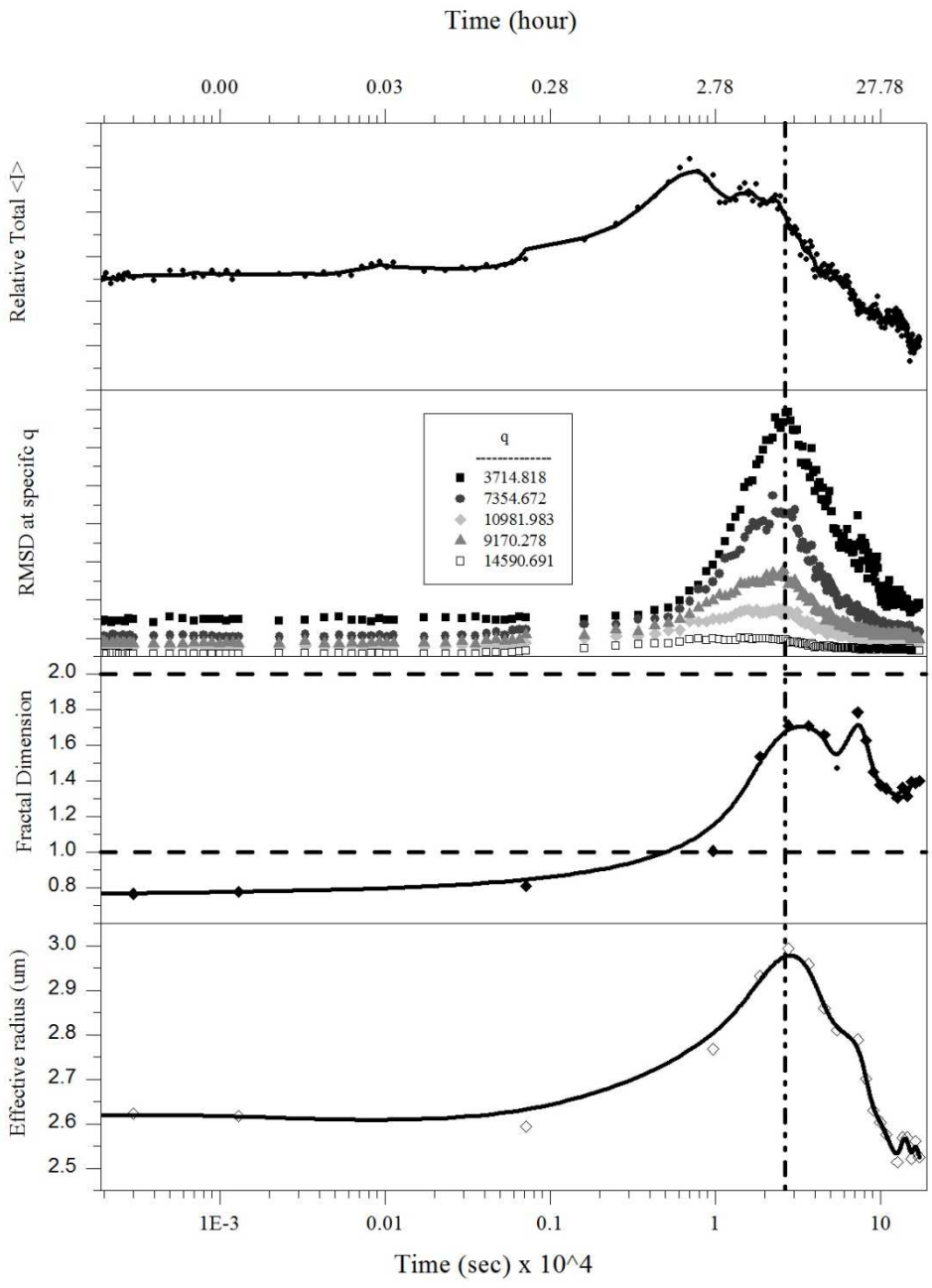

Figure 7-13: Morphological Changes in F-amp *E. coli* Infected with MS2

7.3.4 Nanoparticle Dynamics

Nanoparticles provide unique physical and chemical properties as a result of their particle size (<100 nm). These characteristics allow for applications in numerous biomedical, industrial, commercial, and consumer products. This research observes nanoparticle dynamics using the ARGOS method in an aqueous solution over a period of 2 hours. A method such as this is important because it allows the opportunity to explore biochemical events during a time sequence capable of identifying characteristic behaviors.

Colloidal particles can be observed using existing methodologies (e.g., microscopy, spectroscopy, and the recent addition of magnetic resonance), but each of these techniques contains a certain degree of uncertainty. A high resolution microscopy, such as atomic force microscopy (AFM), can be used to investigate the physical parameters of a particle. Further the study at the individual particle level using image analysis provides information about individual particles, although it is difficult to observe particle dynamics with them; in addition, the sample sizes are small and can only be observed for short periods of time (Brar *et al.*, 2011). Further, sample homogeneity, sample preparation, instrument operating procedures, and statistical practices are likely to add to the complexity of observing nanoparticle characteristics (Brar *et al.*, 2011).

While, DLS instruments (from companies such as Malvern and ALV) use dynamic light scattering to obtain static measurements, such as zeta potentials or hydrodynamic radius, the ARGOs method allows for observation of the kinetic behavior of a nanoparticle. This current research enables the study of nanoparticle dynamics to be implemented in the study of time-dependent relationships such as changing temperatures, disinfection kinetics, and concentration impacts. This research includes methodology that overcomes these limitations, as shown in Figure 7-14, which displays the total intensity of the four nanoparticles and the average total intensity at a specific wave vector (q) regime (10,000) over a 48-hour period. The following figures are included at the log scale in Appendix E. While the total system dynamics of a particle such as MS2 may exhibit a higher total relative intensity for the entire system, when observed at the individual particle level, the uncoated particles have a higher relative intensity at q of 10,000.

The Figure 7-14 demonstrates concentrations based on total intensity, while the RMSD demonstrate kinetics in Figure 7-15. While the concentration of MS2 is higher than ΦX-174as demonstrated by relative total intensity, the RMSD shows that the dynamics are greater and have more variation in ΦX-174 than MS2 and this may be a result of the hydrophobic nature of ΦX-174 and the uncoated particles (Sun *et al.*, 2014).

These differences can be explained through an observation of the differences of RMSD at the q regime of 10,000. The RMSD is a calculations of the changes in dynamics. This figure demonstrates the higher rate of change in particle dynamics at the individual particle level, and therefore impacts the average total intensity at that wave vector. The Figure 7-15 includes the observations for the four types of nanoparticles. Relationships such as these should be further explored, and may reflect relationships such as particle bonds or hydrophobicity. The weak particle bonds, such as hydrogen bonds, may create momentary particle size changes and could impact the particle observed in a specific q regime. Also, interactions between hydrophobic particles may determine the magnitude of the RMSD.

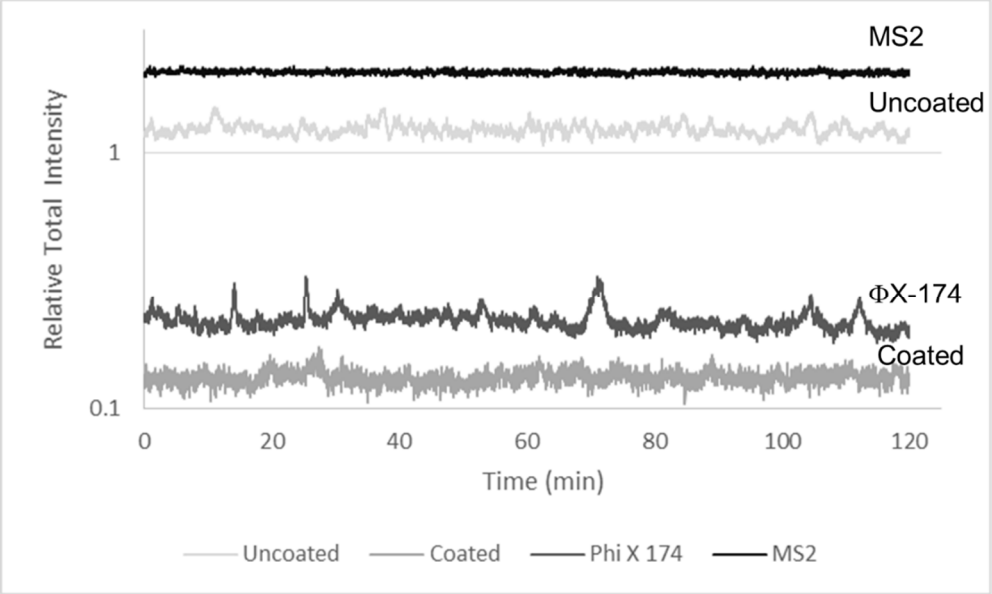

Figure 7-14: Relative Total Intensity of Nanoparticles

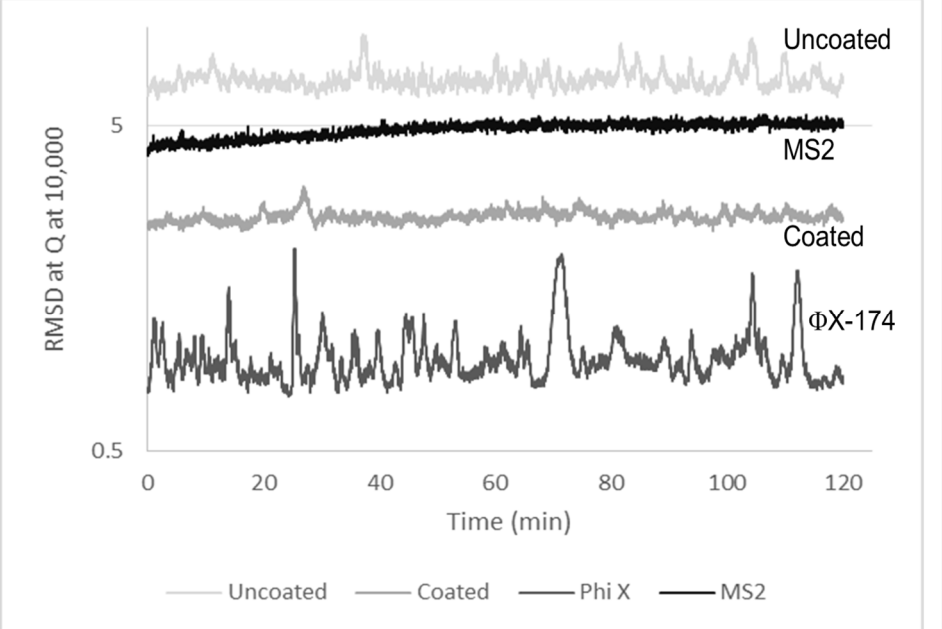

Figure 7-15: Nanoparticle RMSD at q Regime 10,000

The changes observed at the specific wave vector (q) regime <I> reflect the stability of the particles in solution specific to the q range of viruses. The intensity changes are reflected in the dynamics observed when with the calculated RMSD of each nanoparticle. Other methods of time-dependent light scattering have been used, such as methodologies using the Malvern Zetasizer or ALV CGS 3(scattering angle of 90°) use detectors to analyze changes the photos allow additional analysis after the initial measurements

(Nguyen *et al.*, 2005). Each virus type has its unique characteristics and may display different attenuation and transport behaviors and subsequent study of particle dynamics will provide more appropriate models of transport. The ARGOS method provides a new tool to investigate these dynamics.

8.0 CONCLUSIONS

The objective of this research was to examine indicators of viruses in waters systems and identify characteristics of indicators critical for predicting virus behavior. This research considers viruses in water systems at multiple-scales. The results show both that research on viruses at any scale can be difficult and that new methodologies are needed to analyze virus behavior in water systems. The nanoscale analysis included time-dependent light scattering using the ARGOS method to observe phage infection of bacteria and particle dynamics. The ARGOS method was also calibrated to observe the particle dynamics of nanoparticles. This method should be further utilized to predict virus fate and transport in environmental systems and through treatment processes.

Enteric viruses were enumerated in a variety of water systems, including groundwater, wastewater, stormwater and surface water. Traditional water quality indicators such as bacterial coliforms provide limited information regarding enteric viruses in different types of water samples (Gerba *et al.*, 2002; Lipp *et al.*, 2001). The detection of indicator bacteria in untreated drinking waters shows the contribution of fecal matter in source waters and reinforces the need for an adequate treatment processes in drinking water systems. This study evaluated traditional bacterial indicators (coliforms and *E. coli*), viral indicators (male-specific and somatic coliphages), abiotic surrogates (uncoated and protein-coated nanospheres) and viral markers as potential models of enteric viruses in water systems. An ideal indicator should be similar to potentially harmful pathogens in their physical structure, composition, and morphology (Nappier *et al.*, 2006). The results provided by this research add to the physical definitions of indicators and surrogates of enteric viruses by demonstrating that the external surface and morphology could be modeled for time dependent relationships.

8.1 FULL-SCALE WATER SYSTEMS

Multiple full-scale water systems were tested in including surface water, drinking water, stormwater and wastewater systems. The testing was completed in the U.S., Italy and Australia. This research project included the testing of bacteria and bacteriophage indicators, and collaborators at the University of Wisconsin Madison tested the viruses in the U.S. samples. Collaborators in Italy and Australia tested the water quality of samples in their respective countries.

The correlations identified in this research demonstrate that bacteria and viruses are impacted differently by various water systems and have different removal patterns. Viral markers provide additional fecal source information or indicate a fecal contamination event when present above detection limits that may not be captured by bacterial results. In this study, the lack of correlations between adenovirus and that of bacterial indicators suggests that these bacterial indicators are not suitable as indicators of viral contamination. In the wastewater samples, microviridae were correlated to the adenovirus, polyomavirus, and TTV. In wastewater samples, TTV was also correlated to adenovirus and polyomavirus. These results suggest that a viral marker such as microviradae or TTV may be a promising marker of enteric viruses and should be investigated as a representative virus.

While TTV may have some qualities which are consistent with an indicator such as physical similarity to enteric viruses and occurrence in populations worldwide, the use of TTV as an indicator may be limited as a result of the detection occurrence. Out of the total number of samples tested for TTV (324) in this study,

only 33 percent (108) were positive for TTV. As discussed previously, these results were similar to TTV occurrence in similar studies, for example, positive TTV samples were found in limited samples in Germany (56 of 108), Japan (1 of 18), and Brazil (3 of 28) (Hamza *et al.*, 2011b; Haramoto *et al.*, 2005; Vecchia *et al.*, 2012).

While presence of TTV is a potential marker of wastewater contamination, TTV was not consistently present in the water samples. As similarly found by Hamza *et al.* 2011 and Vecchia *et al.* 2012, the limitations of TTV occurrence may impede further analysis. In addition the methodologies are inconsistent for detection of TTV and other markers such as coliphages, and microviridae may be easier to study in the near future due to more developed methodologies.

8.2 LAB SCALE

Abiotic viral surrogates were used as model human enteric pathogens because of the difficulty in using viruses in research. Protein-coated latex nanospheres were used to model bacteriophages (MS2 and ΦX-174) and includes a comparison of the zeta potentials in lab water, and two artificial groundwaters with monovalent and divalent electrolytes. MS2 and ΦX-174 are often used in research and they were selected because while they have similar sizes, MS2 is an RNA bacteriophages with an IEP of 3.5 and ΦX-174 is a DNA bacteriophages with an IEP of 6.7. This research shows that protein-coated particles have higher average $\log_{10}$ removals than uncoated particles.

Studies in this literature and the results of this present study suggest that interfacial relationships are not clearly understood and that further analysis of nanoparticles including colloids and viruses are necessary. Particle characteristics not only impact interfacial relationships, but determine the efficiency of treatment processes. While adsorption of viral surrogates in this study was impacted by media properties, ionic strength, and pH the results were consistent not enough to provide a representative latex sphere with appropriate size and surface characteristics represent viruses in batch scale adsorption tests. The method of fluorescently labeling nanoparticles may not provide consistent data at the nanoscale. Method such as those used by Bradley *et al.* (2007) for radioactively labeling particles or methods such as Pang *et al.* (2012 for DNA labeling particles should be further explored for particles of this size. In addition, further methods such as the ARGOS method developed in this research should also be studied to observe nanoparticle characteristics.

8.3 NANOSCALE

An understanding of nanoparticles, specifically viruses, is important in the assessment of risk and determining potential public health impacts. The combination of physical theory and experiment produced a novel approach to researching viruses. The ARGOS method is a new methodology for the use of dynamic light scattering. The ARGOS method provides morphological dynamics noninvasively over a long time period and allows for a variety of aqueous conditions. A study of motility and adsorption rates on bacteria through porous media found that particle hydrodynamics were better predictors of microbial transport than particle characteristics such as size (Camper *et al.*, 1993).

This method is an improved over current instruments. DLS instruments that obtain measurement from a fixed angle can determine the mean particle size in a limited size range. Scattering techniques such as those

used in the instrument from Malvern are limited by the sensitivity of the DLS and can either work on a nanoscale 90^{o} angle detector, or a sub-nanometer scale with the 173^{o} backscatter detector (Kaszuba *et al.*, 2008). These instruments do not collect the full range of the wave vectors. The ARGOS method allows for a time-dependent analysis of all wave vectors throughout a, 360^{o} scatter detection.

Research such as this provides methods to define characteristics of viruses. Future work should include using the ARGOS method to observe to study time-dependent relationships of nanoparticle and can now be used to study dynamics such as changing temperatures, disinfection kinetics, and concentration impacts. These parameters impact the occurrence and survival of viruses can be incorporated into models that predict the levels of viral contamination in specific types of water because aspects of viral surface structures significantly influence the rate at which viruses are removed from the water phase by filtration (Penrod *et al.*, 1996). A better understanding of virus dynamics will provide the ability to predict and control the transport of viral pathogens in the aquatic environment, which is crucial to predicting public health.

8.4 FUTURE WORK

This research was used to investigate the physical characteristics of viruses which may impact treatment and survival in drinking water treatment. The removal rates and treatment efficiencies of viruses through treatment processes can be different between viruses as discussed in Section 3.2. The recommended next steps for virus fate and transport in water systems would be to group together similar types of viruses, for example RNA, DNA, enveloped and unenveloped and research whether or not each group reacts similarly. The work presented here with coated and uncoated nanoparticles demonstrates that the behavior of particles with different surface characteristics can be studied using ARGOS. Further work comparing lipid coated and protein coated nanoparticles may be able to provide some insight into the differences in environmental behaviors seen between enveloped (lipid containing) and non-enveloped viruses.

In addition, coliphages with similar traits should be investigated and perhaps these coliphages may represent specific viruses more consistently. Ultimately, more than one type of surrogate may need to be used to represent enteric viruses found in water systems.

Determining the dynamic behavior of individual nanoparticles expands the current knowledge of viral transport in treatment processes, which is mainly based on size exclusion. Future work should further define viruses in water systems in terms of size of the particle, the shape of surface structures, particle concentration, and impacts from ions in solution. The new parameters should be added to transport models in order to better predict the levels of viral contamination in water systems. This will allow water quality professionals to better predict and control the transport of viral pathogens in the aquatic environment in order to protect public health.

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APPENDIX A - STANDARD OPERATING PROCEDURES

A.1 COLIFORM AND *E. COLI* ENUMERATION

MATERIALS

(80) 99 mL autoclaved buffered dilution water bottles

((8 bottles/fecal sample × 6 samples) + (6 bottles/wastewater sample × 2 samples) + 2 negative control + 18 extra = 80)

(5) Empty autoclaved dilution bottles for 10^0 wastewater dilutions

(2 bottle/sample × 2 raw samples + 1 extra = 5)

(7) 50 mL autoclaved centrifuge tubes for fecal resuspensions and storing fecal samples

(1 tube/sample × 6 samples +1 extra =7)

(2) Tryptic soy broth shaker flasks, 50 mL in each - autoclaved.

(2) boxes of autoclaved 1 mL pipette tips

(70) Quantitrays

((8 trays/fecal sample × 6 samples) + 1 neg control + 1 pos control + (8 trays/wastewater sample × 2 samlpes) + 4 extra)

(70) Colilert Packets

((8 packets/fecal sample × 6 samples) + 1 neg control + 1 pos control + (8 packets/wastewater sample × 2 samlpes) + 4 extra)

SAMPLE PREPARATION

A. **Prepare log phase *E. coli* (ATCC11775) for positive control**

- Make 2 cultures in laminar flow hood sprayed with 50 percent alcohol. Flame tubes tops, caps and loop in between each use.
    1. Add 50 mL TSB to a labeled flask
    2. Autoclave
    3. Add one looPFUll of frozen *E. coli* stock
    4. Incubate at 35ºC at 100 rpm for 16-18 hours

B. **Prepare Workstation**

1. Turn on microbial hood blower
2. Sterilize microbial hood and workstation with 50 percent ethanol solution

3. Light alcohol burner in hood

C. **Prepare Wastewater Samples**

- Run cap and bottle neck under flame before and after wastewater is transferred, for sterilization purposes

    1. Remove first wastewater sample from refrigerator
    2. Invert bottle a few times to mix contents
    3. If undiluted sample is needed, pour ~100 mL of wastewater into dilution bottle labeled 10^{o}A. Repeat for 10^{o}B.
    4. For dilutions:
        a. From undiluted wastewater, pipette 1 mL into the dilution bottle labeled "10^{-2}A"
        b. Repeat for "B" series.
        c. Cap and invert dilution bottles twice
        d. Dispense used pipette tip
        e. Repeat this dilution series down to 10^{-4} and 10^{-6} for both series A & B.

D. **Prepare Fecal Samples**

1. Label feces samples and fill out work sheets.
2. Check temperature of samples if they are arrived via mail
3. Remove each sample one at a time from refrigerator, and put back in fridge when done
4. Weigh out 1 g of sample into 50 mL centrifuge tube with a flame-sterilized metal scupula
5. Pour some PBS from the 99 mL dilution bottle into centrifuge tube
6. Place on vortex to mix contents
7. Pour contents from centrifuge tube back into dilution bottle. May need to go back and forth a few times between the tube and bottle to ensure full feces sample ends up in the dilution bottle.
    a. This is the 10^{o}A dilution. Fecal results are reported per gram. The entire 100 mL (1 gm) sample will be put on a Quantitray, therefore this resuspension is undiluted
8. Save at least 1 gram of fecal sample and place in fridge.
9. If we collected samples, send at least 1 gram of sample to Wisconsin
10. Prepare dilutions of fecal samples
    a. Make dilutions from 10^{-2} down to 10^{-8} (doing an A and B series) using the dilution process described above for diluting wastewater samples

b. Perform wastewater dilutions first to let fecal samples sit and dissolve

E. **Quantitray preparation**

1. Add one colilert packet to each dilution bottle
2. After adding colilert, recap bottle, and shake until no colilert particles are left in suspension. Allow bottle to sit for 1 – 2 minutes for colilert to dissolve
3. Label all quantitrays as the individual dilution bottles are labeled
4. Slowly invert dilution bottle being careful not to create bubbles, then uncap dilution bottle.
5. Slowly pour contents into quantitray being careful not to create bubbles or turbulence
6. Place quantitrays onto orange quantitray holder and run through quantitray sealer machine.
7. Write the time down on the quantitray and place in incubator

F. **Reading Quantitrays**

1. After 24 hours remove quantitrays from incubator and read under hood.
2. Count and record the number of yellow large and small wells. (total coliform)
3. Turn off lights and using a U.V. light count and quantify the number of wells that fluoresce. (*E. coli*)
4. Use the standard tray as a comparison
5. Upload onto database
6. Use cross reference sheet to quantify bacteria content after adjusting for dilutions

A.2 - **Method 1602: Single Agar Layer (SAL) Method**

Method 1602: Single Agar Layer (SAL) Method
Wastewater - Coliphage Enumeration

A. Prepare Overnight *E. coli*

Check for refrigeration cultures of *E. coli* F-amp and CN-13. If there are none (or if they are more than 1 month old) prepare fresh cultures from frozen stocks. Make new before one month is up. Transfer no more than 6 (8 max.) times.

1. Prepare *E. coli* - CN-13 and F-amp
2. Make two of each
3. Make cultures in laminar flow hood, sprayed with 40 percent reagent alcohol.
4. Flame tube tops and caps and flame loop in between each use, flame loop very carefully between cultures

Overnight E. coli CN-13 (Somatic)

a. Add 50 mL TSB to a flask labeled "Somatic Overnight"
b. Autoclave
c. Add 0.5 mL 100X Nalidixic Acid to Somatic Overnight flask
d. Add 0.5 mL of refrigeration *E. coli* CN-13 to Somatic Overnight flask

Overnight E. coli F-amp (F+)

*more sensitive to time

a. Add 50 mL TSB to a labeled flask: F-amp Overnight
b. Autoclave
c. Add 0.5 mL 100X Strep/Amp to F-amp Overnight Flask
d. Add 0.5 mL of refrigeration *E. coli* F-amp to F-amp Overnight flask

5. Cap overnight flasks and shake/incubate at 36°C at 100-150 rpm for 16-18 hours
6. Use after inoculation (overnight *E. coli*) or save refrigeration flasks in culture fridge (1 month)

B. Prepare Log Phase *E. coli*

1. Start **4 hr** log phase *E. coli* F-amp/ *E. coli* CN-13 hosts from overnight *E. coli*
2. Make Log phase *E. coli*

Log-phase E. coli CN-13 (somatic)

a. Add 100 mL 1X TSB per log-phase flask
b. Autoclave
c. Add 1 mL of 100X Nalidixic Acid to Somatic log-phase flask
d. Add 0.5 mL overnight *E. coli* CN-13 to Somatic log-phase flask

Log-phase E. coli F-amp

a. Add 100 mL TSB per log-phase flask
b. Autoclave
c. Add 1 mL of 100X Strep/Amp to F-amp log-phase flask
d. Add 0.5 mL overnight *E. coli* F-amp to F-amp log-phase flask

3. Incubate at 36°C, shaking at 100-150 rpm for 4 ± 1 hours or until visibly turbid
4. Immediately chill on ice or at 4°C until ready for use
5. Must be used within 2 hours of placing on ice
6. For larger time window, a second set of log-phase cultures can be started an hour after the first

C. Make TSA

1. Turn on Waterbath 48°C
2. 1X TSB + 0.85 percent Bacto agar – See Recipes
3. Autoclave
4. Set in 48°C waterbath

D. Make Sample Dilutions

1. Make resuspensions and dilutions with Phage PBS
2. 1 gm feces in 9 mL PBS (feces reported as PFU/gm feces, plating 1 mL of this resuspensions is equivalent to plating 0.1 gm feces. Therefore, this is designated as the 10^{-1} dilution)
3. Add 1 mL to 9 mL PBS for 10 fold dilution OR 0.1 mL of 10^{-1} dilution in 9.9 mL PBS to make 10^{-3} to make 100 fold dilution

4. 3 plates per dilution (See Target Dilutions Table) of each sample

Target Dilutions						
Wastewater (Influent)			10^{-2}	10^{-3}	10^{-4}	
Wastewater (Effluent)	10^{0}	10^{-1}	10^{-2}			
Source water	10^{0}	10^{-1}	10^{-2}			
Feces*		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}

Dilutions are made with Phage PBS

*Feces Samples make dilutions of 10^{-1} to 10^{-3}, unless "dirty" (ex. Dog 10^{-4}, birds 10^{-5})

100 mm plates – complete F-amp plates first, then CN-13

1. Calculate the number of plates needed.
   a. Number of Feces samples × 4 dilutions × 3 plates × 2 types of *E. coli*
   b. Number of Wastewater samples × 4 dilutions × 3 plates × 2 types of *E. coli*
2. Set up and label 100 mm plates (usually labeled in advance)
3. To the 100 mm plates add:
   a. Sample solution (can vary from 10µL to 1 mL)
   b. 0.5 mL of 4 hour log phase *E. coli* F-amp or CN-13 host
   c. Add host into a different spot on the plate so you don't splash the sample then tilt the plate to mix them together.
4. Make sure sample and host are mixed – allow 1-2 minutes to adsorb – don't wait longer because then the *E. coli* bind to the plate and don't want to swirl
5. Include an MS2/ΦX174, agar, and host/agar plate and matrix spikes for both F-amp (F+) and CN-13 (somatic) enumeration
   a. Make MS2/ΦX174 Dilution (MS2 is for F-amp and ΦX174 is for CN-13)
   b. Agar Negative Control: Add a plate of agar only for each bottle of agar
   c. *E. coli* Positive Control: Add a plate of agar, and host (for both F-amp and CN-13)
   d. Coliphage Positive Control: Add a plate of agar, MS2/ΦX174 and host (for both F-amp and CN-13)
      i. Add 31.3 µL of MS2

ii. Add 59 µL of ΦX174

e. Matrix Spikes: Add a plate of agar, MS2/ΦX174, host (for both F-amp and CN-13), and sample (select one dilution from each sample)

E. Add $MgCl_2$ and Antibiotic to Agar

1. To TSA (1X TSB + 0.85% Bacto agar), add:
   a. 80X (4M) $MgCl_2$ to final concentration

      (1.25 mL into 100 mL TSA; 3.125 mL into 250 mL TSA)

   b. 100X strep/amp (for F-amp) or 100X Nalidixic Acid (for CN-13) to final concentration

      (1 mL into 100 mL TSA; 2.5 mL into 250 mL TSA)

   c. Run each addition down the inside of the bottle
2. Once Antibiotic added, must use in 10 minutes

F. Add TSA to Plate

1. Pipet ~10-12 mL TSA solution onto each of the plates.
2. Add TSA into an empty spot on the plate, so you don't splash the sample
3. After each addition, swirl plates in a figure 8 at least 5 times, then back and forth, then up and down several times to mix (**mixing is very important**).
4. Let sit about 5 minutes with cover slightly askew
5. After agar has solidified, close covers and stack plates upside down
6. Can do all of the F-amp first, then put all in incubator (need to go in within 1 hr of being poured)

G. Incubate plates

1. Seal up plates in bags
2. After plates have solidified, incubate at 36°C for 18-24 hours

H. Count Plates

1. Count and record the number of plaques on each plate after 18-24 hours
2. Calculate % Recovery for Matrix Spikes and OPR:

   % Recovery = (# PFU in Spiked - #PFU in Non-Spiked)/# Coliphages Spiked

Coliphage Enumeration Recipes

***E. coli* :**

Tryptic Soy Broth (TSB) 1X (for small plates)

- Add 30g tryptic soy broth to a sterile 2000 mL bottle
- Add 1000 mL Epure water
- Mix and warm to dissolve, autoclave, store refrigerated for 1 month
- Prior to sampling, autoclave 50 mL and 100 mL quantities as necessary

Antibiotics:

100X Nalidixic Acid

- Materials: Sterile beaker, (2) sterile bottles, sterile serological pipet, sterilization filter apparatus, pump
- Collect at least 100 mL of Epure water in a sterile beaker
- Add 1.0g Nalidixic Acid Sodium Salt to a sterile bottle
- Add 100 mL Epure water using a sterile serological pipet and swirl to dissolve
- Filter sterilize into a sterile bottle
- Freeze 5 mL aliquots at -20°C

100X Streptomycin/Ampicillin

- Materials: Sterile beaker, (2) sterile bottles, sterile serological pipet, sterilization filter apparatus, pump
- Collect at least 100 mL of Epure water in a sterile beaker
- Add 0.15 g ampicillin to a sterile bottle
- Add 0.15 g streptomycin
- Add 100 mL Epure water using a sterile serological pipet and swirl to dissolve
- Filter sterilize into a sterile bottle
- Freeze 5 mL aliquots at -20°C

Agar:

TSA - 1X TSB + 0.85% Agar (100 mL) – TSA for small plates

- Add 3 g tryptic soy broth to a sterile bottle (30g for 1L)
- Add 0.85 g Bacto Agar (8.5 g for 1L)
- Add 100 mL Epure water (1L)
- While stirring, heat to dissolve
- Autoclave
- Place in 48°C waterbath until use

4M (80X) $MgCl_2$

- Add about 1/3 Epure water to 100 mL Volumetric Flask
- Add 81.4 g $MgCl_2 \cdot 6H_2O$
- Bring final volume to 100 mL (Total Volume)
- Autoclave, store refrigerated

Samples and Phage

Phage Phosphate Buffered Saline (PBS) – Label Phage Only

- Add 8.0 g nacl to a sterile 1000 ml bottle
- Add 0.2 g KH_2PO_4
- Add 0.12 g kcl
- Add 0.91 g anhydrous Na_2HPO_4 (or 2.9 g $Na_2HPO_4 \cdot 12H_20$)
- Bring up to 1L with Epure water
- Adjust ph to 7.2-7.4 (with 1N hcl or naoh)
- Autoclave, store refrigerated for 1 year
    a. Wastewater
    b. Drinking Water

A.2 COLIPHAGE PROCEDURE

Method 1602: Single Agar Layer (SAL) Method

Drinking Water

A. **Prepare Overnight *E. coli***

Check for refrigeration cultures of *E. coli* F-amp and CN-13. If there are none (or if they are more than 1 month old) prepare fresh cultures from frozen stocks. Make new before one month is up. Transfer no more than 6 (8 maximum) times.

1. Prepare *E. coli* - CN-13 and F-amp
2. Make two of each
3. Make cultures in laminar flow hood, sprayed with 40% reagent alcohol.
4. Flame tube tops and caps and flame loop in between each use, flame loop very carefully between cultures

   Overnight *E. coli* CN-13 (Somatic)

   a. Add 50 mL TSB to a flask labeled "Somatic Overnight"
   b. Autoclave
   c. Add 0.5 mL 100X Nalidixic Acid to Somatic Overnight flask
   d. Add 0.5 mL of refrigeration *E. coli* CN-13 to Somatic Overnight flask

   Overnight *E. coli* F-amp (F+)

   *more sensitive to time

   e. Add 50 mL TSB to a labeled flask: F-amp Overnight
   a. Autoclave
   b. Add 0.5 mL 100X Strep/Amp to F-amp Overnight Flask
   c. Add 0.5 mL of refrigeration *E. coli* F-amp to F-amp Overnight flask
5. Cap overnight flasks and shake/incubate at 36°C at 100-150 rpm for 16-18 hours
6. Use after inoculation (overnight *E. coli*) or save refrigeration flasks in culture fridge (1 month)

B. Prepare Log Phase *E. coli*

1. Start **4 hour** log phase *E. coli* F-amp/ *E. coli* CN-13 hosts from overnight *E. coli*
2. Make Log phase *E. coli*

Log-phase *E. coli* CN-13 (somatic)

a. Add 100 mL 1X TSB per log-phase flask

b. Autoclave

c. Add 1 mL of 100X Nalidixic Acid to Somatic log-phase flask

d. Add 1 mL overnight *E. coli* CN-13 to Somatic log-phase flask

Log-phase *E. coli* F-amp

e. Add 100 mL TSB per log-phase flask

f. Autoclave

g. Add 1 mL of 100X Strep/Amp to F-amp log-phase flask

h. Add 1 mL overnight *E. coli* F-amp to F-amp log-phase flask

3. Incubate at 36°C, shaking at 100-150 rpm for 4 ± 1 hours or until visibly turbid
4. Immediately chill on ice or at 4°C until ready for use
5. Must be used within 2 hours of placing on ice
6. For larger time window, a second set of log-phase cultures can be started an hour after the first

Make TSA

1. Prepare 2X TSA for Large Plates – See Recipes
2. Autoclave
3. Set in 48°C waterbath

C. Samples

1. Complete the following steps for each sample twice; once for Male-Specific (F-amp) Enumeration and then repeat the steps for Somatic (CN 13) Enumeration.
2. Prepare bottles of samples, and concentrated samples
   a. Prepare samples and a duplicate of each by dispensing 100 mL of each sample into separate sterile 250 mL screw cap bottles.
   b. Prepare concentrated samples and a duplicate of each.
      i. For groundwater, and treated drinking water (25X only)
         1. Prepare a 25X concentrate
            a. Add 50 mL of each HFUF sample into separate sterile 250 mL screw cap bottles.
            b. Add 50 mL PBS
      ii. For surface source water (5X and 25X)

1. Prepare a 5X concentrate
    a. Add 10 mL of each HFUF sample into separate sterile 250 mL screw cap bottles.
    b. Add 90 mL PBS
2. Prepare a 25X concentrate
    a. Add 50 mL of each HFUF sample into separate sterile 250 mL screw cap bottles.
    b. Add 50 mL PBS
    c. Aseptically add 0.5 mL of 4M MgCl2 to each 250 mL sample bottle.
3. Prepare Positive Controls (Matrix spike and OPR) for one of the samples.
    a. Aseptically prepare two sterile 250 mL screw cap bottles.

i. Dispense 100 mL of one of your samples separate sterile 250 mL screw cap bottle.

ii. Dispense 100 mL of PBS into separate sterile 250 mL screw cap bottle

    b. Add Phage

i. For F+ enumeration add a known amount (31.3 µL ~ 80PFU) of MS2 to the positive controls (Sample MS/OPR).

ii. For Somatic enumeration add a known amount (59 µL ~ 80PFU) of ΦX to the positive controls (Sample MS/OPR).

    c. Aseptically add 0.5 mL of 4M $MgCl_2$ to each 250 ml sample bottle.

4. Prepare Temperature Control
    a. Prepare a temperature control by dispensing 100 mL PBS into a separate sterile 250 mL screw cap bottle.
    b. Aseptically add 0.5 mL of 4M MgCl2 to the 250 ml sample bottle.
5. Prepare Centrifuge Tubes for Negative Controls
    a. Add 15mL PBS into two separate sterile 50 mL centrifuge tubes. Label one as “Host” and one as “Agar”
    b. Add 0.075 mL of 4M MgCl2 to each of the two centrifuge tubes.
6. Uncap the temperature control and insert a thermometer.
7. Place the sample bottles (including controls and centrifuge tubes) into a 48°C water bath and shake for 5 minutes or until the temperature control reaches 36°C.
8. Remove bottles/tubes from water bath
9. Add *E. coli* (Should be plated within 20 minutes)
    a. For F+:

i. Add 10 mL log-phase *E. coli* F-amp to each sample bottle (including temperature/positive control).

ii. Add 1.5 mL log-phase host to F+ "host" centrifuge tube.

b. For Somatic:

i. Add 10 mL log-phase *E. coli* CN13 to each sample bottle (including temperature/positive control).

ii. Add 1.5 mL of log-phase host to the Somatic "host" centrifuge tube.

10. Place bottles and centrifuge tubes back into the 48°C water bath and shake until temperature reaches 43°C +/- 1C. Once temperature is reached, transfer to 43°C water bath.

11. Prepare Agar

a. Antibiotic Quantities

i. F+: Add 2.0 mL of 100X Strep/Amp per 100 mL 2X TSA.

ii. Somatic: Add 2.0 mL of 100X nalidixic acid per 100 mL 2X TSA.

iii. Note:

1. 6.0 mL per 300 mL 2X TSA
2. 7.0 mL per 350 mL 2X TSA
3. 12 mL per 600 mL 2X TSA
4. 17 mL per 850 mL 2X TSA

b. Add the antibiotic along the inside of the container to reduce the formation of bubbles,

c. Gently rock the container slowly to mix.

12. Once antibiotics are added, you have 10 minutes to add agar to sample before antibiotics degrade.

13. Add the Agar with antibiotics to the Sample Bottles

a. Pour the agar until the contents of the bottle are approximately doubled (thumb check).

b. Tilt and turn gently to mix – avoid introducing bubbles.

c. For "host" and "agar" tubes, add approximately 15-17 mL agar/antibiotic.

14. Pour the contents of the sample bottle into a series of five – 150 mm Petri dishes. Use the entire solution. For "host" and "agar" tubes pour entire contents into one Petri plate each.

15. Repeat as needed for each of the samples and controls.

16. Leave the tops of the Petri plates askew until agar has hardened (about 5 minutes). Cover, stack, invert, and bag. Incubate at 37C for 16-24 hours.

17. Count all plaque forming units and note any contamination. Plaques can be isolated in 300 µl 20 percent glycerol/TSB in cryotubes for further serotyping or genotyping.

Coliphage Enumeration Recipes

Tryptic Soy Broth (TSB) 1X

- Add 30g tryptic soy broth to a sterile 2000 mL bottle
- Add 1000 mL Epure water
- Mix and warm to dissolve, autoclave, store refrigerated for 1 month
- Prior to sampling, autoclave 50 mL and 100 mL quantities as necessary

100X Nalidixic Acid

- Materials: Sterile beaker, (2) sterile bottles, sterile serological pipet, sterilization filter apparatus, pump
- Add 1.0g Nalidixic Acid Sodium Salt to a sterile bottle
- Add 100 mL Epure water using a sterile serological pipet and swirl to dissolve
- Filter sterilize into a sterile bottle
- Freeze 5 mL aliquots at -20°C

100X Streptomycin/Ampicillin

- Materials: Sterile beaker, (2) sterile bottles, sterile serological pipet, sterilization filter apparatus, pump
- Collect at least 100 mL of Epure water in a sterile beaker
- Add 0.15g ampicillin to a sterile bottle
- Add 0.15g streptomycin
- Add 100 mL Epure water using a sterile serological pipet and swirl to dissolve
- Filter sterilize into a sterile bottle
- Freeze 5 mL aliquots at -20°C

TSA – 2X TSB + 0.85% Agar (100 mL) – TSA for large plates

- Add 6g tryptic soy broth to a sterile bottle (60g for 1L)
- Add 1.8g Bacto Agar (18g for 1L)
- Add 100 mL Epure water (1L)
- While stirring, heat to dissolve
- Autoclave

- Place in 48°C waterbath until use

4M (80X) $MgCl_2$

- Add about 1/3 Epure water to 100 mL Volumetric Flask
- Add 81.4g $MgCl_2 \cdot 6H_2O$
- Bring final volume to 100 mL (Total Volume)
- Stir to dissolve
- Autoclave, store refrigerated

Phage Phosphate Buffered Saline (PBS) – Label Phage Only

- Add 8.0g NaCl to a sterile 1000 mL bottle
- Add 0.2g KH_2PO_4
- Add 0.12g KCl
- Add 0.91g anhydrous Na_2HPO_4 (or 2.9 g $Na_2HPO_4 \cdot 12H_20$)
- Bring up to 1L with Epure water
- Adjust pH to 7.2-7.4 (with 1N HCl or NaOH)
- Autoclave, store refrigerated for 1 year

A.3 COLIPHAGE ENRICHMENT AND CONCENTRATION

Coliphage Enrichment and Concentration

Check for refrigeration cultures of *E. coli* F-amp and CN-13. If there are none (or if they are more than 1 month old) prepare fresh cultures from frozen stocks. Make new before one month is up. Transfer no more than 6 (8 maximum) times.

A. Prepare Appropriate Overnight Host (*E. coli* – CN-13 or F-amp)

1. Make two of each
2. Make cultures in laminar flow hood, sprayed with 40 percent reagent alcohol.
3. Flame tube tops and caps and flame loop in between each use, flame loop very carefully between cultures

Overnight *E. coli* CN-13 (Somatic)

a. Add 50 mL 1X TSB to a flask labeled "Somatic Overnight"
b. Autoclave
c. Add 0.5 mL 100X Nalidixic Acid to Somatic Overnight flask
d. Add 0.5 mL of refrigeration *E. coli* CN-13 to Somatic Overnight flask

Overnight *E. coli* F-amp (F+)

*more sensitive to time

a. Add 50 mL TSB to a labeled flask: F-amp Overnight
b. Autoclave
c. Add 0.5 mL 100X Strep/Amp to F-amp Overnight Flask
d. Add 0.5 mL of refrigeration *E. coli* F-amp to F-amp Overnight flask

4. Cap overnight flasks and shake/incubate at 36°C at 100-150 rpm for 16-18 hours
5. Use after inoculation (overnight *E. coli*) or save refrigeration flasks in culture fridge (1 month)

B. Prepare Log Phase *E. coli*

1. Start **4 hour** log phase *E. coli* F-amp/ *E. coli* CN-13 hosts from overnight *E. coli*
2. Make Log phase *E. coli*

Log-phase *E. coli* CN-13 (somatic)

a. Add 50 mL 1X TSB per log-phase flask

b. Autoclave

c. Add 1 mL of 100X Nalidixic Acid to Somatic log-phase flask

d. Add 0.5 mL overnight *E. coli* CN-13 to Somatic log-phase flask

Log-phase *E. coli* F-amp

a. Add 50 mL TSB per log-phase flask

b. Autoclave

c. Add 1 mL of 100X Strep/Amp to F-amp log-phase flask

d. Add 0.5 mL overnight *E. coli* F-amp to F-amp log-phase flask

3. Incubate at 36°C, shaking at 100-150 rpm for 4 ± 1 hours or until visibly turbid
4. Immediately chill on ice or at 4°C until ready for use
5. Must be used within 2 hours of placing on ice
6. For larger time window, a second set of log-phase cultures can be started an hour after the first

C. Enrich Phage (ΦX-174 or MS2)

1. Dispense 30 mL TSB/Glycerol/Tween into sterile 50 mL centrifuge tube
2. Add 0.375 mL 4M $MgCl_2$, 0.3 mL of appropriate antibiotic, 0.5 mL log phase host, and previous 80 PFU coliphage stock
3. Incubate at 36°C +/-1oC with shaking at 100 rpm, if possible for 6 to 18 hours

D. Prepare Aliquots of Phage

1. Prepare a waste receptacle containing 10 percent bleach (1mL commercial bleach to 9 mL tap water)
2. Tightly seal enrichment and vortex
3. Using a 5 or 10 cc luer-lock syringe, draw up enrichment culture

4. Aseptically attach 0.45 µm MCE syringe filter (Fisher 09719B), and filter into a sterile receiving 50 mL test tube
5. Place used syringe filter in bleach bath, continue until volume needed is filtered.
6. Tightly seal filtered enrichment and vortex
7. Dispense 1.0 mL aliquots into 1.5mL vials
8. Quick freeze in an ethanol bath.
9. Wipe off excess ethanol and store at 80°C for up to 1 year.

E. **Coliphage Concentration**

(Centrifuge RPM is calculated for Fisher Scientific Marathon 21000R Refrigerated Centrifuge with Rotor 04-976-006 4x250 mL sealed swing bucket)

1. Sanitize
2. Spray work area with 50% alcohol solution
3. Prepare Filters (Centricon Plus-70) Spray outside of filters with 50 percent alcohol solution
4. Pre-Rinse
5. Add 70 mL of Phage PBS
6. Centrifuge at 1000xg at 5°C for 7 minutes

a. 2,400 rpm

7. Concentrate Phage Solution
8. Make (2) Phage solution with 49 mL of Phage PBS and 1 mL of phage
9. Prepare two filters
10. Centrifuge at 3,500xg at 5°C for 30 minutes

a. 4,200 rpm

11. Recovery
12. Invert the device
13. Place sample filter cup
14. Centrifuge at 1,00 xg at 5°C for 2 minutes

    a. 2,400 rpm

15. Use syringe to extract concentrated sample

Coliphage Enumeration Recipes

***E. coli* :**

Tryptic Soy Broth (TSB) 1X (for small plates)

- Add 30g tryptic soy broth to a sterile 2000 mL bottle
- Add 1000 mL Epure water
- Mix and warm to dissolve, autoclave, store refrigerated for 1 month
- Prior to sampling, autoclave 50 mL and 100 mL quantities as necessary

Antibiotics:

100X Nalidixic Acid

- Materials: Sterile beaker, (2) sterile bottles, sterile serological pipet, sterilization filter apparatus, pump
- Collect at least 100 mL of Epure water in a sterile beaker
- Add 1.0g Nalidixic Acid Sodium Salt to a sterile bottle
- Add 100 mL Epure water using a sterile serological pipet and swirl to dissolve
- Filter sterilize into a sterile bottle
- Freeze 5 mL aliquots at -20°C

100X Streptomycin/Ampicillin

- Materials: Sterile beaker, (2) sterile bottles, sterile serological pipet, sterilization filter apparatus, pump
- Collect at least 100 mL of Epure water in a sterile beaker
- Add 0.15g ampicillin to a sterile bottle
- Add 0.15g streptomycin
- Add 100 mL Epure water using a sterile serological pipet and swirl to dissolve
- Filter sterilize into a sterile bottle
- Freeze 5 mL aliquots at -20°C

Phage:

TSB 20%, glycerol, 0.1% Tween 80

-0.1g Tween 80

-20 mL glycerol

-3.0g Tryptic Soy Broth

-80 mL Lab water

Adjust pH 7.2-7.4, autoclave for 15 minutes at 121ºC

Store at 4ºC for up to 30 days

Agar:

TSA - 1X TSB + 0.85% Agar (100 mL) – TSA for small plates

- Add 3g tryptic soy broth to a sterile bottle (30g for 1L)
- Add 0.85g Bacto Agar (8.5g for 1L)
- Add 100 mL Epure water (1L)
- While stirring, heat to dissolve
- Autoclave
- Place in 48°C waterbath until use

4M (80X) $MgCl_2$

- Add about 1/3 Epure water to 100 mL Volumetric Flask
- Add 81.4 g $MgCl_2 \cdot 6H_2O$
- Bring final volume to 100 mL (Total Volume)
- Autoclave, store refrigerated

Samples and Phage

Phage Phosphate Buffered Saline (PBS) – Label Phage Only

- Add 8.0g NaCl to a sterile 1000 mL bottle
- Add 0.2g KH_2PO_4
- Add 0.12g KCl
- Add 0.91g anhydrous Na_2HPO_4 (or 2.9 g $Na_2HPO_4 \cdot 12H_20$)

- Bring up to 1L with Epure water
- Adjust pH to 7.2-7.4 (with 1N HCl or NaOH)
- Autoclave, store refrigerated for 1 year

A.4 Viral Marker Methodologies Used By University Partners

Various polymerase chain reaction (PCR) methods were used to enumerate viruses as shown in Table 5-2. The United States viral methodology was developed and conducted by the University of Wisconsin–Madison. A PCR-based TTV detection methodology was developed in this research. The rapid nature of PCR makes it an ideal tool for periodic monitoring of water sources. A number of commercial nucleic acid clean up kits were assessed for DNA clean up and yield. The Mo-Bio DNA Isolation Kit (Mo Bio Laboratories, Inc., Carlsbad, California) consistently demonstrated a positive detection of a number of viruses from environmental samples including adenovirus and TTV. Selected primers are in the highly conserved, untranscribed region (UTR) of the genome and provide detection of a variety of TTV genotypes. The DNA was then extracted and purified prior to the PCR assay. The TTV samples received traditional and touchdown PCR, while the adenovirus was assayed with quantitative PCR.

Table A1: Analytical Conditions for the PCR Analysis of Viral Markers

Location	Virus	Method	Primers and Probes		Cycling Parameters
United States	Torque Teno Virus	Traditional PCR	Forward	GTG CCG IAG GTG AGT TTA	
			Reverse	AGC CCG GCC AGT CC	
	Adenovirus	qPCR	Forward	GGA CGC CTC GGA GTA CCT GAG	
			Reverse	ACI GTG GGG TTT CTR AAC TTG TT	
			Probe	Fam-CTG GTG CAG TTC GCC CGT GCC A-Tamara	
Italy	Torque Teno Virus	Nested PCR	NG133	GTA AGT GCA CTT CCG AAT GGC TGA G	95°C (9 min), followed by 35 cycles at 95°C (30 sec), 60°C (30 sec) and 72°C (40 sec) with a final cycle at 72°C (7 min)
			NG147	GCC AGT CCC GAG CCC GAA TTG CC	
			NG134	50-AGT TTT CCA CGC CCG TCC GCA GC-30	95°C (9 min), followed by 25 cycles at 95°C (30 sec), 60°C (30 sec) and 72°C (40 sec) with a final cycle at 72°C (7 min)
			NG132	50-AGC CCG AAT TGC CCC TTG AC-30	
	Adenovirus	Nested PCR	ADDEG 1	GCC SCA RTG GKCWTA CAT GCA CAT C	95°C (9 min), followed by 25 cycles at 95°C (30 sec), 60°C (30 sec) and 72°C (40 sec) with a final cycle at 72°C (7 min)
			ADDEG 2	CAG CAC SCC ICG RAT GTC AAA	
			ADDEG 3	GCC CGY GCM ACI GAI ACS TAC TTC	
			ADDEG 4	CCY ACR GCC AGI GTR WAI CGM RCY TTG TA	
	Adenovirus	qPCR	AdF	CWTACATGCACATCKCS GG	95°C (10 min), followed by 40-45 cycles at 95°C (10 sec) with a final cycle at 60°C (1 min)
			AdR	CRCGGGCRAAYTGCACC AG	

Location	Virus	Method	Primers and Probes		Cycling Parameters
			AdP1	FAM-CCGGGCTCAGGTACTCCGAGGCGTCCT-TAM	
Australia	Adenovirus	qPCR	Forward	GCC ACG GTG GGG TTT CTA AAC TT	10 min at 95°C, 50 cycles of 15 s at 95 °C and 20 s at 60°C and 20 s at 72°C
			Reverse	GCC CCA GTG GTC TTA CAT GCA	
			Primer	FAM TGC ACC AGA CCC GGG CTC AGG AGG TAC TCC GA BHQ1	
	Polyomavirus	qPCR	Forward	SM2 AGT CTT TAG GGT CTT CTA CCT TT	10 min at 95°C, 50 cycles of 15 s at 95°C and 20s at 55°C and 60 s at 60°C
			Reverse	P6 GGT GCC AAC CTA TGG AAC AG	
			Primer	KGJ3 (FAM)-TCA TCA CTG GCA AAC AT-(MGBNFQ)	
	Torque Teno Virus	qPCR	Forward	CGG GTG CCG DAG GTG AGT TTA CAC	10 min at 95°C, 50 cycles of 20s at 95°C and 20s at 62°C and 20s at 72°C
			Reverse	GAG CCT TGC CCA TRG CCC GGC CAG	
			Primer	FAM-AGTC AAG GGG CAA TTC GGG CTCG GGA-TAMRA	
	Microviridae	qPCR	Forward	TAC CCT CGC TTT CCT GC	10 min at 95°C, 50 cycles of 20s at 95°C and 20 s at 61°C and 20s at 72°C
			Reverse	GCG CCT TCC ATG ATG AG	
			Primer	FAM-CAT TGC TTA TTA TGT TCA TCC CG-TAMRA	

The final method developed in the United States included bead beating to release nucleic acid and the use of a clean-up kit (PowerSoil® DNA Isolation Kit, MO BIO Laboratories, Carlsbad, CA) to reduce inhibitor concentrations. For TTV, amplification of target ssDNA was conducted using a traditional PCR assay. All positive TTV samples and a selected number of negative TTV samples were analyzed for the presence of human adenovirus. A real-time polymerase chain reaction (qPCR) assay was developed with primer and probe sets, master mix conditions, and thermocyler program (Jothikumar *et al.*, 2005; Jothikumar *et al.*, 2010; Liu *et al.*, 2011b; Long *et al.*, 2010).

In the United States, hollow fiber ultrafiltration was utilized to concentrate the drinking water samples, and polyethylene glycol (PEG) precipitation was used as a secondary concentration. PEG was used as the primary concentration for wastewater samples. This procedure can be applied to source and finished waters. Wastewater samples were processed by PEG precipitation directly. Fecal samples were suspended in buffer

and centrifuged; the pellet is treated directly for nucleic acid release and clean up (Long, 2013; Long *et al.*, 2012; Plummer *et al.*, 2014).

In Italy, water samples were concentrated using two-stage tangential flow ultrafiltration. After prefiltration on polypropylene membranes, the samples were filtered through a polysulphone membrane with a 10,000 MW exclusion size. The samples were reconcentrated with a mini-ultrasette apparatus and washed using 15 to 20 mL of 3 percent beef extract at pH 9, obtaining a concentrated sample of 40 mL at pH 7. Then a 1:10 chloroform solution was added for bacterial decontamination. The samples were then shaken for 30 min, centrifuged at 1,200g for 20 min, and the supernatant recovered and aerated for 2 hours (Carducci *et al.* 2006; Carducci *et al.* 2009). The concentrated samples were decontaminated with chloroform, and the nucleic acids were extracted with QIAamp DNA kit (QIAGEN, Germany). In order to process the qualitative analysis by nested PCR, 7 mL of extracted DNA was mixed with 45 mL reaction buffer (50 mM KCl, 0.1% Triton-X-100, 10 mM Tris-HCl pH 8.8, 2mg/mL BSA, 3.5mM $MgCl_2$, 0.2mM of each dNTPs, 2U Taq Polimerase (Promega), 25 pmol of each primer NG133 and NG147). Subsequently, the mixture was added to a microplate well (iCycler system; Bio-Rad Laboratories, Milan, Italy) and incubated. In the second reaction, 5 mL of the first stage was used with the appropriate primers. The amplified product (110 bp) and positive samples were typed by sequencing (ABI PRISM310 Genetic Analyzer; Applied Biosystems, Carlsbad, CA) (Verani *et al.*, 2006).

For qPCR, amplifications were performed in a 25 mL reaction mixture that contained extracted DNA with 1x concentration of the Universal Master Mix (Applied Biosystems, Carlsbad, CA), a 900 nM concentration of each primer, and the appropriate probe. Following activation of the uracil N-glycosylase (2 min, 50°C) and activation of the AmpliTaq Gold for 10 min at 95°C, 40 to 45 cycles (15 seconds at 95°C and 1 min at 60°C) were performed with an ABI 7300 sequence detector system (Applied Biosystems, Carlsbad, CA). Standard curves were generated by using serial dilutions (range 10^2 to 10^7) of known amounts of linearized plasmids containing for adenovirus the entire hexon region of Ad41 and for TTV the highly conserved segment of the nontranslated region (UTR). All samples were run in triplicate. The amount of DNA was defined as the median of the triplicate data calculated by the SDS software and based on correlation with Ct values of the Standard Curve.

In Australia, samples were analyzed by qPCR for TTV, adenovirus, polyomavirus, and microviridae. The samples were concentrated using Hemoflow HF80S dialysis filters (Fresenius Medical Care, Lexington, MA, U.S.). Samples were pumped with a peristaltic pump in a closed loop with high-performance, platinum-cured L/S 36 silicone tubing. Samples were concentrated to approximately 100 mL, and further concentration of sample was carried out by JumboSep with 100 K MWCO filters (Pall, Australia) to a final volume of approximately 10 mL (Sidhu *et al.* 2013). Nucleic acid was extracted from 200 µL of each concentrated sample using the MoBio PowerSoil DNA isolation kit (MoBio Laboratories, Inc., Carlsbad, CA) per manufacturer instructions, and stored at 80°C until processed. Amplifications were performed in 25 µL reaction mixtures using iQ Supermix (Bio-Rad Laboratories, Berkeley, CA). The PCR mixture contained 12.5 µL of Supermix, 400–500 nM each primer, 400–600 nM corresponding probe, and 3 µL of template DNA (Sidhu *et al.* 2013).

A.5 ABIOTIC PARTICLES AND BATCH ADSORPTION

(a) Batch Methods

Abiotic Particles and Batch Adsorption

Prepare Solutions according to SOP Ionic Strength Methodology

Prepare Media according to SOP Sand Methodology

A. Prepare Sample Bottles

40 mL glass bottles wash in 20 percent sulfuric acid solution, autoclave and oven dry at 121ºC for 4 hours

B. Prepare samples of protein-coated 26 nm nanosphere and uncoated 26 nm nanospheres

a. Add 9.9 mL of the appropriate ionic solution (AGW1 and AGW2) or Nanopure lab water (exceeds ASTM Type I, ISO 3696 and CLSI-CLRW Type I standards with uses a four stage deionization process combined UV disinfection, an ultrafilter and a 0.2 micron filter).

b. Add 0.01 mL solutions of phages or nanospheres for a final concentration of 10^5

c. Add 10g of sand (washed or acid/base washed)

C. Prepare dilution series to be used for concentration curve 10^6-10^0 particles per mL

D. Conduct Batch Test

a. Test each combination (12) of surrogate (phages and nanospheres) and ionic solution will be tested at pHs of 4,6 and 8 for both types of sand.

b. Prepare six tubes of each combination will be prepared and placed on a shaker table at 100 rpm in and incubator at 21ºC.

c. Remove samples at 0, 10, 20, 40, 90, and 120 minutes with duplicate samples.

d. Extract 0.3 mL for each sample in duplicate and dispense into a 96 well sample plate

e. Run sample plate twice in the in fluorescence spectrophotometer

(b) Particle Specifications

A.6 IONIC STRENGTH METHODOLOGY

Preparation of High and Low Ionic Strength Solutions

A. Prepare Low Ionic Strength Solutions

The low ionic solution strength is 5mM: 1mM Ca^{2+}, 2mM Na^{+} and 4mM Cl^{-}.

a. Add a small amount of lab water to 1000 mL volumetric flask
b. Add 0.11098g of $CaCl_2$ to the volumetric flask
c. Add 0.11688g of NaCl to the volumetric flask
d. Fill the volumetric flask to 1000 mL
e. Cover the flask and slowly shake until $CaCl_2$ and NaCl have dissolved
f. Transfer the solutions to a 2000 mL beaker for pH adjustment and label low ionic strength

B. Prepare High Ionic Strength Solutions

The high ionic solution strength is 34 mM: 4.8 mM Ca^{2+}, 19.5 Na^{+} and 29.1 mM Cl^{-}.

a. Add a small amount of lab water to a 1000 mL volumetric flask
b. Add 0.5377g of $CaCl_2$ to the volumetric flask
c. Add 1.139g of NaCl to the volumetric flask
d. Fill the volumetric flask to 1000 mL
e. Cover the Flask and slowly shake until $CaCl_2$ and NaCl have dissolved
f. Transfer the solution to a 2000 mL beaker for pH adjustment and label high ionic strength

C. Adjusting pH

Carry this procedure out for pH 2.5, 4, 6, 8, 10

a. Place a 2000 mL low ionic strength beaker on the stir plate
b. Insert a stir bar into the solution and turn the stir plate on
c. Insert a clean pH probe to measure the pH of the solution
d. Raise pH by adding HCL or lower pH by adding NaOH in small .01-.1 increments until pH is stable at desired pH
e. Transfer the solution to two 1000 mL autoclave bottles, 500 mL in each bottle, and label the bottles.

f. Autoclave the bottles.

Solutions for Altering pH

A. Sodium Hydroxide, NaOH

Use Fisher Chemical SS254-4 certified 50 percent w/w to make the 1 normality NaOH solution.

a. Add 25 mL lab water to a 250 mL amber bottle

b. Slowly Add 5.81mL NaOH stock

c. Add 69.19 mL lab water

d. Cover and slowly turn the bottle to mix the solution

e. Label the bottle and store for use

B. Hydrochloric Acid

Use Fish Chemcial A5085SK2-L 37% HCl to make one normality solution of HCl

a. Add 25 mL lab water to a 250 mL amber bottle

b. Add 8.212 mL HCl

c. Add 66. 788 mL lab water

d. Cover and slowly turn the bottle to mix the solution

e. Label the bottle and store for use

A.7 SAND PREPARATION

Sand Preparation

Part 1: Detergent Washing

A. Wash Sand

1. In a 2 L Pyrex beaker add:
    a. Approximately 500 mL of Sand (685g)
    b. 1000 mL of 10 percent soap solution
2. Wash sand in soap solution for 3 hours, stirring in 30 minute increments
3. Pour the soap solution out of the beaker
4. Rinse the sand in lab water until there are no bubbles
5. Rinse the sand five extra times after the bubbles are gone and drain the water

B. Sterilize Sand

1. Add lab water to the sand until the water level reaches 1600 mL
2. Autoclave the sand and lab water
3. Decant the sand and pour it into an autoclaved porcelain dish.
4. Dry the sand in an oven at 80°C overnight

C. Storing Sand

1. Pour dried sand into an autoclaved 1000 mL beaker
2. Cover the beaker with aluminum foil and store until use

Part 2: Nitric Acid/Sodium Hydroxide Washing

A. Prepare Sand

** Observe Safety Protocol**

a. Pour washed/autoclaved/dried sand into a 2L beaker
b. Fill beaker to 1500 mL with 70 percent nitric acid.
c. Cover the beaker with a watch glass
d. Place the beaker on the heat plate
e. Suspend a thermometer in the nitric acid solution approximately an inch above the sand.
f. Hold the thermometer in place with a stand.

B. Boiling Sand

a. Start heating at 250°C

b. Heat until solution is 83°C, occasionally stirring.

c. Slowly begin to reduce the temperature while maintaining solution temperature of 83°C minimum. (A final temp of 220°C held then solution temperature at 83°C)

d. Boil the solution at 83°C for 24 hours, stirring every 4 hours.

C. Cleaning Sand

a. Dispose of the nitric acid in the appropriate hazardous waste bottle

b. Fill the beaker to 1500 mL with lab water and stir

c. Dump lab water into nitric acid hazardous waste bottle.

d. Repeat steps a-c until the pH of the lab water is above pH 3

e. Decant the sand, inserting solution into a hazardous waste bottle.

f. Fill the beaker to 1500 mL with lab water and place it on the hot plate.

g. Boil the sand/lab water solution at 200°C for 2 hours.

h. Decant the sand, pouring the solution into a hazardous waste bottle.

i. Transfer the sand into a porcelain dish

j. Dry the sand in the oven at 80°C overnight.

k. Transfer the sand back into a 2L beaker

l. Rinse the sand with lab water

m. Decant the sand

n. Rinse sand with 0.1 N NaOH solution, place on shaker plate and let sit for minimum of 12 hours

o. Fill the beaker to 1500 mL with lab water and let sit overnight.

p. Decant the sand

q. Rinse the sand with lab water

r. Test the pH of the water in the beaker.

s. Repeat the drying, rinsing, testing and boiling procedure until the water in the beaker reaches a pH greater than 6 and less than 10.

Recipes

- 10 percent Soap Solution

  100 mL of MPbio ES7X Phosphate Free soap

  900 mL Labwater

A.8 MICROBIAL ADHESION TO HYDROCARBONS

Standard Operating Procedure:
Microbial Adhesion to Hydrocarbons

Utilize single layer procedure SOP for coliphage enumerations and use ionic strength SOP for solutions

Hydrophobicity Colloid Methodology

1. Make dilutions with corresponding Ionic Solution (using ionic strength SOP) with Phage
2. Suspend colloids in ionic solution. 1mL of the phage sample to 9mL Ionic Solution.
3. Vortex vigorously to break aggregates
4. Transfer 4 mL colloidal suspension to a glass rounded bottom test tube
5. Add 1 mL of dodecane to the colloidal suspension
6. Vortex the solution for 2 minutes
7. Leave solution undisturbed for 15 minutes at room temp. This creates a phase separation with a emulsion layer on top and a aqueous layer on the bottom.
8. Extract 1mL from the aqueous layer with pipette

Make Sample Dilutions

1. Add 1 mL to 9 mL PBS for 10 fold dilution. Therefore, this is designated as the 10^{-1} dilution)
2. OR 0.1 mL of 10^{-1} dilution in 9.9 mL PBS to make 10^{-3} to make 100 fold dilution
3. 3 plates per dilution of each sample
4. Enumerate coliphages using standard procedure

Count Plates

3. Count and record the number of plaques on each plate after 18-24 hours
4. Calculate % Recovery for Matrix Spikes and OPR:

   % Recovery = (# PFU in Spiked - #PFU in Non-Spiked) / # Coliphages Spiked

5. Calculate hydrophobicity % = $\frac{Acont-Amath}{Acont} * 100$

A.8 ZETASIZER AND ELECTROPHORETIC MOBILITY

Standard Operating Procedure:
ZetaSizer and Electrophoretic Mobility

1. Prepare samples
    a. Prepare 3 glass round bottomed test tubes of each ionic solution.
    b. Add sample to test tube
        i. Bacteriophage – 1 mL to 9mL
            1. Place test tubes on ice
        ii. Sand – 10 grams to 20 mL
        iii. Microsphere
2. Prepare ZetaSizer Capillary Cell
    a. Use two 10 mL sterilized syringes to flush the cell with ethanol
        i. Flush a minimum of 10 times and repeat two times using new water each time
    b. Use two 10 mL sterilized syringes to flush the cell with Lab Water
        i. Flush a minimum of 10 times and repeat two times using new water each time
3. Fill Capillary Cell
    a. Fill a 5 mL leur lock syringe with sample.
    b. Place 0.2 um syringe filter onto the syringe.
    c. Invert the cell.
    d. Slowly inject the sample from the syringe through the filter and into the cell.
    e. Fill the "U" tube to just over half way.
    f. Check that no air bubbles form in the cell and tap the cell gently to dislodge any that have formed.
    g. Turn the cell upright and continue injecting slowly until the liquid reaches the fill area.
    h. Check again for air bubbles.
    i. Check that the electrodes are completely immersed.
    j. Remove the syringe and fit one stopper into the cell firmly and the other stopper in loosely.
    k. Wipe gently with Kimwipe.
    l. When inserting the cell into the Zetasizer, ensure that the Malvern logo faces toward the front of the instrument.

4. Run Program for Electrophoretic Mobility and Size
    a. Run duplicate samples with 5 runs with a minimum of 10 trials

A.9 DYNAMIC LIGHT SCATTERING

Standard Operating Procedure
Dynamic Light Scattering

A. Prepare Samples for Dynamic Light Scattering

1. Determine sample type Bacteria Only or Bacteria and Virus
2. If bacteria only,
    a. Add 8.9 mL of Phase PBS
    b. Add 0.1 mL antibiotic
    c. Add 1.0 mL bacteria
3. If bacteria and virus,
    a. Add 8.8 mL of Phage PBS
    b. Add 0.1 mL phage
    c. Add 0.1 antibiotic
    d. Add 1.0 mL Famp
4. Please in beaker of water with an ice pack
5. Prepare cuvette and set up for DLS
6. If bacteria with virus, add 0.1 mL MS2 immediately prior to running the DLS
7. Run sample according to pre-determined duration

Samples were measured in Spectrosil® Quartz cuvettes, 12.5 mm (width), 12.5 mm (length), and 45 mm (height) (Vernier Software & Technology, Beaverton, OR). The room temperature was maintained at 23 °C, unless otherwise noted.

B. Utilize ARGOS Methodology

a. Place Cuvette in holder
b. Adjust setting according to particle size
c. Review the scattering output to ensure no glare and appropriate initial intensity
d. Run LabView Program "Taking Photos"
    i. Complete inputs for settings (time, number of photos, etc.)

C. Analyze Photos

a. Analyze photos using LabView Program "Main Analysis Program"
b. Transfer output to Excel

APPENDIX B - DATA

All data can be provided electronically. The data is itemized here and noted where not included.

Electronic data can be supplied by emailing:

Abigail Charest, WIT, charesta@wit.edu

Or

Jeanine Plummer, WPI, jplummer@wpi.edu

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B.4 Zeta Potential Data

- Table – Sand – Zeta Potentials of Sand and Acid-washed Sand
- Table – Nano – Zeta Potentials of Uncoated and Protein Coated Nanoparticles
- Table – Phage – Zeta Potentials of MS2 and ΦX-174

Data Files

- Excel 2013 file – Zeta Potentials
- SPSS statistic 17.0 file – Zeta Potentials

B.5 DLS Bacteria Analysis

Data Files

- Excel 2013 file –Data DLS F-amp
- Data F-amp 6 5 14 - Photos – .tiff files
- Excel 2013 file – Data DLS F-amp and MS2
- Data F-amp and MS2 6 14 14 - Photos – .tiff files

B.6 DLS Nanoparticle Analysis

Data Files

- Excel 2013 file – Data DLS Nanoparticles
    - Sheets
        - Uncoated
        - Coated
        - MS2
        - ΦX-174
- Data MS2 7 30 14 - Photos – .tiff files
- Data Φ X 174 7 30 14- Photos – .tiff files
- Data Uncoated Nanoparticles7 30 14 - Photos – .tiff files
- Data Coated Nanoparticle 7 31 14 - Photos– .tiff files

APPENDIX C - SPSS CORRELATION RESULTS

All data can be provided electronically. The data is itemized here and noted where not included.

Electronic data can be supplied by emailing:

Abigail Charest, WIT, charesta@wit.edu

Or

Jeanine Plummer, WPI, jplummer@wpi.edu

Data Files

- Excel 2013 file – Final Full Scale Correlations

  SPSS statistic 17.0 file Final Full Scale Correlations

- Excel 2013 file – Zeta Potential Totals

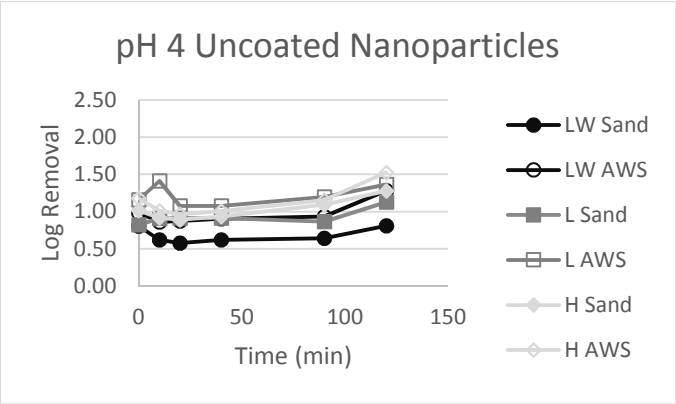
  SPSS statistic 17.0 file Zeta Potential Totals

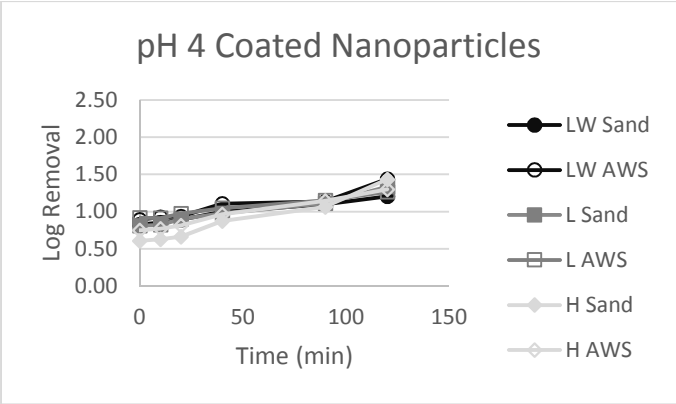
Drinking Water Correlations											
			Coliform (CFU per 100 mL or g)	***E. coli* (CFU per 100 mL or g)**	Enterococci **(CFU per 100 mL)**	**Male-Specific Coliphage (PFU per 100 mL or g)**	**Somatic Coliphage (PFU per 100 mL or g)**	**Microviradae (genomic copies per mL)**	**TTV (genomic copies per mL)**	**Adenovirus (genomic copies per mL)**	**Polyomavirus (genomic copies per mL)**
Spearman's rho	Coliform (CFU per 100 mL or g)	Correlation Coefficient	1.000	1.000**	–	.956**	.746**	–	–	–	–
		Sig. (2-tailed)	–	–	–	.000	.000	–	–	–	–
		N	20	20	0	20	20	0	0	0	0
	E. coli (CFU per 100 mL or g)	Correlation Coefficient	1.000**	1.000	–	.956**	.746**	–	–	–	–
		Sig. (2-tailed)	–	–	–	.000	.000	–	–	–	–
		N	20	20	0	20	20	0	0	0	0
	Enterococci (CFU per 100 mL)	Correlation Coefficient	–	–	–	–	–	–	–	–	–
		Sig. (2-tailed)	–	–	–	–	–	–	–	–	–
		N	0	0	0	0	0	0	0	0	0
	Male-Specific Coliphage (PFU per 100 mL or g)	Correlation Coefficient	.956**	.956**	–	1.000	.693**	–	–	–	–
		Sig. (2-tailed)	.000	.000	–	–	.001	–	–	–	–
		N	20	20	0	20	20	0	0	0	0
	Somatic Coliphage (PFU per 100 mL or g)	Correlation Coefficient	.746**	.746**	–	.693**	1.000	–	–	–	–
		Sig. (2-tailed)	.000	.000	–	.001	–	–	–	–	–

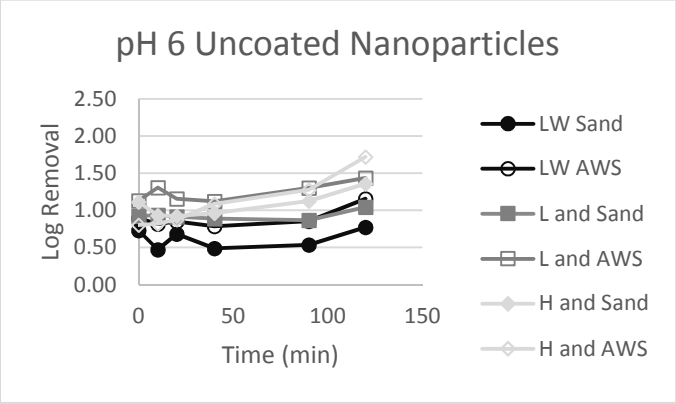
Drinking Water Correlations											
			Coliform (CFU per 100 mL or g)	***E. coli* (CFU per 100 mL or g)**	Enterococci **(CFU per 100 mL)**	**Male-Specific Coliphage (PFU per 100 mL or g)**	**Somatic Coliphage (PFU per 100 mL or g)**	**Microviradae (genomic copies per mL)**	**TTV (genomic copies per mL)**	**Adenovirus (genomic copies per mL)**	**Polyomavirus (genomic copies per mL)**
		N	20	20	0	20	20	0	0	0	0
	Microviradae (genomic copies per mL)	Correlation Coefficient	–	–	–	–	–	–	–	–	–
		Sig. (2-tailed)	–	–	–	–	–	–	–	–	–
		N	0	0	0	0	0	0	0	0	0
	TTV (genomic copies per mL)	Correlation Coefficient	–	–	–	–	–	–	–	–	–
		Sig. (2-tailed)	–	–	–	–	–	–	–	–	–.
		N	0	0	0	0	0	0	0	0	0
	Adenovirus (genomic copies per mL)	Correlation Coefficient	–	–	–	–	–	–	–	–	–
		Sig. (2-tailed)	–	–	–	–	–	–	–	–	–
		N	0	0	0	0	0	0	0	0	0
	Polyomavirus (genomic copies per mL)	Correlation Coefficient	–	–	–	–	–	–	–	–	–
		Sig. (2-tailed)	–	–	–	–	–	–	–	–	–
		N	0	0	0	0	0	0	0	0	0

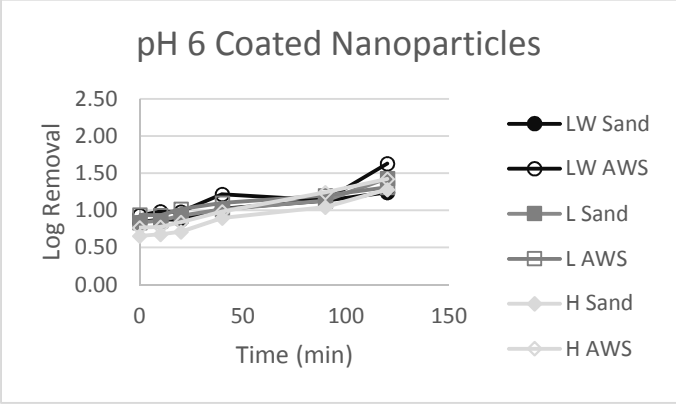
		L_Uncoated	**L_Coated**	**L_MS2**	**L_ΦX174**
L_Uncoated	Spearman Correlation	1.000	-.169	-.241	-.263
	Sig. (2-tailed)	.	.477	.306	.262
	N	20	20	20	20
L_Coated	Spearman Correlation	-.169	1.000	.681**	.565**
	Sig. (2-tailed)	.477	.	.001	.009
	N	20	20	20	20
L_MS2	Spearman Correlation	-.241	.681**	1.000	.716**
	Sig. (2-tailed)	.306	.001	.	.000
	N	20	20	20	20
L_ΦX174	Spearman Correlation	-.263	.565**	.716**	1.000
	Sig. (2-tailed)	.262	.009	.000	.
	N	20	20	20	20

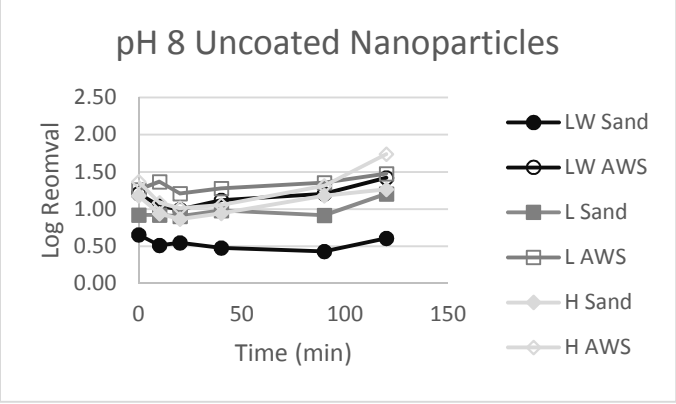
APPENDIX D - BATCH ANALYSIS TABLES - NANOPARTICLES BY PH

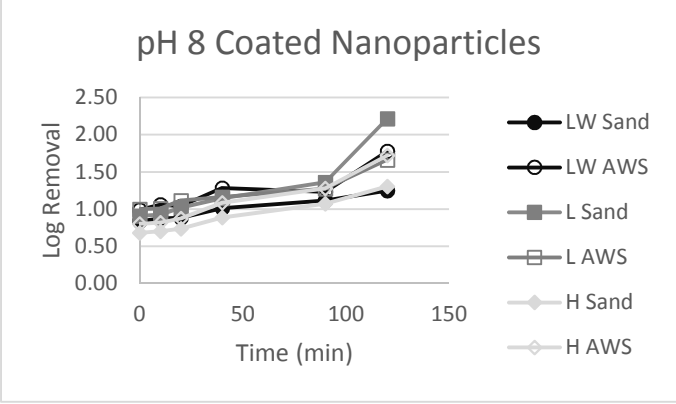




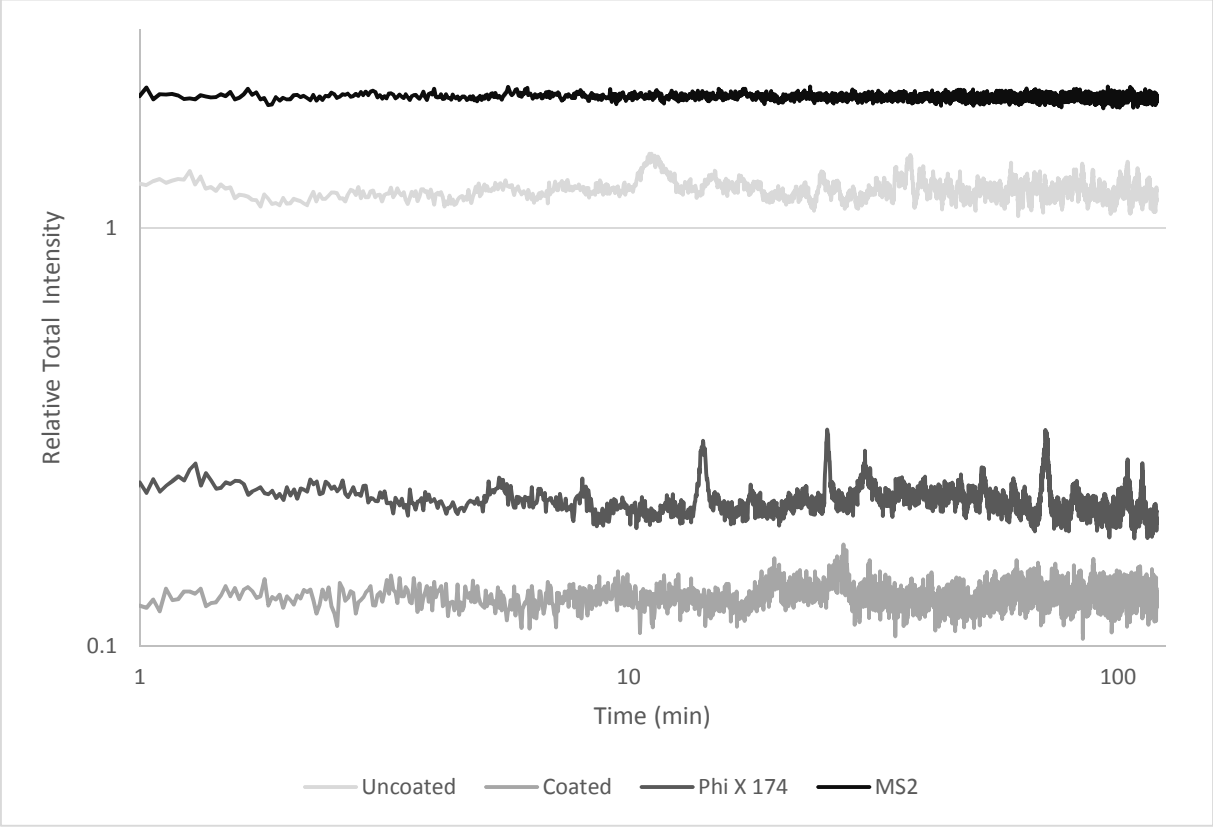




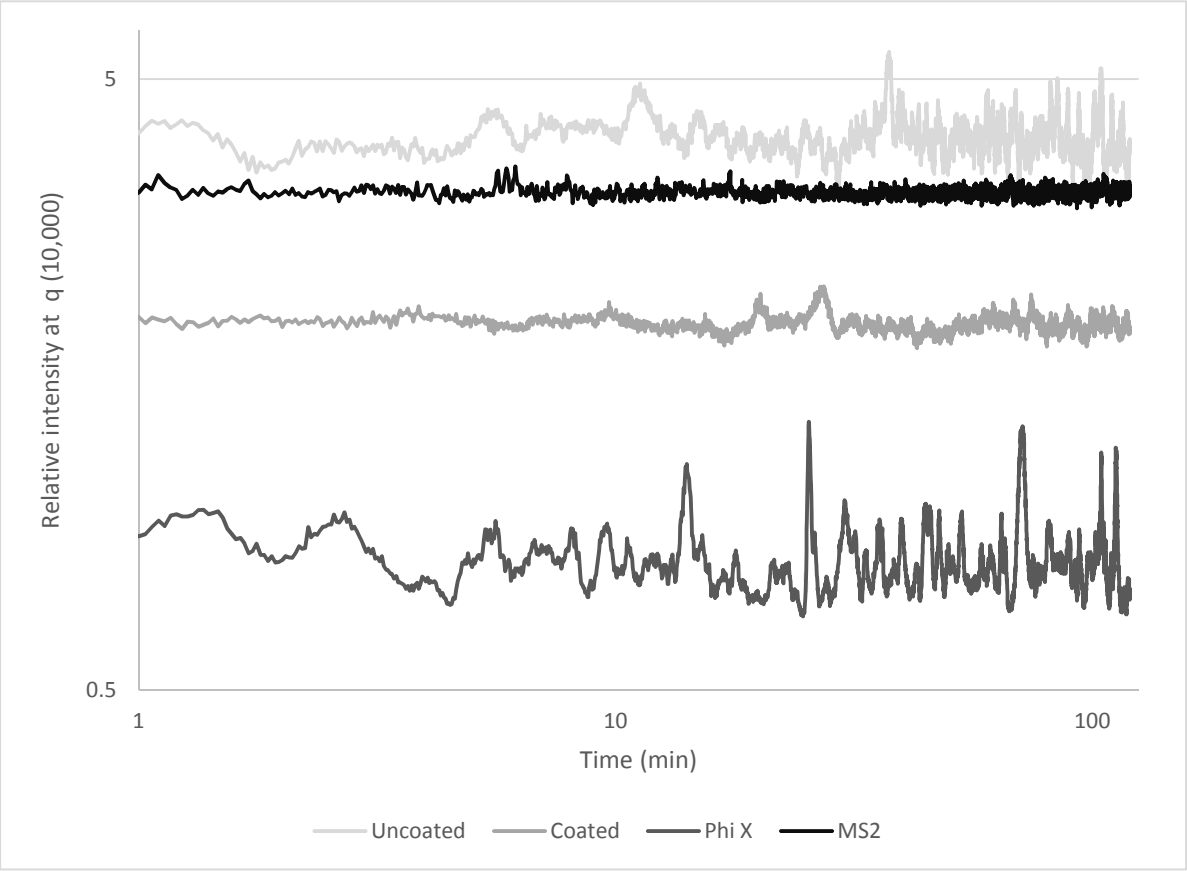




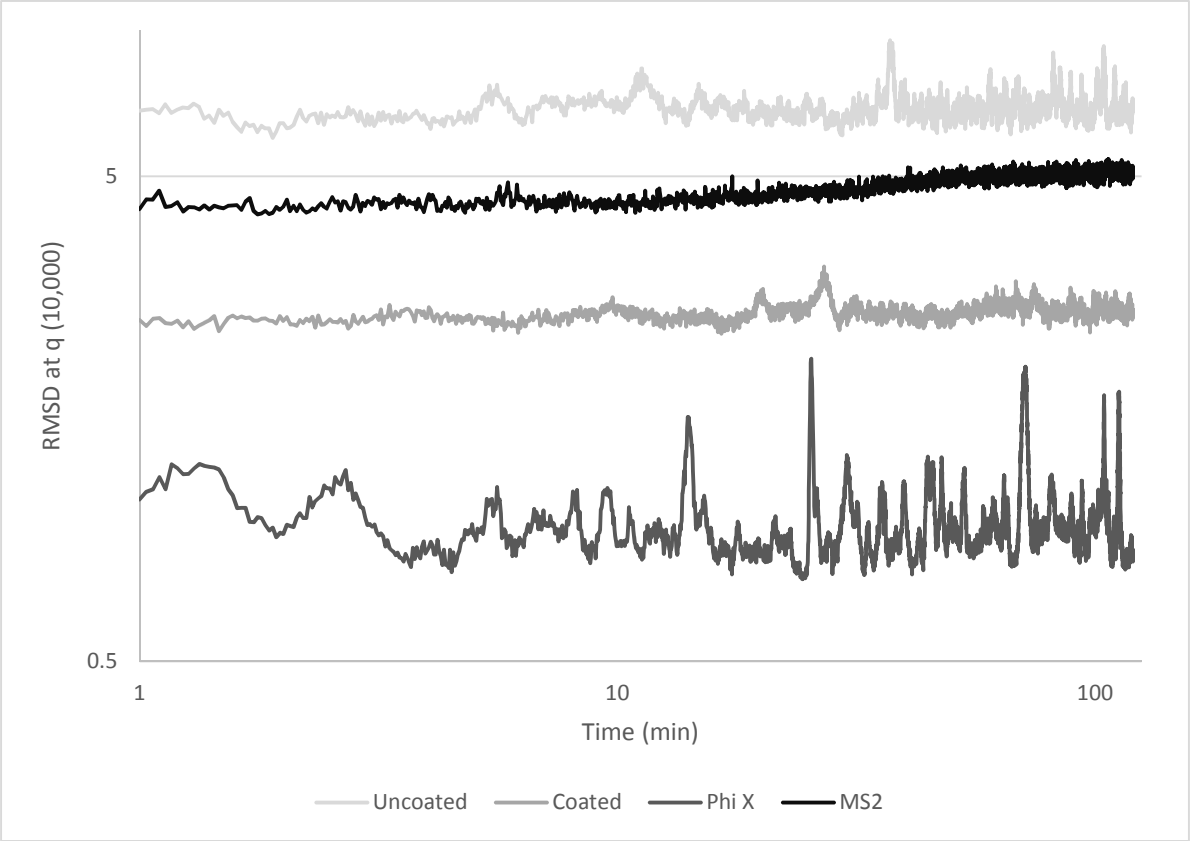
APPENDIX E - NANOPARTICLE DATA LOG-LOG SCALE



Appendix Figure E-1: Nanoparticle Relative Total Intensity



Appendix Figure E.2: Nanoparticle Relative Intensity at q (10,000)



Appendix Figure E.3: Nanoparticle RMSD at q (10,000)