EVALUATION AND APPLICATION OF ENTERIC VIRUS RECOVERY TECHNIQUES FOR ENVIRONMENTAL WATER MONTIORING

by

ANNIE REBECCA PHILLIPS

(Under the Direction of Jennifer L. Cannon)

ABSTRACT

Virus recovery techniques must preserve enteric viruses present in environmental waters and remove co-concentrated PCR inhibitory compounds. The aim of this study was to evaluate and apply virus recovery techniques for use in environmental water monitoring. For each technique, the percent recovery was determined for multiple enteric viral pathogens and process control viruses. Poly-ethylene glycol precipitation was shown to be the main source of viral loss, with percent recoveries ranging from 0.0% to 4.7%. Chloroform extraction and ultrafiltration had percent recoveries ranging from 277.2% to 0.6% and 62.5% to 9.7% respectively. The ultrafiltration technique was the most effective at removing PCR inhibitory compounds. Stream samples from Gwinnett County, Georgia were concentrated using the combined methods to test for multiple human enteric viral pathogens, with one sample testing positive for Sapovirus. This study highlights the need for more efficient recovery techniques to avoid underestimating possible public health risks.

INDEX WORDS: environmental water monitoring, enteric virus, polyethylene glycol precipitation, process control virus, real-time PCR, sapovirus

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ANNIE REBECCA PHILLIPS

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ANNIE REBECCA PHILLIPS

Major Professor:	Jennifer L. Cannon
Committee:	Mussie Y. Habteselassie
	Xiangyu Deng

Electronic Version Approved:

Suzanne Barbour
Dean of the Graduate School
The University of Georgia
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CHAPTER 1

INTRODUCTION

Surface and groundwater are both capable of being contaminated with a wide array of chemical and microbial pollutants, including human bacterial and viral pathogens (Tran et al. 2015). During infection, human enteric viruses are shed in high numbers in human feces and can enter environmental water through the discharge of sewage or from other sewage contaminated water (Atmar et al. 2008; Bosch 2010). Due to the small infectious dose of enteric virus pathogens, even the presence of low concentrations in the environment can pose a public health risk (Teunis et al 2008). Enteric viruses are host-specific, and are not capable of replicating outside of the host in the environment (Gibson 2013). This means that detecting human pathogenic viruses in the environment is a clear indication of human fecal pollution.

Detecting enteric viruses in environmental water samples can be challenging due to the low concentration of virus particles present. To increase the chances of detection, large volumes (multiple liters) of water are collected and then subjected to concentration methods to reduce the volume to an amount appropriate for screening via real-time reverse transcriptase polymerase chain reaction (RT-PCR) (Dalla Vecchia et al. 2015). To date, there is not a clear consensus as to which concentration method is the best for environmental samples, with each method having their own advantages and disadvantages (Dalla Vecchia et al. 2015). In this study, the virus percent recovery will be determined for multiple pathogens and process control viruses for a set of concentration and purification techniques both individually and in succession to assess their

applicability to environmental monitoring for enteric viruses. These methods will be used to prepare a series of environmental stream samples taken from Gwinnett County, GA for screening via real time RT-PCR for numerous human enteric viral pathogens and a fecal-indicator bacteriophage.

The viruses selected for the study are the most commonly implicated pathogens in food and water gastroenteritis outbreaks and include, GII.4 and GI.1 Norovirus, Hepatitus A Virus, Enteroviruses, Group A Rotavirus, Adenovirus Type 40 and 41, Sapovirus, and Astrovirus (Gibson 2013). MS2 bacteriophage was included as a fecal indicator. The study also includes two commonly used process control viruses; Murine Norovirus and Mengovirus.

On-site wastewater treatment systems (OWTS) are a commonly used method in urban and rural residential areas to eliminate microbial pathogens and excess nutrients from household effluent without transferal to a municipal water treatment facility (Ahmed et al. 2005). OWTS are also one of the most common sources of groundwater contamination in the United States (Nicosia et al. 2000). To ensure the treatment system is sufficiently removing pathogens, government agencies have implemented required minimum setback distances between septic tanks and drinking water wells (Yates 1989) as well as lakes, streams and wetlands. However, studies have shown these distances may not be sufficient to remove viral pathogens present in the effluent (Gerba 1984; Keswick and Gerba 1980). One of the aims of this study is to use the presence or absence of viral pathogens as an indicator of influence OWTS have on environmental streams. Five unique streams in Gwinnett Co. GA, two residing in low-density OWTS areas and three residing in high-density OWTS areas, were screened for six of the most common enteric viral pathogens and a fecal indicator bacteriophage. By comparing the amount

of viruses found in low-density and high-density locations, we hope to elucidate the relationship OWTS have with environmental water quality.

CHAPTER 2

LITERATURE REVIEW

Enteric Viruses

Historically human enteric viruses are have been frequently implicated in waterborne gastroenteritis outbreaks in both industrialized and developing parts of the world (Okoh et al. 2010). Enteric viruses enter environmental water through the discharge of sewage or from other sewage contaminated water (Bosch 2010). Enteric viruses are host-specific, and are not capable of replicating outside of the host in the environment (Gibson 2013). This means that detecting human pathogenic viruses in the environment is a clear indication of human fecal pollution. The most commonly implicated viruses in waterborne outbreaks include Noroviruses, Sapovirus, Rotavirus, Enteroviruses, Hepatitis A virus, and Astrovirus (Gibson 2013).

Noroviruses

Human Norovirus (NoV) is currently recognized as the leading cause of foodborne illness in the United States, causing 5.4 million illnesses each year (Hall et al. 2011). Not limited to foodborne infections, NoV causes 21 million cases of acute gastroenteritis annually in the United States (Hall et al. 2011). The high rates of NoV infections are predominantly due to its high infectivity and high levels of virus shedding during illness (Thornton et al. 2004). During infection, a host can shed up to 10^{11} viruses per g of feces and 10^{7} viruses per 30 ml of vomitus (Atmar et al. 2008), and it has been observed that as few as 18 virions are sufficient to cause infection (Teunis et al. 2008).

NoV, previously called Norwalk-like virus, is described as a small, non-enveloped virus, with virions measuring 27-35 nm in diameter. Its genome consists of 7–7.5 kilobases of poly-adenylated, positive sense, single-stranded RNA. NoV belongs to the genus *Norovirus* and to the family *Caliciviridae*. The genus has been divided into six genogroups (de Graaf et al. 2016). Humans are affected by strains from genogroups I, II, and IV, with strain GII.4 causing the majority of outbreaks, however, a newly emerged genotype, GII.P17–GII.17, has recently become the predominant strain in some parts of Asia (de Graaf et al. 2016).

Symptoms of NoV infection include abdominal cramps with or without nausea, vomiting, and diarrhea. The average incubation period is 12–48 hours. Symptoms of illness usually resolve within 12–72 hours, however, hosts may continue to shed virus asymptomatically for 22 days or longer (Thornton et al. 2004).

NoV outbreaks can occur due to foodborne transmission, person to person contact, as well as both recreational and drinking water contamination. Outbreaks have been associated with drinking water sources including those from private wells and community municipal water systems (Waarbeek et al. 2010). In August 1980, a NoV outbreak occurred in the Lindale community of Rome, Georgia, affecting approximately 1,500 persons. The source of the outbreak was traced to the one of the community's municipal water systems (Kaplan et al. 1982). Outbreaks have also been documented to originate from contaminated surface water such as lakes, swimming pools and recreational fountains (Waarbeek et al. 2010). A survey of NoV outbreaks revealed that 6% of all outbreaks occur due to transmission by contaminated water (Fankhauser et al. 1998). GI NoV strains are more often associated with outbreaks due to waterborne transmission than GII NoV strains (Lysén et al. 2009).

Sapoviruses

Sapoviruses (SaV) are another type of enteric virus that can cause acute gastroenteritis in humans and other mammals. They are very similar to NoV in both physicality and clinical manifestation. They are of the genus *Sapovirus*, and are from the family *Caliciviridae*, of which NoV are also members. The virion particles are non-enveloped, and range from 30-38 nm in diameter (Oka et al. 2005). The SaV genome is comprised of positive-sense single-stranded RNA that is 7.1-7.7 kilobases in length. The symptoms of SaV infection in humans are identical to those of NoV infection, and laboratory testing is required to distinguish between the two infection types, although illness due to SaV infection is often milder than that of NoV (Oka et al. 2005).

Outbreaks of the virus typically occur in semi-closed settings, and have been reported as originating from person to person transmission in a variety of locations, such as child day care centers, schools, colleges, hospitals, nursing homes, restaurants, hotels, wedding halls, and ships (Oka et al. 2015). SaV has been implicated in numerous outbreaks in long-term care facilities for the elderly where they can cause significant illness and death (Lee et al. 2012). In a study on all reported gastroenteritis outbreaks that occurred from 2002-2009 in Minnesota and Oregon, 21 (23%) of the 93 outbreaks in which NoV was not implicated were found to be positive for SaV (Lee et al. 2012). From these outbreaks, one hospitalization and one death occurred in patients who tested positive for SaV.

SaV has also been implicated in outbreaks from food or waterborne transmission, and are usually caused by infected food handlers shedding the virus (Oka et al. 2015). SaV is known to be present in environmental waters, especially during the peak outbreak season in the winter. A study in Japan found viral concentrations up to 100 genome copies (GC) per liter of river water

and viral concentrations ranging from 2.8 $x10^3$ - 1.3 $x10^5$ GC per liter in wastewater treatment plant influent. It should also be noted that 58% of the wastewater treatment plant effluent samples tested positive for SaV genetic material, with concentrations nearly reaching 1000 GC per liter in some winter and spring months (Haramoto et al. 2008).

Astroviruses

Similar to NoV and SaV, Astroviruses (AsV) are also small and non-enveloped. The virion particle size ranges from 28 to 30 nm in diameter, and they have a 6.8-7.9 kilobase genome consisting of positive sense, single stranded RNA (De Benedictis et al. 2011). Human AsV infections occur most commonly in children and infants, and studies have suggested that they are the second most common cause of gastroenteritis in children after rotavirus (De Benedictis et al. 2011). The primary symptom of AsV infection is watery diarrhea that lasts 2-3 days. Vomiting, fever, anorexia, and abdominal pain may also be associated with the infection (Bosch et al. 2014).

AsV belongs to the family *Astroviridae* and have been classified into two genera, Mamastroviruses, consisting of mammalian AsV, and Avastroviruses, consisting of avian AsV (Mendez and Arias 2007). Until 2008, human AsV infections were thought to be limited to only eight genotypes, HAstV 1-8, which are now referred to as the ‘‘Classic human Astroviruses” (De Benedictis et al. 2011). HAstV 1 is the most prevalent serotype worldwide, followed by HAstV 3 and HAstV 5 (De Grazia et al. 2011; Gabbay et al. 2007).

AsV transmission occurs via the fecal-oral route, and outbreaks can occur due to various sources. Contaminated food and water, as well as contaminated fomites, have all been implicated in the spread of the virus (Bosch et al. 2014). Both drinking and recreational water can become contaminated with AsV, as they are shed in high numbers and wastewater treatment facilities do

not ensure the complete removal of viral pathogens from treated effluent (Bosch et al. 2014). It should also be noted that chlorine and other commonly employed water disinfectants are ineffective at deactivating the waterborne virus (Bosch et al. 2014). After deposition from the host, it has been reported that the virus can remain infectious in ground water for several months (Espinosa et al. 2008).

Rotavirus

As previously mentioned, RV continues to be a major global cause of childhood diarrhea and a source of infant mortality. Estimations indicate that approximately 39% of all diarrhea deaths in children under 5 years of age are due to RV (Munos et al 2010). RVs are sub-grouped into seven types, named groups A-G (Santos and Hoshino 2005). The most common type affecting humans, group A, is estimated to cause 2 million hospitalizations and 325,000–592,000 deaths per year among children under the age of 5 (Parashar et al. 2003). To date, two live, oral RV vaccines are available internationally, RotaTeq (Merck), a pentavalent human-bovine reassortant vaccine, and Rotarix (GlaxoSmithKline), a monovalent attenuated human RV vaccine. Recently, a second oral human bovine monovalent vaccine has been made available in India (Kollaritsch et al. 2015). It has been demonstrated that these vaccines prevent up to 74% of severe RV episodes, which is used as an indicator of the prevention of RV mortality (Walker and Black 2011). After including RV vaccines in childhood immunization programs, there has been an over 90% reduction of in the number of RV associated hospitalizations in both industrialized and resource-deprived countries (Kollaritsch et al. 2015). A meta-analysis conducted in 2015 indicated that RV vaccination has already produced a herd immunity effect in children under one year of age in the United States and Latin American countries (Pollard et al. 2015).

RVs are a genus in the family *Reoviridae*. The RV particle is non-enveloped, but is significantly larger than those of NoV and AsV, approximately 75 nm in diameter (Estes and Cohen 1989). The viral genome is comprised of 11 segments of double stranded RNA (Estes and Cohen 1989). The hallmark symptom of rotavirosis is severe diarrhea that, if untreated, can lead to severe dehydration and possibly death (Leclerc et al. 2002). After the incubation period of 4-7 days, the illness presents as diarrhea, in some cases accompanied by vomiting, that lasts for approximately 7 days (Carter 2005).

Water contamination has been implicated in multiple RV outbreaks, including those in the United States. In March 1981, a community waterborne gastroenteritis outbreak occurred in Eagle-Vail, Colorado, which was determined to be caused by RV infection (Hopkins et al. 1984). Waterborne outbreaks can cause persons of all ages to be infected and exhibit severe symptoms (ST Timenetsky et al. 1996). Increased observance of symptoms in adults is assumed to be caused by the high virus load, which is often present in water sources contaminated with sewage. This was observed in a rotavirus outbreak associated with drinking water in Finland, which resulted in severe cases in both young and older children (Räsänen et al. 2010).

Enteroviruses

Enteroviruses (EV), a collective term, corresponds to the genus *Enterovirus*, which contains multiple viral species. They are members of the family *Picornaviridae*, and the genus contains ten species, which each have their own subgroups. Seven of the species; Human EV (HEV)-A, HEV-B, HEV-C, HEV-D, Human Rhinovirus A (HRV-A), HRV-B and HRV-C are known to infect humans, and the other three species infect monkeys (Smura 2011). All virus types are non-enveloped, have a viral particle around 27 nm in diameter, and have a positive

sense, single stranded, nonsegmented RNA genome, approximately 7.5 kilobases in length (Okoh et al. 2010).

EV infections can manifest very differently depending on the virus subgroup. The infection typically occurs in the epithelial cells of the host respiratory or gastrointestinal tract. However, secondary infection can result in a wide variety of illnesses, such as acute hemorrhagic conjunctivitis, aseptic meningitis, acute flaccid paralysis, myocarditis and neonatal sepsis-like disease (Okoh et al. 2010). Poliovirus is considered the prototype for the genus, and was the first virus proven to be subject to foodborne transmission, specifically by contaminated water and unpasteurized milk (Sair et al. 2002). Although they are associated with contaminated food and water, foodborne disease outbreaks caused by coxsackie and echoviruses are considered rare (Sair et al. 2002).

The World Health Assembly launched The Global Polio Eradication Initiative in 1988, with oral poliovirus vaccine (OPV) as the primary tool for efforts to eradicate the virus. Subsequent progress towards eradication has been remarkable, including eradication of one of the three strains of PV, wild type PV 2 in 1999. In comparison to 350,000 cases of polio in 1988, 1352 cases of polio were reported in 2010, with a further reduction to 650 cases in 2011 (Abzug 2014). In January 2012, it was declared that India was no longer polio-endemic, after having had no wild-type disease in the preceding year (Abzug 2014). A small number of countries still struggle with ongoing endemic disease including Afghanistan, Pakistan, and Nigeria. Recently, importations led to reestablished transmission of the disease in some neighboring countries, particularly on the African continent (Abzug 2014).

Hepatitis A Virus

Hepatitis A Virus (HAV) is another member of the *Picornaviridae* family, and belongs to the *Hepatovirus* genus (Sánchez 2015). Similar to the other viruses in the family, their virions are small (27-32 nm) in diameter and non-enveloped. Their genome consists of positive-sense single-stranded RNA. Currently there is only one serotype of HAV, but this serotype has been divided into six genotypes based on differences in the VP1X2A region of the genome (Sánchez 2015). Genotypes I, II, and III are known to infect humans, while types IV, V, and VI are infectious to simians (Sánchez 2015).

HAV infections account for approximately half of all cases of hepatitis globally (Sánchez 2015). The World Health Organization reports that there are 1.4 million new cases of HAV worldwide annually (WHO 2012). Infection occurs via the fecal-oral route with possible methods of transmission including person-person contact, ingestion of contaminated food or water, or contact with contaminated fomites (Lemon 1997). Symptoms of infection appear gradually, and include loss of appetite, nausea, vomiting, headache and fever (Sánchez 2015). In 1-2 weeks post the onset of initial symptoms, jaundice occurs, but with no associated chronic illness (Sánchez, 2015). The duration of illness can range from a few weeks to several months. Illness associated with infection is typically more severe in adults than children, who may present asymptomatically or subclinically (Sánchez 2015). However, overall mortality rates for the disease are only 0.1-0.3%, and 1.8% in the elderly (ECDC 2014).

Vaccines for HAV became commercially available in the 1990s, and include two types, inactivated and live attenuated (Ott et al. 2014). Currently, four inactivated monovalent HAV vaccines are commercially available: Havrix®, Vaqta®, Avaxim®, and Epaxal®(Ott et al 2014). Before the availability of vaccines, 25000 to 35000 cases of acute HAV were reported each year

in the United States (Shapiro et al. 1996) After the implementation of vaccination programs, HAV incidence rates have drastically declined in the United States (Hopkins et al. 2005). In an analysis of nationwide data, HAV declined by much higher proportions than would be expected if the vaccine were only protecting the individual vaccine recipients (Samandari et al. 2004), suggesting that children and adults who did not receive the vaccine are being protected by the effect of herd-immunity.

The incidence of HAV infections are strongly correlated to access to safe water and proper sanitation (Gossner et al. 2014). Infections are most common in areas that have poor hygiene practices and do not properly treat sewage, causing the virus to be endemic (Lemon 1997). In these areas, the majority of residents is infected in early childhood, and therefore acquire immunity that lasts through adulthood (Gossner et al. 2014). However there have been incidences of HAV associated outbreaks in industrialized nations, including the United States. In 1982, an outbreak of hepatitis A occurred in a north Georgia trailer park served by a private well, affecting 16 residents (Bloch et al. 1990). In 1972, an outbreak occurred due to sewage contaminated drinking water in a rural elementary school in Colbert County, Alabama, causing illness in 49 children and 1 adult (Baer et al. 1977).

Mengovirus

Mengovirus (MgV) is also a member of *Picornaviridae* and shares structural characteristics with HAV (Hennechart-Collette et al. 2015). It is a member of the genus Cardiovirus and is a close relative of Columbia SK virus, Mouse Eberfield virus and Encephalomyocarditisvirus (Dick 1949). The natural host is the mouse (Dick 1949). Due to

these attributes, it has gained acceptance as a process control virus for projects involving the detection of Norovirus or HAV in shellfish and food matrices (Hennechart-Collette et al. 2015).

Adenoviruses

Adenoviruses (AdV) belong to the family *Adenoviridae,* which has been divided into two genera, *Mastadenovirus,* containing mammalian Adenoviruses, and *Aviadenovirus,* containing the avian types. Human AdVs are classified into six subgenera, named groups A-F (Mena and Gerba, 2009). The AdV particle is non-enveloped, and relatively large compared to the other enteric viruses, being 70-100 nm in diameter (Mena and Gerba 2009). The viral genome consists of double stranded DNA, and is 34-48 kilobases in size (Okoh et al. 2010).

AdV infections can cause a variety of symptoms and illnesses, depending on the virus type. Illnesses associated with AdV infections include upper and lower respiratory illnesses, conjunctivitis, cystitis and gastroenteritis (Mena and Gerba 2009). Infection by strains Adv40 and Adv41 is associated with gastroenteritis due to consumption of contaminated food or water (Okoh et al. 2010). Most infections are self-limiting and short lived, however, as with most enteric virus infections, the host can continue to shed the virus long after the symptoms of infection have dissipated (Foy 1997). Human AdV is one of the top three leading causes of childhood gastroenteritis globally (Mena and Gerba 2009).

There have been multiple water-related human AdV outbreaks. Outbreaks have occurred from both recreational and drinking water. Multiple outbreaks have been traced back to contact with community swimming pools, including an outbreak in the state of Georgia that caused 72 cases of pharyngoconjunctival fever in 1979 (D'Angelo et al. 1979). The drinking water outbreaks occurred in Europe, however, in all of the outbreaks, multiple viral agents were isolated from the water, and the exact roles of AdV in the spread of illness were unclear (Mena

and Gerba 2009). Due to their long environmental persistence and absence in animal wastewaters and slaughterhouse effluents, human AdVs have been proposed as indicators of viral contamination in environmental waters (Okoh et al. 2010).

MS2 Bacteriophage

Male specific bacteriophage, fraction 2, or MS2, is a bacteriophage virus that infects male Escherichia coli (Golmohammadi et al. 1993). The phage is termed "male specific" due to the fact that they are only able to infect and replicate in the host bacterium if it has a F+, or sex pilus (Miller et al. 1998). The virus particle is 21-30 nm in diameter, and has a single-stranded RNA genome (Miller et al. 1998). Because they infect fecal coliforms such as *E. coli*, bacteriophages such as MS2 have been reported to be present in high numbers in untreated sewage and wastewater, and therefore can be used as indicators for fecal pollution. Furthermore, coliforms only produce the pilus necessary for virus infection when in the temperature range of 30-45° C. This means that the phage cannot multiply outside of its host and is unlikely to reproduce in environmental *E. coli* (Miller et al. 1998), although in tropical regions it is possible. Since it is found in feces and has a similar heat and chlorine resistance as the enteric viruses, the male specific bacteriophage can be an indicator of the possible presence of human viral pathogens in water (Havelaar and Nieuwstad 1985). It should be noted that, unlike the enteric pathogens, MS2 is not human specific, and therefore is not a clear indicator of human fecal pollution.

On-Site Wastewater Treatment Systems

Onsite wastewater treatment systems (OWTS), commonly known as septic systems, are one of the most common sources of groundwater contamination in the United States (Nicosia et al. 2000). OWTS are common in both urban and rural non-sewered residential areas as the sole

method of wastewater treatment (Ahmed et al. 2005) These systems are designed to eliminate microbial pathogens and excess nutrients from household effluent so it can be safely returned to the groundwater without being processed by a municipal water treatment facility (Ahmed et al. 2005).

OWTS are comprised of a tank and a soil absorption field, also referred to as the drainfield. The tank provides preliminary treatment of the household waste by allowing the solids to sediment and the lipid material to form a surface layer. After passing through the tank, the liquid effluent is released to the soil absorption field via one or more perforated pipes, where the bulk of microbial pathogen removal and biological stabilization occurs (Tracy 1989; Ahmed et al. 2005). These processes occur in the vadose zone, or the soil that spans from the ground surface to the underlying water table, as the effluent interacts with the soil and its associated biofilms (O'Luanaigh et al. 2012). The percolation of the system effluent through the vadose zone is most effective at removing pathogens when the flow is unsaturated and the effluent has a long residence time (O'Luanaigh et al. 2012). Factors that can increase the effluent residence time in the vadose zone include presence of moisture deficits, development of a biomat layer, and the soil being well drained (O'Luanaigh et al. 2012).

The biomat associated with the drainfield is integral to system integrity and removal of pathogens. It should be noted that it operates most efficiently at a certain thickness. If the mat is less than a few centimeters thick, it does not sufficiently retain the system effluent or trap microbes. However, if the biomat is too thick or dense, the effluent cannot enter the soil and begins to pool, first underground then spilling out onto the soil surface (Hagedorn et al. 1981). The biomat layer is especially integral to increasing effluent retention in highly permeable soils, such as sand or gravel (O'Luanaigh et al. 2012). To ensure the drainfield is long enough to

sufficiently remove pathogens, government agencies have implemented required minimum setback distances between septic tanks and drinking water wells (Yates 1989) as well as lakes, streams and wetlands. These distances range from 15 to 91 meters, with typical values near the lower end of the range, 15-30 m. However, studies have shown that these distances may not be sufficient. It has been reported that viruses can migrate as far as 1600 m in karst terrain (Gerba 1984) and 400 m in sandy soil (Keswick and Gerba 1980)

It has been observed that there are numerous factors that affect the distance viral particles can travel in soil. The distance is determined by the level of adsorption of the viruses to the soil, and is influenced by surface level characteristics (Schijven and Hassanizadeh 2010). The rate of adsorption is influenced by virus type, soil type, pH, multivalent cations, ionic strength, and the amount of organic matter present in the soil (Schijven and Hassanizadeh 2010). Virus adsorption increases with increased soil clay content, soil cation exchange capacity, and specific surface area, and decreased amounts of organic material (Nicosia et al. 2001). This is due to the fact that organic matter competes with the viral particles for adsorption sites on mineral surfaces (Rao and Melnick 1986). Since the level of adsorption differs with soil type, a setback distance that delivers the necessary level of viral removal in one region may not produce safe water in another region. For example, a clay rich area could operate on shorter distances than an area with sandier soil. Studies have also reported high failure rates in areas that have high densities of OWTS (Jelliffe 1995). In June 2007, 229 patrons and employees of a restaurant in northeastern Wisconsin reported acute gastroenteritis, with 6 people requiring hospitalization (Borchardt et al. 2011). GI.2 NoV was determined to be the etiologic agent, and the outbreak was associated with drinking the restaurant well-water, which was determined to be contaminated from the

restaurants septic-tank. Both the septic treatment system and well were in accordance to state building codes.

Methods for Concentrating Viruses in Environmental Water Samples

Detecting enteric viruses in environmental water samples is always challenging due to the low concentration of viral particles often present in the water. Therefore, to increase the chances of detection, large volumes of water are collected and then subjected to concentration methods to reduce the volume to an amount appropriate for screening via RT-PCR (Dalla Vecchia et al. 2015). The basic criteria for a good concentration method include being technically simple, non-time consuming, cost-effective and appropriate for a wide range of viruses, and providing high viral recovery and a small volume of concentrate (Albinana-Gimenez et al. 2009). Commonly used techniques to concentrate the viral particles include adsorption-elution with charged membranes, flocculation, polyethylene glycol precipitation, ultrafiltration, and ultra-centrifugation (Dalla Vecchia et al. 2015). To date, there is not a clear consensus as to which method is the best for environmental samples, with each method having their own advantages and disadvantages (Dalla Vecchia et al. 2015).

The adsorption-elution method, the most common method for large volumes, involves passing the water through a charged membrane to which the viral particles adsorb, then using the appropriate reagents to elute the particles off the membrane into a smaller volume, increasing the concentration (Cashdollar and Dahling 2006). It has been shown that there is no difference in recovery rates between positively and negatively charged filters, but since the use of negatively charged filters requires pH adjustment of the water, it is a more time consuming method (Rose et al. 1984). The main disadvantage of this method is the variability of recovery rates. It is known

that the rates can be influenced by numerous factors such as differences in pH, salt concentration, and the presence of organic materials or humic compounds (Olszewski et al. 2005). One study utilizing a negatively charged membrane produced generally high recovery rates, but the rates differed between viral species and were also altered by species coupling (Dalla Vecchia et al. 2015). Additionally, it should be noted that the high cost of filters make this method cost-prohibitive for use in routine monitoring (Cashdollar and Dahling 2006).

Flocculation is another commonly used method for large-volume environmental samples. The basis of the method is the entrapment of viral particles in protein flakes, then release of the particles upon dissolution of the protein material (Prata et al. 2012). However, similarly to the adsorption elution method, since the method relies on the electrostatic interaction between the virus and the surface of the protein flakes, variations in water chemistry can affect viral recovery rates and may necessitate sample modification (Prata et al. 2012). Iron, aluminum, and polyelectrolytes have all been used as flocculants to remove viruses from wastewater, with rates of greater than 99% removal (Zhu et al. 2005; Chaudhuri and Engelbrecht 1970; Johnson et al. 1967).

To further increase the viral concentration, passage through the charged filter is often followed by a second concentration step, either ultrafiltration or ultracentrifugation (Schultz et al. 2011). Ultrafiltration concentrates the virus via size exclusion, employing a filter with a pore size ranging from 0.02 to 0.1 um in diameter (Olszewski et al. 2005). Small molecules and ions can easily pass through the membrane, but larger molecules, such as virus particles, are concentrated in the device retentate (Olszewski et al. 2005). Ultracentrifugation employs a strong g-force over a sustained time to pellet out the viruses in the sample (Prata et al. 2012). This method requires

little sample modification and is time efficient, but is not suitable for large volumes of water (Prata et al. 2012).

Polyethylene glycol (PEG) precipitation is a method that utilizes phase separation to concentrate the virus (Lewis and Metcalf 1988). PEG and NaCl are added to the sample and then centrifuged to produce the phase separation (Lewis and Metcalf 1988). The virus particles localize in the PEG pellet, and after supernatant removal, can be re-suspended in a smaller volume of liquid (Lewis and Metcalf 1988).

Use of Process Control Viruses in Detection Studies

Although the inclusion of a concentration step is often necessary when testing large volumes of water, these processing techniques also result in some viral loss (Gentry-Shields and Jaykus 2015). Therefore, it is beneficiary to include a process control virus to estimate the amount of viral loss associated with all techniques employed, including those relating to recovery, concentration, and nucleic acid extraction (Gentry-Shields and Jaykus 2015). Excluding a process control can lead to underestimation of sample pathogen load or the occurrence of false negatives (Gentry-Shields and Jaykus 2015). To determine extraction efficiency, the process control virus is added to the sample at the earliest possible step during sample processing, then quantified from the post-processing product (Hennechart-Collette et al. 2015). In order to be a good candidate for a process control, the virus needs to have morphological and physicochemical properties and environmental persistence similar to those of the target pathogens of the study. This is to ensure they will give the most accurate estimation of the target virus behavior (Hennechart-Collette et al. 2014). They should also be easy to detect,

non-pathogenic to humans, and unlikely to be naturally found in the sample-type being evaluated (Hennechart-Collette et al. 2014).

Mengovirus strain MC0 has been used as a process control for the detection of HAV and Noroviruses in shellfish in several studies (Costafreda et al. 2006; Le Guyader et al. 2009; Uhrbrand et al. 2010). It has also been used as a process control in analyses of drinking water, waste water and sewage sludge, and was included in the development of the Technical Specification ISO/TS 15216 as a process control virus for the testing of bottled water (Amdiouni et al. 2013: Hennechart-Collette et al. 2015).

MNV-1, a murine Norovirus, has also been used as a process control and surrogate organism for human Norovirus and other enteric viruses such as HAV and HEV (Hennechart-Collette et al. 2015). It has been included in studies detecting human Noroviruses and HAV in water, shellfish, fruits, lettuce, and ready to eat foods (Coudray et al. 2013; Hennechart-Collette et al. 2014; Martin-Latil et al. 2012; Stahls et al. 2011a, 2011b). Feline calicivirus (FCV) has also been used as a surrogate organism for human Noroviruses, however, studies have shown MNV-1 to be perform better in stability studies (Cannon et al. 2006).

Use of Amplification Controls in Testing Environmental Water Samples by RT-PCR

Real-time reverse transcription quantitative polymerase chain reaction (RT-qPCR) is a sensitive and specific method commonly used to detect the presence of enteric viruses in environmental water samples (Jothikumar et al. 2009). However, this method can be affected by various inhibitory compounds found in the environment (Murray et al. 2013). Inhibitory compounds present in water samples are co-concentrated with the viral nucleic acids during the extraction process (Murray et al. 2013). Inhibition of DNA amplification results in a positive

shift in the RT-PCR cycle threshold (CT) values or the complete absence of CT values (Murray et al. 2013). This inhibition can result in an underestimation of the number of virus particles present in the sample, and the severity of the corresponding health risk (Murray et al. 2013). If viral concentrations are low, as they commonly are in environmental samples, inhibition may result in the PCR reporting false negatives (Diez-Valcarce et al. 2010).

To determine if inhibition is occurring, an amplification control is utilized, either an external amplification control (EAC) or an internal amplification control (IAC). To perform the external amplification approach, two separate reactions are performed for each sample, one containing just the sample nucleic acids and the other containing the sample and control nucleic acids (Murray et al. 2013). To perform the internal method, the control nucleic acid is amplified simultaneously in the same reaction well as the sample nucleic acid (Diez-Valcarce et al. 2010). IACs can either be endogenous or exogenous to the sample. Endogenous controls are usually a "house keeping gene" that would be present in a sample that also contains the target nucleic acid sequence, but utilizes a different set of primers than those that correspond to the target (Hoffmann et al. 2009). Invariably, it is difficult to select such a gene that would be naturally present in all samples, so the endogenous method is not commonly used for environmental samples (Murray et al. 2013). Exogenous controls are added to the reaction mixture along with the sample nucleic acid, and can be either competitive or noncompetitive (Murray et al. 2013). The distinction is that competitive IACs use the same primers as the target sequence, and noncompetitive IACs require their own primer set (Murray et al. 2013).

The interpretation of all amplification controls in RT-PCR is similar. If the data reports no amplification of the target sequence, but shows expected Cycle Threshold values for the control, then the result is a true negative (Murray et al. 2013). However if there is no

amplification of the target sequence and no amplification of the control, inhibition may be causing the data to report a false negative (Diez-Valcarce et al. 2010). Without the inclusion of a control, it is impossible to distinguish between negative results due to the absence of the target sequence and negative results due to amplification inhibition, making utilization of these controls crucial when testing environmental samples.

CHAPTER 3

MATERIALS AND METHODS

Virus source and propagation

For determining viral loss from Poly-Etheylene Glycol (PEG) precipitation (ppt), stream water was inoculated with a virus cocktail comprised of ~6 log particles of GII.4 Norovirus (GII.4 NoV), Enterovirus 69 (EV-69) (ATCC VR-1077), Murine Norovirus 1 (MNV-1), and Adenovirus 41 (Ad41), ~7 log particles of Mengovirus (MgV), and ~8 log of MS2 phage (ATCC 15597-B1). The GII.4 NoV was strain GII.4- Sydney gifted from Dr. Jan Vinjé at the Centers for Disease Control, Atlanta, GA. Adenovirus stock was type Ad41, gifted from Dr. Dean Erdman at the Centers for Disease Control, Atlanta, GA. MgV stock was from the CeeramTools™ Mengo Extraction Control kit (Ceeram S.A.S, La Chapelle-sur-Erdre, France). MS2 phage was acquired freeze dried from ATCC® and propagated according to distributer's instructions in the recommended host. MNV-1 gifted from Dr. Herbert "Skip" Virgin, at the Washington School of Medicine. MNV-1 was propagated in RAW 264.7 cells (ATCC TIB-71). The Hepatitis A virus (HAV) stock was prepared by propagating HAV (ATCC VR-1402) in FRHK-4 cells (ATCC CRL-1688). Cell culture and virus stock preparation was performed as previously described (Fang et al. 2016). RNA positive controls for real-time RT-PCR testing for each virus assay were comprised of viral RNA extracted from the respective virus stock. RNA extraction procedure was performed as described subsequently with the exceptions of using 100 µL volumes for lysis buffer and sample and using 50 µL volumes of eluent. Rotavirus (RV) RNA

was extracted from strain Wa (ATCC® VR-2018). PCR positive controls for GI.1 NoV consisted of in-house made GI NoV RNA transcript generated as previously described (Afolayan et al. 2016). Sapovirus (SaV) type V transcript and heat treated Human Astrovirus (HuAsV) was gifted from Dr. Jan Vinjé at the Centers for Disease Control, Atlanta, GA. GI and GII NoV internal amplification control (IAC) templates, gifted from Dr. Lee-Ann Jaykus at North Carolina State University, were included in some assays to assess the presence of PCR inhibitors when performing RT-PCR assays.

Experiments to quantify viral loss associated with methods to recover viruses from water

Water collection

For preliminary laboratory experiments to evaluate the virus recovery procedures, stream water was collected from Big Haynes Creek in Grayson, GA at coordinates 33° 52' 4" N 83°59' 7" W on November 24, 2015. A second set of water samples for preliminary virus recovery experiments were collected from the same location on April 29, 2016. During both collection events, stream water was collected in a series of sterile 1 L polypropylene copolymer screw-cap bottles. At the first collection, a total of 30 L was collected, and on the second collection date, 12 L was collected. The bottles were transported in a cooler back to the lab, where they were combined and stored in a 4° C walk-in refrigerator for the duration of the experiments.

Poly-ethylene glycol precipitation

Duplicate stream water samples (850 mL) were transferred to individual sterilized, polypropylene copolymer 1 L centrifuge bottles before 8% PEG 8000 (Omnipur, Calbiochem) and 3M NaCl were added to each. Samples were then shaken on an Innova 2000 platform shaker at 35 rpm, overnight at 4°C to allow the PEG and NaCl to completely dissolve. The liquid was

dispensed into a set of twenty 50-mL centrifuge tubes and centrifuged for 30 minutes at 9000 x *g* at 4°C using a Sorvall™ Legend™ X1R centrifuge (Thermo Fisher). After centrifugation, the supernatant was removed from each tube, sans 1-2 mL, which was used to redistribute each pellet by vortexing. The pellets from each set of twenty tubes were combined into two 50 mL tubes, each with a final volume of 20-30 mL. One sample of each duplicate was then stored at 4°C and designated the "primary only treatment" sample. The other sample of each duplicate was designated as the "primary and secondary treatment" sample and subjected to secondary PEG precipitation by adding 5X PEG 8000 solution, prepared as described in ISO/TS 15216-1 2013, in a volume that equaled 5% of the total sample volume. Samples were then shaken on an Innova 2000 platform shaker at 40 rpm at 4°C for 1 hour, followed by centrifugation for 30 minutes at 9000 x *g* at 4°C and removal of the supernatant, sans 1-2 mL, which was vortexed to redistribute the pellet.

To test the virus recovery efficiency (or virus loss) associated with the primary and secondary PEG precipitation methods, after the overnight incubation but prior to centrifugation, each 1 L sample was inoculated with the virus cocktail previously described (Inoculation 1 on Figure 1). After completion, 1 mL was then removed for nucleic acid extraction from both the "primary only" and "secondary" sets of samples. Samples were stored overnight in a -70°C freezer, and nucleic acid extraction was performed within 24 hours. The PEG precipitation procedure alone was evaluated by performing three experimental replicates using water collected on the November, 24, 2015 sampling date.

Chloroform extraction

Two 1L volumes of stream water were concentrated to 20-30 mL using the PEG precipitation method described. One of the samples was concentrated to a final volume of 2-3

mL via a secondary PEG precipitation. Post PEG precipitation, the virus cocktail inoculum (Inoculations 2A and 2B on Figure 1) was added and the sample was vortexed to evenly distribute the virus particles. The virus cocktail was identical to that previously described with the exception that it contained 6 log particles of MgV and 5 log particles of each of the other viruses. Chloroform was then added in equal volume to the sample, then the tube was vortexed for 30 s. Sample tubes were centrifuged for 10 minutes at 3000 x *g* using a Sorvall™ Legend™ X1R centrifuge (Thermo Fisher). The top aqueous phase was removed and deposited into a clean 50 mL centrifuge tube. From each sample, 1mL was removed for immediate nucleic acid extraction. This procedure was performed for 3 replicates using water collected on the November, 24, 2015 sampling date.

Ultrafiltration

PEG precipitation and chloroform extraction treatments were performed as previously described on two 1L volume samples. Immediately following completion of chloroform extraction, the virus cocktail (Inoculations 3A and 3B on Figure 1) was added and briefly vortexed to mix. A Millipore Amicon® Ultra Ultracel-15® 100 KDa centrifugal filter and an Amicon® Ultra Ultracel® 100 KDa centrifugal filter were first pre-treated with 3 mL of nuclease-free water (IBI Scientific, Peosta, IA). A second pre-treatment was performed on each filter with 3% Beef extract solution. For the first centrifugation cycle, half of the 20-30 mL sample was added to the 15 mL filter and the entirety of the 2-3 mL sample was added to the standard centrifugal filter. The filtration tubes were centrifuged at 3000 x *g* for 5 minutes. Additional centrifugation cycles were added as needed to produce a retentate volume between 100-250 µL for each sample. The retentate was then removed from each filter and placed in a

respective clean 1.5 mL centrifuge tube for immediate RNA extraction. This procedure was performed for three replicates using water collected on the November, 24, 2015 sampling date.

Simple Removal of Suspended Solids

Two 1 L samples were concentrated via PEG precipitation and one samples was subjected to a secondary PEG precipitation as previously described. After the PEG precipitation, the virus cocktail was added (similar to Inoculations 2A and 2B in Figure 1 if the suspended solids method was replaced with the chloroform extraction method). Sample tubes were then vortexed for 10 s to adequately distribute the viruses throughout the samples. The inoculated samples were then centrifuged at 9000 x *g* for 10 minutes to precipitate the suspended solid material. The supernatant was poured into a clean tube and 1 mL was removed for immediate RNA extraction. This procedure was performed for three replicates using water collected on the November, 24, 2015 sampling date.

Combined Methods

Two 1 L volumes were prepared as described for PEG precipitation replicates with the exception that they were inoculated with the virus cocktail prepared as previously described for chloroform extraction replicates. Inoculation occurred after overnight incubation but prior to PEG precipitation centrifugation. A third 1L sample was prepared minus the inoculum to serve as a negative control. Primary PEG precipitation was performed to completion as previously described for both inoculated samples and the negative control. Secondary PEG precipitation was performed for one inoculated sample. Chloroform extraction and ultrafiltration were performed subsequently for both treatments and the negative control. Two replicates were performed with water collected on November 24, 2015 and two replicates were performed using water collected on April 29, 2016.

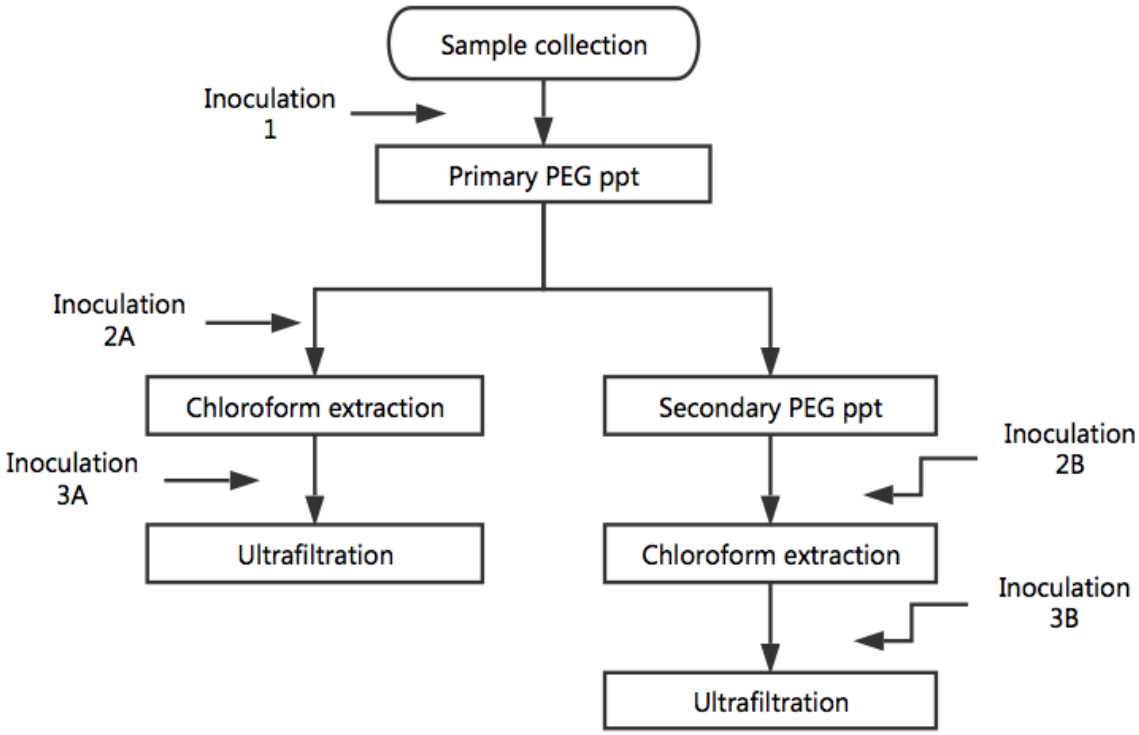

Figure 1 Flow chart depicting sequence of methods evaluated for enteric virus recovery. Points in the procedure where the inoculum was applied for different sets of experiments (1, 2A, 2B, 3A and 3B) are indicated.

Testing of environmental stream samples for presence of enteric viruses

Stream water collection

To test for the presence of enteric viruses in natural stream waters, duplicate 1 L samples were collected from 5 unique locations (Table 1) in Gwinnett Co. GA, northeast of Atlanta, GA on 7 dates ranging from March 22, 2012 to July 30, 2014 during baseflow. The selected streams are typical of urban watersheds in the southeastern Piedmont region of the USA. The selected locations included streams from low to high density of On-site Wastewater Treatment Systems (OWTS), a suspected source of human fecal pollution. Sites 5 and 6 are characterized as having a low density (LD) of OWTS, and sites 12, 14, and 19 are considered to have a high density (HD) of OWTS. An arbitrary threshold of <38 OWTS per km^2 was defined for LD watersheds and >77

OWTS per km^2 for HD watersheds (Landers and Ankcorn 2008). These threshold values were based on the U.S. EPA's designation of areas with >15 units per km^2 as regions of potential groundwater contamination (U.S. EPA 1977). However, considering advancements in treatment system technology, it is justified to increase the threshold values (Sowah et al., 2014).

Stream baseflow conditions were determined using long-term discharge measurements at two USGS stream gages (http://waterdata.usgs.gov/ga/ nwis/uv/?site_no=02205522; http://waterdata.usgs.gov/ga/ nwis/uv/?site_no=02207385) proximal to the study sites. Stream samples were collected from each location in duplicate in 1 L sterile high-density polypropylene, screw-capped bottles and transported back to the lab on ice. Samples were stored at 4° C for 24-48 hours before being subjected to PEG precipitation.

Processing of stream water samples in the laboratory

Environmental water samples collected during the March 22, 2012 to July 30, 2014 sampling dates were concentrated via PEG precipitation using an identical procedure to that listed above, with the exception that sample volumes of either 1 L or 50 mL were centrifuged using a Sorvall™ RC-3B Superspeed Centrifuge or a Sorvall™ Legend™ X1R centrifuge respectively. All other steps were identical to the PEG precipitation protocol previously described. Secondary PEG precipitation treatments were not performed on any of the samples. Post PEG precipitation, samples were placed in sterile 15 mL centrifuge tubes and stored at -70° C for 2-4 years until being thawed for subsequent processing.

Immediately upon thawing, each sample was inoculated with 6 log particles of MNV-1 and MgV to serve as process controls. To further purify and concentrate the samples, chloroform extraction and ultrafiltration via centrifugal filter were performed as described aboe. The nucleic-extraction procedure was identical to that described above with the exception that nucleic acids

were eluted in 75 µL of nuclease- free water in order to have an adequate volume for PCR testing. Each sample was tested for GI and GII NoV, HAV, EV, RV, SaV, AsV, AdV40 and AdV41, and MS2 phage via real-time RT-PCR. Samples that tested positive were then subjected to real-time RT-qPCR to quantify the amount of viral particles present. The amount of MNV-1 and MgV process control viruses present in the processed samples was quantified using real-time RT-qPCR.

Nucleic acid extraction

RNA extractions were performed using an in-house made Guanidine thiocyanate, GuSCN, based lysis buffer. The lysis buffer was made by dissolving 60 g of guanidine thiocyanate in 50 ml 0.5X TE (Tris-EDTA) buffer, followed by the addition of 5.5 ml 5 M sodium chloride, 5.5 ml sodium acetate, and 1.1 ml polyadenylic acid potassium salt. Lysis buffer was added to samples in a 1:1 ratio and let incubate for 10 minutes. Two times the sample volume of 100% ethanol was added and then vortexed. Each sample was added to a respective RNA silica membrane mini column (Omega Bio-Tek, Norcross, GA), and spun in a microcentrifuge at 14,000 rpms for 1 minute. Multiple centrifugation cycles were necessary to pass the entire sample volume through the column for some extractions. Once the entire sample had been passed through the column, a wash step was performed using 75% ethanol. A dry spin was included to remove any remnants of ethanol from the column. Nucleic acids were then extracted using 50 µL of nuclease-free water (IBI Scientific, Peosta, IA). Nucleic acid extracts were stored at -70°C until use for PCR testing.

Quantification of viruses via real-time RT-qPCR

Each virus was quantified using a TaqMan real-time PCR assay. The total reaction volume was 20 μL containing 2 μL of RNA template. ROX was used as a reference dye. Cycling conditions were performed using a Stratagene Mx3005P qPCR System (Aligent Technologies, Santa Clara, CA). Sample data collection and analysis was performed using the MxPro software based on a standard curve, derived from 10-fold serial dilutions of viral RNA. The PCR efficiency of the standard curve was deemed acceptable if it fell within the range of 90-110%. GI and GII NoV, MgV, MNV-1, AdV-41, and MS2 assays were performed using the Qiagen QuantiTect Probe PCR kit with 400 nM of the appropriate primers (Table 2), 200 nM of the appropriate probe (Table 2), QuantiTect RT Mix, 1 μl per reaction of IAC template and 0.5 μL per reaction of Rnase inhibitor. The GII NoV IAC template was also included in the GII assay. The cycling conditions used were as follows: one 30 minute cycle at 50°C for reverse transcription, one 15 minute cycle at 95°C for the initial PCR activation, and 50 cycles of 95° C for 10s, 55° C for 30 seconds with endpoint data collection, and 72° C for 30s for denaturation, annealing and extension steps, respectively. For MS2, cycling conditions varied slightly and were as follows: 30 minutes at 50° C, 15 minutes at 95° C, and 50 cycles of 95° C for 15s and 56° C for 1 minute. EV69, GI NoV, RV, SaV, HAV, and AsV assays were performed using the Quanta Biosciences qScript ™ XLT 1-Step RT-qPCR ToughMix® Low ROX master-mix with 800 nM of appropriate primers (Table 2), 200 nM of probe (Table 2), and 0.5 μL per reaction of Rnase inhibitor. For most viruses, cycling conditions used were as follows: one 30 minute cycle at 48°C, one 10 minute cycle at 95° C, followed by 45 cycles of 95° C for 15s and 60° C for 1 minute. For RV, the cycling conditions used were as follows: 30 minutes at 50° C, 15 minutes at 95° C, and 45 cycles of 95° C for 10s, 55° C for 30 seconds, and 72° C for 30s. For SaV, cycling

conditions used were as follows: 30 minutes at 50° C, 15 minutes at 95° C and 50 cycles of 94° C for 10s and 62° C for 1 minute. For AdV, the cycling conditions were as follows 50°C for 2 min, 95° C for 15 min, and 45 cycles of 95° C for 15 s and 60°C for 1 min.

The amount of virus present in each sample was calculated by multiplying the sample genome copy (GC) number reported by the realtime RT-PCR software by the appropriate dilution factor. GC values were determined using a standard curve of known GC values assigned using end-point dilution. RNA positive controls for real-time RT-PCR testing for each virus assay were comprised of viral RNA extracted from the respective virus stock with the exceptions of GI NoV and SaV, which were RNA transcript, and AsV, which was heat-treated virus stock (viral RNA is released after heat treatment of viruses). Cycling conditions and data collection were performed as described above. Negative PCR control samples, consisting of RNase-free water, were also tested for each assay during each experiment. For some assays, IAC viruses were included in order to test for the presence of inhibitors to RT-PCR. ΔCT values were calculated by subtracting the IAC control CT values from the sample CT values. IAC controls were selected from the RNA standard dilution values, with the dilution with the template quantity closest to that of the sample being selected as the IAC control.

Percent Recovery Calculations and Statistical Analysis

For experiments evaluating enteric virus recovery methods, the quantity of each virus in the inoculum cocktail (RNA extraction positive control) was determined during each experimental trial via a standard curve generated by end-point dilution. These quantities were used for calculating the virus recovery percentages for each experimental replicate. The amount of each virus recovered after each treatment was thus divided by the concentration determined

for each RNA extraction positive control virus and multiplied by 100%. For experiments recovering viruses from natural stream waters, process control viruses (MgV and MNV-1) were added to each sample after the PEG precipitation step, but the inoculum concentration was not determined at the time of sample processing. Therefore, the process control virus concentration was determined by calculating the average concentration of MgV and MNV-1 recovered in the RNA extraction positive control samples from experiments evaluating enteric virus recovery methods. Care was taken to only include experiments where the MgV and MNV-1 inoculum concentrations were identical; namely the PEG precipitation loss replicates for MNV-1 and the ultrafiltration and combined methods replicates for MgV.

All statistical tests were performed using JMP 12 (SW) (SAS Institute Inc., Cary, NC). One-way ANOVA was performed for each set of experimental replicates to determine if there were significant differences in recovery rates between treatment groups and to assess differences in PCR inhibition observed between experiments when different RT-PCR master-mixes were used. Two-way (factorial) ANOVA was performed on the recovery rates of the process control viruses from the stream sample to determine the effect virus type and processing date had on recovery rates, and also the interaction between the processing date and virus type variables. Tukey's honest significance difference test was performed to assess differences in virus recovery within the treatment groups. Student's T test was performed to compare recovery values from PEG precipitation replicates and the combined methods replicates. Statistical significance was determined at the $\alpha = 0.05$ level.

Table 1 Site information for stream water sample collection locations

Site ID	**Coordinates**	**OWTS[a] Density**
5	33° 56’ 26.6” N 83° 50’ 21.4” W	LD[b]
6	33° 55’ 58.3” N 83° 48’ 54.6” W	LD[b]
12	33° 54’ 31.4” N 83° 55’ 41.9” W	HD[c]
14	33° 54’ 5.6” N 83° 55’ 54” W	HD[c]
19	33° 52’ 19.2” N 84° 0’ 13.9” W	HD[c]

[a] On-site Wastewater Treatment System
[b] Low-Density
[c] High-Density

Table 2 List of primers and probes used for target or IAC amplification during real-time RT-PCR

Virus or	Name	Sequence	References
GI.1 NoV	Cog1F	CGY TGG ATG CGI TTY CAT GA	(Kageyama et al. 2003)
GI.1 NoV	Cog1R	CTT AGA CGC CAT CAT CAT TYA C	(Hill et al. 2010)
GI.1 NoV	Ring1C	(FAM)-AGA TYG CGI TCI CCT GTC CA-(BHQ2)	
GII.4 NoV	Cog2F	CAR GAR BCN ATG TTY AGR TGG ATG AG	(Kageyama et al. 2003)
GII.4 NoV	Cog2R	TCG ACG CCA TCT TCA TTC ACA	
GII.4 NoV	Ring2	(FAM)-TGG GAG GGC GAT CGC AAT CT-(BHQ2)	
GII.4 NoV	JJV2F	CAA GAG TCA ATG TTT AGG TGG ATG AG	(Jothikumar et al. 2005)
IAC	IAC-Probe	(Cy5)-ATC TCA GTT CGG TGT AGG TCG TTC GCT CC-(BHQ2)	(Liu et al. 2013)
EV	/	CCCTGAATGCGGCTAATCC	(Verstrepen et al. 2001)
EV	/	ATTGTCACCATAAGCAGCCA	
EV	/	AACCGACTACTTTGGGTGTCCGTGTTTC	
RV	JVKF	CAGTGGTTGATGCTCAAGATGGA	(Jothikumar et al. 2009)
RV	JVKR	TCATTGTAATCATATTGAATACCCA	
RV	JVKP	(FAM)-ACAACTGCAGCTTCAAAAGAAGWGT-(BHQ)	
MgV	Mengo110	GCG GGT CCT GCC GAA AGT	(Pinto et al. 2009)
MgV	Mengo209	GAA GTA ACA TAT AGA CAG ACG CAC AC	
MgV	Mengo147	(FAM)-ATC ACA TTA CTG GCC GAA GC-(MGB-NFQ)	
MNV	G54763F	TGA TCG TGC CAG CAT CGA	(Park et al. 2010)
MNV	G54863R	GTT GGG AGG GTC TCT GAG CAT	
MNV	G54808P	(FAM)- CTA ACC ACC AGA ACC CCT TTG AGA CTC-(ZEN)	
MS2	MS2F	TGGCACTACCCCTCTCCGTATTCACG	(Rolfe et al. 2007)
MS2	MS2R	GTACGGGCGACCCCACGATGAC	
MS2	$MS2_{Hex}$	(HEX)-CACATCGATAGATCAAGGTGCCTACAAGC-(BHQ2)	
SaV	SaV124F	GAYCASGCTCTCGCYACCTAC	(Kitajima et al. 2010)
SaV	SaV1F	TTGGCCCTCGCCACCTAC	
SaV	SaV5F	TTTGAACAAGCTGTGGCATGCTAC	
SaV	SaV1245R	CCCTCCATYTCAAACACTA	
SaV	SaV124TP	(FAM)-CCRCCTATRAACCA-(MGB-NFQ)	
SaV	SaV5TP	(FAM)-TGCCACCAATGTACCA-(MGB-NFQ)	
AsV	AsFF	GGC CAG ACT CAC AGA AGA GCA	(Grant et al. 2012)
AsV	AsFr	GTC CTG TGA CAC CTT GTT TCC TGA	
AsV	AstZFb*	(HEX)-CCA TCG CAT TTG GAG GGG AGG ACC AGC GA-(BHQ)	
HAV	HAV 68F	TCA CCG CCG TTT GCC TAG	(Bosch et al. 2001)
HAV	HAV 240R	GGA GAG CCC TGG AAG AAA G	
HAV	HAV 150	(FAM)- TTAATTCCTGCAGGTTCAGG-(MGB	(Costafreda et al. 2006)

CHAPTER 4

RESULTS

Virus percent recovery after primary and secondary PEG precipitation

Poly-ethylene glycol precipitation (PEG ppt) used as a single treatment (primary) or combined with a secondary PEG precipitation step was evaluated for its ability to concentrate multiple enteric viruses and process control viruses from artificially inoculated 1 L stream water samples. Overall, the performance of PEG precipitation was poor. Average enteric virus and process control virus recovery percentages ranged from 0.02% ± 0.02% for MgV to 4.70% ± 2.92% for EV69 after primary treatment alone and from 0.00% for MgV to 0.12% ± 0.05% for EV69 after the combined primary and secondary PEG treatments were applied (Table 3). The average recovery of GII.4 NoV was 0.70% ± 0.55 and 0.01% ± 0.01 for the primary treatment group and secondary treatment group, respectively. Of the two process control viruses, the recovery rates for MNV-1 were higher than MgV, with average MNV-1 percent recovery values of 0.89% ± 0.71 for the primary only treatment group and 0.01% ± 0.02 for secondary treatment group (Table 3). For the indicator virus, MS2 phage, average recovery rates were determined to be 3.02% ± 4.16 and 0.10%± 0.08 for primary treatment and secondary treatment groups, respectively. However, most likely due to the high variability observed between replicates, no significant differences were observed when the individual mean percent recoveries for each virus were compared after PEG precipitation treatments ($\alpha = 0.05$). However, when all enteric and process control viruses were considered together, statistical analysis indicated that the

performance of the secondary PEG precipitation resulted in significantly lower percent recovery values ($p=0.0074$).

Virus percent recovery following chloroform extraction

Virus recovery was determined after chloroform extraction, a technique used to remove particulate matter and inhibitors of PCR. After treatment by PEG precipitation (primary and secondary treatment groups), the virus cocktail was added to each set of samples and the viruses were quantified from 1 mL of the aqueous phase resulting from chloroform extraction. Average recovery rates obtained after chloroform extraction ranged from 15.01% ± 19.77 for MNV-1 to 277.20% ± 347.57 for MgV for samples in the primary PEG precipitation group and from 0.56% ± 0.88 for MNV-1 to 43.34% ± 24.26 for MS2 in the secondary PEG precipitation group (Table 3). The chloroform extraction replicates were unique in that they were only experiment set to have the average rates obtained for the pathogens be lower than the rates obtained for a process control virus and MS2. The average recovery percentages determined for GII.4 NoV were 33.55% ± 29.18 and 5.31% ± 2.34 for samples in the primary PEG precipitation group and secondary PEG precipitation group, respectively (Table 3) In contrast to the results obtained in the primary and secondary PEG precipitation recovery experiments, the average recovery rates determined for MgV were higher than those obtained for the pathogens and MNV-1, with MgV recovery values of 277.20% ± 347.57 for samples in the primary PEG precipitation group and 21.53% ± 123.70 for samples in the secondary PEG precipitation group (Table 2). Similar to the results obtained for MgV, the average recovery percentages obtained for MS2 exceeded 100%, with specific recovery values of 246.83% ± 263.56 and 43.34 %± 24.26 for samples in the primary PEG precipitation group and secondary PEG precipitation groups, respectively. Due to

the high variance between replicates, statistical analysis showed no statistical differences between individual mean percent recoveries for each virus after the chloroform extraction treatment ($\alpha = 0.05$). There was also no significant difference between the mean percent recoveries of viruses extracted by chloroform when samples in the primary PEG precipitation and secondary PEG precipitation groups were compared.

Virus percent recovery from ultrafiltration

Next, the performance of ultrafiltration was evaluated for its ability to concentrate viruses into a volume that could be easily used for RNA extraction after samples were first processed by PEG precipitation and chloroform extraction. The virus recovery percentages obtained after ultrafiltration ranged from 9.71% ± 7.38 for MgV to 39.32% ± 17.13 for GII.4 NoV for samples in the primary PEG precipitation group and from 20.79% ± 12.73 for MgV to 62.49% ± 35.29 for EV69 for samples in the secondary PEG precipitation group (Table 3). For the samples that were processed first using only the primary PEG precipitation step, GII.4 NoV had significantly different average recovery rates when compared to those of MgV ($\alpha = 0.05$ level), indicating a difference in behavior between this pathogen and the process control. Similarly, in the secondary PEG precipitation group, the pathogens were recovered at higher rates than the process control viruses and indicator virus; however, no statistically significant differences were observed in the mean virus recovery rates when these groups were compared ($\alpha = 0.05$). Statistical analysis also indicated there was no significant difference between the mean percent recoveries of samples processed by ultrafiltration when samples in the primary PEG precipitation and secondary PEG precipitation groups were compared.

Virus percent recovery after suspended-solid removal

The suspended-solid removal method was included as an alternate method for removing particulate matter accumulated after PEG precipitation. This method was hypothesized to have less opportunity for viral loss when compared to chloroform extraction, but may not be as effective as chloroform extraction in removing PCR inhibitors. After suspended-solid removal, virus average recovery rates ranged from 5.04% ± 5.81 for MS2 to 334.31% ± 266.80 for GII.4 NoV for samples in the primary PEG precipitation group and from 1.00% ± 0.64 for MNV-1 to 51.87% ± 24.62 for GII.4 NoV for samples in the secondary PEG precipitation group (Table 3). For samples in both treatment groups, the average recovery rates determined for the pathogens were higher than those of the process control and indicator viruses. GII.4 NoV mean percent recovery values were also significantly different from those of the other viruses for samples in the secondary PEG precipitation group. No significant differences were observed between mean rates of virus recovery in the primary PEG precipitation treatment group. Statistical analysis indicated there was no significant difference between the mean percent recoveries of samples in the primary PEG precipitation and secondary PEG precipitation groups.

Virus percent recovery using the combined method

Virus recovery rates were determined using a method that combined PEG precipitation, chloroform extraction, and ultrafiltration. Samples were inoculated with the virus cocktail prior to the PEG precipitation step (Inoculation 1 on Figure 1) to assess if recovery rates could be improved after removing PCR inhibitors by chloroform extraction and lowering the limit of detection using ultrafiltration to concentrate the samples. Replicates 1 and 2 and replicates 3 and 4 were grouped separately because these replicates were performed with water samples collected

at two different times. For replicates 1 and 2, recovery rates were highest for EV in both the primary and secondary PEG precipitation treatment groups with average percent recovery values of 0.84% ± 0.54 and 0.94% ± 0.36 in the primary and secondary PEG precipitation treatment groups respectively. For replicates 3 and 4, the highest rates were obtained for GII.4 NoV in both treatment groups, 0.25% ± 0.19 in the primary treatment group and 0.20% ± 0.22 in the secondary treatment group (Table 4). Similarly to previous experiment replicates sets, the process control viruses were recovered at the lowest rates (Table 4). There were no significant differences between the individual virus recovery rate means in the primary PEG precipitation treatment group and second PEG precipitation treatment group when grouped by replicate set (1-2 and 3-4). When the recovery rates for all viruses were considered together, significantly lower virus recovery values were obtained when water from the second water collection event was used with the combined method ($p=0.0057$) (Table 4).

To assess if the methods performed after PEG precipitation positively or negatively influenced virus recovery rates, comparisons between the virus recovery results obtained for samples in the PEG precipitation only and the combined method groups were compared. This comparison was performed for virus recovery values obtained from samples in replicates 1-2 of the combined method since the water from these replicates was collected on the same date as the water used in the PEG precipitation only experiments. Overall the performance of the combined method failed to drastically improve virus recovery rates, with average values ranging from 0.00% for MNV-1 to 0.84% ± 0.54 for EV69 for samples in the primary PEG precipitation treatment group and from 0.00% for MgV to 0.94 ± 0.36% for EV69 for samples in the secondary PEG precipitation group (Table 4). When considering samples that were processed only by the primary PEG precipitation step, there was no significant difference between the

average virus recovery values obtained for samples in the PEG precipitation only treatment group and those of the combined method treatment group. However, when samples were processed by both the primary and secondary PEG precipitation treatments, the inclusion of the subsequent processing techniques (as is performed in the combined method) appeared to significantly increased average virus percent recovery ($p=0.045$), although a larger sample size is needed to understand if this difference is real.

PCR inhibition assessed by inclusion of IACs

PCR inhibitors are an important consideration when choosing which concentration and purification methods to use when recovering viruses from environmental water samples. Internal amplification controls (IAC) were included in one of the assays for each of the PCR master-mixes used in the study in order to assess the influence the processing techniques had on PCR inhibitors. Different master-mixes were used for different viruses because preliminary experiments indicated that quantification of EV69 was best performed using the Quanta Biosciences qScript ™ XLT 1-Step RT-qPCR ToughMix compared to the QuantiTect Probe master-mix (data not shown).

Significant inhibition (defined as a change in average CT value (ΔCT) of 3 or higher when the IAC controls were compared to the IACs included in environmental water samples) was observed in at least one treatment group for all of the processing techniques except for the ultrafiltration and the combined method replicates 1-2 (Table 5). Statistical analysis indicated there was no significant difference between the mean ΔCT values obtained from the different PCR master-mixes. Inhibition from PEG precipitation only treatment was significantly higher than that determined for replicates 1 and 2 of the combined methods ($p=0.0425$), suggesting the treatments performed after PEG precipitation were successful in removing PCR inhibitors. The

combined methods replicates 3 and 4 did not behave similarly, and had significantly more PCR inhibition compared to the ultrafiltration replicates ($p=0.0251$) and the combined methods replicates 1 and 2 ($p=0.0089$). Performance of the secondary PEG precipitation step did not result in significantly different ΔCT values when compared to the primary PEG only treatment group values. Overall, this indicates the significant inhibition seen in PEG precipitation treated only samples can be reduced or removed by performing the subsequent techniques of chloroform extraction and ultrafiltration.

Screening of environmental stream samples for enteric viruses and fecal indicator virus

To further assess their value in applications related to environmental water monitoring, the combined methods were applied to a set of environmental stream samples, which were then tested for a host of human enteric viral pathogens and MS2 phage, which was used as a fecal indicator virus. All samples tested negative for GI.1 NoV, GII.4 NoV, HAV, EV, AsV, RV, and MS2. One sample, from site 19 (HD), collected on March 21, 2014, tested positive for SaV. The average sample CT value obtained for this sample was 39.57. This corresponded to 147.75 SaV particles per 1L of water. PCR results were not able to be obtained for AdV40 and AdV41, likely as a result of the nucleic acid extraction procedure not efficiently capturing DNA viruses. Due to the low occurrence of viruses in the sample, comparisons between high-density and low-density samples could not be performed.

Process control viruses MgV and MNV-1 were added to each sample after PEG precipitation and before chloroform extraction and ultrafiltration treatment. Process control viruses were added to assess the ability of the chloroform extraction and ultrafiltration methods to concentrate enteric viruses possibly present in the environmental samples. MgV was

recovered at a higher rate than MNV-1 when recovery results for all processing dates were considered together ($p<0.001$). Significant differences in process control virus recovery values were also obtained for the difference processing dates ($p<0.001$) (Table 6). There was interaction effect between virus and processing date variables that was also significant, indicating the "processing date" variable is influencing the two viruses differently ($p<0.001$). ΔCT values determined from the GI NoV and GII NoV assays indicated significant PCR inhibition did not occur in any of the samples. Based on the recovery rates of MgV, the treatment of samples by chloroform extraction and ultrafiltration should not have affected the ability detect enteric virus pathogens from 1 L environmental water samples.

Table 3 Average percent recovery of enteric viruses as determined by RT-qPCR after primary and/or secondary Poly-Ethylene Glycol (PEG) precipitation (ppt) and subsequent processing techniques.

		Average percent recovery ± StDev[a]	
Processing technique following PEG precipitation (ppt)	**Virus**	**Primary PEG ppt only[b]**	**Primary + Secondary PEG ppt[c]**
No additional treatment	GII NoV	0.70 ± 0.55 (A)	0.01 ± 0.01 (A)
	EV69	4.70 ± 2.92 (A)	0.12 ± 0.05 (A)
	MS2	3.02 ± 4.16 (A)	0.10 ± 0.09 (A)
	MgV	0.02 ± 0.02 (A)	0.00 (A)
	MNV-1	0.89 ± 0.71 (A)	0.01 ± 0.02 (A)
Chloroform Extraction	GII NoV	33.55 ± 29.18 (A)	5.31 ± 2.34 (A)
	EV69	23.40 ± 7.26 (A)	15.06 ± 2.79 (A)
	MS2	246.83 ± 263.56 (A)	43.34 ± 24.26 (A)
	MgV	277.20 ± 347.57 (A)	21.53 ± 123.70 (A)
	MNV-1	15.01 ± 19.77 (A)	0.56 ± 0.88 (A)
Ultrafiltration	GII NoV	39.32 ± 17.13 (A)	27.10 ± 11.79 (A)
	EV69	26.11 ± 14.94 (A,B)	62.49 ± 35.29 (A)
	MS2	26.69 ± 5.09 (A,B)	48.53 ± 14.29 (A)
	MgV	9.71 ± 7.38 (B)	20.79 ± 12.73 (A)
	MNV-1	23.42 ± 11.99 (A,B)	51.39 ± 59.51 (A)
Suspended Solid Removal	GII NoV	334.31 ± 266.80 (A)	51.87 ± 24.62 (A)
	EV69	31.53 ± 10.22 (A)	10.17 ± 2.52 (B)
	MS2	5.04 ± 5.81 (A)	1.43 ± 2.38 (B)
	MgV	17.43 ± 9.26 (A)	1.85 ± 1.30 (B)
	MNV-1	13.51 ± 13.34 (A)	1.00 ± 0.64 (B)

[a] Values in different letter groupings indicate significant differences between the means when samples in each processing technique group were compared within each PEG precipitation treatment group the $\alpha = 0.05$ level.

[b] Primary PEG precipitation performed for all samples prior to subsequent processing treatments

[c] Primary and secondary PEG precipitation performed for all samples prior to subsequent processing treatments

Table 4 Average percent recovery of enteric virus as determined by RT-qPCR following primary and/or secondary PEG precipitation, chloroform extraction and ultrafiltration performed in succession.

		Average percent recovery ± StDev	
	Virus	**Primary PEG ppt only[b]**	**Primary + Secondary PEG ppt[c]**
Replicates 1&2[a,d]	GII.4 NoV	0.39 ± 0.26 (A)	0.05 ± 0.09 (A)
	EV69	0.84 ± 0.54 (A)	0.94 ± 0.36 (A)
	MS2	0.35 ± 0.40 (A)	0.72 ± 0.72 (A)
	MgV	0.39 ± 0.20 (A)	0.33 ± 0.30 (A)
	MNV-1	0.00 (A)	0.00 (A)
Replicates 3&4[a,d]	GII.4 NoV	0.25 ± 0.19 (B)	0.20 ± 0.22 (B)
	EV69	0.21 ± 0.06 (B)	0.08 ± 0.03 (B)
	MS2	0.06 ± 0.13 (B)	0.09 ± 0.18 (B)
	MgV	0.00 (B)	0.00 (B)
	MNV-1	0.16 ± 0.32 (B)	0.03 ± 0.06 (B)

[a] The pairs of replicates were performed with water collected from the same location, but different dates.
[b] Only primary PEG precipitation was performed
[c] Primary and secondary PEG precipitations were performed
[d] Values in different letter groupings indicate significant differences between the means when average percent recovery values from the two replicate sets were compared at the $\alpha = 0.05$ level

Table 5 PCR inhibition observed in samples subjected to primary and/or secondary Poly-Ethylene Glycol (PEG) precipitation (ppt) and subsequent processing techniques.

Processing technique following PEG ppt[d]	Δ CT[a]			
	Primary PEG ppt only[b]		Primary+secondary PEG ppt[c]	
	Quanta mix	QuantiTect Probe mix	Quanta mix	QuantiTect Probe mix
None (A,B)	/[e]	5.44	/[e]	9.40
Chloroform extraction (A,B,C)	7.60	0.93	5.54	2.29
Ultrafiltration (B,C)	-0.065	1.43	0.21	2.85
Suspended solid removal (A,B,C)	0.29	0.34	6.03	0.98
Combined methods Reps 1&2 (C)	-0.61	0.95	-0.37	0.68
Combined methods Reps 3&4 (A)	11.33	4.59	9.69	4.46

[a] ΔCT values of 3 or higher as compared to the IAC control indicates significant PCR inhibition
[b] Only primary PEG precipitation was performed
[c] Primary and secondary PEG precipitations were performed
[d] Techniques in different letter groupings indicate significant differences between the ΔCT means when the techniques were compared the $\alpha = 0.05$ level
[e] Data not obtained

Table 6 Average percent recovery of process control viruses from environmental water grouped by processing date.

Sample Date[a]	Processing Group[b]	Virus	Percent recovery
3/22/2012	A	MNV-1	14.91 ± 8.41
		MgV	4.21 ± 1.07
7/19/2012	B	MNV-1	6.47 ± 3.95
		MgV	4.42 ± 1.54
11/29/2012	C	MNV-1	0.06 ± 0.10
		MgV	1.69 ± 0.69
4/23/2013	D	MNV-1	5.15 ± 3.92
		MgV	57.29 ± 18.55
11/14/2013	E	MNV-1	0.72 ± 0.49
		MgV	41.44 ± 18.43
3/21/2014	F[c]	MNV-1	1.23 ± 0.67
		MgV	118.31 ± 72.47
7/30/2014	F[c]	MNV-1	2.27 ± 1.05
		MgV	146.02 ± 46.66

[a] Date in which environmental water samples were collected
[b] Each set of samples, grouped by collection date, were subjected to secondary processing on a unique date
[c] Samples collected on 3/21/2014 and 7/30/2014 were subjected to secondary processing on the same date

CHAPTER 5

DISCUSSION

Viral percent recovery was determined for a series of established concentration and purification techniques for application in environmental water samples for two enteric pathogens, GII.4 NoV and EV69, two process control viruses, MNV-1 and MgV, and one fecal indicator virus, MS2 bacteriophage. Comparing the recovery rates of the viruses from different techniques, it is apparent that the PEG precipitation step is the main source of virus loss. Other studies that had successful results using the method included 1.5-3% Beef extract or 1% BSA to the solution, suggesting the protein content may have a significant influence on virus recovery (Schwab et al 1996: Lambertini et al. 2008; Kahler et al. 2015; Lewis and Metcalf 1988).

Overall, there was a trend that the two pathogenic viruses were recovered at higher rates than the process control viruses and the indicator bacteriophage. When determining virus loss from ultrafiltration, in the primary PEG precipitation treatment group, MgV was recovered at significantly lower rates than GII.4 NoV. Similarly, in a study comparing the recovery rates of MNV-1 and MgV to those of GII NoV from three food matrices, it was observed that GII NoV was recovered similarly to MNV-1 from bottled water, but the rates were significantly higher than those of MgV (Hennechart-Collette et al. 2015). Recovery rates obtained for GII.4 NoV were also significantly higher than those of the other viruses in the suspended solid removal loss replicates of the primary PEG precipitation treatment group. However, the validity of the trends seen in these experiments is weak due to there being few statistically significant differences

between the virus means, most likely due to high variance between replicates. This effect was at least partially due to there being variability in the genome copy values (concentrations) obtained for the RNA extraction positive controls. When the percent recovery data from the virus recovery experiments and environmental stream samples are viewed as a whole, there does not seem to be any elucidation as to which process control virus behaves most similarly to the pathogens and therefore is best suited for use in environmental water monitoring.

If the assumption that, when using the set of methods employed in this study, pathogens are recovered at rates higher than those of process control viruses is correct, it suggests that the results obtained for the environmental stream samples are true negatives. However, a major limitation of making such an interpretation from this study is that the process control viruses were not inoculated into the samples until after the PEG precipitation step. The ISO 15216-1 method defines a cut off value of 1% process control virus recovery for valid samples, and states if recovery rates are below 1% the sample should be retested (ISO/TS 15216-1, 2013). MgV was recovered from environmental water samples at average rates higher than 1% for all processing dates, indicating results are valid and the samples are true negatives for the enteric pathogens. The process control recovery values obtained for MNV-1 were statistically different and did not meet this process control recovery cut off value, suggesting the recovery results obtained for the environmental water samples should be rejected. The significant difference between MgV and MNV-1 recovery suggests that MgV may be the more a more suitable process control virus for this application. However, the environmental water samples underwent PEG precipitation prior to process control inoculation. Considering how much virus loss was associated with this processing step alone, it is highly unlikely, based on data obtained from the PEG precipitation only replicates, that either of the process control viruses would be recovered from environmental

water samples at rates >1% if the samples were inoculated with the process control viruses before the PEG precipitation step.

During the PEG precipitation treatments performed to determine viral loss, an average of 99.3% of a ~6 log inoculum of GII.4 NoV was lost. If the recovery rates of pathogens were the same for the PEG precipitation treatments performed on the stream sample set, this would indicate a strong possibility for false negatives, especially considering the low virus concentrations observed in the environment. One sample tested presumptive positive from real-time RT-PCR for SaV, and currently the sample awaits confirmatory results by DNA sequencing. Assuming SaV behaves similarly to NoV, this would mean the number of particles determined to be present in the 1L sample was underestimated by 99.3%, with the possibility of actual virus concentrations being as high as 2,111 particles per liter. Considering the low infectious dose of enteric viruses (18-1000 particles) (Teunis et al. 2008: Glass et al. 2009) a considerable public health risk could be present if these estimations were correct.

There were several unforeseen limitations that arouse during the course of the study. A third pathogen, AdV41, was included in the virus cocktail inoculum, however, the virus DNA did not survive well in the nucleic acid extraction buffer and could not be quantified. Because all the other viruses have RNA as their genetic material, water was used as the extraction eluent. It is known that Tris-EDTA or TAE buffer with a pH of 8-9 is the preferred eluent for DNA storage since DNA extracts survive longer in alkaline environments. Even in only slightly acidic solutions, DNA is sensitive to degradation via depurination, depyrimidination, deamination and hydrolytic cleavage (An et al. 2014; Jeong et al. 2008; Knight 1963). The ionic strength of the solution also affects depurination rates, so storage in salt containing solutions will further slow the nucleic acid degradation rate (Lindahl and Nyberg 1972). RNA is not stable in alkaline

solutions because bases can easily deprotonate the hydrogen in the hydroxyl group located on the C_2 carbon, making pure water the best choice for storage (Nelson and Cox 2013).

Due to the low occurrence of viruses in the sample set, comparisons between high-density and low-density samples could not be performed. This resulted in failure to complete one of the aims of the study, which was to use the enteric virus data to elucidate the influence of on-site wastewater treatment systems on environmental water quality.

There was significant variation in the recovery rates of the process control viruses recovered from environmental waters on different processing dates. Percent recovery values calculated for the process control viruses inoculated into the set of environmental stream samples would be more accurate if RNA extraction positive controls had been included for each virus during the time the environmental samples were processed. Instead, the average concentration of the MgV and MNV-1 inoculum material tested during the preliminary recovery experiments was used (RNA extraction positive controls from duplicate samples of at least three experimental replicates). This is not the ideal because there could have been slight differences in the inoculum levels, possibly from varying numbers of freeze/thaw cycles of virus stock aliquots. Including positive controls using the same virus stock on the same date would likely give a better estimation of the actual amount of virus particles present in the starting inoculum for each processing period. The significant effect of processing date on virus recovery is likely due to the aforementioned slight differences in the concentration of process control viruses for inoculums processed on different days. If positive controls of the process control viruses were included during stream sample RNA extraction, this effect may not have been observed since there was not any indication PCR inhibition was influencing virus quantification. Alternatively, this effect could also be due to slight day-to-day differences in the performance of the techniques due to the

experimenter. This is supported in the results from the virus recovery experiments, in which RNA positive extraction controls were included, but high variation between replicates (performed on different days) was observed.

It was also apparent that PCR inhibitors that remained after treatment in the virus recovery experiment samples reduced the sensitivity of the PCR assays, especially for the experiments performed with water collected on April 29, 2016 (reps 3 and 4 of the experiments evaluating the combined method). Humic and fulvic acids are found in soil and sediment and are the main PCR inhibitors associated with environmental water samples (Schrader et al. 2012; Abbaszadegan et al. 1993). Humic acid inhibits the PCR reaction through sequence-specific binding to the cDNA, which leads to a reduction in the amount of available template (Opel et al. 2010). It has also been observed that humic acids chelate magnesium ions, which are required by Taq polymerase, leading to inhibition (Tsai and Olsen 1992). These compounds are extracted with nucleic acids and will remain in the sample unless the proper purification technique is performed (Faber et al. 2012). Liquid-liquid extractions, such as chloroform extraction, have been shown to remove some humic substances (Tsai and Olsen 1992); however, it has also known that this treatment may not be sufficient to remove all traces of the compounds from the sample (Pachner and Delaney 1993). Humic acid and fulvic acid are both water soluble at neutral or alkaline pH and fulvic is also soluble in acidic conditions. This is a possible explanation as to why the chloroform extraction technique successfully removed particulate matter from the samples, but because some of these acids remained in the aqueous phase, PCR inhibition was still observed.

The significant PCR inhibition seen after chloroform extraction could also be a result of the RNA extraction procedure. For the chloroform extraction replicates, 1 mL of sample was

passed through the Silica spin column compared to only 250 µL for the ultrafiltration replicates. During ultrafiltration, the humic and fulvic acids would have been able to pass through the 100 KD filter and would not have been co-concentrated with the viruses. Assuming the inhibitors were evenly dispersed throughout the sample, this would mean there would be significantly less inhibitor molecules in the ultrafiltration retentate than would be present in 1 mL of the aqueous phase from chloroform extraction. Overall, from the data collected it appears that ultrafiltration is the more valuable tool for removing PCR inhibitors from environmental samples; however it should be noted that without the performance of chloroform extraction, the particulate matter concentrated during PEG precipitation would clog the filtration device and render it useless. Therefore, ultrafiltration must be performed subsequently from chloroform extraction, or another purification technique, in order to be successful.

Water collected on April 29, 2016 (used for the rep 3 and 4 samples of the combined method evaluation experiments) was collected during low-flow rate, which forced us to collect the water slightly downstream from the location used to collect the water in November 2015 (used for reps 1 and 2 of the combined method evaluation experiments and all other experiments evaluating virus recovery processing techniques). At this location, the stream width narrows and the water is funneled under a concrete bridge where it then passes over a rocky cascade into a deep pool. On the collection date, the section of stream prior to the bridge was too shallow to collect clean samples, so the samples were drawn by standing on shoreline rocks and dipping into the collection pool. After the performance of PEG precipitation it was apparent the second batch of water contained much more sediments than the first batch of water. Post PEG precipitation, the combined method replicates 3 and 4 samples were yellow to orange in color in contrast to samples treated in replicates 1 and 2, which had little to no discoloration. The

funneling and presence of rocks near the collection site caused the water to be more turbulent, which has shown to be correlated with increased sediment loads (Ruzycki et al. 2014). It is likely that the increased sediment load resulted in an increase in soil-associated PCR inhibitors. Similar results, in which more colored stream samples exhibited more PCR inhibition in comparison to clear samples, have been observed (Jane et al. 2015). This resulted in difficulty drawing conclusions from percent recovery values obtained from the combined methods. If only replicates 1 and 2 were compared to the PEG precipitation only treated replicates, the statistical conclusions are not robust due to small sample size. If replicates 3 and 4 are included in analysis, results could be skewed due to the presence of PCR inhibitors, exaggerating the amount of virus loss that occurred. It was ultimately decided that the PCR inhibitors were influencing the results in these replicates too greatly to use them in method comparisons.

In conclusion, the evaluation of the PEG precipitation, chloroform extraction, and ultrafiltration methods revealed that due to the high amount of virus loss associated with the PEG precipitation step, this method may not be the best choice for concentrating environmental water with low organic content. Performance of chloroform extraction and ultrafiltration after PEG precipitation failed to significantly increase virus recovery for when only primary PEG precipitations are performed, but resulted in significantly higher recovery if primary and secondary PEG precipitations are performed. Further experimentation, in which it is determined if the addition of beef extract or BSA could improve the virus recovery from stream water concentrated by PEG precipitation is suggested. From each of the treatment techniques, recovery rates for two viral pathogens, two process control viruses, and a fecal indicator virus were compared and the findings were that the pathogenic viruses were recovered at higher rates than the process control and indicator viruses in multiple experiments. The determination of the level

of PCR inhibition that occurred during quantification revealed that the significant inhibition seen in PEG precipitation treated only samples can be reduced or removed by performing the subsequent techniques of chloroform extraction and ultrafiltration. The alternate technique of suspended solid removal failed to eliminate significant PCR inhibition in all samples. The recovery of two process control viruses were compared from the inoculated stream samples and MgV was recovered at significantly higher rates than MNV-1. However, it is believed that if the environmental water samples underwent PEG precipitation prior to process control inoculation, neither virus would have been recovered. Despite these methods leading to the detection of SaV in one of the samples, the high possibility of false negatives may result in an under estimation of public health risk from contaminated ground or recreational water if employed in environmental monitoring efforts.

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