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Human Enteric Viruses in Recreational Coastal Waters

A Dissertation Presented

by

Alexandra Valdés Wochinger

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Abstract of the Dissertation

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Human enteric viruses (HEV) are recognized by the CDC as common etiological agents for waterborne outbreaks and they have been detected in recreational waters in the US and around the world. Current concentration and detection methods present challenges to study of HEV in coastal waters and have prevented the routine monitoring of HEV in recreational or shellfish harvesting waters. In this dissertation, various methods of concentrating viruses from water were investigated. A two-step viral concentration method that included viral adsorption-elution and ultrafiltration was found to be effective for the concentration of total viruses and improved detection limits for HEV in coastal waters. This method was then applied in a time series study of human viral contamination to coastal recreational waters. RT-PCR was used to screen for three types of HEV: enteroviruses (EV), hepatitis A viruses (HAV) and noroviruses (NoV) in viral concentrates from surface waters of Port Jefferson Harbor (PJH) NY, which receives point and nonpoint sources of human waste. No HAV or NoV were detected in any samples, but EV

was detected after precipitation events. Cloning and sequencing of the EV-positive samples revealed that the amplicons derived from strains of poliovirus. The results suggest that storm water runoff, which is discharged directly into the harbor, results in human viral contamination. Future studies of HEV sources and occurrence in the environment will help reduce public health risk to human viral pollution and transmission into coastal recreational waters, particularly after precipitation events.

To my mother, Margaret Wochinger Figueroa,
who loved the ocean and taught me how to swim far away in it.

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CHAPTER 1

INTRODUCTION AND BRIEF LITERATURE BACKGROUND

Human pathogens are introduced into coastal waters through discharges of treated and untreated sewage (Bosch et al., 2006). Current wastewater treatment plants do not provide complete removal of viral pathogens (Rao and Melnick, 1986). Worldwide, each year approximately 120 million cases of gastrointestinal disease and 50 million cases of severe respiratory disease are caused by swimming and bathing in wastewater-polluted coastal waters (Shuval, 2005). These gastrointestinal diseases are partly attributable to fecal contamination in the marine environment (Cabelli et al., 1982). Monitoring of the viral quality of coastal waters used for recreation and seafood harvesting is imperative for the prevention of diseases transmitted via the oral-fecal route, and reducing economic losses due to closures of beach resorts and shellfish fisheries (Bosch et al., 2006).

Dissertation Objectives. My objectives were to evaluate and optimize methods for detecting human enteric viruses in coastal recreational waters and to apply these methods to determine where and under what conditions these pathogens are present. I chose as my sampling sites several locations along the north shore of Long Island, New York, which receive point and nonpoint sources of human viral contamination. In the first part of the project, I evaluated several commonly used viral concentration methods and devised a protocol to improve total virus recovery. I then applied conventional reverse transcriptase PCR (RT-PCR), quantitative RT-PCR (qRT-PCR), and molecular cloning and sequencing to detect HEV in surface water samples

collected under varying environmental conditions. The physicochemical characteristics of the sampling sites were determined at each sampling to determine which environmental factors affect the prevalence of HEV in recreational waters.

MOTIVATION

Importance of Water Quality. Water is one of the most valuable resources on our planet. Unfortunately, not everyone has access to “healthy water”. The Center for Disease Control and Prevention (CDC) reports that over 900 million people in the world lack access to clean water (<http://www.cdc.gov/ncezid/>) and waterborne infectious diseases are still a major cause of death in developing countries. Even in developed countries, which have widespread wastewater treatment, extensive vaccination programs, and routine monitoring of recreational water quality, millions of cases of waterborne illness are reported every year (Colford et al., 2006; Messner et al., 2006).

HUMAN VIRAL PATHOGENS IN THE ENVIRONMENT

Human Enteric Viruses (HEV) and Infectious Diseases. Enteric viruses are the most common cause of gastroenteritis worldwide, and most often transmitted via the fecal-oral route (Bosch et al., 2006). They are an important and diverse group of viruses found in the intestinal tract of humans that can also cause other maladies like hepatitis, meningitis, fevers, rashes, and respiratory diseases (Bosch et al., 2006). HEV belong to the families Adenoviridae (adenoviruses), Picornaviridae (polioviruses, coxsackieviruses, echoviruses, enteroviruses and hepatitis A viruses), Reoviridae (reoviruses and rotaviruses), and Caliciviridae (noroviruses, caliciviruses, and astroviruses) (Fong and Lipp, 2006). Although waterborne viral diseases can be transmitted via drinking water, they can also be transmitted by immersion in recreational

water or by contact through skin or inhalation of human sewage contaminated water (Percival et al., 2004). It is particularly difficult to confirm and quantify whether contamination of water with human sewage has occurred (Percival et al., 2004), and only a small number of studies have shown epidemiological evidence of waterborne viral transmission (Craun et al., 2002).

The CDC reports a continuous increase in waterborne outbreaks in recreational waters (Figure 1.1). In the last *Surveillance Summaries of Waterborne Outbreaks in Recreational Waters* by the CDC (Yoder et al., 2008), a total of 78 outbreaks associated with recreational waters were reported during the years of 2005-2006, in which 4,412 people were affected and five died. This is in contrast to the years of 2003-2004, when they reported 62 outbreaks, i.e. an increase of 16 events associated with recreational waters in the last two years (Dziuban et al., 2006). For the most part, outbreaks reported by the CDC have been associated with gastroenteritis (61.5%), skin conditions (14.1%) and acute respiratory (14.1%) diseases. Although most recent CDC-reported gastroenteritis outbreaks were due to parasites or bacteria rather than viruses in treated water, untreated water showed higher cases of viral gastroenteritis (Figure 1.2). However, many outbreaks were of unknown etiology and viral outbreaks tend to be underreported because testing for viruses in stool and water samples is not widely practiced.

Transmission of HEV into Environmental Waters. Figure 1.3 illustrates possible routes of transmission of human enteric viruses in aquatic environments. Viruses are discharged in extremely high numbers from the feces of infected individuals, e.g. 10^5 to 10^{11} virus particles per gram of stool (Bosch, 1998). Current wastewater treatment plants fail to entirely remove viral pathogens (Rao and Melnick, 1986). HEV can be transported in the environment through groundwater, estuarine water, seawater, rivers, aerosols emitted from sewage treatment plants, insufficiently treated waters, drinking water, and private wells that receive treated or untreated

wastewater (Fong and Lipp, 2005). Humans are exposed to viral pathogens via the consumption of contaminated shellfish and immersion in recreational waters.

Sources of HEV in Coastal Environments. HEV are discharged into the environment via point and non-point sources of human fecal waste. Point source contamination describes a single localized source such as a sewage outfall where contaminants are introduced in relatively high concentrations from a wastewater treatment plant. The Environmental Protection Agency (EPA) defines non-point sources as pollution coming from a variety of scattered sources such as land runoff, precipitation, atmospheric deposition, drainage, seepage or hydrologic modification. It is caused by rainfall or snowmelt that transports natural and human-made pollutants to lakes, rivers, wetlands, coastal waters and ground waters (<http://water.epa.gov/polwaste/nps/whatis.cfm>).

It is important to determine sources of viral pollution to avoid contamination of recreational waters in general, and more specifically contamination of shellfish because they represent potential vectors of human enteric diseases. Rainfall and sewage discharges have been key environmental factors responsible for human viral contamination in bathing and shellfish harvesting waters (Bosch et al., 2006). The Department of Environmental Conservation of NY (DEC) orders emergency closures of shellfish harvesting areas after a rainfall event of more than three inches, based on monitoring data that shows degradation of water quality (<http://www.dec.state.ny.us/website/dfwmr/marine/shellfish/sfspec/index.html>). Jiang et al. (2001) reported viral contamination in coastal waters in Southern California beaches associated with urban runoff and concluded that a higher level of human viral contamination could be expected during heavy rainfall. Nevertheless, only a few field studies have been able to show

statistical evidence of a link between rainfall events and occurrence of HEV (Gersberg et al., 2006; Lipp et al., 2001).

Survival and Persistence Strategies. As obligate parasites, viruses depend on hosts for their reproductive success. One survival strategy can be categorized as “hit and run”, in which viruses evade immune destruction by infecting successive new hosts without persisting in any one host (Hilleman, 2004). These cytolytic viruses destroy the host cell in which they multiply, are highly infective and easily transmitted to a susceptible new host, and include viruses such as influenza, rhinoviruses, and enteroviruses. Human enteric viruses that employ the “hit and run” strategy can be transmitted within coastal environments provided that they remain infectious for long enough periods that they can come in contact with another susceptible host (Gerba, 2006). Other viruses employ a “hit and stay” survival strategy by persisting in the same host, from whom there may be frequent or infrequent transmission to successive hosts (Hilleman, 2004).

Environmental Stressors. Given the fact that HEVs employ the “hit and run” strategy, environmental conditions presumably would be of major importance when they are between hosts (note that environmental conditions that affect the host may also affect its associated viruses). Once discharged from the human host into coastal waters, HEV are unable to multiply. However, these viruses are very stable and can survive for as long as 130 days outside their host (Fong and Lipp, 2005; Percival et al., 2004). A range of environmental factors may influence HEV survival, including temperature, light, pH, salinity, suspended particulate matter, nature of bottom sediments, and other microorganisms (Gerba, 2006).

Temperature and UV light. Viruses survive longer at lower temperatures (Gerba, 2006) while high temperatures cause denaturation of proteins and/or nucleic acids causing viral inactivation. While most researchers have found higher concentrations of HEV during winter

months (Fattal et al., 1983; Fong et al., 2005; Gersberg et al., 2006; Lipp et al., 2001), others have reported high concentrations of enteric viruses during the summer (Jiang et al., 2007). UV light can also inactivate viruses by damaging their nucleic acids (Gerba, 2006).

pH and Salinity. Most enteric viruses have been found to be unaffected by pH values of natural waters (pH 5 to 9), but alkaline conditions (pH 9 to 12) may inactivate viruses by releasing their nucleic acid or disassembling the capsid (Gerba, 2006). Salinity has also been mentioned as a controlling factor in viral abundance with the higher concentration of cations in solution seeming to stabilize the structure of the viral capsid (Gerba, 2006). However, higher salinities and greater ionic strength can affect replication ability and adsorption to particles (Maranger, 1995; Wommack and Colwell, 2000; Jiao, 2006).

Sediments. Association with suspended particulate matter appears to prolong viral survival and protect against virus inactivation, while serving as a transportation mechanism (Gerba, 2006). In a meticulous study of inactivation mechanisms of the T4 coliphage in seawater Finiguerra et al. (2011) found that inorganic clay (kaolinite and montmorillonite) and organic particles (phytoplankton debris) did not promote phage inactivation; on the contrary, sediment particles appear to enhance viral survival by adsorption. It has been found that enteric viruses are more abundant in sediments than in the water column of sewage-polluted coastal marine environments (Goyal et al., 1978; Rao et al., 1984; Gerba, 2006), reflecting the association of viruses with suspended particles that settle out of the water column to the seabed. Clay may protect viruses in seawater by adsorbing enzymes and other substances thereby enhancing the stability of the viral capsid (Gerba, 2006).

However, these findings are mostly based on clinical or laboratory studies and the few field studies on environmental prevalence of HEV have proven to be greatly complex and not definitive.

Current Detection Methods. Although exposure assessments could be improved by virus monitoring in recreational waters, the quantification of human viruses in water remains technically difficult, time consuming, and expensive. In fact, the viruses of primary concern are difficult or impossible to quantify. Therefore, many public agencies rely on bacterial indicator organisms to establish a qualitative association between water quality data and risk to public health (Parkin et al., 2003)

Indicator Microbes. Most countries, including the United States, use only bacterial indicators to monitor the microbiological quality of water. The main problems with bacterial indicators are (1) they do not necessarily reflect the risk for other pathogens, such as viruses and parasites, (2) they reflect an unknown source of contamination because indicator bacteria can be found naturally in both human and warm-blooded animal feces, and (3) they don't adequately capture the fact that many pathogens reproduce in the environment after being excreted by their host (Fong and Lipp, 2005). In some cases, where assays have been within the limits recommended for bacterial indicators in water, human enteric viruses have been found at dangerously high concentrations (Bosch, 1998; Ehlers et al., 2005). Wetz et al. (2004) found infectious enteroviruses in surface waters of the Florida Keys while levels of microbial water quality indicators (enterococci or fecal coliform bacteria) did not exceed recommendations. Current microbial indicators (e.g. enterococci, fecal streptococci and fecal coliform bacteria) simply do not give information on the viral quality of water. In spite of studies supporting the use of bacterial organisms as indicators for viral pathogens (Gersberg et al., 2006), other studies

suggest the use of alternative indicators of viral pathogens, such as bacteriophages, (Brion et al., 2002; Ogorzaly et al., 2009), or adenoviruses (Wyn-Jones et al., 2011). Still there is no consensus on the best indicator for viral pathogens in the environment.

CHAPTERS OUTLINE AND HYPOTHESES.

To address the objectives mentioned above this dissertation is divided into the following sections,

Chapter Two: Viral Concentration Methods. This chapter reports on the optimization and comparison of two major viral concentration methods, tangential flow filtration (TFF) and adsorption-elution (VIRADEL) using six different approaches. Viral concentration methods were evaluated on the basis of their (1) suitability for detection of HEV, (2) total extracted RNA yields (3) viral-like particle (VLP) recoveries, and (4) concentration factors in final volume. The following hypotheses were tested:

H1: The TFF concentration method will yield concentrations of viruses from surface waters sufficient to detect HEV.

H2: TFF (which is based on physical size selection) will be more consistent in VLP recoveries than the VIRADEL method (which requires chemical manipulation of the sample).

H3: Since the VIRADEL method has been optimized elsewhere for the detection of HEV rather than concentration of total viruses in environmental waters, it will yield higher HEV detection than the TFF method.

H4: The use of a secondary concentration method, such as centrifugal ultrafiltration, will increase the likelihood of successful detection of HEV in surface waters.

Chapter Three: Detection of HEV in recreational waters. This chapter reports on multi- season and multi-year efforts to detect and quantify enteroviruses, hepatitis A viruses and noroviruses from surface waters and wastewater treatment plant samples to determine point sources of human viral contamination. Techniques applied include SYBR Green Quantitative Reverse Transcriptase PCR (qRT-PCR), conventional Reverse Transcriptase PCR (RT-PCR), and cloning and sequencing.

The following hypotheses were tested:

H1: HEV will be present at the Nissequogue River and Port Jefferson Harbor surface waters due to the presence of point sources of human pollution.

Chapter Four: Environmental factors affecting HEV. This chapter examines the relationship between environmental factors and HEV occurrence in coastal recreational waters. For this, a suite of environmental factors, including UV Index, in situ water temperature, salinity, pH, precipitation, and tidal height were examined in relation to human enterovirus (EV) occurrence. Samples were also collected during rainfall events to evaluate the role of land runoff discharges as a source of viral pollution.

The following hypotheses were tested:

H1: HEV will be more prevalent during the winter months than the summer.

H2: Since HEV are used to acidic, low pH from the human digestive system, alkaline pHs will affect negatively EV occurrence.

H3: Due to the enhance adsorption of viral particles to other particles in higher salt concentrations EV occurrence will be negatively affected by salinity in the water column.

H4: HEV will be present after a rainfall event.

Chapter Five: Final Conclusions.

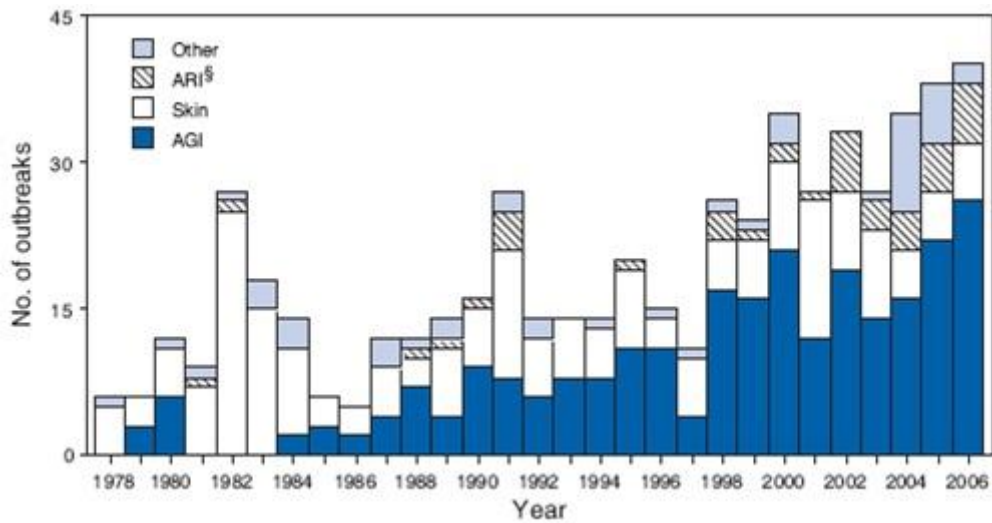


Figure 1.1 Time series of recreational waterborne outbreaks (n = 557) by illness in the US during the years of 1978-2006. Legend of illnesses: acute gastrointestinal illness (AGI); illness, condition, or symptom related to skin (SKIN); acute respiratory illness (ARI); and keratitis, conjunctivitis, otitis, bronchitis, meningitis, meningoencephalitis, hepatitis, leptospirosis, and combined illnesses (Other) (modified from Yoder et al. 2008).

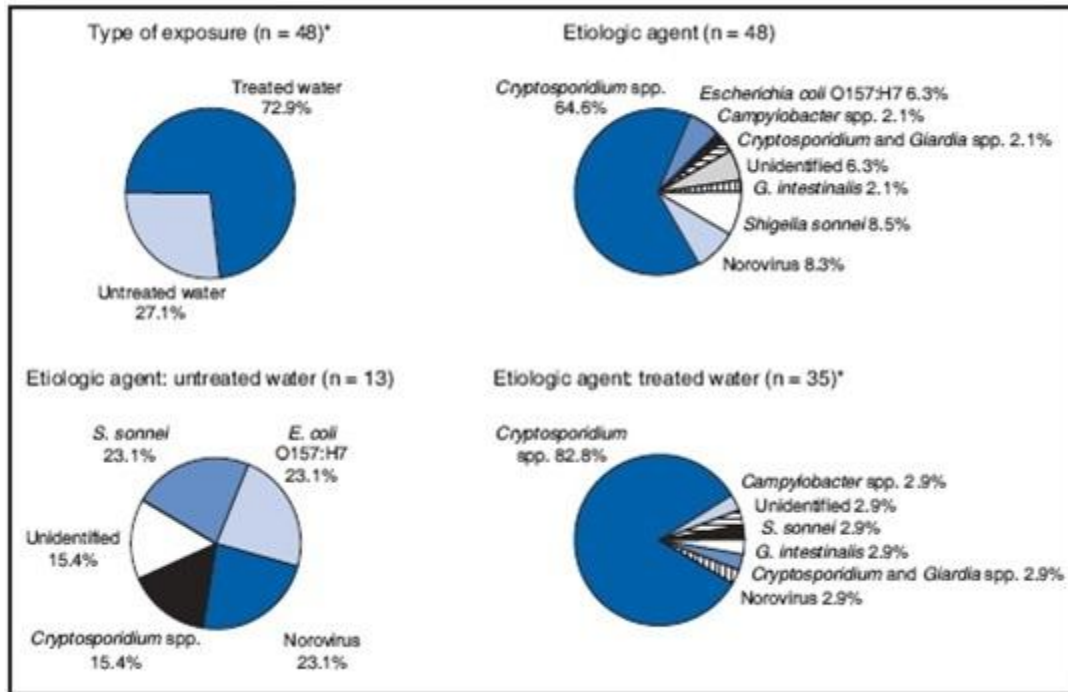


Figure 1.2 Reported recreational waterborne outbreaks of gastroenteritis by type of water exposure and etiological agent in the US during 2005-2006. Note that most gastroenteritis outbreaks were due to exposure to treated water (72.9%) compared to untreated water (27.1%), however most viral identified outbreaks were seen in untreated water (23.1%) rather than in treated waters (2.9%) (modified from Yoder et al., 2008).

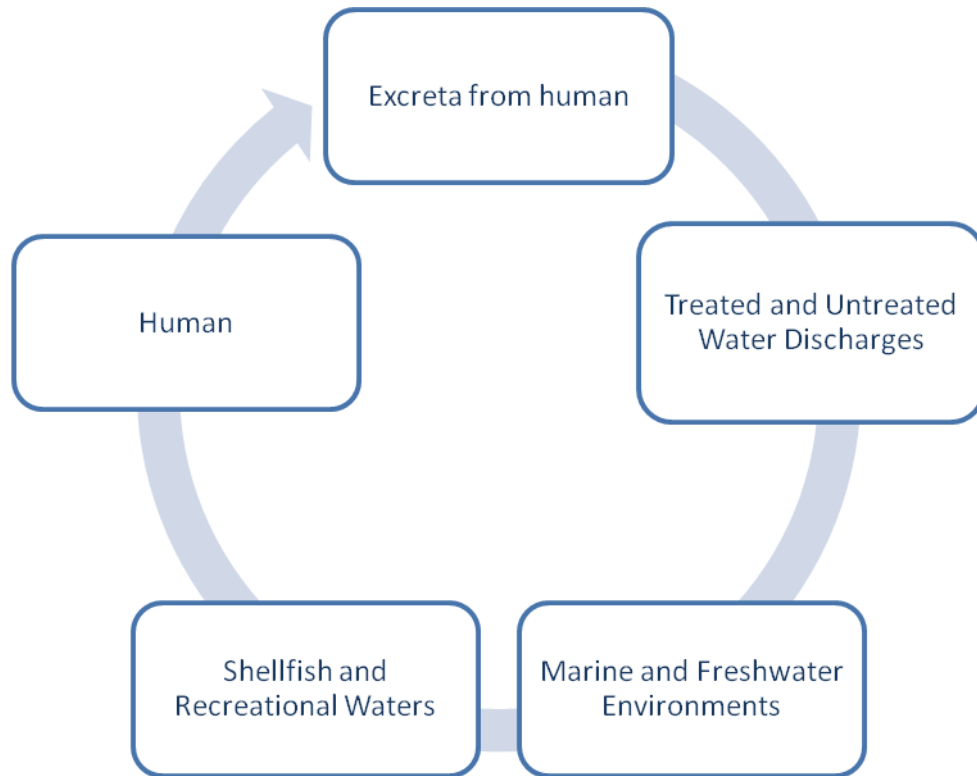


Figure 1.3 Routes of transmission of human enteric viruses in aquatic environments (modified from Bosch et al., 2006)

CHAPTER 2

VIRAL CONCENTRATION METHODS TO STUDY HUMAN ENTERIC VIRUSES IN RECREATIONAL COASTAL WATERS

INTRODUCTION

Research on human viral pollution in recreational coastal waters has been hampered by technical difficulties (Bosch et al., 2006). Natural water assemblages are complex mixtures of macroorganisms, microorganisms, inorganic and organic compounds. Viruses must be both concentrated and isolated for downstream applications, specifically those using molecular methods. These technical challenges are particularly significant for the detection of human enteric viruses (HEV) in surface waters. HEV are single stranded RNA viruses that are relatively dilute in aquatic environments (Fong and Lipp, 2005), perhaps as few as 10 viral particles per ml (Donaldson et al., 2002). A number of studies have detected naturally occurring HEV in surface coastal waters as reviewed by Griffin et al. (2003) in the US and Wyn-Jones et al. (2011) in Europe. Many other studies have focused on optimizing viral concentration methods due to the difficulties in detection of naturally occurring HEV and have opted to spike HEV into natural waters to validate their methods (Albinana-Gimenez et al., 2009; Calgua et al., 2008; Di Pasquale et al., 2010; Haramoto et al., 2007; Jothikumar et al., 1995; Katayama et al., 2002; Victoria et al., 2009; Li et al., 1998). Optimization of viral concentration methods is a critical initial step for the detection of HEV in natural waters.

Viral Concentration Methods. A good viral concentration method has the following characteristics: it is technically straightforward; rapid; provides high recoveries; is adequate for recovering a wide range of viruses; provides as small a volume of final concentrate as possible; and is inexpensive (Bosch, 1998). Several approaches have been used to concentrate viruses from natural waters, including ultrafiltration (Wommack et al., 2010), ultracentrifugation (Lawrence and Steward, 2010), and adsorption-elution techniques (Katayama et al., 2002). Ultrafiltration and ultracentrifugation have been used by microbial ecologists to concentrate native viruses from large sample volumes (~100L) of natural water (Lawrence and Steward, 2010; Steward and Culley, 2010; Wommack et al., 2010). Adsorption-elution methods have been used by public health scientists for water quality assessments and detection of human viral pathogens, because of the small sample volume required (~1L), the simplicity of the equipment needed, and the straightforward nature of the procedure.

Methods vary in a number of critical aspects, including the mechanism used to concentrate viruses (e.g. filtration vs. adsorption of viral particles), minimum initial sample volume, filtration time, final volume of concentrate, and cost. In addition, a secondary concentration may be needed to attain sufficient viral material for downstream applications. Given the lack of consensus regarding an optimal method for detection of HEV in environmental waters, I found it necessary to optimize and compare viral concentration methods. The goal was to detect three specific human enteric viruses (HEV) groups in recreational surface waters along the north shore of Long Island, NY. Viral concentration methods were evaluated on the basis of: (1) HEV detection, (2) total RNA extractions, (3) VLP recoveries (4) concentration factors, and (5) final volume of viral concentrate.

Approach. I used both one- and two-step viral concentration methods. These included ultrafiltration via tangential flow filtration (TFF) and viral adsorption-elution (VIRADEL), as primary concentration techniques, and ultracentrifugation (Uc) and centrifugal ultrafiltration (Uf) as secondary concentration techniques. A total of six method combinations were compared: (1) tangential flow filtration (TFF), (2) viral adsorption-elution (VIRADEL), (3) tangential flow filtration followed by ultracentrifugation (TFF and Uc), (4) VIRADEL followed by ultracentrifugation (VIRADEL and Uc), (5) TFF followed by centrifugal ultrafiltration (TFF and Uf), and finally (6) VIRADEL followed by centrifugal ultrafiltration (VIRADEL and Uf) (Figure 2.1).

METHODS

Collection Sites. Two different types of recreational coastal water sites were selected for sampling on the central north shore of Long Island, New York. These are representative of coastal aquatic environments where human enteric viruses would be expected to be found. The Nissequogue River (NR; n=7) is a freshwater environment where leakage from domestic septic systems along the river can introduce HEV and there is direct discharge of land runoff into the river. The Port Jefferson Harbor Sites 1 (PJH1; n=5) and 2 (PJH2; n=39) are seawater environments that receive discharges from the Port Jefferson wastewater treatment facility (PJWTP), storm water runoff, and vessel wastewater (Figure 2.2). In addition, one sample was collected from Conscience Bay (CB), an embayment adjacent to Port Jefferson Harbor that does not receive surface discharges from the PJWTP.

Surface Water Sample Collection. A total of 52 surface water samples were collected from March 2006 through December 2009. Sampling involved the collection of 200-20,000 ml of

water (a variety of volumes were collected for filtration and concentration optimizations) with a hand pump or by holding containers immediately below the surface. Sterilized carboys or bottles were rinsed three times with surface water prior to final collection. Samples were immediately transferred to the laboratory.

Tangential Flow Filtration (TFF). TFF was conducted using a Quixstand Benchtop System® (AGT Technology Model QSM-02S, Serial no. 01QS1350) with a Masterflex® peristaltic pump (Cole Parmer Model no. 7520-35) and a polysulfone hollow-fiber ultrafiltration membrane cartridge of 100,000 NMWC (Amersham Biosciences GE Healthcare®, Catalog no. UFP-100-C-4A). Before analysis of samples, the entire system including the membrane cartridge was rinsed with one liter of distilled water, followed by one liter 0.1N NaOH, and again with one liter of distilled water. This three-step rinse was repeated until retentate was visually clear, and required between one to four hours before a sample could be processed. In general, 1L of surface water sample was filtered in approximately 30 minutes with a pressure of no more than 5 psi per the manufacturer's recommendation. The filter was reverse flushed with ~50 ml of sample water to recover the viral concentrate. This concentrate was stored at -80°C prior to further concentration and/or RNA extraction.

Virus Adsorption-Elution (VIRADEL). The VIRADEL method was adapted from protocols by (Fong et al., 2005; Gentry et al., 2009; Katayama et al., 2002). Surface water samples of 1,000 ml of were adjusted to pH 4 using a 1N of CH₃COOH or 30nM AlCl₃. Samples were then filtered through a 90 mm, 0.45 µm pore size Type HA MF™ Millipore membrane filter. Viruses adsorbed to the filter membrane. The filter was rinsed with 100 ml of 0.5 mM H₂SO₄ and viruses were eluted from the filter with 10 ml of 1mM NaOH into a sterile polypropylene tube that contained 100 µl of 50 mM H₂SO₄ and 100 µl of 100 x TE buffer.

Surface water samples (1L) were filtered in ~ 60 minutes using vacuum pressure of no more than 200 mm Hg. The final volume of viral concentrate was approximately 10 ml which was stored at -80°C for further concentration and/or RNA extraction. All solutions were freshly made prior to sample processing.

Ultracentrifugation. Primary viral concentrates (1 – 10 ml), obtained either by TFF or by VIRADEL, were further concentrated using a Beckman ultracentrifuge (TL-100 Ultracentrifuge) and a Beckman Coulter rotor (model TLA-120.2). Samples were spun at 10,600-434,500 x g (50,000-100,000 rpm) for 2 h at 4°C and no gradient solution was used. Approximately 800-900 µl of supernatant was discarded and the remaining 100-200 µl was stored at -80°C for RNA extraction.

Centrifugal Ultrafiltration. Centriplus YM 100,000 MWCO (Cat. No. 4424) or Ultracel 50,000 MWCO (Cat. No. UFC905024) centrifugal filter units (both by Amicon Millipore) were used to further concentrate 10-15 ml of viral concentrate, obtained from TFF or VIRADEL. Samples were loaded into the sample reservoir (maximum capacity 12-15 ml) and centrifuged using an Eppendorf centrifuge (model 5804 R) and Eppendorf rotor (model F-34-6-38) at 2,055 x g (4000 rpm) for 15 minutes to a final volume of 200-500 µl. The concentrates were stored at -80°C for RNA extraction.

Viral Direct Counts. Direct counts of stained viral-like particles (VLP) pre- and post-concentration were obtained using epifluorescence microscopy following protocols modified from Chen et al. (2001) and Feng and Wang (2005). Briefly, 1 ml of water sample or viral concentrate was filtered thru a 0.02µm pore size anodisc (Millipore) membrane filter on a backup membrane filter using a hand pump with vacuum <15 kPa. After no sample water remained on the anodisc (by visual examination), the dry filter was stained with 10 µl of SYBR®

Gold (10x) dye (Invitrogen) and placed in a sterile aluminum foil-covered Petri dish at room temperature in the dark for at least 15 minutes. The filter was mounted on a drop of immersion oil on a glass slide and covered by a drop of a commercial antifade solution (Pro Long® Gold antifade reagent (Invitrogen, no. P36934). VLPs were counted under epifluorescent illumination.

VLP Recoveries. To determine the efficiency of recovery of VLP the following equation was used:

$$\text{VLP Recovered (\%)} = [(V_f \times \text{VC}) / (V_i \times \text{VDC})] \times 100$$

where

V_f = Final Volume of viral concentrate

VC = Viral direct count of VLP per ml from the viral concentrate

V_i = Initial Volume of sample concentrated

VDC = Viral direct counts of VLP per ml in the initial sample before being concentrated

Total VLP recovered was expressed as a percent.

Total RNA Extractions. RNA was extracted from the viral concentrates using the RNeasy Mini Kit (QIAGEN). Following the kit's handbook (fourth edition April 2006) and supplementary protocol, the procedure for the "purification of total RNA from bacteria" was used with the following modifications since there was not a specific procedure for purification of viral RNA. Briefly, an initial volume of 100 μl from the viral concentrate ($\geq 10^9$ VLP ml^{-1}) was used for the extraction. Then, 350 μl of Buffer RLT (containing 1% β -mercaptoethanol (β -ME), i.e., 10 μl of β -ME per one ml of Buffer RLT) was added to the viral concentrate and vortexed for approximately 30 seconds, followed by an incubation of one minute at room temperature. The rest of the procedure followed the kit's handbook instructions. The final filtrate (50 μl total volume) was stored as in aliquots of 10 μl at -20°C and -80°C freezers for further analysis.

RNA Concentrations. RNA concentrations were measured using the commercial kit Quant-iT Ribogreen (Invitrogen, catalog num R11490) according to the manufacturer's instructions for the 2,000-fold dilution low range, or directly using a Nanodrop spectrophotometer to measure absorbance of RNA at λ 260 nm.

Statistical Analysis. From the descriptive statistics the mean values are reported with standard deviation (SD). A t-test, a Mann-Whitney Rank Sum test, and a Kruskal-Wallis One Way Analysis of Variance on Ranks were used to calculate significant differences between concentration methods. A Spearman rank order analysis was used to calculate significant correlations. Rank tests were used for non-normal data. All statistical analyses were performed using Sigma Plot (version 11.0) and Excel (version 2007) software.

RESULTS

Primary Concentration Methods. Among all samples concentrated using the TFF method (n=27) the mean VLP recovery was 40 % (SD 43), the mean concentration factor was 74 (SD 175), and the mean final volume was 41 ml (SD 13). In comparison, those that were concentrated using the VIRADEL method (n=25) had values of 34 % (SD 30), 101 (SD 140), and 5 ml (SD 2), respectively (Table 2.1). Using a Mann-Whitney Rank Sum test, I found no significant difference ($p = 0.842$) between VLP recoveries by the TFF and the VIRADEL methods. However, using the same statistical analysis I found significant differences ($p = < 0.001$) in both concentration factor and final volume between the TFF and the VIRADEL methods.

In addition, seawater samples had higher mean recoveries using both TFF and VIRADEL methods, 45 % (SD 44) and 39 % (SD 30), compared to freshwater samples which had VLP

recoveries of 4 % (SD 4) and 8 % (SD 10), respectively. Using a Mann-Whitney Rank Sum test, a significant difference ($p = 0.010$) in VLP recoveries was found between freshwater samples (NR, $n = 3$) and seawater samples (PJH, $n = 24$) using the TFF and a significant difference ($P = 0.018$) in VLP recoveries found between freshwater samples (NR, $n = 4$) and seawater samples (PJH, $n = 21$) using the VIRADEL method.

A subset of paired samples ($n=13$) were analyzed to compare differences between the TFF and VIRADEL viral concentration methods (Table 2.2). Using a Signed Rank test, there was no significant difference ($p = 0.414$) between VLP recovered by the TFF method compared to the VIRADEL method (means were 64 % (SD 52) and 41 % (SD 34), respectively; Table 2.2). TFF had values for concentration factor of 19 (SD 7) and a final volume of viral concentrate of 50 ml (SD 11), while VIRADEL had values for concentration factor of 66 (SD 100) and final volume of viral concentrate was 5 ml (SD 0.4). Using a Signed Rank test I found a significant difference ($p = <0.001$) in concentration factors between the TFF and the VIRADEL methods and a significant difference ($p = <0.001$) in final volumes of viral concentrate between the TFF and the VIRADEL method.

Secondary Concentration Methods. For ultracentrifugation ($n=8$), the mean VLP recovered was 45 % (SD 59), the mean concentration factor was 5,953 (SD 4,536), and the mean final volume of viral concentrate was 0.3 ml (SD 0.3). By contrast, the values for centrifugal ultrafiltration ($n=13$) were 157 % (SD 145), 2,692 (SD 1,316), and 0.4 ml (SD 0.1), respectively (Table 2.3). Using a t-test, no significant difference ($p = 0.284$) was found between VLP recovered by centrifugal ultrafiltration or ultracentrifugation. Mann-Whitney Rank Sum tests found no significant difference ($p = 0.396$) in concentration factors but a significant difference ($p = 0.012$) in final volume of concentrate between ultrafiltration and ultracentrifugation.

RNA Extractions. Highest mean values of total RNA yields were found in the nine samples concentrated using the TFF and Uf method, 1,847 ng (SD 1275) (Table 2.4), followed by three samples concentrated using VIRADEL and Uf, 304 ng (SD 281). Using a Kruskal-Wallis One Way Analysis of Variance on Ranks a significant difference ($p = <0.001$) in RNA extracted from samples concentrated between the six different combinations was determined. Using a Mann-Whitney Rank Sum Test a significant difference ($p = 0.016$) in RNA extracted between the TFF and UF and the VIRADEL and UF methods was confirmed. Likewise, HEV were detected in samples (details in Chapter 3) that were concentrated using both two-step methods of TFF and Uf and VIRADEL and Uf (Table 2.5). However, a Spearman rank order analysis indicated no significant correlation ($r_s = 0.079$, $p = 0.687$) between RNA concentrations and HEV detection ($n = 28$).

DISCUSSION

The concentration of enteric viruses by adsorption-elution followed by centrifugal ultrafiltration (VIRADEL and Uf) has been used successfully by others for the recovery and detection of various HEV (Haramoto et al., 2007; Victoria et al., 2009; Villar et al., 2006). However to my knowledge only a single recent study has evaluated the use of the TFF method followed by centrifugal ultrafiltration (TFF and UF) for the detection of HEV (Gibson et al., 2011). In my study, one- and two-step approaches to concentrate viruses were compared using six methods that included tangential flow filtration, viral adsorption-elution, ultracentrifugation, and centrifugal ultrafiltration techniques with the final aim to detect HEV in surface coastal waters.

Primary Concentration Methods. Although the TFF and VIRADEL yielded significantly concentration factors and final volumes of viral concentrate, they did not differ

significantly in their ability to recover total VLP. This finding was unexpected since the methods rely on different mechanisms to concentrate viruses.

The TFF method uses particle size exclusion via tangential flow filtration to concentrate viruses, while the VIRADEL method relies on chemical manipulation to adsorb viruses from environmental samples to a membrane filter (Bosch et al., 2006; Steward and Culley, 2010). It has been reported that different concentrations of cations used to adsorb the viruses onto the membrane and the presence of natural salts in the sample can affect VIRADEL recoveries. Victoria et al. (2009) tested different types of waters and varying concentrations of $MgCl_2$ using VIRADEL and centrifugal ultrafiltration methods and found lower recoveries from seawater (0.8%) than from freshwater (17.8%) at 5mM $MgCl_2$ but at 50mM $MgCl_2$ the recoveries for seawater (5.9%) were higher than for freshwater (3.7%). In contrast, others have reported that in seawater samples the attachment of viruses has been hampered due to a high ionic strength, lowering the efficiency of the method (Calgua et al., 2008; Lukasik et al., 2000). However, in my results I found significantly ($p = 0.018$) higher recoveries for seawater than for freshwater samples using the VIRADEL method with concentration of 1N of CH_3COOH or 30nM $AlCl_3$ as cations. More so, this significant difference ($p = 0.010$) in recovery was also seen for samples concentrated by the TFF method, where higher recoveries were seen again for seawater than for freshwater samples.

Because I could not determine if the concentration of salts in the sample was affecting the recoveries of the VIRADEL method, as has been reported elsewhere, I decided to further examine which method was better to concentrate viruses from seawater surface samples. Thus I analyzed paired samples from PJH using both methods (Table 2.2). However, there was no significant difference between methods using the same sample. As a consequence I added

secondary concentration steps, since no HEV were detected using a one-step viral concentration approach. For this I used ultracentrifugation and centrifugal ultrafiltration techniques.

Secondary Concentration Methods. Positive detection of HEV was seen only in samples that were further concentrated using centrifugal ultrafiltration (Table 2.5). This result was expected, since the concentration efficiency after the primary concentration was ≤ 100 -fold and after the secondary concentration $\geq 1,000$ -fold using ultracentrifugation and centrifugal ultrafiltration. Likewise, other researchers have been able to detect HEV in surface waters after 1,000 to 25,000-fold concentration using TFF and centrifugal ultrafiltration (Gibson et al., 2011), VIRADEL and centrifugal ultrafiltration (Gentry et al., 2009; Gersberg et al., 2006), and even just centrifugal ultrafiltration (Jiang et al., 2007) (Table 2.6). However, these recoveries were measured using specific strains of human enteric viruses and not direct counts of VLP.

Ideally, a specific viral strain of the virus of interest would be employed to measure efficiency of the concentration method used. On the other hand, it is apparent that an inexpensive and non-specific method using direct counts of VLP by epifluorescence microscopy (EFM) and SYBR® Gold can be used to measure total viral concentration efficiencies of the VIRADEL and TFF methods for surface water samples. Direct counts have been used elsewhere (Fuhrman et al., 2005) to measure the recovery efficiencies of methods to concentrate and detect poliovirus particles stained with SYBR Green II. Additionally, Suttle and Fuhrman (2010) suggest that although SYBR® Gold is known to stain double-stranded and single-stranded DNA and RNA (Chen et al., 2001; Feng and Wang, 2005), not all viral particles, especially ss RNA viruses, are stained equally well. This approach is low cost, feasible for most laboratories, can be used to provide estimates of total recovery of VLP, and can be applied for any environmental virology

studies, but it is not optimal to specifically estimate the recovery of human enteric viruses, in particular single-stranded RNA viruses.

Total RNA Extractions. A higher total RNA yield can be used to determine the best viral concentration method for HEV detection. The method that yielded the highest RNA concentration was TFF combined with UF (Table 2.4). However there was no significant correlation ($r_s = 0.079$, $p = 0.687$) between HEV detection and RNA concentration. In addition, due to the small number of positive HEV samples ($n=3$), these results did not allow me to examine the HEV detection and total RNA concentration relationship rigorously.

To conclude, I was able to detect HEV (further details in Chapter 3) in samples that were concentrated using a two-step approach, either using TFF and UF or VIRADEL and UF. Since there was not a significant difference between HEV detection, total RNA extracted, and VLP recoveries using either the TFF or the VIRADEL methods, I would recommend the use of the VIRADEL because it is technically simpler, it requires a small initial volume (500 ml), and it yields a small final volume of viral concentrate (0.2 ml). However, the critical step to concentrate viruses efficiently was the use of the centrifugal ultrafiltration technique. This second step assures that environmental surface water sample are at least concentrated 1,000-fold and the viral concentration is sufficient to determine presence or absence of HEV using molecular methods.

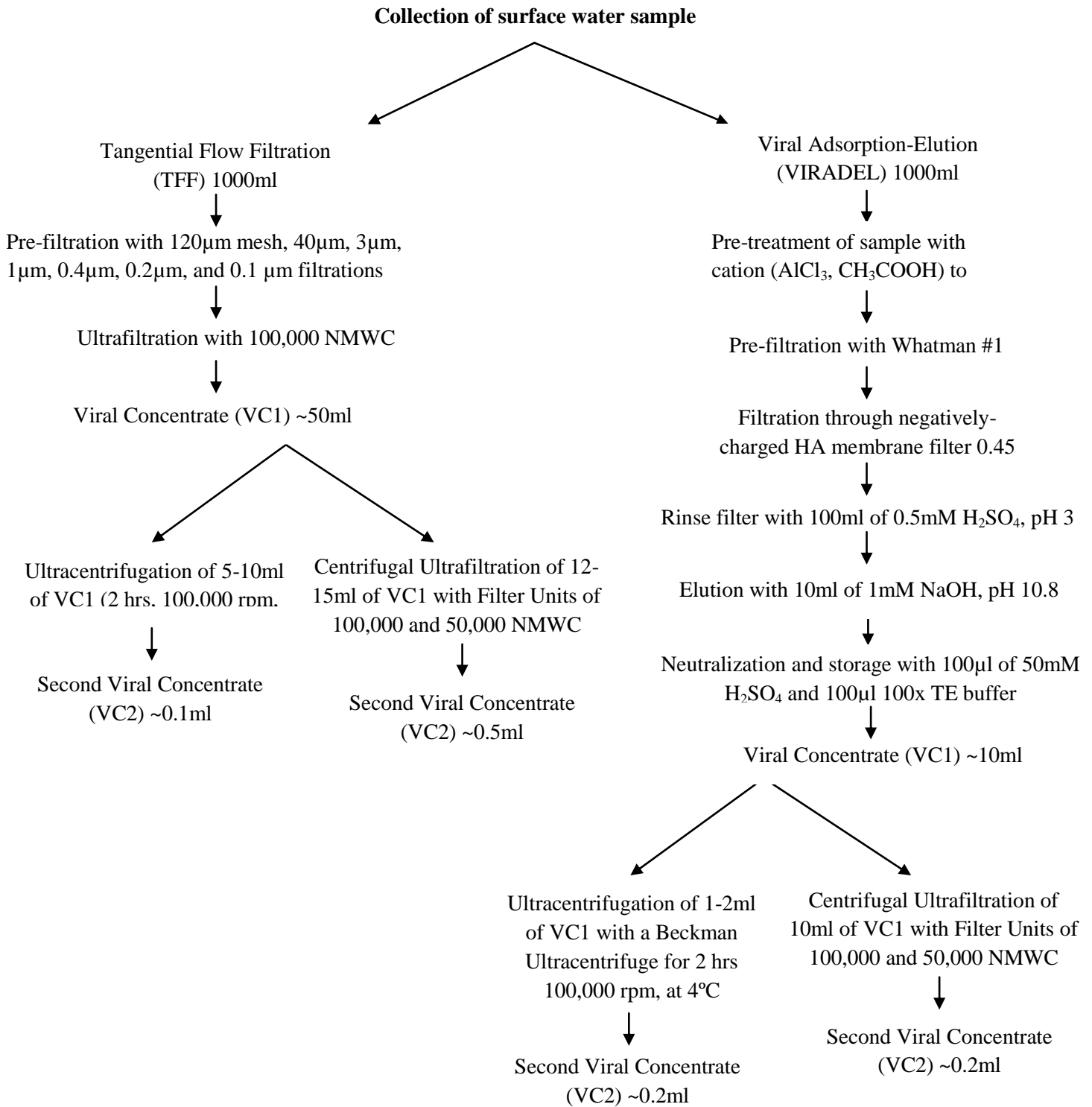


Figure 2.1 Flowchart of the six protocols tested for the concentration of total viruses from recreational surface waters using 1-step (primary concentration) and 2-step approaches (primary and secondary concentrations): (1) Tangential Flow Filtration (TFF), (2) Tangential Flow Filtration and Ultracentrifugation (TFF/Uc), (3) Virus Adsorption-Elution (VIRADEL), (4) Virus Adsorption-Elution and Ultracentrifugation (VIRADEL/Uc), (5) Tangential Flow Filtration and Centrifugal Ultrafiltration (TFF/Uc), and (6) Virus Adsorption-Elution and Centrifugal Ultrafiltration (VIRADEL/Uc). VC1 is the first viral concentrate and VC2 is the second viral concentrate.



Figure 2.2 Maps of study sites located along the North Shore of Long Island, NY (a). Surface water samples were collected from the Nissequogue River (NR), Conscious Bay (CB), and the Port Jefferson Harbor (PJH) (b). PJH is a semi- enclosed tidal urban estuary (c) that receives surface discharges from a local Wastewater treatment Plant (WWTP), various storm water pipes, and private boats (d). The majority of samples were collected at the Port Jefferson Harbor Site 2 (PJH2), where there is a small recreational park and access to the harbor water. During summer 2008 samples were also taken from Site 1 (PJH1) (d). Bold squares symbolized areas zoomed in. Images were modified from Google maps.

Table 2.1. Descriptive statistics of primary viral concentration methods, TFF and VIRADEL, used for surface water samples (n=52) collected at the Nissequogue River (NR), Conscious Bay (CB,) and the Port Jefferson Harbor Site 1 (PJH1) and Site 2 (PJH2). VC 1 stands for the first viral concentrate and VLP stands for viral-like particle. SD = standard deviation

TFF (n=27)	Max	Min	Median	Mean (SD)
Initial Volume (ml)	20000	200	1000	2026 (3998)
Final Volume VC 1(ml)	55	13	35	41 (13)
Concentration Factor	769	6	27	74 (175)
Initial Virus (VLP 10 ⁷ ml ⁻¹)	21	0.3	4	5 (5)
Final Virus VC 1 (VLP 10 ⁸ ml ⁻¹)	24	0.1	3	4 (5)
VLP Recovered (%)	157	0.8	23	40 (43)
VIRADEL (n=25)	Max	Min	Median	Mean (SD)
Initial Volume (ml)	2000	200	200	401 (425)
Final Volume VC 1 (ml)	10	1	5	5 (2)
Concentration Factor	500	37	50	101 (140)
Initial Virus (VLP 10 ⁷ ml ⁻¹)	17	0.4	4	6 (5)
Final Virus VC 1 (VLP 10 ⁸ ml ⁻¹)	48	0	7	9 (11)
VLP Recovered (%)	118	0.1	22	34 (30)

Table 2.2 Descriptive statistics for paired samples (n=13) surface water collected at the Port Jefferson Harbor Site 1 (PJH1) and Site 2 (PJH2) using the TFF and VIRADEL primary viral concentration methods. VC 1 stands for the first viral concentrate and VLP stands for viral-like particle. SD = standard deviation.

TFF	Max	Min	Median	Mean (SD)
Initial Volume (ml)	1000	200	1000	900 (252)
Final Volume VC 1(ml)	55	25	55	50 (11)
Concentration Factor	40	6	18	19 (7)
Initial Virus (VLP 10 ⁷ ml ⁻¹)	21	1	6	7 (5.4)
Final Virus VC 1 (VLP 10 ⁸ ml ⁻¹)	8	1	5	5 (2.5)
VLP Recovered (%)	157	7.5	48	64 (52)
VIRADEL	Max	Min	Median	Mean (SD)
Initial Volume (ml)	2000	200	200	342 (498)
Final Volume VC 1 (ml)	6	4	5	5 (0.4)
Concentration Factor	400	37	37	66 (100)
Initial Virus (VLP 10 ⁷ ml ⁻¹)	21	1	6	7 (5.4)
Final Virus VC 1 (VLP 10 ⁸ ml ⁻¹)	33	1	7.1	8.5 (8)
VLP Recovered (%)	118	0.4	29	41 (34)

Table 2.3 Descriptive statistics for surface water samples (n=21) collected at the Port Jefferson Harbor Site 1 (PJH1) and Site 2 (PJH2) that were further concentrated using the secondary viral concentration methods, ultracentrifugation and centrifugal ultrafiltration, after the primary concentrations. VC2 stands for second viral concentrate and VLP stands for viral-like particle. SD = standard deviation.

Ultracentrifugation (n= 8)	Max	Min	Median	Mean (SD)
Initial Volume (ml)	1000	200	1000	800 (370)
Final Volume VC 2 (ml)	0.9	0.1	0.2	0.3 (0.3)
Concentration Factor	10000	513	7500	5953 (4536)
Initial Virus (VLP 10 ⁷ ml ⁻¹)	8	2	4	4 (2)
Final Virus VC2 (VLP 10 ¹⁰ ml ⁻¹)	0.5	0	0.2	0.3 (0.2)
VLP Recovered (%)	112	3	21	45 (59)
Centrifugal Ultrafiltration (n=13)	Max	Min	Median	Mean (SD)
Initial Volume Concentrated (ml)	1000	1000	1000	1000
Final Volume VC2 (ml)	0.5	0.2	0.5	0.4 (0.1)
Concentration Factor	5000	2000	2000	2692 (1316)
Initial Virus (VLP 10 ⁷ ml ⁻¹)	21	1	5	6 (6)
Final Virus VC2 (VLP 10 ¹⁰ ml ⁻¹)	24	7.6	14.3	15 (8)
VLP Recovered (%)	312	26	133	157 (145)

Table 2.4 Descriptive statistics for total RNA yield from viral concentrates using the following six protocols combination: tangential flow filtration (TFF); viral adsorption-elution (VIRADEL); tangential flow filtration and ultracentrifugation (TFF and Uc); tangential flow filtration and centrifugal ultrafiltration (TFF and Uf); viral adsorption-elution and ultracentrifugation (VIRADEL and Uc); and viral adsorption-elution and centrifugal ultrafiltration (VIRADEL and Uf). SD stands for standard deviation.

Viral Concentration Methods	Total RNA yield (ng)			
	Max	Min	Median	Mean (SD)
TFF	305	8.5	115	138 (117)
VIRADEL	NA	NA	715	715
TFF and Uc	305	0	115	125 (122)
TFF and Uf	4045	816	1107	1847 (1275)
AE and Uc	NA	NA	100	100
AE and Uf	619	76	219	304 (281)

Table 2.5 Comparison summary between the six viral concentration protocol combinations tested: tangential flow filtration (TFF); viral adsorption-elution (VIRADEL); tangential flow filtration and ultracentrifugation (TFF and Uc); tangential flow filtration and centrifugal ultrafiltration (TFF and Uf); viral adsorption-elution and ultracentrifugation (VIRADEL and Uc); and viral adsorption-elution and centrifugal ultrafiltration (VIRADEL and Uf). VLP stands for viral-like particles and VC stands for viral concentrate. ND = no data. *Median values are presented.

Viral Concentration Methods	HEV Detection	Total RNA yield (ng)*	VLP Recovered (%)*	Concentration Factor*	Final Volume of Viral Concentrate (ml)*
TFF	No	115	23	27	35
VIRADEL	No	715	22	50	5
TFF and Uc	No	115	3	10000	0.1
TFF and Uf	Yes	1107	ND	2000	0.5
AE and Uc	No	100	67	756	ND
AE and Uf	Yes	219	133	5000	0.2

Table 2.6 Comparison between viral concentration methods evaluated by other researchers and this study using centrifugal ultrafiltration (Uf) for the successful detection of HEV in seawater.

Reference	Type of virus	Spiked	% Recovered	Concentration Methods	Initial Vol. (L)	Final Vol. (ml)	Concentration Factor
Current Study	Natural VLP	No	26-312 %	VIRADEL and Uf	1	0.2	5000
Current Study	Natural VLP	No	ND	TFF and Uf	1	0.5	2000
Gibson et al. (2011)	NA	NA	NA	TFF and Uf	100	4	25000
Gersberg et al. (2006)	Strains of HAV and EV	Yes	11 % and 71 %	VIRADEL and Uf	1	0.5	2000
Victoria et al. (2009)	Strain of NoV	Yes	0.8-5.9 %	VIRADEL and Uf	2	2	1000
Jiang et al. (2007)	Bacteriophage ϕ HSIC	Yes	60 %	Uf	0.5	0.5	1000

CHAPTER 3

DETECTION OF HUMAN ENTERIC VIRUSES IN RECREATIONAL COASTAL WATERS

INTRODUCTION

Human Enteric Viruses (HEV). Enteric viruses are the most common cause of gastroenteritis worldwide, and most often transmitted via the fecal-oral route. They are an important and diverse group of viruses found in the intestinal tract of humans and can cause maladies like hepatitis, meningitis, fevers, rashes, and respiratory diseases (Table 3.1). Enteric viruses are divided into a number of families, including Adenoviridae (adenoviruses), Picornaviridae (polioviruses, coxsackieviruses, echoviruses, enteroviruses and hepatitis A virus), Reoviridae (reoviruses and rotaviruses), and Caliciviridae (noroviruses, caliciviruses, and astroviruses) (Bosch et al., 2006). Although some of these viruses have been detected in the natural environment and specifically in coastal recreational waters (Griffin et al., 2003; Wyn-Jones et al., 2011), no indicator is currently available to monitor viral contamination. This complete lack of monitoring is alarming given their potential to cause a variety of infectious diseases, to remain infectious for long periods outside their host (up to 130 days) and infect at a low dose compared to pathogenic bacteria (Fong and Lipp, 2005), and to be constantly discharged into the environment. Human risk exposure assessment would be improved by virus monitoring in recreational waters. However, the quantification of human viruses in aquatic environments remains technically difficult, time

consuming, and expensive. In addition, the viruses that are the etiological agents of main concern for environmental exposures are difficult or currently impossible to quantify in water (Parkin et al., 2003).

Current Detection Methods. HEV have been detected using cell culture methods. However, due to the technical and time demands of this approach, the most popular detection approach today is to use molecular methods such as the polymerase chain reaction (PCR)(Fong and Lipp, 2005). Detection protocols involve a series of steps including (1) nucleic acid extraction, (2) reverse transcriptase, and (3) detection and amplification of a conserved region of the viral genome (Girones et al., 2010). Although these methods are efficient for the detection of HEV, confirming infectivity of the viral particles requires the presence of the viral host. Cell culture and plaque assays are still the preferred techniques to measure infectivity, but cell culture assays require a specific host cell for each viral strain, and this technology has not been developed for many viruses. Important infectious viruses such as noroviruses are not cultivable at present. In addition, environmental viral concentrates must be highly purified in order to remove contaminants prior to testing on cell lines, and cell culture assays are extremely time consuming and costly. These practical restrictions on the plaque assay approach have made molecular methods more popular for the detection and monitoring of HEV in recreational waters.

Approach. In this study, recreational estuarine waters were monitored for the occurrence of three groups of human enteric viruses using a combination of molecular methods: enterovirus (EV), hepatitis A virus (HAV), and norovirus (NoV). The targeted viruses were chosen because they represent a public health risk, they have previously been detected in surface coastal waters (Griffin et al., 2003; Wyn-Jones, et al., 2011), and environmental detection of viral RNA may indicate a recent viral contamination event of infectious viruses (Tsai et al., 1995; Wetz et al.,

2004). In this study, after total RNA was extracted from concentrated viral samples we attempted to detect and quantify EV, HAV and NoV using SYBR Green Quantitative Reverse Transcriptase PCR (qRT-PCR), conventional Reverse Transcriptase PCR (RT-PCR), and cloning and sequencing techniques. In addition, samples were collected from wastewater treatment effluent and the benthos, and analyzed to determine sources of human viral contamination and their possible reservoirs, respectively.

METHODS

Collection Sites. Sites along the north shore of Long Island, New York were selected to represent a range of environments where native and non-native viruses would be expected to be found. These included the Nissequogue River (NR) in Smithtown (where viruses enter from cesspools which border the small river), the Stony Brook Harbor (SBH) (which is well flushed and has limited shoreline discharges), Conscience Bay (CB) (a small embayment adjacent to the Port Jefferson Harbor with reduced circulation and potential inputs from cesspools belonging to homes along the shoreline), and the Port Jefferson Harbor (PJH) (receives direct discharges of land runoff pipes and vessels' waste and from the Port Jefferson Wastewater Treatment Plant, PJWTP) (Figure 3.1). The majority of surface water samples were from the Port Jefferson Harbor Site 2 (PJH2; n= 34) located in the innermost harbor area in front of a storm water runoff pipe discharge (Figure 3.2).

Surface Water Samples (n=55). Surface water samples were collected during March 2006 and December 2009. Sampling was done by collecting 1L of water by dipping containers just below the surface at each site. Sterilized carboys and sample bottles were rinsed three times with surface water prior to final collection. Samples were put on ice and taken immediately to the laboratory.

Wastewater Treatment Plant Samples (n=5). Two samples from activated sludge treatment were collected from the Port Jefferson Wastewater Treatment Plant on March 27 2008 and July 23 2008. On March 27 2008, three grab samples were collected from the effluent, post-chlorination, and post-UV tanks (one per tank).

Benthic Animal Samples (n=3). Mussels and polychaetes were collected on November 3, 2009 and December 4, 2009 from the Port Jefferson Harbor pier, near PJH Site 2. Animal samples were transported to the laboratory on ice and then stored at -20°C before processing.

Viral Concentration from Surface Water and Effluent Samples. Viruses were concentrated using one and two-step viral concentration approaches, which consisted of tangential flow filtration, adsorption-elution, ultracentrifugation and centrifugal ultrafiltration. Concentration methods are described in detail in Chapter 2.

Sludge Sample Processing. A protocol modified from Monpoeho et al. (2001) was used. Sludge samples (400 ml) were centrifuged (5,000 rpm, 15 min) using an Eppendorf centrifuge. Supernatant was then discarded and the pellet was re-suspended in 5 ml of 10% beef extract (pH 9.0). The suspension was mixed (30 min, maximum speed) using an incubator shaker, followed by centrifugation (5,000 rpm, 1h, 4°C). Supernatant was then transferred to a sterile tube and the same volume of PEG 8% in phosphate buffer (pH 7.2) was added. Samples were vortexed and stored overnight at 4°C. On the following day, samples were centrifuged (3h, 5,000 rpm, 4°C) after which the supernatant was discarded and the pellet re-suspended in 12 ml of phosphate buffer (pH 7.2). Samples were stored at -80°C until RNA extraction.

Separation of VLP from animal tissue. Five grams of mussel digestive tract tissue and 1.5 g of polychaete entire worm tissue were processed to separate viruses from tissue using a modified protocol from Gentry et al. (2009b). Briefly, tissue was finely chopped with a sterile razor and an

equal volume of phosphate buffered saline (PBS) buffer containing $100 \mu\text{g ml}^{-1}$ proteinase K, was added to the tissue suspension. The mixture was incubated at 37°C for 1h with shaking at 320 rpm. Sample was incubated at 65°C for 15 min to inactivate proteinase K, then vortexed and centrifuged at $3000 \times g$ for 5 minutes. The supernatant was removed by pipetting and stored in cryovials at -80°C .

RNA Extraction. The commercial RNeasy Mini Kit (QIAGEN) was used for the majority of RNA extraction following the manufacturer's manual procedure for "purification of total RNA from bacteria" with some modifications. Details are given in Chapter Two.

RNA Concentration. RNA concentrations were measured either using a Quant-iTTM RiboGreen[®] RNA Assay Kit (Invitrogen, catalog num. R11490) according to the manufacturer's "low range standard curve" protocol, or directly using a NanoDropTM 1000 spectrophotometer (Thermo Scientific) to measure absorbance of RNA at λ 260 nm.

Detection and Quantification by SYBR Green qRT-PCR. Enteroviruses, hepatitis A viruses, and noroviruses, were detected by a one-step SYBR Green Real-Time PCR (Brilliant II SYBR Green QRT-PCR Master Mix Kit, 1-Step from Stratagene) in a Mx3000P Thermal Cycler (Stratagene, Inc.). Five pairs of primers were chosen based on the manufacturer's recommendations (Ambion, Inc. and Cenetron Diagnostics) for the commercial positive controls Armored RNATM. Four different Armored RNA controls were used for all three different groups, EV, HAV and NoV Genegroup I and Genegroup II, and have been previously used in a number of studies by Ando et al. (1995); Griffin et al. (1999); Rotbart (1990); and Schwab et al. (1995) to detect EV, HAV and NoV, respectively (Table 3.2). These primers included two pairs for the detection of 25 different enteroviruses, a pair for the detection of HAV, and two pairs for the

detection of NoV Genegroup I and Genegroup II. All primers were synthesized by Integrated DNA Technologies, Inc.

The one-step reaction was carried out in a final volume of 25 μ l that contained 1.125 μ l of nuclease-free PCR-grade H₂O, 12.5 μ l of 2x SYBR Green QRT-PCR master mix, 200nM (2.5 μ l) forward primer, 200 nM (2.5 μ l) reverse primer, 0.375 μ l of diluted reference dye (Rox, 1:500), 1.0 μ l of RT/RNase block enzyme mixture, and 5 μ l of extracted RNA as the template. A standard curve (10 – 10⁸ copies in 10X increments) was created from the appropriate Armored RNA. Given that each virus carries one specific amplified target per genome, it was assumed that one genome copy (amplicon) was equivalent to a single virus (Gregory et al., 2006). The reverse transcription was performed for 30 min at 50°C, followed by 10 min incubation at 95°C for *Taq* activation and inactivation of the RT enzymes. Then, the viral cDNA was amplified by 40 cycles of denaturation (95°C for 30 s) and annealing/extension (60°C for 1 min). Real product quantification fluorescence was confirmed based on the melting temperature (T_m) of product amplicon (Table 3.2) in the dissociation and standard curves for EV, HAV and NoV using the Mx3000P Thermal Cycler (Stratagene, Inc.) data analysis software.

Detection of HEV by conventional RT-PCR. Enteroviruses, hepatitis A viruses, and noroviruses were detected by a one-step Reverse Transcriptase-PCR amplification kit (QIAGEN, catalog num), using the same primers and controls as those used for qRT-PCR. RT-PCR was carried out in a Mastercycler in a final volume of 50 μ l that contained: 29.0 μ l of Nuclease-free PCR-grade H₂O, each primer at a concentration of 600 nM (1.0 μ l), 10 μ l of 5x Buffer, 2.0 μ l of dNTP mix, 2.0 μ l of enzyme mix, and 5.0 μ l of RNA sample. The reaction mixture was subjected to the following conditions: 30 min at 50°C for reverse transcriptase; an activation step for 15 min at 95°C; followed by 40 cycles of three steps: denature for 1 min at 94°C, annealing

for 1 min at 50°C, and extension for 1 min at 72°C; and a final incubation for 10 min at 72°C. A no-template negative control (nuclease-free water) was included in all assays. PCR products were visualized by means of 2.5% agarose gel electrophoresis stained with 1.0 µg per ml of Ethidium Bromide on a UV light transilluminator. A QIAGEN GelPilot 50-bp ladder was used to confirm expected amplicon size (Table 3.2).

Cloning and Sequencing. Specific and non-specific PCR amplicons from summer 2008 samples were cloned and sequenced in order to confirm presence or absence of enteroviruses, and identify EV if present. 3' A-overhangs were added to the fresh PCR amplicons (to maximize cloning between the T-ended plasmid vectors and the now A-ended PCR products) and 4µl of aliquots were then used immediately for cloning following instructions in the TOPO TA Cloning Kit for Sequencing (Invitrogen, Version O). Approximately 2-6 white colonies per sample were picked and cultured overnight in LB broth containing 150 µg/µl ampicillin. Plasmids were then isolated using the Invitrogen PureLink™ Quick Plasmid Miniprep Kit. Plasmid DNA was eluted in 50 µl of TE buffer, quantified spectrophotometrically, and sequenced using the vector derived T7 primer at the DNA Sequencing Facility, Stony Brook University, Stony Brook, NY.

Sequences BLAST Search Identification and Alignment Analysis. Nucleotide sequences of enteroviruses from samples collected were edited and aligned using the program BioEdit Sequence Alignment Editor (Hall, 1999). Primer and vector sequences were removed. Sequence identification was determined by a BLAST search of amplicon sequence using the nt-database against Genbank at the National Center for Biotechnology Information (NCBI; <http://www.ncbi.nlm.nih.gov/>). Subsequently, a multiple alignment was made using the program ClustalW2 (Larkin et al. 2007) of sequences that were identified as enteroviruses and positive controls.

RESULTS

A total of 55 surface water samples were tested for detection and quantification of EV, HAV and NoV. EV were detected in three surface water samples. In addition, we tested five samples from the Port Jefferson wastewater treatment plant where EV were only detected and quantified in the sludge sample. No HAV or NoV were detected in any environmental samples. No HEV were detected in any of the three animal tissue samples.

Quantification and Detection by SYBR Green quantitative reverse transcriptase polymerase chain reaction (qRT-PCR).

Enteroviruses (EV). A standard curve was created to quantify EV with a range of 6.5×10^2 - 6.5×10^4 genome copies ml^{-1} (Figure 3.3). However other non-specific products were also amplified and were interfering with the fluorescence signal of the lowest standards. To address this issue a careful analysis of the dissociation curves of the products melting temperature (T_m) and controls was needed to determine true EV amplification. From these, the T_m for EV was at 84°C while other non-specific products had a T_m of 72°C and/or 77°C (Figure 3.4). These non-specific products were also seen in the negative controls of no template (NTC) and no reverse transcriptase controls (NoRT) (Figure 3.5). Thus only products that had a T_m of 84°C were considered true EV detection. In addition, there was possible inhibition of the PCR reaction by substances in the RNA environmental samples since there was no product in the dissociation curves for the NoRT PJWTP Sludge Sample 072308 (Figure 3.5) and for the PJWTP Sludge Sample 072308 undiluted (Figure 3.6). On the contrary, the diluted and spiked (with the Armored EV RNA standards) subsamples of PJWTP Sludge Sample 072308 had products with T_m of 72°C , 77°C and 84°C (Figure 3.6). Thus, EV were detected in sludge samples 032708 and

072308 from the PJWT. The former sample had 2.68×10^4 genome copies ml^{-1} (Figure 3.7), while the other sample could not be quantified due to interference of the fluorescence signal by these other non-specific products. No other samples showed detection or quantification of EV.

Hepatitis A viruses (HAV). A standard curve was created to quantify HAV with a range of 5.5×10^1 - 5.5×10^3 genome copies ml^{-1} (Figure 3.8). Based on the dissociation curve of products T_m only our desired HAV product amplified at T_m 75.5°C (Figure 3.9). No HAV were detected in any environmental samples.

Noroviruses (NoV). A standard curve was created to quantify NoV with a range of 2.1×10^5 - 2.1×10^7 genome copies ml^{-1} (Figure 3.10). There was no amplification of Armored RNA NoV standards that were below 2.1×10^5 genome copies ml^{-1} . Based on the dissociation curve of products T_m there was only our desired NoV product amplified at T_m 79°C (Figure 3.11). Most negative controls showed no products amplification with the exception of the no RT Armored NoV RNA Standard 4 control, which had a small (below 400) fluorescence signal at T_m 79°C (Figure 3.12). Environmental samples were diluted (1:10 and 1:100) and spiked with Armored RNA standards to corroborate inhibition. However there was no amplification for samples that were diluted, only those that were spiked with the highest standard concentrations as seen with sample 032708PJWTPSludge (Figure 3.13). After subtraction of the standards used for spiking the environmental samples, no NoV were detected in any environmental samples, including the 032708PJWTOEffluent samples (Figure 3.14).

Detection of HEV by reverse transcriptase polymerase chain reaction (RT-PCR).

Initially, surface water samples concentrated via a one-step viral concentration approach did not reveal any specific detection of EV, HAV and NoV using RT-PCR. The only amplifications seen

from environmental samples were from the EV RT-PCR assays and showed non-specific products (~450-500 base pairs) amplifications (Figure 3.15). However, after a two-step viral concentration approach three samples showed specific detection of EV: 062308PJH2 amplifications at ~195 base pairs (Figure 3.16); and, 100909PJH2 and 12049PJH2 amplification at ~152 base pairs (Figure 3.17), although other non-specific amplification was also evident (~450-500 base pairs).

HAV and NoV were not detected in any surface water sample. Furthermore, no HEV was detected in animal tissue samples (Figure 3.17).

Identification of enteroviruses by sequencing. PCR amplicon samples from the EV RT-PCR assays were further analyzed to identify enteroviruses by means of cloning and sequencing. After a BLAST search of all PCR amplicon sequences, three samples sequences (062308PJH2, 101109PJH2, and 120309PJH2) matched to the 5' UTR sequences of human poliovirus and to the Armored RNA EV positive controls (Figure 3.18).

DISCUSSION

Enteroviruses (EV) were the only human enteric viruses detected in environmental samples with RT-PCR and sequencing techniques. HAV and NoV were not present in any sample.

Detection and quantification of EV by SYBR Green qRT-PCR. Detection of human viral pathogens in the environment is hampered by inadequate techniques for viral concentration and detection (Bosch, 1998; Fong and Lipp, 2005). In this study EV were detected in three samples of the surface waters of Port Jefferson after a secondary viral concentration approach, and in each sample were present at over 10^3 viral genome copies ml^{-1} . The qRT-PCR detection limits

for EV were 6.5×10^2 - 6.5×10^4 genome copies ml^{-1} using Armored RNA EV positive controls (Figure 3.3). This range was higher compare to other studies that have successfully detected EV in surface coastal waters and had detection limits in the range of: 1.3×10^1 - 1.3×10^3 copies μl^{-1} (Donaldson et al., 2002); 6.6×10^1 - 6.6×10^4 copies μl^{-1} (Fuhrman et al., 2005); 10^1 - 10^5 copies μl^{-1} (Gregory et al., 2006). The detection limits of my EV qRT-PCR were hampered by other non-specific amplification products and/or PCR inhibitory substances concentrated from the environmental samples that interfered with the fluorescence signal (Figures 3.4, 3.5, 3.6 and 3.7). These non-specific amplifications were also seen in gel figures from the EV RT-PCR assays (Figures 3.15, 3.16 and 3.17).

Detection of Enteroviruses by RT-PCR. Initially no EV amplification was detected using RT-PCR, (Figure 3.15). However, HEV were detected from using a two-step viral concentration method along with RT-PCR. In 13 out of 55 surface water samples, viruses were sufficiently concentrated for possible detection of EV by RT-PCR (details in Chapter 2 of this dissertation). EV were actually detected in three of the 13 samples, a finding similar to other reports (Table 3.4). Along with the positive EV amplifications there were other non-specific amplification products in qRT-PCR assays (Figure 3.16 and Figure 3.17), thus requiring apparent EV amplification to be confirmed by cloning and sequencing.

Enteroviruses sequence. In this study three samples (062308PJH2, 101908PJH2, and 120409PJH20) had sequences identified as 99% similar to human poliovirus sequences from the UTR region (Figure 3.18). Gersberg et al. (2006) isolated specimens from the Tijuana river mouth ~75% similar to Poliovirus 1, ~51% similar to Poliovirus 3, and ~75% similar to Poliovirus 2. In another study, Rose et al. (2006) isolated samples ~90% similar to Poliovirus 1, and ~64% similar to Poliovirus 2. It is possible that the enteroviruses found in Long island

coastal waters were indeed polioviruses, although this is clearly not a certainty given the small number of positive samples, and the fact that polio has been virtually eradicated in the US. An alternative explanation to this finding is the possibility of detection of a polio vaccine in environmental waters. This been reported previously by Mueller et al. (2009) and Zurbriggen et al. (2008), and there is a major regional medical facility, the Stony Brook University hospital which is connected to the Port Jefferson wastewater treatment plant.

Armored RNA™ as a positive control. Armored RNA is a noninfectious viral surrogate that encapsulates specific RNA targets in a coat protein of *E.coli* bacteriophage MS2 and is designed for standard controls of RNA amplification and calibration of standard curves in RT-PCR (Yu et al., 2008; Pasloske et al., 1998; Walker and Pasloske, 2004). Armored RNA has been used by others to detect HEV in natural waters but as an internal control (Gibson et al., 2011; Hewitt et al., 2007). In this study the commercial Armored RNA for EV, HAV and NoV were used as positive controls for RT-PCR and to establish standard curves for qRT-PCR assays. Although the Armored RNA technology is accessible, inexpensive, and safe for most laboratories it is restricting in its primer selection based on the manufacturers' recommendations and the length of the encapsulated RNA target (~200bp). In addition, it is optimized to be a specific standard to detect presence or absence of EV, HAV and NoV when concentrations are in the order of $\geq 10,000$ copies ml⁻¹. This is because the designed primers are optimized for amplifying viral genome concentration of 6.5×10^6 copies ml⁻¹ for Armored RNA EV, 2.75×10^4 copies ml⁻¹ for Armored RNA HAV, and 2.1×10^9 copies ml⁻¹ for Armored RNA NoV genegroup II, respectively. These limitations may be a problem when non-specific products are formed, as they did in this study for the amplification of EV. As both seen in the results from EV qRT-PCR (Figures 3.9, 3.10, 3.11, and 3.12) and EV RT-PCR (Figures 3.15, 3.16, and 3.17) non-specific

amplification products interfere with the detection of EV. These products were suspected to be either primer-dimers or non-specific products from the primers chosen (poor specificity). To address these issues we used other primers to amplify EV (Table 3.2), changed primer concentrations, and even add a step to melt dimers before generating fluorescence signal acquisition in the case of the qRT-PCR assay. However, our attempts were unsuccessful and we had no other choice as positive control than Armored RNA technology. In addition, the presence of inhibitory substances from the environmental samples made our assay more complicated.

Inhibition of the PCR. One of the main problems in using qRT-PCR to monitor environmental samples is that substances inhibitory to PCR may be concentrated and extracted along with viral RNA (Gregory et al., 2006). Therefore, many researchers have opted to spike their environmental samples with an internal control to measure inhibition and validate their method (Fuhrman et al., 2005), or to dilute samples and any inhibitory substances they may contain (da Silva et al., 2007). In this study, both strategies were used and samples were diluted (1:10, 1:100, and 1:1,000) and spiked with the respective positive control Armored RNA for each assay (EV, HAV and NoV).

Nevertheless, naturally occurring HEV were not detected in surface water samples after all samples were diluted and spiked with respective controls. Therefore, either no HEV were present or the dilution of the sample reduced their concentration below detection limits.

Wastewater treatment plant and animal tissue samples. Since no HEV were detected initially in surface water samples, wastewater treatment plant samples were tested to validate human viral contamination sources. In these samples, EV were detected in two and quantified in one of the two sludge samples (Table 3.3). In addition, given the filtering capability of mussels and

consumption of surface sediments by polychaetes (sediments are well-known to act as viral reservoirs of viruses: Gerba and Schaiberger, 1975), these two groups of benthic animals were examined as possible viral reservoirs of HEV but no HEV were detected (Figure 3.17). The absence of HEV was unexpected given that mussels and other bivalves are known to concentrate viral pathogens from waters (Asahina et al., 2009), in particular noroviruses, and viruses have been detected in mussel tissue from coastal waters (Myrmel et al., 2004). However in this study detection and viral isolation methods may not have been optimal to detect HEV in mussel tissues. The absence of HEV in polychaetes tissue was not unexpected since they are not filter feeders and are less likely to concentrate viruses. Nevertheless, polychaetes worms have been reported to be passive vectors of other viruses, like shrimp viruses (Vijayan et al. 2005). No study has examined polychaetes or benthic organisms, except bivalves, as possible vectors of human viruses.

In summary, HEV were detected in surface waters via cloning and sequencing but not by RT-PCR. Of the three groups of HEV, enteroviruses were the only ones detected. In spite of the fact that HEV were detected in only a few natural samples, this study reconfirms the importance of method development and optimization for the accurate and successful detection and quantification of HEV in the environment. Adequate detection and quantification methods will be crucial for studying HEV occurrence in the environment, and hence for evaluating and minimizing human health risks from waterborne HEV.

Table 3.1 Characteristics of human enteric viruses (HEV) that may be water transmitted. In bold are the three groups that were detected in this study, enteroviruses, hepatitis A viruses, and noroviruses. Modified from (Bosch et al., 2006); http://www.virology.net/Big_Virology/BVDNAadeno.html; ICTVdB - The Universal Virus Database, version 4. <http://www.ncbi.nlm.nih.gov/ICTVdb/ICTVdB/>; <http://vm.cfsan.fda.gov/~mow/chap31.html>)

Virus Name, Synonyms	Family Genus Human Groups	Structure	Nucleic Acid	Diameter (nm)	Capsid Proteins, Structural Proteins	Disease caused
Adenovirus	Adenoviridae Mastadenovirus HAd group A HAd group B HAd group C HAd group D HAd group E HAd group F	Non-enveloped and icosahedral	dsDNA 30000-42000 nucleotides long	70-90	252 capsomeres per nucleocapsid (8-9 nm in diameter)	Keratoconjunctivitis, Pharyngoconjunctival fever, Pharyngitis,
Rotavirus	Reoviridae Rotavirus Group A Group B	Non-enveloped and icosahedral	dsRNA 16500-21000 nucleotides long,	60-80	Three proteins layers, two outer layer consist of the proteins VP4 and VP7	Diarrhea in infants (less than 2 yrs old) Gastroenteritis
Norovirus Norwalk-like virus	Caliciviridae <i>Calicivirus</i> <i>Norovirus</i>	Non-enveloped and icosahedral	(+)ssRNA, 7300-7700 nucleotides long,	35-39	Capsid protein molecular mass 58-60 kDa	Gastroenteritis in children and adults
Human hepatitis A virus	Picornaviridae Hepatovirus	Non-enveloped and icosahedral	(+)ssRNA 7500 nucleotides long	27	Capsids of 12 capsomers	Hepatitis A; viral liver disease; infectious dose is unknown but presumably is 10-100 virus particles
Poliovirus Human enterovirus, A, B, C, D, E	Picornaviridae <i>Enterovirus</i>	Non-envelope, round with icosahedral symmetry	(+)ssRNA, 7400 nucleotides long	28-30	Capsid of 12 capsomers	Herpangina Hand-foot-mouth disease, Infectious myocarditis, Infectious pericarditis, Meningitis, aseptic, Polio, Poliomyelitis, Pleurodynia



Figure 3.1 (a) Maps of sampling sites located along the north shore of Long Island, NY. (b) Surface water samples were collected from the Nissequogue River (NR), Stony Brook Harbor (SBH), Conscience Bay (CB) and Port Jefferson Harbor (PJH). (c) PJH is a semi- enclosed tidal urban estuary that receives surface discharges from a local wastewater treatment plant (WTP), various storm water pipes, and pleasure boats especially in the summer. (d) The majority of samples were collected at the Port Jefferson Harbor Site 2 (PJHS2) and during summer 2008 samples were also taken from Site 1 (PJHS1)). Bold squares indicate areas zoomed in following images. Images were modified from Google maps.



Figure 3.2 Storm water runoff direct discharges into two recreational coastal estuaries, (a) the Nissequogue River in Smithtown, NY and (c) the Port Jefferson Harbor in Port Jefferson, NY (c). Surface water samples were collected near these two pipes discharges. (a) At the Nissequogue River (NR) viruses may enter from cesspools which line the small river via (b) storm water runoff discharges while at Port Jefferson Harbor human viral contamination may come from point sources, local wastewater treatment plant (PJWTP), and nonpoint sources, (c) storm water runoff pipes and pleasure boats discharges, especially in the summer.

Table 3.2 Primers pairs used for the specific detection of enteroviruses, hepatitis A virus, and the norovirus groups using RT-PCR and quantitative RT-PCR in this study. *T_m stands for melting temperature of amplicon product.

Name (Ref.)	Sequence (5'-3')	RT-PCR Amplicon Length (bp)	T _m (°C)*	Genomic Sequences Targeted
EV1-forward (EV1F)	CCT CCG GCC CCT GAA TG			UTR
EV1-reverse (EV1R) (Schwab et al., 1995)	TTG GAT TGG CCA TCC GG	195	84	VP4 and VP2 for Human coxsackievirus
EV2-forward (EV2F)	CCT CCG GCC CCT GAA TGC GGC TAA T			UTR
EV2-reverse (EV2R) (Rotbart et al., 1990)	ATT GTC ACC ATA AGC AGC CA	152	84	
Entarm-forward (designed by JCR)	CCC TGA ATG CGG CTA ATC	146	84	
EV2-reverse (EV2R) (Rotbart et al., 1990)	ATT GTC ACC ATA AGC AGC CA			
HAV-forward	CAG CAC ATC AAA AAG GTG AG			
HAV-reverse (Schwab et al., 1995)	GTT GGA GAT GAT TCA GGA G	192	76	VP1 and VP3
NoV(I)-forward	GTG AAC AGC ATA AAT CAC TGG			
NoV(I)-reverse (Ando et al., 1995)	GGT GAT GAT GAG ATT GTG TCA	123	ND	RNA polymerase gene
NoV(II)-forward	TGG AAT TCC ATC GCC CAC TGG			
NoV(II)-reverse (Ando et al., 1995)	GGT GAT GAA ATT GTC AGT	123	79	RNA polymerase gene

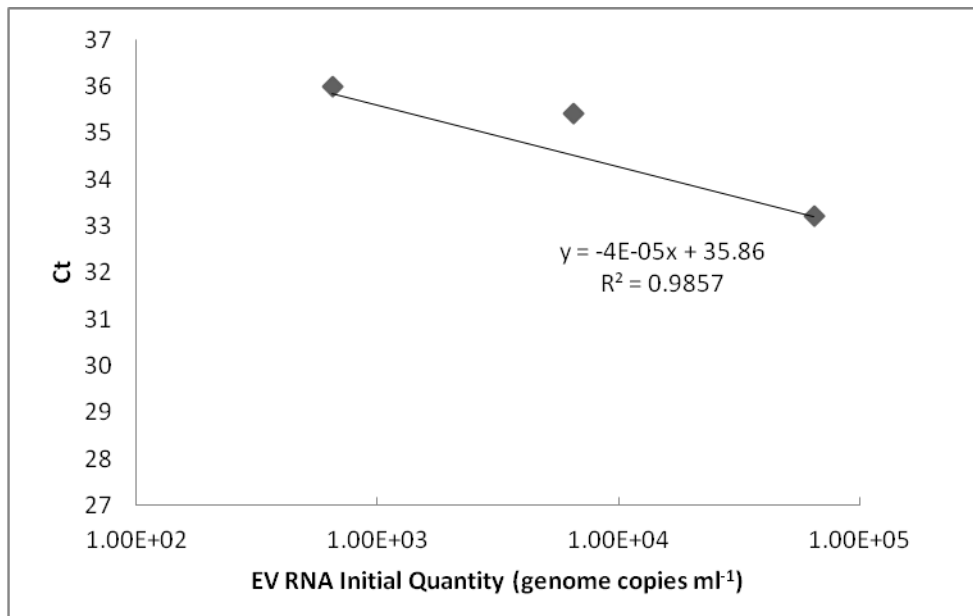


Figure 3.3 Standard curve of enteroviruses (EV) using positive controls Armored RNA EV. Legend: x-axis is EV RNA Initial Quantity, later converted to cDNA by the RT reaction; and the y-axis is the cycle threshold (Ct), which is the cycle number that indicates when the amplification is quantifiable based on a fluorescence threshold signal. The greater the amount of initial DNA template in the sample, the earlier the Ct value for that sample.

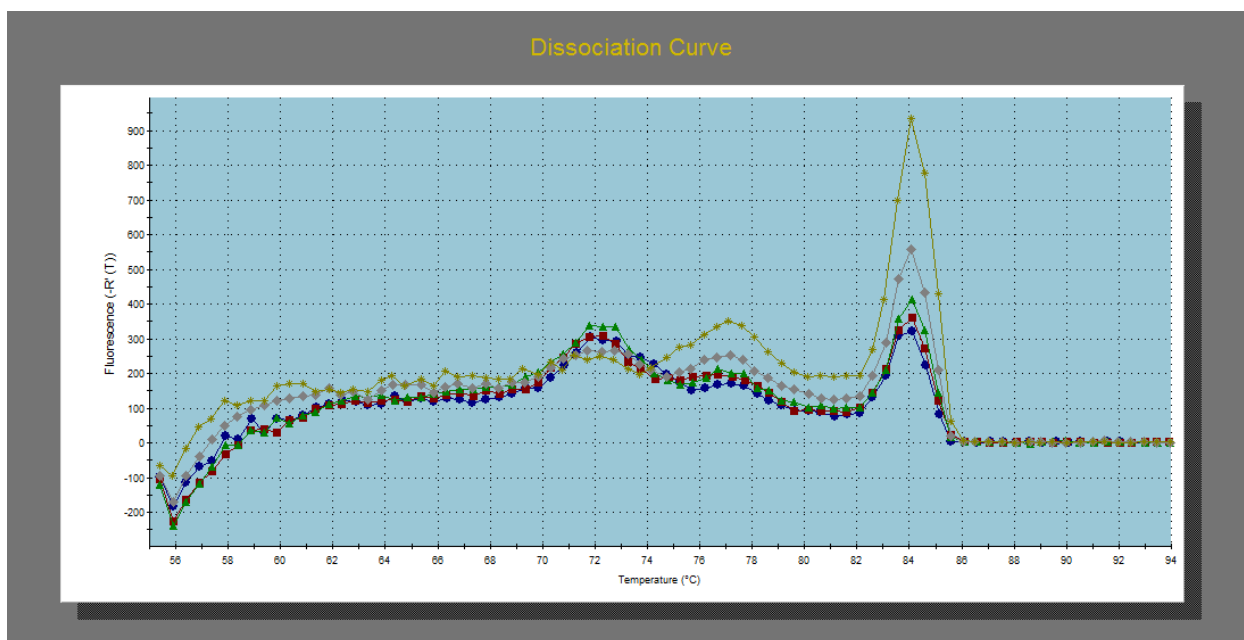


Figure 3.4 Enterovirus (EV) Armored RNA Standards Dissociation Curve for Amplicons Products using SYBR Green qRT-PCR. Legend: X-axis is Temperature; y-axis Fluorescence signal; yellow stars line is Armored RNA EV Standard 5, 6.5×10^5 genome copies ml^{-1} ; gray diamonds line is positive control Armored RNA EV Std. 4, 6.5×10^4 genome copies ml^{-1} ; green triangle lines is positive control Armored RNA EV Std. 3, 6.5×10^3 genome copies ml^{-1} ; red squares line is positive control Armored RNA EV Std. 2, 6.5×10^2 genome copies ml^{-1} ; and the blue circles line is positive control Armored RNA EV Std. 1, 6.5×10^1 genome copies ml^{-1} . All EV positive controls standards amplified and had the desired amplicon product melting temperature, T_m at 84°C . However, other specific products were also amplifying that had a T_m of 72°C and 77°C . We suspected these other products were due to non-specific amplification and/or primer-dimer. To confirm presence of primer-dimer we examined negative controls with no template (NTC). Nevertheless, the fluorescence signals from second products were interfering with the desired amplification products' ones. This was particularly an issue for the lowest concentration of enterovirus RNA. Thus, only amplification products that had a T_m of 84°C were considered as true enterovirus detection. However our quantification was compromise by other non-specific amplification products.

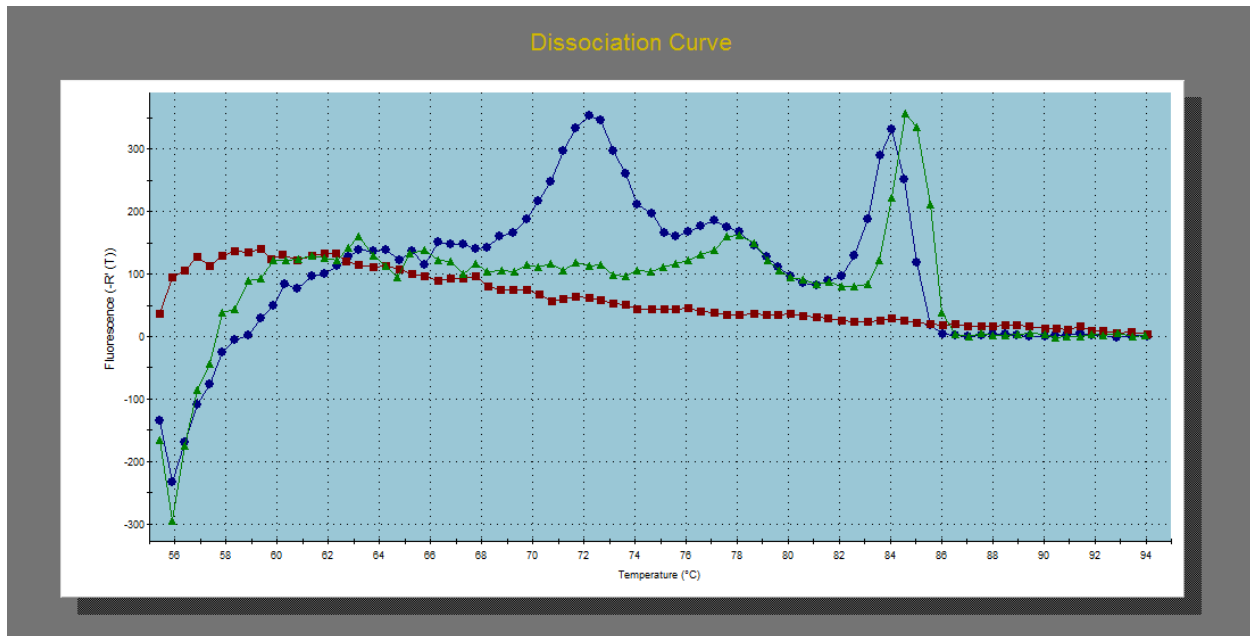


Figure 3.5 Negative controls for Enterovirus (EV) Dissociation Curve. Legend: X-axis is Temperature; y-axis Fluorescence signal; blue circles line is no template control (NTC); green triangles line is no RT Armored EV RNA Standard 5 (NoRT Std) control; and red squares line is no RT 072308PJWTPSludge (NoRT Sludge) control. Although no amplification products were expected from the negative controls, both NTC (blue circles line) and NoRT Std (green triangles line), showed products with T_m of 72°C and 84°C. These results suggested three things: first, possible contamination in the NTC with positive controls due to fluorescence signal from the product with T_m of 84°C ; secondly, non-specific product at a T_m of 72°C was from the chemicals added to qRT-PCR, since in the NTC there were no nucleic acids added to the PCR; and thirdly, the fluorescence signal from the product with T_m of 84°C was from the initial Armored EV RNA Standard 5 concentration used to prepare the NoRT Std control, since there was no reverse transcriptase to convert RNA to cDNA. Therefore in the absence of enterovirus genome templates for the PCR, primers form a product that had a T_m of 72°C. However primer-dimers product was not seen in the NoRT Sludge negative sample must probably due to inhibition. In any case, fluorescence signals that were below 300 were not considered true amplification of EV since these non-specific products were interfering with quantification values below 400.

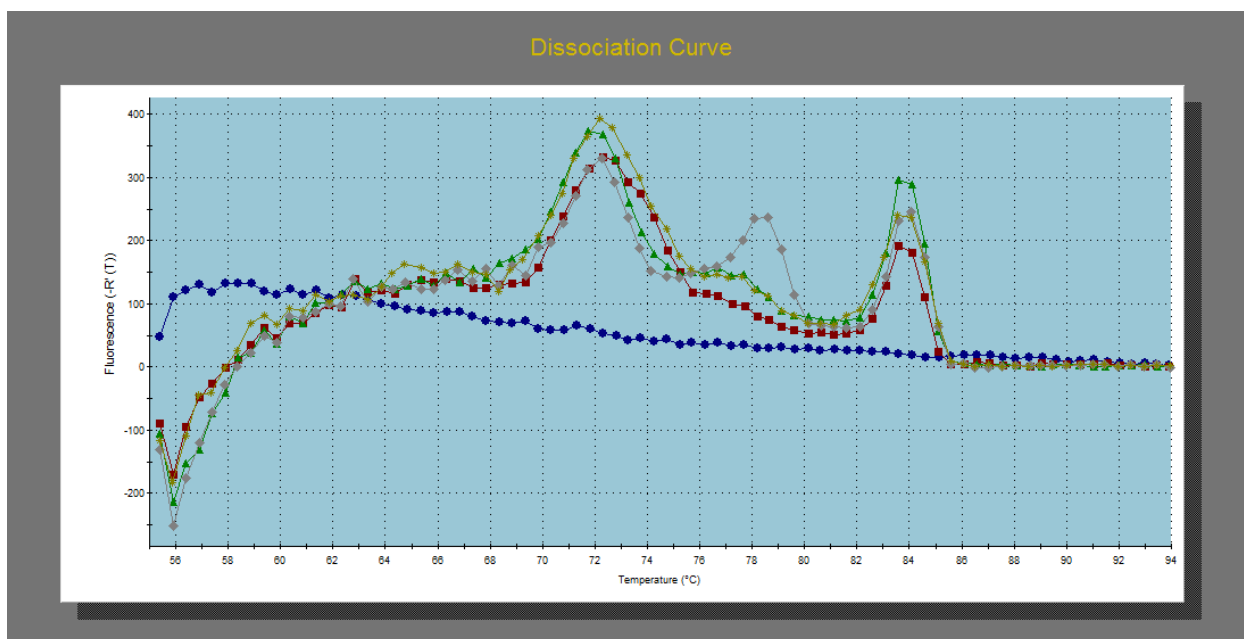


Figure 3.6 PJWTP Sludge Sample 072308 Dissociation Curve for Enteroviruses Amplicon Products. Legend: X-axis is Temperature; y-axis Fluorescence signal; yellow stars line is PJWTP Sludge Sample 072308 dilution 1:10,000; gray diamonds line is PJWTP Sludge Sample 072308 dilution 1:1,000; green triangles line is PJWTP Sludge Sample 072308 dilution 1:100; red squares line is PJWTP Sludge Sample 072308 dilution 1:10; and blue circles line is PJWTP Sludge Sample 072308 undiluted. All diluted samples amplified, however the first amplification product had a melting temperature (T_m) of 72°C, while our desired enterovirus amplicon product T_m is at 84°C. We suspected this non-specific amplification product was due to (1) non-specific amplification, (2) primer-dimer, and/or (3) inhibition of the PCR from substances in the environmental samples RNA. To address the latter issue we run a serial dilution of our samples. The sample without dilution did not amplify (blue circles line) while the ones diluted did amplify (green triangles line, gray diamonds line, yellow stars line, and red squares line). Although, we could not quantify enteroviruses in the environmental samples due to higher fluorescence signal from the non-specific product at T_m of 72° we determine true enterovirus amplification due to amplicon products formation at T_m of 84°C. Thus using qRT-PCR we needed to examine the dissociation curves analysis to establish true amplification of desired products.

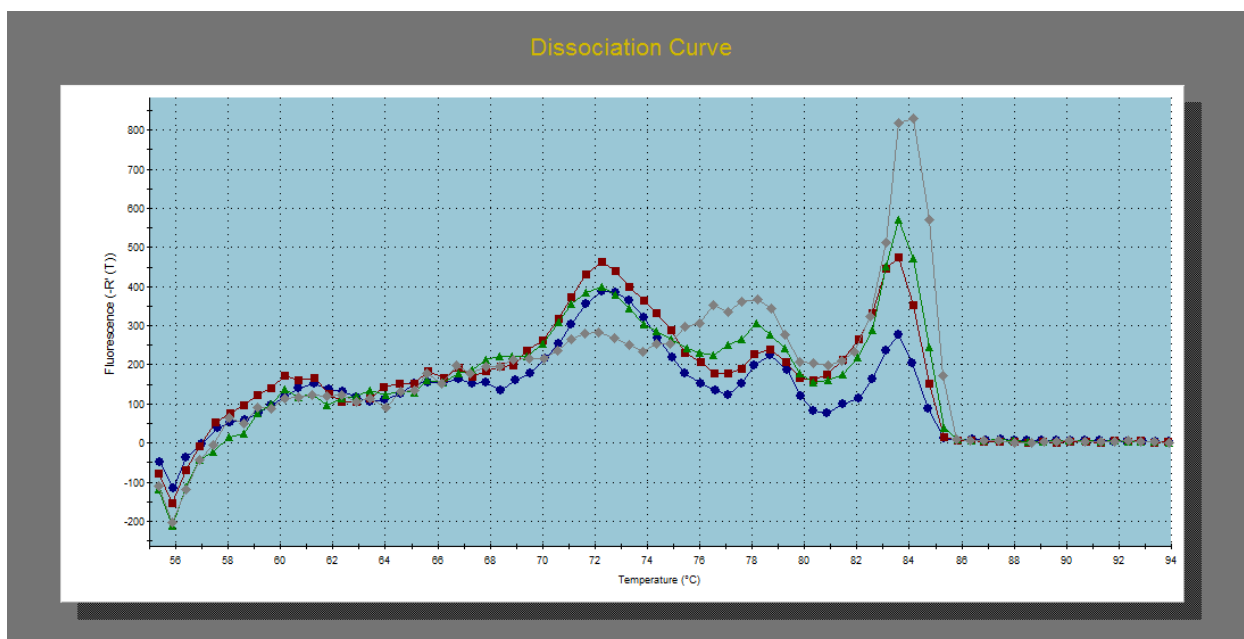


Figure 3.7 PJWTP Sludge Sample 032708 Dissociation Curve for Enteroviruses Amplicon Products. Legend: X-axis is Temperature; y-axis Fluorescence signal; gray diamonds line is PJWTP Sludge Sample 032708 dilution 1:100 spiked with Armored EV RNA Standard 5, 6.5×10^5 genome copies ml^{-1} ; green triangles line is PJWTP Sludge Sample 032708 dilution 1:10 spiked with Armored EV RNA Standard 5, 6.5×10^5 genome copies ml^{-1} ; red squares line is PJWTP Sludge Sample 032708 dilution 1:100; and blue circles line is PJWTP Sludge Sample 032708 dilution 1:10. All samples amplified at our desired enterovirus amplicon product melting temperature (T_m) of 84°C ; however there was also fluorescence signal from a product with a T_m of 72°C . We suspected this was due to a non-specific amplification or primer-dimer formation. Nevertheless, there was enterovirus quantification (2.68×10^4 genome copies ml^{-1}) of the 032708PJWTP Sludge sample (red squares line). Other samples that showed true enterovirus amplification was due the spike standard added to test inhibition.

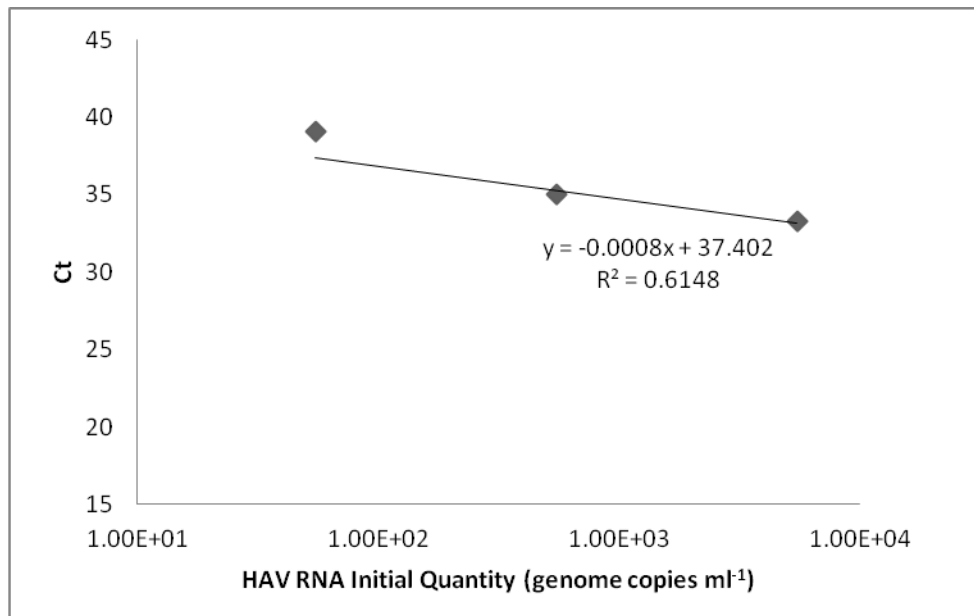


Figure 3.8 Standard curve for Hepatitis A virus (HAV) using positive controls Armored RNA HAV. Legend: x-axis is HAV RNA Initial Quantity, later converted to cDNA by the RT reaction; and the y-axis is the cycle threshold (Ct), which is the cycle number that indicates when the amplification is quantifiable based on a fluorescence threshold signal. The greater the amount of initial DNA template in the sample, the earlier the Ct value for that sample.

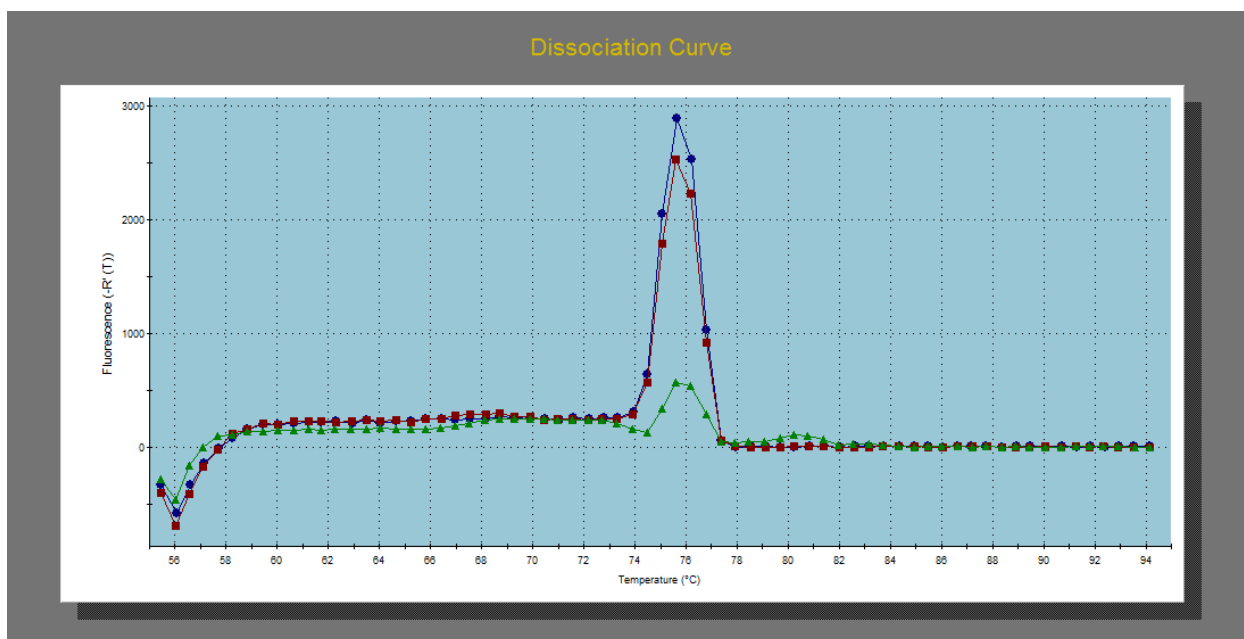


Figure 3.9 Hepatitis A Virus (HAV) Armored RNA Standards Dissociation Curve for Amplicons Products. Legend: X-axis is Temperature; y-axis Fluorescence signal; the blue circles line is positive control Armored RNA HAV Std. 3, 5.5×10^3 genome copies ml^{-1} ; red squares line is positive control Armored RNA HAV Std. 2, 5.5×10^2 genome copies ml^{-1} ; green triangle lines is positive control Armored RNA HAV Std. 1, 5.5×10^1 genome copies ml^{-1} . All HAV positive controls standards amplified and had the desired amplicon product melting temperature, T_m at 76°C . No other products amplified. HAV were not detected in any environmental samples.

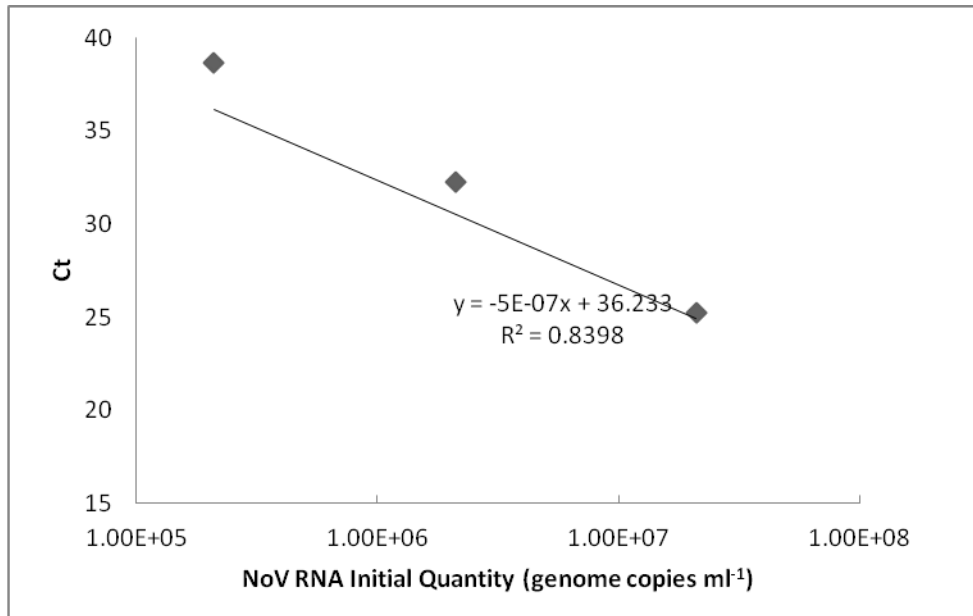


Figure 3.10 Standard curve for Norovirus using Armored RNA NoV. Legend: x-axis is NoV RNA Initial Quantity, later converted to cDNA by the RT reaction; and the y-axis is the cycle threshold (Ct), which is the cycle number that indicates when the amplification is quantifiable based on a fluorescence threshold signal. The greater the amount of initial DNA template in the sample, the earlier the Ct value for that sample.

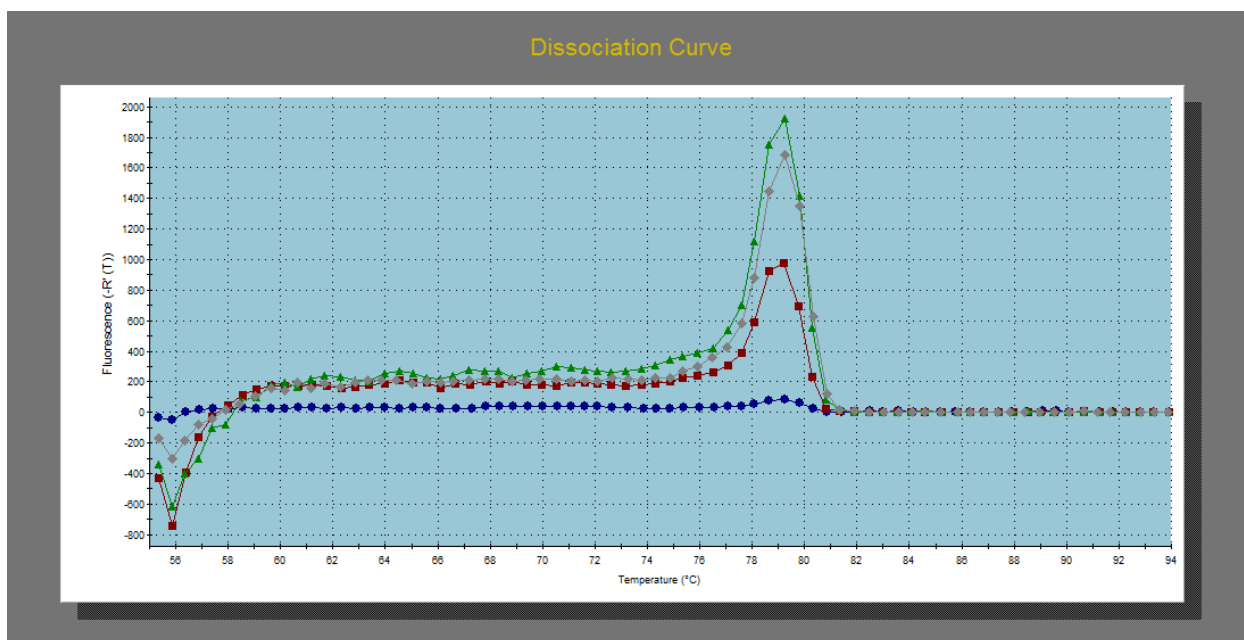


Figure 3.11 Norovirus Armored RNA Standards Dissociation Curve for Amplicons Products. Legend: X-axis is Temperature; y-axis Fluorescence signal; green triangles line is positive control Armored RNA NoV Standard 4, 2.1×10^8 genome copies ml^{-1} ; gray diamonds line is positive control Armored RNA NoV Std. 3, 2.1×10^7 genome copies ml^{-1} ; red squares lines is positive control Armored RNA NoV Std. 2, 2.1×10^6 genome copies ml^{-1} ; and the blue circles line is positive control Armored RNA NoV Std. 1, 2.1×10^5 genome copies ml^{-1} . All positive standards controls had true norovirus amplification and a product amplicon melting temperature T_m of 79°C . No other product was formed, discarding the possibility of primer-dimer for the NoV assay. NoV were not detected at any environmental samples.

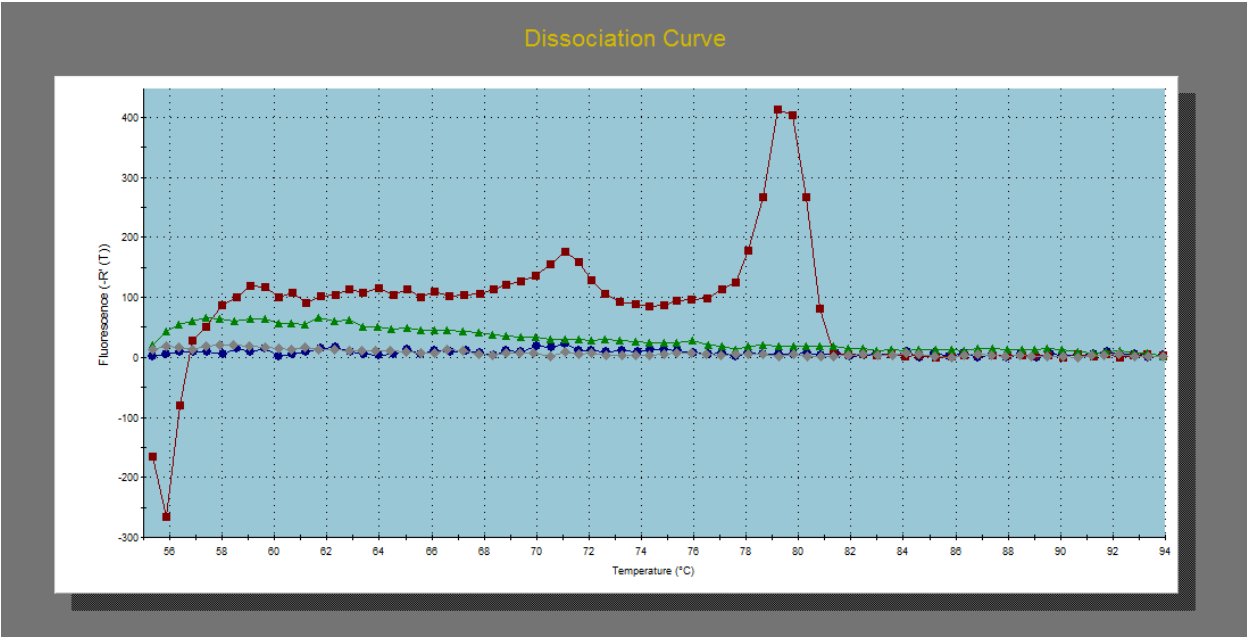


Figure 3.12 Negative controls for Norovirus Dissociation Curve. Legend: X-axis is Temperature; y-axis Fluorescence signal; red squares line is No RT Armored NoV RNA Standard 4 (NoRT Std) control; green triangle lines is No RT 032308Sludge 1:10 (NoRT Sludge1:10) control; gray diamonds line is No RT 032308Sludge 1:100 (NoRT Sludge1:100) control; and blue circles line is No template control (NTC). There was no amplification from the negative controls as expected except for a small (below 400) fluorescence signal from the initial quantity of Armored NoV RNA Standard 4 used in the NoRT Std control.

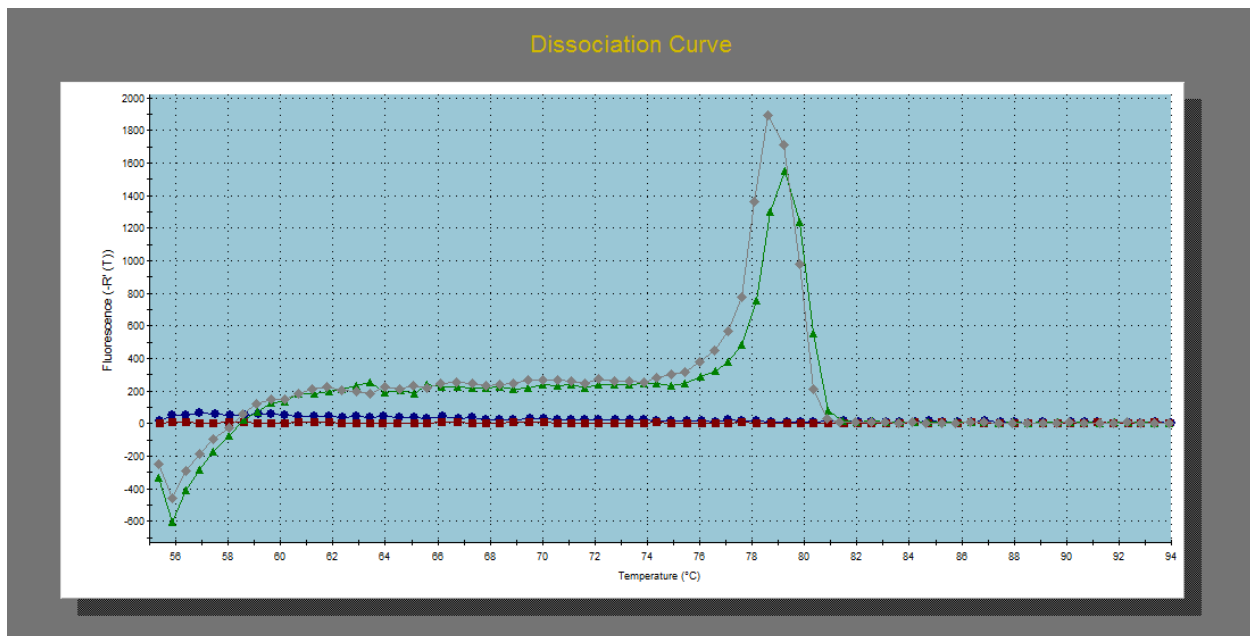


Figure 3.13 PJWTP Sludge Sample 032708 Dissociation Curve for Noroviruses Amplicon Products. Legend: X-axis is Temperature; y-axis Fluorescence signal; gray diamonds line is PJWTP Sludge Sample 032708 dilution 1:100 and spiked with Armored RNA NoV Standard 4, 2.1×10^8 genome copies ml^{-1} ; green triangles line PJWTP Sludge Sample 032708 dilution 1:10 and spiked with Standard 4; red squares line is PJWTP Sludge Sample 032708 dilution 1:100; and blue circles line is PJWTP Sludge Sample 032708 dilution 1:10. Samples that were spiked and diluted (gray diamonds and green triangle lines) did amplify and had a desired T_m of 79°C , while those that were only diluted did not amplify. Thus there was no PCR inhibitions after the environmental sample were diluted; nevertheless there was no NoV present at the PJWT Sludge Sample 032708. The only amplification that occurred was from the standard positive controls used to spike the environmental samples.

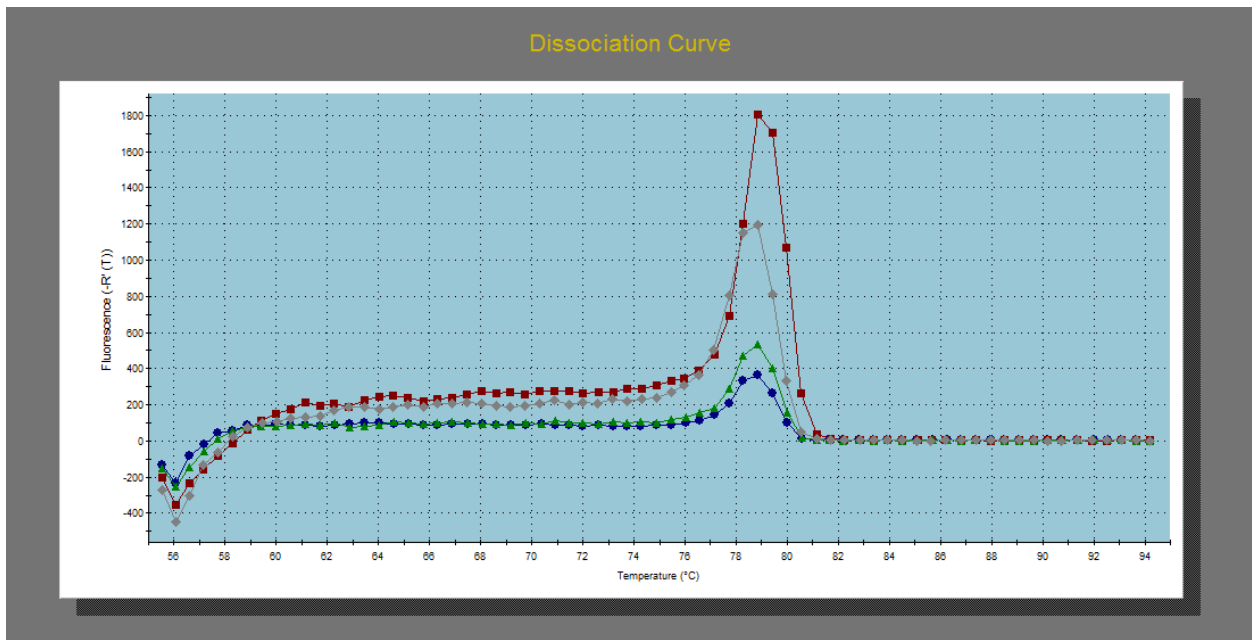


Figure 3.14 PJWTP Effluent Sample 032708 Dissociation Curve for Norovirus Amplicon Products. Legend: X-axis is Temperature; y-axis Fluorescence signal; red squares line is PJWTP Effluent Sample 032708 dilution 1:100 spike with Armored RNA NoV Standard 4, 2.1×10^8 genome copies ml^{-1} ; gray diamonds line is PJWTP Effluent Sample 032708 dilution 1:10 and spiked with Armored RNA NoV Standard 4; green triangles line PJWTP Effluent Sample 032708 dilution 1:100 and spiked with Armored RNA NoV Standard 3, 2.1×10^7 genome copies ml^{-1} ; and blue circles line is PJWTP Effluent Sample 032708 dilution 1:10 and spiked with Armored RNA NoV Standard 3. All samples were spiked and diluted and did amplify for norovirus and had a desired T_m of 79°C , however this true amplification was from the standard positive controls used to spike the environmental samples and not from the PJWTP Effluent 032708 sample.

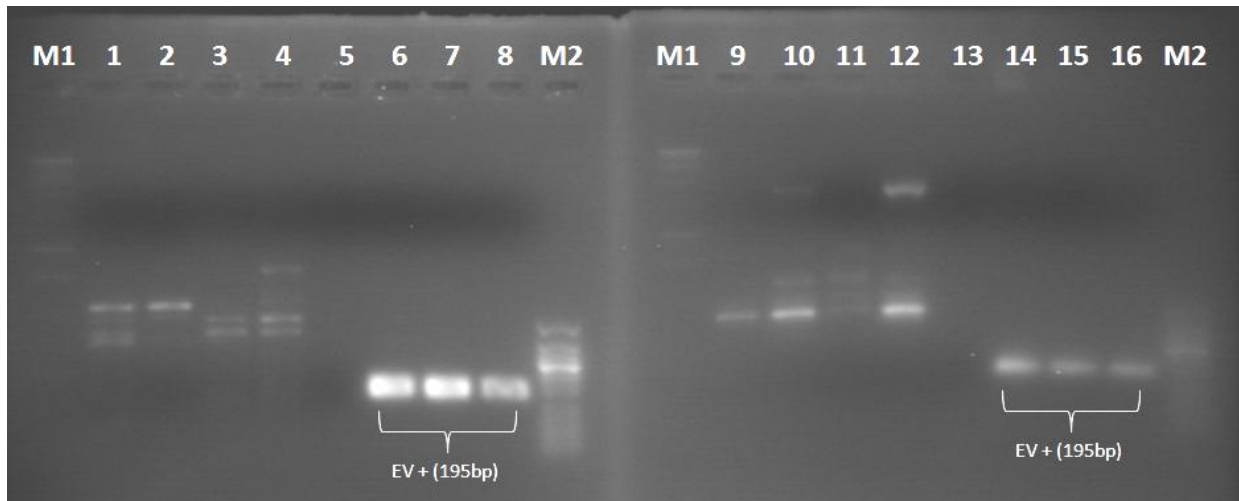


Figure 3.15 Gel electrophoresis of the RT-PCR products of enterovirus specific amplification from eight surface water samples at the PJH, that were concentrated using a one-step viral concentration approach, tangential flow filtration (TFF) and virus adsorption-elution (VIRADEL). The conserved genomic region has 195 bp (EV+). Lanes: M1, 1 Kb DNA Ladder with five visible fragments ranging from the bottom-up: 1500, 2000, 4000, 5000 and, 6000; M2, 50 bp DNA Ladder with nine visible fragments ranging from the bottom-up: 50, 100, 150, 200, 250, 300, 350, 400 and, 500; 1, 062308PJH1; 2, 062308PJH2; 3, 063008PJH2; 4, 070708PJH1; 5, NC, Negative control (water); 6, Positive control enteroviruses (Armored RNA 10^7 copies ml^{-1}); 7, Positive control enteroviruses (Armored RNA 10^6 copies ml^{-1}); 8, Positive control enteroviruses (Armored RNA 10^4 copies ml^{-1}); 9, 070708PJH2; 10, 0701508PJH1; 11, 071508PHJ2; 12, 072108PJH1; 13, NC, negative control (water); 14, Positive control enteroviruses (Armored RNA 10^7 copies ml^{-1}); 15, Positive control enteroviruses (Armored RNA 10^6 copies ml^{-1}); 16, Positive control enteroviruses (Armored RNA 10^4 copies ml^{-1}). Sample code as date of collection and location, e.g., 062308PJH1, sample collected on June 23, 2008 at the Port Jefferson Harbor Site 1.

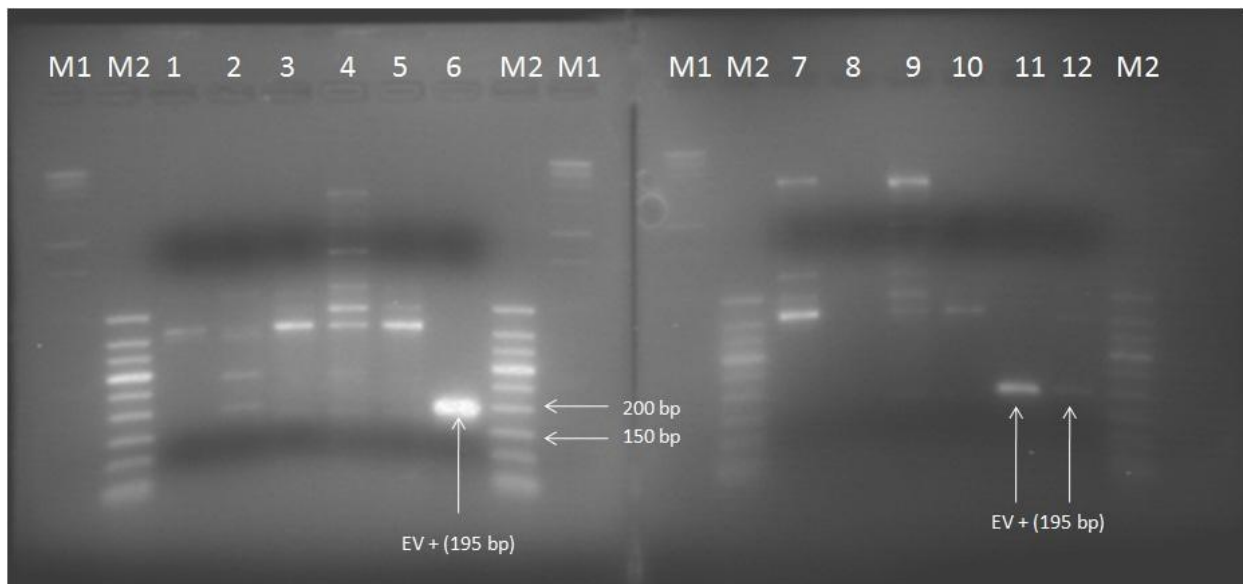


Figure 3.16 Gel electrophoresis of the RT-PCR products of enterovirus specific amplification from eight surface water samples at the PJH after second concentration, ultrafiltration. The conserved genomic region has 195 bp (EV+). Lanes: M1, 1 Kb DNA Ladder with six visible fragments ranging from the bottom-up: 500, 1500, 2000, 4000, 5000 and, 6000; M2, 50 bp DNA Ladder with nine visible fragments ranging from the bottom-up: 50, 100, 150, 200, 250, 300, 350, 400 and, 500; 1, 062308PJH1; 2, 062308PJH2; 3, 063008PJH2; 4, 070708PJH1; 5, 070708PJH2; 6, PC1: Positive control enteroviruses (Armored RNA 10^6 copies μl^{-1}); 7, 0701508PJH1; 8, 071508PHJ2; 9, 072108PJH2; 10, NC, negative control (water); 11, PC1: Positive control enteroviruses (Armored RNA 10^6 copies μl^{-1}); 12, PC2: Positive control enteroviruses (Armored RNA 10^3 copies μl^{-1}).

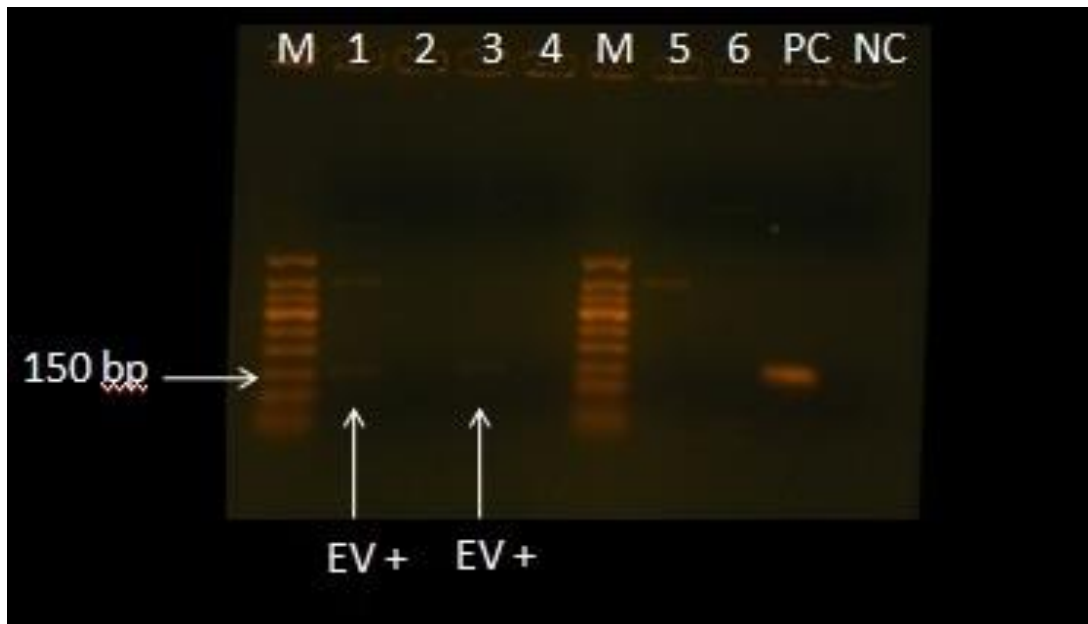


Figure 3.17 Gel electrophoresis of the RT-PCR products of enterovirus specific amplification from three water samples and animal tissue samples. The conserved genomic region has 150 bp (EV+). Lanes: M, 50-bp DNA Ladder with nine fragments ranging from the bottom-up: 50, 100, 150, 200, 250, 300, 350, 400, and 500 bp; 1, 100909PJH2; 2, 110309PJH2; 3, 120409PJH2 4, 110309PJMussels; 5, 120409PJMussels; 6, 120409PJWorms; PC, Positive Control (Armored RNA Enterovirus 10^6 copies μl^{-1}); NC, Negative Control.

Table 3.3 Detection and quantification of EV, HAV and NoV using SYBR Green qRT-PCR in wastewater plant samples: sludge, effluent, post UV, and post UV and chlorination treatments (final effluent) at the Port Jefferson Harbor. “0” indicates absence and “1” presence of HEV.

Sample	Viral Concentrate (x 10 ⁷ VLP ml ⁻¹)	Total RNA yields (ng)	HEV Detection (viral genomes copies ml ⁻¹)		
			EV	HAV	NoV
032708PJWTPSludge	285	3550	2.68x10 ⁴	0	0
032708PJWTPEffluent	758	6550	0	0	0
032708PJWTPPostUV	238	2780	0	0	0
032708PJWTPPostUVChlorination	0.425	275	0	0	0
072308PJHWTPSludge	ND	1953	1	0	0


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      .....|.....| .....|.....| .....|.....| .....|.....| .....|.....|
                10         20         30         40         50
062308PJH2  CGGCTAATCC CAACCTCGGA GCAGGTGGTC ACMAACCAGT GGTTGGCCTG
101908PJH2  CGGCTAATCC CAACCTCGGG GCAGGTGGTC ACAAACCAGT GATTGGCCTG
120409PJH2  CGGCTAATCC CAACCTCGGA GCAGGTGGTC ACAAACCAGT GATTGGCCTG
Schwab 199  CGGCTAATCC CAACCTCGGG GCAGGTGGTC ACAAACCAGT GATTGGCCTG
Rotbart 19  CGGCTAATCC CAACCTCGGG GCAGGTGGTC ACAAACCAGT GATTGGCCTG
AJ783731.1  CGGCTAATCC CAACCTCGGG GCAGGTGGTC ACAAACCAGT GATTGGCCTG
AJ783730.1  CGGCTAATCC CAACCTCGGG GCAGGTGGTC ACAAACCAGT GATTGGCCTG

      .....|.....| .....|.....| .....|.....| .....|.....| .....|.....|
                60         70         80         90         100
062308PJH2  TCGTAACGCG CAAGTCCGTG GCGGAACCGA CTACTTTGGG TGTCCGTGTT
101908PJH2  TCGTAACGCG CAAGTCCGTG GCGGAACCGA CTACTTTGGG TGTCCGTGTT
120409PJH2  TCGTAACGCG CAAGTCCGTG GCGGAACCGA CTACTTTGGG TGTCTGTGTT
Schwab 199  TCGTAACGCG CAAGTCCGTG GCGGAACCGA CTACTTTGGG TGTCCGTGTT
Rotbart 19  TCGTAACGCG CAAGTCCGTG GCGGAACCGA CTACTTTGGG TGTCCGTGTT
AJ783731.1  TCGTAACGCG CAAGTCCGTG GCGGAACCGA CTACTTTGGG TGTCCGTGTT
AJ783730.1  TCGTAACGCG CAAGTCCGTG GCGGAACCGA CTACTTTGGG TGTCCGTGTT

      .....|.....| .....|.....| .....|.
                110        120        130
062308PJH2  TCCTTTTATT TTATTGTGGC TGCTTATGGT GACAAT
101908PJH2  TCCTTTTATT TTATTGTGGC TGCTTATGGT GACAAT
120409PJH2  TCCTTTTATT TTATTGTGGC TGCTTATGGT GACAAT
Schwab 199  TCCTTTTATT TTATTGTGGC TGCTTATGGT GACAAT
Rotbart 19  TCCTTTTATT TTATTGTGGC TGCTTATGGT GACAAT
AJ783731.1  TCCTTTTATT TTATTGTGGC TGCTTATGGT GACAAT
AJ783730.1  TCCTTTTATT TTATTGTGGC TGCTTATGGT GACAAT

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Figure 3.18 Alignment of positive sequences for human polioviruses 5' UTR. EV positive samples from this study 062308PJH2, 101908PJH2, and 120409PJH2 identified as 99% similar to positive controls Armored RNA EV (Schwab 1995 and Rotbart 1990) and human poliovirus 5' UTR sequences from the NCBI database (Accession numbers AJ783731.1 and AJ783730.1).

Table 3.4 Human enteric viruses' detection in environmental waters by RT-PCR.

Type of HEV	Type of Water	% Positive Detection by RT-PCR	Reference
EV, HAV and NoV	Surface marine water	23% (3/13), 0% (0/13), and 0% (0/13)	Current study
NoV	Surface Estuarine Water	8.3% (6/72)	Gentry et al. (2009a)
EV	Surface coastal water	4.8% (10/206)	Jiang et al. (2007)
HAV and EV	Coastal seawater	55% (11/20) and 65% (14/20)	Gersberg et al. (2006)
EV	Environmental waters	14.8% (4/27)	Gregory et al. (2006)
EV	Surface water samples	56.6% (17/30)	Fong et al. (2005)
EV	Seawater, freshwater	17.6% (3/17)	Fuhrman et al. (2005)
EV, HAV and NoV	River water	88%, 1.5 % and 1.5%	Hot et al. (2003)
EV	Surface water	60% (9/15)	Donaldson et al. (2002)
EV, HAV and NoV	Seawater	79% (15/19), 63% (12/19) and 10% (2/19)	Griffin et al. (1999)

CHAPTER 4:

HUMAN VIRAL SOURCES AND ENVIRONMENTAL FACTORS AFFECTING HUMAN ENTEROVIRUSES IN A RECREATIONAL COASTAL WATER

INTRODUCTION

Viral Pollution Sources. Human enteric viruses (HEV) are transported into the marine environment via point and nonpoint sources of human waste including sewage, land runoff, and vessel discharges (Gerba, 2006). Non-point sources may be affected by seasonal changes; for example, land runoff after a rainfall event can increase the discharge of microbial pathogens into coastal waters (Lipp et al., 2001). However, few field studies have identified such nonpoint sources of HEV and correlations between HEV occurrence and rainfall events are contradictory. Once discharged in the marine environment, any risk of disease would require that these viruses persist long enough and in sufficiently high concentrations (Bosch et al., 2006). HEV have the capacity to remain infectious outside a human host for long enough to cause viral water- and food-borne outbreaks (Rzezutka and Cook, 2004). Greater occurrence in the environment increases the chance of exposure between HEV to a new host; however HEV occurrence and persistence will be affected by environmental factors.

Environmental Factors. The inactivation of enteric viruses and the environmental factors that influence their persistence in the marine environment has been studied extensively (Gerba, 2006). Factors such as Ultraviolet (UV) light, temperature, pH, and salts have all been shown to affect HEV persistence in aquatic environments. UV light can inactivate human viruses by damaging their nucleic acids by configuration changes among nucleotides (Gerba, et al., 2002; Lamont et al., 2007). Human viruses survive longer at lower temperatures (Gerba, 2006) while high temperatures cause denaturation of proteins and/or nucleic acids causing viral inactivation. Salo and Cliver (1976) showed a slower inactivation rate of enteroviruses at 2°C than at 30° C when the pH is acidic, and they reported that pH is a major factor in poliovirus inactivation mechanisms when combined with salt concentration and temperatures.

Although persistence per se of enteric viruses can only be measured with plaque assay methods, enteric viral RNA detected in the environment may indicate a recent viral contamination event (Gantzer et al., 1999; Wetz et al., 2004). Monitoring of HEV RNA via Reverse Transcriptase-PCR has the potential to provide a better understanding of the behavior and persistence of HEV in the environment. Still, only a few in situ field studies have shown significant correlations between HEV occurrence and environmental factors in natural waters, and field data seems to be contradictory or particular to each geographical location. Additional field studies of HEV occurrence and its correlation to environmental factors are needed to better characterize the exposure risk of these viruses in a particular marine environment.

Approach. In an effort to identify HEV sources and environmental factors affecting the occurrence of human viruses in local coastal recreational waters, a suite of environmental factors were examined in relation to human enterovirus (EV) occurrence. EV are a group of human enteric viruses (HEV) that include the groups of polioviruses, coxsackieviruses, and echoviruses,

they are the second group of viruses after the rhinoviruses to cause most common viral infections in humans, they transmitted via the oral-fecal route and most non-polio diseases occur during the summer and fall season (http://www.cdc.gov/ncidod/dvrd/revb/enterovirus/non-polio_entero.htm). Surface water samples were collected and environmental factors that have shown to affect HEV (specifically UV, temperature, salinity, and pH) were measured. Samples were also collected at different tidal phases (measured via tidal height) and during dry and rainy periods (measured via precipitation) to evaluate exposure risks and non-point sources of viral pollution.

METHODS

Collection site. Samples were collected from the Port Jefferson Harbor (PJH), where there are surface waters discharges from a wastewater treatment (WWTP) plant located within the harbor and from storm sewer overflow pipes in the innermost harbor area (Figure 4.1).

Surface Water Samples. Surface water samples (n=8) were collected during summer 2008 (days of June 23, 2008, June 30, 2008, July 7, 2008, July 15, 2008, July 21, 2008) and fall 2009 (days of October 19, 2009, November 3, 2009, and December 4, 2009). Sampling involved the collection of 1,000 ml of water with a hand pump or by holding containers immediately below the surface. Sterilized carboys and bottles were rinsed three times with surface water prior to final collection. Samples were put on ice and taken immediately to the laboratory.

Physicochemical Factors. A YSI Sonde was used to measure salinity, water temperature, and pH in situ. Ultraviolet Index (UVI) data was collected from the NOAA website

(http://www.cpc.ncep.noaa.gov/products/stratosphere/uv_index/Bulletin/) for each day of collection.

Viral Pollution Sources. To identify human enteric viruses sources into the harbor samples were also collected during dry (summer) and rainy/wet (fall) periods A rainfall event was defined as ≥ 5.0 mm of precipitation, consistent with previous studies (Brooks et al., 2005; Gersberg et al., 2006), where a “rainfall event” was defined as precipitation of 0.5 cm or more in a 72 hour period, comparable to the basis used for advisory warnings of possible water contamination by urban runoff, issued by the San Diego County Department of Environmental Health. Precipitation was recorded as the total rainfall in the 72 hours preceding sample collection. In addition, samples were collected during periods of low tide (outgoing tide) and high tide (incoming tide) to establish human exposure risks to viral pathogens. Based on a previous study of contamination from the PJ wastewater treatment plant (P. Rose, pers com), circulation patterns in the harbor are such that more treatment plant effluent should reach our surface water site during incoming tides than during outgoing tides (Figure 4.1c). Data on actual tidal heights and precipitation were obtained from:

<http://www.mobilegeographics.com:81/locations/4177.html?y=2007&m=5&d=14>

<http://www.saltwatertides.com/>

and the NOAA website <http://water.weather.gov/precip/>.

Viral Concentration. Viruses were concentrated using a two-step viral concentration approach, which consisted of tangential flow filtration, adsorption-elution, ultracentrifugation and centrifugal ultrafiltration. Details are in Chapter 2.

Enteroviruses (EV) detection. Enteroviruses were detected using reverse transcriptase PCR (RT-PCR) and sequencing techniques. Details are discussed in Chapter 3.

Statistical analysis. Spearman's rank correlation was used to evaluate associations between enterovirus occurrence and environmental factors, including total viral abundance, total bacterial abundance, temperature, salinity, pH, tidal height, and precipitation. Significance was declared at a p value of ≤ 0.050 .

RESULTS

Enteroviruses (EV) were detected in three of eight samples collected from the PJH (Table 4.1). Among the environmental factors measured, only precipitation and salinity showed significant correlations with EV occurrence (Table 4.2).

UV Index (UVI). The UVI at the PJH during summer 2008 and fall 2009 were between 0-9 (Figure 4.2). Summer UVI values were 8-9 compared to fall UVI values of 0-3. Enteroviruses were detected at UVI of 9, 3 and 0 during the dates of June 23, 2008, October 19, 2009, and December 4, 2009, respectively, and there was no significant correlation between EV occurrence and UVI ($r_s = -0.423$, $p = 0.260$).

Temperature. In situ measurements of water temperature at the PJH during summer 2008 and fall 2009 were 21-23.7°C and 8.7-11.2°C, respectively (Figure 4.3). Enteroviruses were detected at temperatures of 21°C, 11.2°C and 11.1°C during the dates of June 23, 2008, October 19, 2009, and December 4, 2009, respectively. There was no significant correlation between EV occurrence and temperature ($r_s = -0.455$, $p = 0.233$).

pH. In situ measurements of water surface pH at the PJH during summer 2008 and fall 2009 were between 7.75-7.98 (Figure 4.4). Summer had higher pH (7.88-7.98) than the fall (7.63-

7.88). Enteroviruses were detected at pH of 7.88, 7.63 and 7.75 during the dates of June 23, 2008, October 19, 2009, and December 4, 2009, respectively. There was no significant correlation between EV occurrence and water pH ($r_s = -0.283$, $p = 0.460$).

Salinity. In situ salinities at the PJH surface waters during summer 2008 and fall 2009 were from 26.0 to 27.6 and there was little or no seasonal variation (Figure 4.5). Enteroviruses were detected at salinities of 26.0, 26.8 and 26.7 during the dates of June 23, 2008, October 19, 2009, and December 4, 2009, respectively. There was a significant negative correlation between EV occurrence and salinity ($r_s = -0.680$, $p = 0.047$).

Precipitation and Rainfall Events. Cumulative precipitation in the last 72 hrs prior to collection of surface waters at the PJH showed variations between 0-38.0 mm during summer 2008 and fall 2009 (Figure 4.6). Enteroviruses were detected at cumulative precipitations of 6.40, 15.45, and 38.00 during the dates of June 23, 2008, October 19, 2009, and December 4, 2009, respectively. Enteroviruses were detected only during and after rainfall events of more than 0.5 mm. There was a significant positive correlation between EV occurrence and 72-hr precipitation ($r_s = 0.850$, $p = 0.001$).

Tidal Height. Samples were collected during low tide (≤ 0.17 meters) and high tide (≥ 0.17 meters). Tidal height at collection time for surface water samples at the PJH during summer 2008 and fall 2009 varied from -0.12 to 2.12 meters (Figure 4.7). Enteroviruses were detected at 0.55, -0.12, and -0.18 m tidal heights during the dates of June 23, 2008, October 19, 2009, and December 4, 2009, respectively. Enteroviruses were detected both during low tide and high tide. There was no significant correlation between EV occurrence and tidal height ($r_s = -0.453$, $p = 0.233$).

DISCUSSION

The effects of UV, temperature, salinity, pH, precipitation, and tidal height were all studied in relation to EV occurrence in surface recreational waters of the Port Jefferson Harbor, Long Island, New York.

Temperature. In my study enteroviruses were detected twice at lower temperatures, $\leq 11.0^{\circ}\text{C}$, during the months of October and December, and once at a higher temperature, 21°C , during late June (Figure 4.3). Higher survival at low temperatures ($4\text{-}10^{\circ}\text{C}$) compared to higher temperatures ($20\text{-}25^{\circ}\text{C}$) has been reported in the laboratory for enteroviruses (Lo et al., 1976), polioviruses and hepatitis A viruses (Bosch, 1995), and feline caliciviruses (noroviruses) (Kadoi and Kadoi, 2001). Similarly, in an environmental study (Gentry et al., 2009) reported an inverse association between noroviruses concentrations and water temperature, and higher noroviruses occurrences during the winter months compared to the summer, but no significant relationships. Other researchers have also found inverse relationship using logistic regression analyses between presence or absence of HEV and water temperature, where HEV are mostly detected during low temperatures or winter months (Fong et al., 2005; Gersberg et al., 2006; Lipp et al., 2001). In contrast, Jiang et al. (2007) reported higher detection of enteroviruses during the summer than the winter season, although they suggested that their viral detection assay was considerably hampered by the presence of PCR inhibitors from storm waters during the winter months. Nevertheless, given the small number of samples ($n=8$) in my study I can't conclude any seasonal trend or any effect of temperature over EV occurrence.

pH. While enteroviruses have been found stable at lower pH ranges, pH 3-5, most probably due to their native human digestive system pH environment, they have also been found stable between pH 5-9 in the laboratory (Gerba, 2006). In a recent study conducted in the Netherlands,

Lodder et al. (2010) reported that noroviruses were more abundant in seawater samples with higher pH values, however their pH range (7.4-8.0) was very narrow. In this study there was very little variation in pH and no correlation between EV occurrence and pH.

Salinity. In this study there was a significant negative correlation between EV occurrence and salinity ($r_s = -0.680$, $p = 0.047$). In situ salinity measurements were taken directly in front of an outfall and were most probably influenced by discharges following rainfall events. However, other researchers have reported higher stability of enteroviruses at higher salt content (Kapuscinski and Mitchell, 1980) while others have reported no significant relationships between salinity and occurrence of enteroviruses (Jiang et al., 2007).

Rainfall Events. Land runoff discharges after rainfall events have been mentioned as nonpoint sources of human viral contamination into the coastal ocean (Bosch et al., 2006), but only a few studies have found statistical relationship between viral pathogens and rainfall events (Lipp et al., 2001). In a recent study by Futch et al., (2010) enteric viruses were detected more frequently in groundwater in south Florida during the wet season than the dry season, and the authors attribute this to rainfall events. In another study, Lipp et al., (2001) found a strong correlation between precipitation (preceding 7 days' rainfall) and the presence of enteroviruses in recreational waters of the Charlotte Harbor, Florida. Similarly, in our study we found a significant positive correlation ($r_s = 0.850$, $p = 0.001$) between EV occurrence and precipitation during the 72 hours preceding sampling (Table 4.2).

Tidal Phase. Tidal phase was used as an indirect measure of harbor circulation. Based on a previous study of contamination from the PJ wastewater treatment plant (P. Rose, pers com), circulation patterns in the harbor are such that more treatment plant effluent should reach our

surface water site during incoming tides (high tides) than during outgoing tides (low tides). Most samples collected during years 2007 and 2008 were during high tide. However, after a summer seasonal weekly collection series (summer 2008), it became apparent that more viruses were present at low tide (Table 4.1). In addition, other researchers have reported collection of samples during a falling spring tide (Gentry et al., 2009) or on an outgoing tide to ensure the maximum likelihood of detecting land runoff pollutants (Lipp et al., 2001). Consequently, during late fall and winter 2009 samples were taken during low tide. My finding is similar to Gersberg et al., (2006) who reported no correlation between tidal height and occurrence of viruses.

In conclusion, the occurrence of enteroviruses was affected by rainfall events more than any other environmental factor at the Port Jefferson Harbor. Thus the occurrence of human viruses in the marine environment is highly influenced by its host sources, in this case the discharge of viral contamination after rainfall events. One might reasonably speculate that during summer months increased boating activity may lead to human viral contamination into recreational waters, via illegal discharges of waste tanks from recreational boats, leaks of vessel waste pump-out stations, and dock and marine wash down activities (Jiang et al., 2007). However, I found no evidence to support this. Although my results could be inconclusive due to the small number of samples (n=8) analyzed, Brooks et al., (2005) detected hepatitis A viruses after rainfall events based on eight samples of ocean water collected from the Tijuana River in California. In spite of the small number of samples which positively detected HEV, this study confirms land runoff discharges after rainfall events as sources of human viral contamination.



Figure 4.1 (a) Maps of sampling site located along the north shore of Long Island, NY. (b) Surface water samples were collected from the Port Jefferson Harbor (PJH). (c) PJH is a semi-enclosed tidal urban estuary that receives anthropogenic surface discharges from a local wastewater treatment plant (WWTP), various storm water discharge pipes, and pleasure boats especially in the summer. (d) Inner portion of the PJH and where samples were collected during the years 2008 and 2009. Images were modified from Google Maps.

Table 4.1 Environmental factors and EV occurrence at PJH surface water samples (n=8) collected during summer 2008 and fall 2009.

Collection Date	EV ^a	UVI ^b	Temp. (°C)	pH	Salinity	Tidal Height (m)	Tidal Phase	Precipitation (mm)	Rainfall Events ^c
6/23/2008	+	9	21	7.9	26	0.5	High	6.4	Yes
6/30/2008	-	8	23.5	8	26.7	0.4	High	0.3	No
7/7/2008	-	9	21	7.8	27.9	0	Low	2.5	No
7/15/2008	-	9	22	7.9	27.1	1.8	High	0.5	No
7/21/2008	-	9	23.7	7.8	27.6	2.1	High	0	No
10/19/2009	+	3	11.2	7.6	26.8	-0.1	Low	15.5	Yes
11/3/2009	-	3	8.7	7.4	27	-0.1	Low	0.3	No
12/4/2009	+	0	11.1	7.8	26.7	-0.2	Low	38	Yes

^a EV presence, "+"; EV absence, "-".

^b UVI, ultraviolet index

^c Rainfall events were ranked on precipitation accumulated of ≥ 5.1 mm in the 72 hours preceding sampling.

Table 4.2 Spearman rank correlations analyses for the occurrence of EV in surface recreational water samples at the PJH (n=8) using environmental factors.

Factors	EV	
	r_s	p value
UV Index	-0.423	0.260
Temperature	-0.455	0.233
pH	-0.283	0.460
Salinity	-0.680	0.047*
Precipitation ^a	0.850	0.001*
Tidal Height ^b	-0.453	0.233

^a Precipitation is the total rainfall 72 hrs preceding the sampling was used.

^b The water level at the time of sampling was used.

* An asterisk indicates the value is significant.



Figure 4.2 UV light index (UVI) at the PJH (n=8) during summer 2008 and fall 2009. There was a seasonality difference for UVI: summer indexes were 8 and 9 compare to the fall, 0 and 3. Enteroviruses (white circles) were detected at UVI of 9, 3 and 0 during the dates of June 23, 2008, October 19, 2009, and December 4, 2009, respectively. UVI data was collected from the NOAA website (http://www.cpc.ncep.noaa.gov/products/stratosphere/uv_index/Bulletin/)

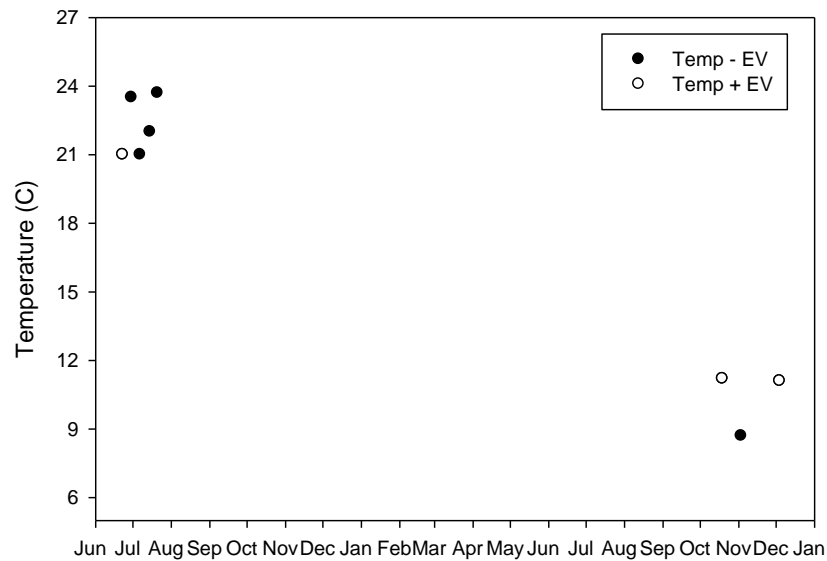


Figure 4.3 Water temperatures at the PJH (n=8) during summer 2008 and fall 2009. There was a seasonality differences during the collection dates among water temperatures: summer temperatures were between 21-23.7°C, and fall temperatures were between 8.7-11.2°C. Enteroviruses (white circles) were detected at temperatures of 21°C, 11.2°C and 11.1°C during the dates of June 23, 2008, October 19, 2009, and December 4, 2009, respectively.

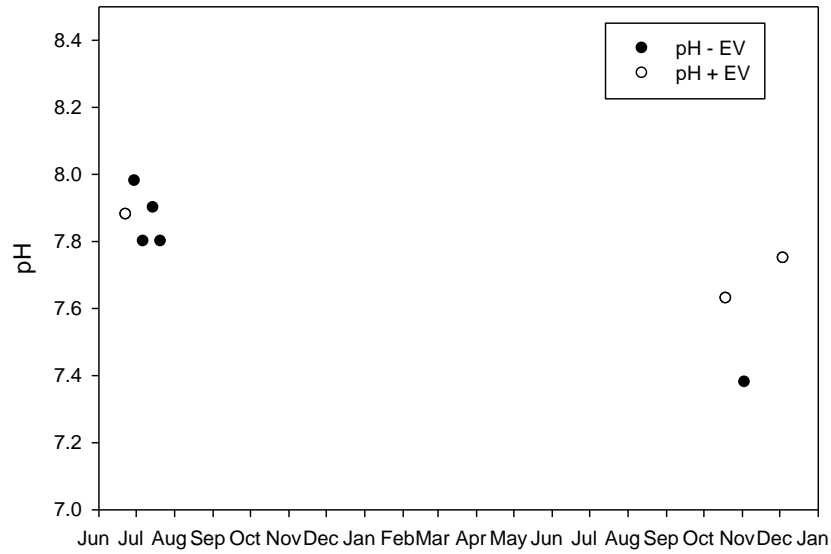


Figure 4.4 Water surface pH at the PJH during summer 2008 and fall 2009. During the collection dates pH were between 7.75-7.98, summer had higher pH than the fall. Enteroviruses (white circles) were detected at pH of 7.88, 7.63 and 7.75 during the dates of June 23, 2008, October 19, 2009, and December 4, 2009, respectively.

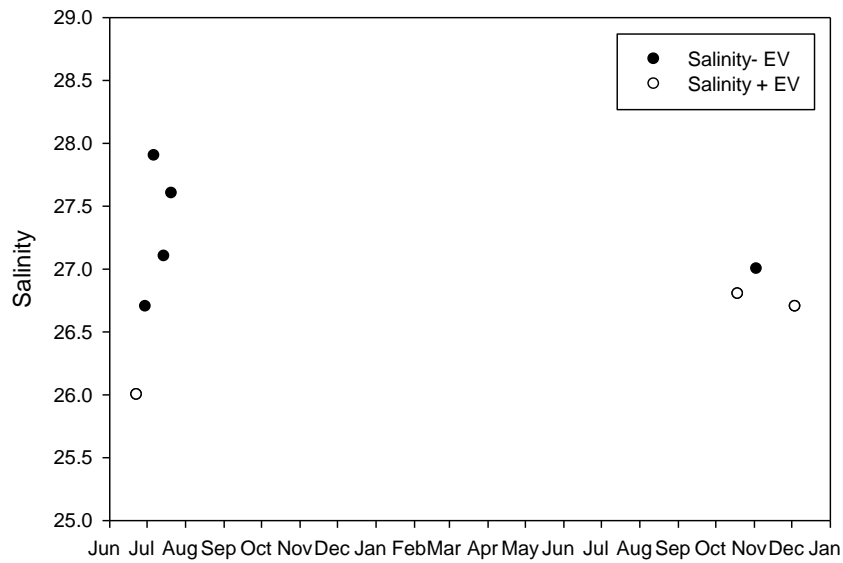


Figure 4.5 In situ salinities at the PJH surface waters during summer 2008 and fall 2009. Salinity values were from 26.0 to 27.6, there was no variation between summer and fall salinities. Enteroviruses (white circles) were detected at salinities of 26.0, 26.8 and 26.7 during the dates of June 23, 2008, October 19, 2009, and December 4, 2009, respectively.

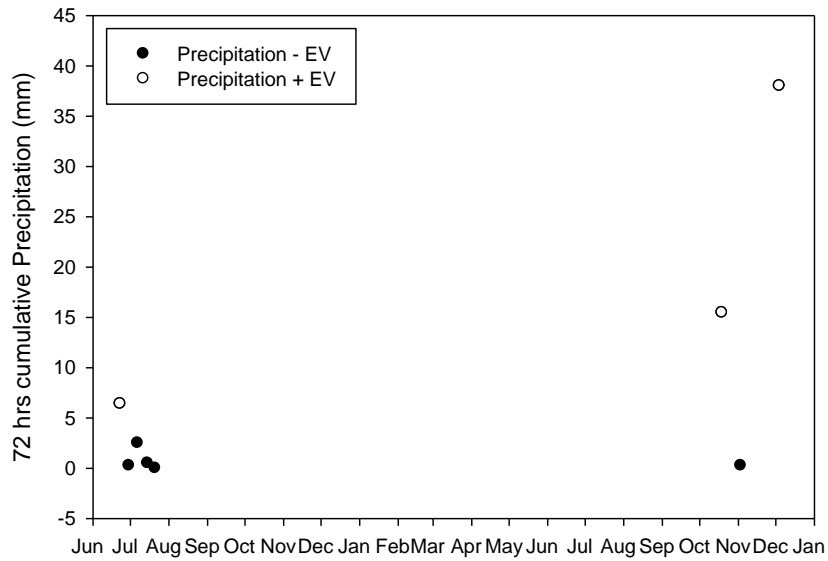


Figure 4.6 Cumulative Precipitation in the last 72 hrs prior collection of surface waters during summer 2008 and fall 2009 at the PJH. Precipitation varied from 0.0 to 38.0 mm. Enteroviruses (white circles) were detected at cumulative precipitations in the last 72 hrs prior collection of 6.4, 15.5, and 38.0 mm during the dates of June 23, 2008, October 19, 2009, and December 4, 2009, respectively. Enteroviruses were detected only during and after rainfall events of more than 0.5 mm of cumulative precipitation in the last 72 hrs.

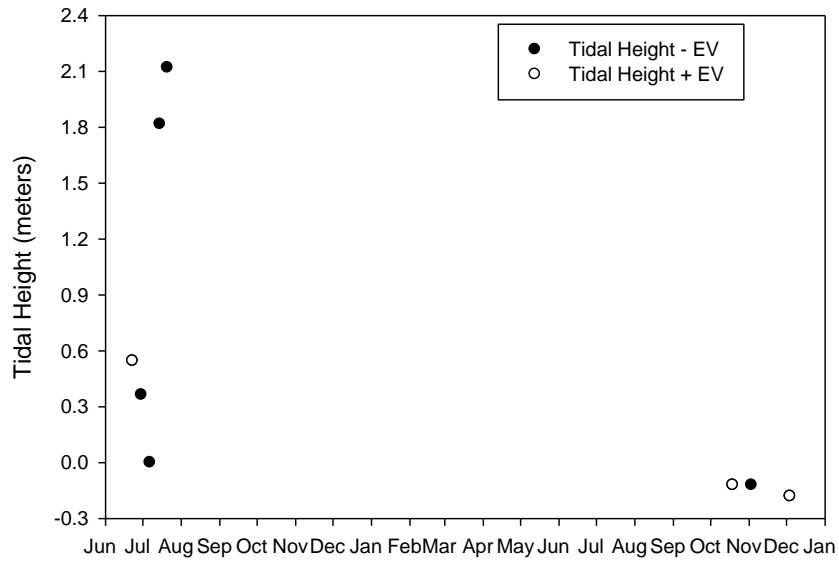


Figure 4.7 Tidal height at collection time for surface water samples at the PJH during summer 2008 and fall 2009. Samples were collected during low tide (≤ 0.17 meters) and high tide (≥ 0.18 meters). Tidal height varied from -0.12 to 2.12 meters. Enteroviruses (white circles) were detected at 0.55, -0.12, and -0.18 meters during the dates of June 23, 2008, October 19, 2009, and December 4, 2009, respectively. Enteroviruses were detected during low tide and high tide.

CHAPTER 5:

CONCLUSIONS

In this dissertation it was hypothesized that human enteric viruses (HEV) were present in recreational surface waters that had discharges of human waste via point and nonpoint sources. Previous reports indicate that sewage and rainfall can be key factors responsible for human viral contamination in the marine environment (Bosch et al., 2006), but few studies have shown significant correlations between rainfall and HEV contamination (Lipp et al., 2001). Therefore, a specific hypothesis was that after a rainfall event HEV would be present in surface waters via inputs from point and non-point sources. The Port Jefferson Harbor was chosen as the main location since it receives discharges from a wastewater treatment plant and combined sewer overflow (CSO) outfall pipes directly into surface waters. While initially no HEV could be detected at any location, after careful modifications of our viral concentration and detection methods, enteroviruses were detected in surface water at the Port Jefferson Harbor.

One of the main objectives in this dissertation was the optimization of viral concentration and detection methods to quantify HEV. Once this was accomplished I evaluated the following two objectives: identify sources responsible for human viral contamination; and identify environmental factors that affect human enteric virus presence in the Port Jefferson Harbor surface waters.

Chapter two describes and evaluates viral concentration methods with the goal of detecting three specific human enteric viruses (HEV) groups in recreational surface waters. While much effort went into obtaining higher recoveries of total extracted RNA and VLPs by primary concentration methods, in particular utilizing ultrafiltration via tangential flow filtration (TFF), the addition of a secondary concentration method proved to be the most successful approach. The two-step approach of adsorption-elution followed by centrifugal ultrafiltration was the simplest and most practical technique to concentrate viruses from surface waters and to detect specific group of human enteric viruses.

Chapter three describes efforts to detect and quantify enteroviruses, hepatitis A viruses and noroviruses using SYBR Green Quantitative Reverse Transcriptase PCR (qRT-PCR). I was unable to quantify enteroviruses, hepatitis A viruses or noroviruses from surface utilizing qRT-PCR. I was successful in detecting EV using conventional Reverse Transcriptase PCR (RT-PCR) and cloning and sequencing techniques. In addition, I quantified enteroviruses from sludge samples of the Port Jefferson wastewater treatment plant. However, I could not conclude that wastewater discharges are in fact a point source of viral contamination into the surface waters of the Harbor, since enteroviruses were only detected after rainfall events.

In chapter four, environmental factors that are known to affect persistence of human viruses were compared to EV occurrence. These included: UV Index, temperature, salinity, pH, precipitation and tidal height. It was hypothesized that HEV occurrence would be inversely correlated with UV, temperature, pH, and salinity, based on laboratory results (Gerba, 2006). In my study only salinity and rainfall had statistically significant correlations with EV presence. Rainfall events, defined as precipitation of more than 5.0 mm in a period of 72 hrs, resulted in decreased salinity and appeared to lead to EV occurrence in PJH surface recreational waters.

These results must remain inconclusive given the small number of comparable samples (n=8) and even smaller number of EV-positive samples (3).

Viral concentration and detection methods needed to monitor human RNA viruses in surface waters remain problematic. However, we found that monitoring of HEV in surface waters can be accomplished with current technology. In addition, it was determined that viral contamination in the Port Jefferson Harbor is driven more by inputs of contaminants from point sources (i.e. surface runoff drain pipes) including land runoff discharges after a rainfall even than any other source including direct discharge effluent from the sewage treatment plant, or faunal viral reservoirs.

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