

FATE OF ENTERIC VIRUSES IN ADVANCED AND CONVENTIONAL
WASTEWATER TREATMENT

BY

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ABSTRACT

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Human enteric viruses are currently listed on the United States Environmental Protection Agency Contaminant Candidate List (USEPA CCL) as emerging contaminants and have been linked to several waterborne diseases, such as acute gastroenteritis, conjunctivitis and respiratory illness. In this study, a total of 82 wastewater samples using two different electropositive filters and 6 biosolids samples were analyzed from five separate full-scale wastewater treatment plants (WWTPs) in Michigan using real-time PCR and cell culture assays. Adenovirus (HAdV), enterovirus (EV) and norovirus genogroup II (NV2) were detected in approximately 100%, 84% and 37%, respectively of all wastewater samples using real-time PCR. Cytopathic effect (CPE) was present in 100% of the cell culture samples for influent, pre and post disinfection and biosolids with an average concentration of 2.2×10^4 , 5.9×10^1 , 6.2×10^0 and 2.9×10^7 MPN/100L, respectively. Based on real-time PCR data, we concluded a MBR system is able to achieve approximately 2 log higher reduction of HAdV (average 4.1 log units) as compared with conventional wastewater treatment (average 2.2 log units). However, similar EV log removal values (3.6 for MBR and 2.9 for conventional) were observed between the two types of treatment processes. In addition, there is a significant log reduction (2.3 - 4.5) in infectious viruses throughout the wastewater treatment process before being discharged into natural waterways.

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This work is dedicated to J, L and Z

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Table of Contents

LIST OF TABLES	ix
LIST OF FIGURES	x
CHAPTER 1	1
INTRODUCTION	1
References.....	3
CHAPTER 2	5
REMOVAL OF HUMAN ENTERIC VIRUSES BY A FULL-SCALE MEMBRANE BIOREACTOR DURING MUNICIPAL WASTEWATER PROCESSING	5
Abstract.....	5
Introduction.....	7
Methods and Materials.....	9
Sample Collection.....	9
Virus elution process for filters.....	10
Nucleic Acid Extraction.....	10
Real-Time PCR Standard Curves and Detection Limits.....	11
Molecular detection for EV, NV1 and NV2	12
Human Adenovirus Data.....	13
Inhibition Control.....	13
Calculations for Virus Concentration	14
Membrane Influent Concentration	14
Log Removal.....	15
Statistical Analysis.....	16
Results.....	16
Quantification of Enteric viruses	16
Inhibition Control.....	18
Enteric Virus Removal by MBR.....	18
Distribution of Viruses between Settled Particles and Supernatant.....	19
Discussion.....	20
Enteric Virus Occurrence.....	20
Enteric Virus Removal by MBR.....	22

Different removal efficiency by MBR treatment among viruses.....	25
Association of viruses with solid particles.....	26
Conclusion	28
Tables and Figures	29
References.....	38
CHAPTER 3	43
RELEASE OF INFECTIOUS HUMAN ENTERIC VIRUSES BY FULL-SCALE WASTEWATER UTILITIES.....	43
Abstract	43
Introduction.....	44
Methods and Materials.....	47
Wastewater Treatment Plants	47
Wastewater and biosolids sampling.....	48
Virus elution process for filters and biosolids	48
Nucleic acid extraction	49
Real-time PCR standard curves, sequencing and detection limit	49
Quantitative real-time PCR assays.....	50
Inhibition control	51
Calculations of enteric virus concentration.....	52
Biosolids Conversion	52
Cell culture.....	53
Log Removal.....	53
Statistical analysis.....	54
Results.....	54
Inhibition control	54
Quantification of human enteric viruses in WWTPs	54
Quantification of human enteric viruses in biosolids	56
Infectivity of viruses	57
Removal of infectious viruses.....	58
Inactivation of infectious viruses using UV and Cl.....	58
MBR and conventional WWTP	58
Discussion.....	59
Release of viruses by WWTPs.....	59
Inactivation of infectious viruses between UV and Chlorine	63

Comparing the removal of HAdV and EV in MBR and conventional WWTPs	64
Conclusions.....	66
Acknowledgements.....	67
Tables and Figures	68
References.....	78
 CHAPTER 4	 84
CONCENTRATION OF ENTERIC VIRUSES FROM WASTEWATER USING NANOCERAM ELECTROPOSITIVE FILTERS	84
Abstract.....	84
Introduction.....	86
Methods and Materials.....	87
Results.....	91
Discussion.....	92
Conclusions.....	94
Acknowledgements.....	95
Tables and Figures	96
References.....	101
 CHAPTER 5	 104
CONCLUDING REMARKS.....	104

LIST OF TABLES

Table 2-1. List of the eight different sampling events and the volumes sampled at each point during this study. The Mem Inf consists of two volumes (Membrane Influent as Supernatant / Membrane Influent as Activated Sludge (total volume)).	29
Table 2-2. Cloning primers used for the creation of the standard curves. Degenerate base code: B = C, G or T; D = A, G or T; H = A, C, or T; M = A or C; N = A, C, G or T; R = A or G; Y = C or T.....	30
Table 2-3. Primers, probes and real-time PCR conditions used for the standard curves and molecular detection assays.....	31
Table 3-1. Characteristics of the different WWTPs used in this study.....	68
Table 3-2. List of enteric virus primer and probes, gene regions, reaction conditions and references used in this study	69
Table 3-3. Real-time PCR and A549 cell culture results for WWTPs 1, 2 and 5. The samples represent both the final effluent and biosolids infectivity that are released into the environment. ± - presence versus absence for CPE and ND – not detected. Values are e	75
Table 4-1. List of the WWTPs sampled from	96
Table 4-2. List of enteric virus primer and probes, gene regions, reaction conditions and references used in this study	97

LIST OF FIGURES

Figure 2-1. Overall HAdV distribution within the Influent (n=8), Pri Eff - Primary Effluent (n=8), Mem In - Membrane Influent (n=8) and Mem Eff - Membrane Effluent (n=8) for all eight sampling events.....	32
Figure 2-2. Overall EV distribution within the Influent (n=8), Pri Eff - Primary Effluent (n=8), Mem In - Membrane Influent (n=8) and Mem Eff - Membrane Effluent (n=8) for all eight sampling events.....	33
Figure 2-3. Overall NV2 distribution within the Influent (n=8), Pri Eff - Primary Effluent (n=8), Mem In - Membrane Influent (n=8) and Mem Eff - Membrane Effluent (n=8) for all eight sampling events.....	34
Figure 2-4. Virus removal by the MBR (membrane influent and effluent). HAdV (n=8), EV (n=8) and NV2 (n=8).	35
Figure 2-5. Virus removal between the influent and MBR effluent. HAdV (n=8), EV (n=8) and NV2 (n=8).	36
Figure 2-6. Overall distribution of HAdV (left side) and EV (right side) for the entire eight month sampling period. Inf-Membrane Influent (n=8), Sludge - Settled Sludge (n=8) and Super - Filtered Supernatant (n=8) for each HAdV and EV.	37
Figure 3-1. Average (n=2) HAdV real-time PCR virus concentration detected at the five different WWTPs at each sampling point and their respective biological and disinfection processes.	70
Figure 3-2. Average (n=2) EV real-time PCR virus concentration detected at the five different WWTPs at each sampling point and their respective biological and disinfection processes. All samples are expressed as viruses/L. The detection limit was used for the pre	71

Figure 3-3. Real-time PCR data showing the distribution of HAdV (left side) and EV (right side) in WWTPs 1, 2 and 5. The values were compiled for the influent (n=6), pre disinfection (n=6), post-disinfection (n=6) and biosolids (n=6)..... 72

Figure 3-4. Virus infectivity distribution using BGM cell line throughout the three different sampling points from all 5 WWTPs (n=5 for Influent, Pre and Post Disinfection). The values are expressed as MPN/100L. 73

Figure 3-5. BGM cell culture data showing the concentration of viruses being released into the environment from WWTPs 1, 2 and 5. All results are expressed as MPN/100L (n=3 for influent, pre and post-disinfection and biosolids). 74

Figure 3-6. Infectious virus reduction between WWTPs using UV and Cl disinfection. Pre and Post-Disinfection UV (n=4), Pre and Post-Disinfection Cl (n=2). 76

Figure 3-7. Comparison of HAdV and EV log removal values using real-time PCR data for MBR and conventional wastewater treatment. (MBR removal n=8, Con removal n=8). 77

Figure 4-1. Comparison in average real-time PCR concentration of HAdV for the influent, pre and post disinfection between the NanoCeram and 1MDS cartridge filters for all sampling events. Error bars were used to show the standard deviation within the data. Sampl 98

Figure 4-2. Comparison in average real-time PCR concentration of EV for the influent, pre and post disinfection between the NanoCeram and 1MDS cartridge filters. Error bars were used to show the standard deviation within the data. Sample size was n=6 for influent 99

Figure 4-3. Comparison of virus infectivity using BGM cell line between the NanoCeram and 1MDS filters for influent, pre and post disinfection samples. Values are expressed in MPN/100L. Inf. – Influent, Pre – Pre-Disinfection and Post – Post-Disinfection..... 100

CHAPTER 1

INTRODUCTION

Human enteric viruses are abundant in all aquatic environments from river to sea-water, ranging in concentration (Castignolles et al., 1998). A large number of enteric viruses are excreted in human feces and urine, which makes wastewater one of the most concentrated sources of these viruses (Puig et al., 1994; Castignolles et al., 1998). Due to their low infectious dose, survival in environmental media and their detrimental effect on human health, enteric viruses pose considerable threats to the public (Xagorarakis et al., 2007; Castignolles et al., 1998). Excreted enteric viruses are continually being detected in wastewater, rivers, recreational water, sea water, ground water and even in municipal drinking water (Pusch et al., 2005; Lee and Kim, 2002). Human enteric viruses have been related to several waterborne diseases, such as acute gastroenteritis, conjunctivitis and respiratory illness. The severity of the conditions can vary depending on the species, serotypes, concentration, age of individual, and exposure rates of these viruses (Gerba, 1984). In the US, human enteric viruses are the main etiologic agents of childhood gastroenteritis, resulting in several hospitalizations and deaths each year. The particular viruses often responsible for these symptoms include adenovirus, enterovirus, norovirus and rotavirus (Puig et al., 1994). There are several routes whereby the public can become infected, including direct contact, food borne illness, contamination and vectors (Godfree et al., 2005).

One of the main goals of environmental engineers is to restrict the presence of infectious pathogens, such as viruses found in the environment. This usually requires close observation of the occurrence, level, fate and the exposure to the public associated with the various human pathogens found in the air, soil and water (Guzman et al., 2007). Virus removal from wastewater continues to receive attention due to the epidemiological significance of viruses as waterborne pathogens and because of the high diversity that is excreted in human waste (Rose et al., 1996). The focus of this work is on addressing the fate of emerging biological contaminants found in wastewater (influent, primary settling, secondary biological treatment and final effluent following disinfection) and biosolids. Various techniques were used to detect enteric viruses in this study which included both molecular and culture methods.

The specific objectives of this study are to (i) quantify Human Adenovirus F40/41 (HAdV), Human Enterovirus (EV) and Norovirus genogroup I and II (NV1) and (NV2) within the wastewater treatment process using real-time PCR data, (ii) compare virus removal efficiency between advanced (MBR treatment) and conventional (activated sludge, oxidation ditch and rotating biological contactors) wastewater treatment process using real-time PCR data, (iii) determine the release of infectious viruses found in the final effluent and biosolids samples, (iv) describe the solid-liquid distribution of enteric viruses (HAdV, EV and NV) in the membrane influent, (v) compare the effectiveness of two different disinfection processes (ultraviolet light and chlorination) for virus inactivation and (vi) compare the performance of two different electropositive cartridge filters (NanoCeram® SOE Series and 1MDS) used for sampling, quantification and infectivity determination of human enteric viruses.

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CHAPTER 2

REMOVAL OF HUMAN ENTERIC VIRUSES BY A FULL-SCALE MEMBRANE BIOREACTOR DURING MUNICIPAL WASTEWATER PROCESSING

Abstract

In the US, human enteric viruses are the main etiologic agents of childhood gastroenteritis, resulting in several hospitalizations and deaths each year. These viruses have been linked to several waterborne diseases, such as acute gastroenteritis, conjunctivitis and respiratory illness. The removal of human enterovirus (EV) and norovirus genogroup II (NV2) was studied in a full-scale membrane bioreactor (MBR) wastewater treatment plant (WWTP) and compared with the removal of human adenovirus (HAdV). In total, 32 samples were quantified using real-time reverse transcription-PCR (RT-PCR) from four separate locations throughout the treatment process; influent, primary settling effluent, membrane influent (which includes the MLSS) and membrane effluent. EV was detected in all 32 samples (100%) with an average concentration of 1.1×10^7 and 7.8×10^1 viruses/L for the membrane influent and membrane effluent, respectively. NV2 was detected in 20 of 32 samples (63%) with an average membrane influent and membrane effluent concentration of 2.8×10^5 and 1.2×10^1 viruses/L, respectively. HAdV was detected in all 32 samples with an average membrane influent concentration of 5.2×10^8 and 2.7×10^3 viruses/L in the membrane effluent. Our findings indicate that this particular full-scale MBR WWTP was able to reduce the viral loads by approximately 5.1 and 4.0 log units for EV and NV2 as

compared to 5.5 log units for HAdV. This full-scale MBR system outperformed the removal observed in previous pilot and bench scale studies by 1-2 log units. To the best of our knowledge, this is the first study that has looked at the removal of EV in a full-scale MBR WWTP using quantitative real-time RT-PCR.

Introduction

Membrane filtration is the physical process that separates particles and colloidal material present in the raw feed water from the permeate effluent. Membrane bioreactors (MBR) are a modification of the activated sludge process in which separation of solids is achieved without the requirement of a secondary clarifier as compared to conventional activated sludge systems. In the past decade, MBRs have been increasingly used in the wastewater treatment industry as an advanced treatment technology to improve treated water quality especially when water reuse is required (Ahn et al., 2001). As of 2003, there were over 1,000 MBR's in operation around the world, 66% of these are used throughout Japan, while the rest a found in Europe and the US (Cicek, 2003). As the population worldwide continues to rise, there is a greater demand and increased pressure from the public to be able to treat wastewater efficiently and effectively while trying to minimize the risk of exposure to biological contaminants.

Human enteric viruses are one of the main pathogens on the United States Environmental Protection Agency Contaminant Candidate List (USEPA CCL) of emerging contaminants. Wastewater effluent discharge is often the source of enteric viruses detected in natural waterways (Kuo et al., 2010; Kitajima et al., 2009; da Silvia et al., 2007; Haramoto et al., 2007; Kageyama et al., 2003). Presently, the USEPA does not require wastewater treatment plants (WWTPs) to monitor the concentration of these viruses in the final effluent. Human Adenovirus (HAdV), Human Enterovirus (EV), Norovirus genogroup 1, 2 and 4, (NV1) and (NV2) are some of the enteric viruses of concern because of their low infectious dose. Human enteric viruses have been linked to several waterborne diseases, such as acute gastroenteritis, conjunctivitis and respiratory illness (Kuo et al., 2010; Kitajima et al., 2009; da Silvia et al., 2007; Haramoto et al., 2007; Kageyama et al., 2003). The main pathways of exposure are often direct fecal-oral route or

dermal contact through secondary exposure (Godfree et al., 2005). In the US alone, enteric viruses are the main origin of gastroenteritis detected in children whereby averaging roughly 100 deaths per year (Gerba et al., 2002). The degree of viral infection can often vary depending on the species, serotypes, concentration, age of individual, high risk category individuals and exposure rates to these viruses (Gerba et al., 2002; Rose et al., 1996).

Determining the concentration of enteric viruses throughout the treatment process helps to monitor any increases in influent concentration and ensures safe levels of pathogens in the final effluent. In the past, several studies have used norovirus, enterovirus, poliovirus and viral indicators (i.e., coliphage or bacteriophage) to determine overall removal capabilities of membranes in both bench and pilot-scale MBR systems (Ueda et al., 2000; Hu et al., 2003; Ottoson et al., 2006; Zheng et al., 2006; Zhang et al., 2007). In addition, two past studies have determined the performance of full scale MBR's for adenovirus and norovirus (Kuo et al., 2010; da Silva et al., 2007). The focus of this study is to (i) quantify Human Enterovirus (EV) and Norovirus genogroup I and II (NV1) and (NV2) and determine their removal by a full-scale MBR WWTP, (ii) compare with HAdV removal in the same plant (Kuo et al., 2010) and (iii) describe the solid-liquid distribution of enteric viruses (HAdV, EV and NV) in the membrane influent.

Methods and Materials

Sample Collection

Eight sampling events took place at the Traverse City Wastewater Treatment Plant (TCWWTP) monthly (except April, which had two events and no samples taken in June) between January and August 2008.

The WWTP is described in Kuo (2010). Briefly, the TCWWTP is designed to treat maximum monthly wastewater loads of 9200 kg/day biological oxygen demand (BOD) (20,200 lb/day) 16,550 kg/day total suspended solids (TSS), and 1000 kg/day ammonia at a flow of 32,000m³/day (8.5 mgd), with peak flows up to 64,000m³/day (17 mgd). The MBR system is combined with a biological nutrient removal technology. The membranes for the MBR system are Zenon's ZeeWeed 500c cassettes (Zenon Environmental Inc., Oakville, Ontario, Canada) made by hydrophilic and non-ionic proprietary polymer. They are immersed, hollow-fiber ultrafiltration membranes with a nominal pore size of 0.04 micron (or micrometer) and absolute pore size of 0.1 micron.

There were four sampling locations; influent, primary settling effluent, membrane bioreactor influent (which includes the MLSS) and membrane effluent. In total there were 32 viral samples collected using 1MDS cartridge filters as explained in the USEPA Manual of Methods for Virology (USEPA, 2001). An average of 20 L of influent, 30 L of primary settling effluent, 50 L of membrane influent an average 400 L of membrane effluent were sampled through the 1MDS electropositive cartridge filter (Table 2-1). Each sample was pumped through the apparatus at a rate of about 11-12 L/min (3 gal/min) except the membrane influent.

50 gallons of membrane influent was collected in a large tank and allowed to settle for 30 min due to the high amount of MLSS (~2000mg/L). After that time, the supernatant was passed through the 1MDS filter and 15 mL of sludge was collected for analysis. All samples collected were stored on ice and shipped the same day using overnight delivery to the Michigan State University Water Quality Engineering Laboratory in East Lansing, MI. Upon delivery, samples were placed in a 4°C cooler for 12-24 hours before processing.

Virus elution process for filters

All samples collected were eluted 12-24 hours after initial sampling according to the Concentration and Processing of Waterborne Viruses by Positive Charge 1MDS Cartridge Filters and Organic Flocculation (USEPA, 2001) and explained in our previous study (Kuo et al., 2010).

Nucleic Acid Extraction

All viral samples (except the activated sludge) were extracted using the MagNa Pure Compact System automatic machine (Roche Applied Sciences, Indianapolis, IN). The extraction kits used were the MagNA Pure Compact Nucleic Acid Isolation Kit-Large Volume. The program used required 1000µL of sample to be extracted and concentrated for a final volume of 100µL. Immediately following the completion of the extraction, all samples were placed in -80°C freezer to preserve the integrity of the RNA molecule. However, due to the high concentration of suspended solids in the activated sludge samples the viral nucleic acids were hand extracted using the QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA) which incorporates the spin column protocol listed in the manufacturer's handbook. Following extraction the quantity of

viral nucleic acid extracts from all samples were checked using the NanoDrop Spectrophotometer (NanoDrop[®] ND-1000, Wilmington, DE).

Real-Time PCR Standard Curves and Detection Limits

The standard curves for the molecular detection of EV, NV1 and NV2 were created using stock cultures of Coxsackie virus B5 (ATCC VR-1036AS/MK), and NV1 and NV2 stool samples were supplied by the Ingham County Health Department following a confirmed outbreak. The cloning primers used in this study are summarized in Table 2-2. All standard curve assays were run in the LightCycler[®] 1.5 Instrument (Roche Applied Sciences, Indianapolis, IN). Briefly, the PCR amplicons from EV and NV2 from pure culture and stool sample extracts were cloned into a plasmid vector (i.e., pCR[®]4-TOPO[®]) which follows the one-shot chemical transformation described in the manufacturer instructions (TOPO TA Cloning[®] Kit for Sequencing, Invitrogen, Carlsbad, CA). The plasmids carrying the cloned EV, NV1 and NV2 were purified using Wizard[®] Plus SV Minipreps DNA Purification System (Promega, Madison, WI) and sent for sequencing at the Research Technology Support Facility at Michigan State University. All target gene sequences were compared with those published in the National Center for Biotechnology Information (NCBI) database by using the program of Basic Local Alignment Search Tool (BLAST). The concentrations were determined by using the NanoDrop spectrophotometer and then the samples were serial diluted 10-fold and used for creating the standard curves for all target viruses. All standard curve reactions were run in triplicate and the detection limit of EV and NV2 is 10¹ genomic copies/reaction (10¹ viruses/rxn).

Molecular detection for EV, NV1 and NV2

The extracted samples were reverse transcribed before the qPCR was performed for the three parameters (EV, NV1 and NV2). Each reverse transcription reaction mix included 2.5 μL of 10 μM reverse primer (for each target), 1 μL of reverse transcriptase (Promega Corporation, Madison, WI), 4 μL of 5X transcriptor reaction buffer (Roche), 0.5 μM of protector Rnase inhibitor (Roche Applied Sciences, Indianapolis, IN), and 2 μL of 10 mM deoxynucleotide, all RT reactions were run in a Bio-Rad thermal cycler (iCycler™, Bio-Rad, Hercules, CA) and the conditions are as follow: 55°C for 30 minutes and then 85°C for 5 minutes to inactivate the enzyme and a final hold cycle at 4°C for indefinite (the RT conditions are the same for all three target viruses). The RT samples were then placed into -20°C until further analysis.

The primers and probes used for the standard curve and molecular detection are summarized in Table 2-3. EV and was run using both the one-step and two-step approach for detection of the target gene. The conditions for the separate assays, EV and NV2 are as previously described (Dierseen et al., 2007; Kageyama et al., 2003). Briefly, each assay condition is as follow: EV one-step, reverse transcription 50°C for 30 min, denaturation at 95°C for 15 min, 45 cycles of amplification 60°C for 1 min and 95°C for 10s, for two-step, denaturation at 95°C for 10 min, 50 cycles of amplification 95°C for 10s, 58°C for 30s and 72°C for 30s, followed by 1 cooling cycle at 40°C for 30s. NV2, denaturation at 95°C for 10 min followed by 45 cycles of amplification at 95°C for 15s with annealing and extension at 56°C for 1 min. The real-time PCR reaction master mix for two-step included 10 μL of 2X LightCycler 480 TaqMan Master Mix (Roche Applied Sciences, Indianapolis, IN), appropriate volume of

primers and probes to obtain the concentration described in Table 3, 5 μL of cDNA sample, and appropriate volume of PCR-grade water to make up a final 20 μL reaction mix. However, for EV one-step, the qPCR reaction master mix included 10 μL of QuantiTect Probe RT-PCR Master Mix, 0.2 μL QuantiTect Probe RT-Mix.

All samples were run in triplicate and included a negative control reaction (PCR-grade H_2O without template) and a positive control reaction for all targets.

Human Adenovirus Data

Overall HAdV data published in Kuo (2010) are also reported in this paper for comparison purposes. The HAdV data were obtained from the analysis of the same samples presented in the current study.

Inhibition Control

To determine if inhibition occurred during viral analysis, the methods previously explained (Viau et al., 2009; Rajal et al., 2007) were used. Bovine Enterovirus (BEV) was chosen as the virus to spike all samples to determine if inhibition was present, BEV was quantified following the methods previously published (Jimenez-Clavero et al., 2005). Prior to the inhibition check, all samples were initially analyzed for BEV using real-time PCR. Next, all extracted samples and molecular grade H_2O were spiked with a final concentration of 10^5 viruses/rxn of BEV.

Following the analysis, the C_p values of the extracted water and wastewater samples were compared to see if inhibition was present in the samples. If the C_p values of both the spiked

water and wastewater samples were within an acceptable level (5%), then inhibition did not affect our analysis.

Calculations for Virus Concentration

All samples were quantified using the following equation:

$$\frac{Viruses}{L} = \frac{\frac{Viruses}{rxn} \times \frac{1 rxn}{5 \mu L} \times 100 \mu L \times \frac{1}{1000 \mu L} \times 30,000 \mu L}{Volume} \quad (2 - 1)$$

Where the 5 μL is the amount of sample per reaction tube, the 1000 and 100 μL are the amounts of sample extracted and the volume of the extract, respectively. The 30,000 μL is the amount of concentrated eluent after the final filtration through a sterilized 0.22 μm PVDF (polyvinylidene fluoride) syringe filter (Millipore, Billerica, MA) from the elution process stated above. Lastly, divide the numerator by the initial sample volume in the denominator which varied from each sampling point to determine the concentration in each sample.

Membrane Influent Concentration

During each sampling event, 50 gallons of activated sludge was collected and allowed to settle. The supernatant was passed through the 1MDS filter and 15mL of settled sludge was also collected. According to the mass balance for each membrane influent sample, concentrations were calculated by adding up the virus concentration in the supernatant and the settled activated sludge part volumetrically. The equation is listed as follows,

$$\left[\begin{array}{c} \text{membrane} \\ \text{influent} \end{array} \right] = \frac{([\text{Supernatant}] \times V \text{ Supernatant}) + ([\text{Sludge}] \times V \text{ Sludge})}{(V \text{ Supernatant} + V \text{ Sludge})} \quad (2 - 2)$$

In the equation above (2), [Supernatant] / V Supernatant and [Sludge] / V Sludge are the virus concentration and volume of the supernatant and settled sludge, respectively.

Log Removal

Following the quantification of viruses, the overall log removal achieved by the MBR and the entire WWTP's was calculated using equation 3 and 4 for HAdV, EV and NV.

$$\text{Log Removal} = \log_{10} \left(\frac{\text{Membrane Influent Concentration}}{\text{Membrane Effluent Concentration}} \right) \quad (2 - 3)$$

$$\text{Log Removal} = \log_{10} \left(\frac{\text{Influent Concentration}}{\text{Secondary Effluent Concentration}} \right) \quad (2 - 4)$$

For membrane and post-secondary treatment samples that were below the detection limit, the log removal values were calculated by using the detection limit of the individual assays. This indicates that certain removal values may be greater than reported. However, this will allow for proper calculations when this value is needed for comparison. It was assumed that using the detection limit would give the lowest removal value when it may be higher.

Statistical Analysis

Log removal values for each WWTP was analyzed using t-test in Microsoft Excel ® using an alpha value (α -value of 0.05), showing a 95% confidence interval.

Results

Quantification of Enteric viruses

HAdV - HAdV quantification was previously reported by Kuo (2010). The HAdV data used in the current study is to compare with the occurrences and removal of both EV and NV. The results showed 32/32 samples were positive for HAdV. Figure 2-1 shows the overall log concentration of HAdV that was detected over the course of the entire study. The influent concentration ranged from 5.8 – 6.7 (average 6.5) log units, and the primary effluent ranged from 5.7 – 7.2 (average 6.2) log units. There was an increase in the membrane influent samples ranging from 7.9 – 9.1 (average 8.9) log units and the membrane effluent ranged from 2.7 – 4.5 (average 3.4) log units. It was concluded that no significant seasonal variation (p -value > 0.05) was observed during the 7 month sampling period.

EV - EV was detected in all 32 samples. As shown in Figure 2-2, an average log influent concentration of 6.5 viruses/L (0.7 standard deviation (std)) with a range of 5.8 – 6.7 was observed for all 8 sampling events. The highest and lowest log concentrations were detected in the August and April #2 samples at 6.1 and 4.1 viruses/L, respectively. However the average concentration for the winter months was approximately 5.7 log units. According to the data (Figure 2-2), the detection in the influent ranged from 4.1– 6.1 log units with an average 5.8 (0.7 std.). The average log concentration for the primary effluent samples were approximately 5.0 log

units (0.8 std). The highest concentrations were observed in the February and August samples, both sampling events concentrations were approximately 5.4 log units. However, the April #2 sample had the lowest log concentration approximately 3.2 log units.

Primary settling only accounted for approximately 0.6 log unit reduction before reaching the secondary biological process. The membrane influent concentration was significantly higher ($p\text{-value} < 0.05$) as compared to the primary effluent concentration. The elevated concentration occurred because the membrane influent sample was collected at the activated sludge tank after the point of addition of returned sludge (Kuo et al., 2010). The average EV log membrane influent concentration was 7.1 log units (0.6 std) indicating that the return activated sludge has increased the concentration to the membrane by 2 log units. The membrane effluent log concentration averaged 1.9 log units (0.5 std) ranging between 0.9 – 2.4 log units for both April #2 and May samples, respectively. Overall, the concentration of EV throughout the WWTP remained relatively stable for the eight month sampling period. No significant ($p\text{-value} > 0.05$) seasonal variation was observed during the current study for EV quantification.

NV - All 32 samples were analyzed for both NV1 and NV2 using the methods described above. NV1 was not detected in any of the samples (00/32) but NV2 was detected in 20/32 samples. Figure 2-3 shows the overall distribution of NV2 that was detected for all sampling events during the current study. NV2 was detected in 8/8, 4/8, 4/8 and 0/8 samples in the influent, primary effluent, membrane influent and effluent, respectively. The average log concentration of the influent samples were 7.7 log units (1.0 std) and the highest log concentration was detected in the January sample (8.6 log units). The lowest concentration (5.1 log units) was detected in the July sample was approximately 3 log units below the highest sample (January) and 2 log units below

the average. However, this concentration level was also reported in the February, March, April#2 and May samples. The average concentration in the primary settling effluent, membrane influent and membrane effluent was approximately 7.7 (2.0 std), 5.5 log units (0.6 std) and below detection limit, respectively.

Inhibition Control

BEV was not initially detected in the 32 wastewater (0/32) samples. All 32 samples collected were then spiked with 10^5 viruses/rxn of BEV following extraction including a PCR grade- H_2O . The Cp values for both WWTP samples (average Cp value 26.13, std 0.09) and H_2O (average Cp value 26.23, std 0.05) were within 2% of each other. This indicates that any inhibition that may be present in the extracted samples was not able to suppress the detection of the viruses in this study. This was concluded based on the average Cp values for all samples spiked with BEV and a *p-value* > 0.05.

Enteric Virus Removal by MBR

The log removal values for HAdV (Kuo et al., 2010), EV and NV2 were calculated from the MBR alone (membrane influent and effluent sample points) and the entire WWTP (influent and membrane effluent sample points). Figure 2-4 shows the log removal values between the membrane influent and effluent from the eight different samples for HAdV, EV and NV2. As shown in the Figure, HAdV removal ranged from 4.1 – 6.3 (average 5.5 and 0.8 std) as compared to 4.1 – 6.8 (average 5.1 and 0.9 std) and 3.5 - 4.8 (average of 3.9 and 0.5 std) log units for EV and NV2, respectively for removal by the MBR. In addition to the membrane removal efficiency,

we also determined the removal achieved by the entire WWTP. As shown in Figure 2-5, HAdV removal ranged from 2.2 – 3.6 (average 3.0 and 0.5 std) and the entire WWTP ranged from 1.9 – 4.6 log units (average 3.6 log units and 0.9 std) and log units for the MBR. According to our results, NV2 was able to achieve a higher overall removal (4.7 log units) as compared to HAdV (3.1 log units) and EV (3.6 log units) for the entire treatment process. There was no significant difference ($p\text{-value} > 0.05$) observed between HAdV and EV removal by the MBR for all eight sampling events.

Distribution of Viruses between Settled Particles and Supernatant

The viral nucleic acids from 16 different samples were analyzed for overall virus concentration detected in the membrane influent (settled sludge and filtered supernatant) by real-time PCR and calculated using Equation (2-2) for both HAdV and EV. The overall HAdV (Kuo et al., 2010) membrane influent concentration ranged from 7.6 – 9.1 log units (average 8.5). The concentration in the settled sludge had an average of 9.0 (range of 8.5 – 9.2 log units) and 4.9 log units (range of 4.9 – 5.9 log units) for the filtered supernatant. Figure 2-6 shows the overall distribution for the eight samples of HAdV concentration of the membrane influent (average 8.7 log units), settled sludge (9.1 log units) and filtered supernatant (5.2 log units).

Figure 2-6 also shows the distribution for the 24 individual samples analyzed for EV. The membrane influent ranged from 5.9 – 7.6 log units (average 6.8 log units), while the concentration in the settled sludge and filtered supernatant ranged from 6.2 – 7.9 (average 7.0 log units) and 2.3 – 5.6 log units (average 4.1 log units), respectively. The results were compiled over the duration of eight months to observe if any fluctuations occurred in the concentration of HAdV and EV as compared to the concentrations for each individual sampling event. Due to

inconsistent detection in the membrane influent, NV2 was not included in the comparison between the settled sludge and the filtered supernatant.

Discussion

Enteric Virus Occurrence

The results presented in the current study provide conclusive evidence of the removal efficiencies of EV and NV with the use of membrane technology. Our study is one of few (Kuo et al 2010; da Silvia et al., 2007) that have looked at the removal of enteric viruses in full-scale MBR WWTP systems. To the best of our knowledge, this is the first study to determine the concentration and removal of EV in full-scale MBR WWTP. In this study we evaluated the removal of EV and NV2 over a period of eight months (January – August 2008) from a WWTP located in Traverse City, Michigan. All samples were analyzed below detection limit for NV1 throughout the study.

Previous full-scale conventional wastewater utilities reports (Katayama et al., 2008; Pusch et al., 2005; Rodriguez et al., 2009) have detected EV in 65 – 89% in both the influent and effluent samples (with an average of 76% of samples) using conventional PCR as compared to 100% of the samples in the present study using quantitative real-time PCR. In the current study, EV was consistently detected during the eight months of sampling. The average influent concentration was 5.6 log units (3.6×10^5 viruses/L) (0.7 std) and no significant ($p\text{-value} > 0.05$) seasonal variation was observed. Furthermore, it was concluded that EV persisted throughout the treatment process at average concentration of 6.0 ($\sim 9.4 \times 10^5$ viruses/L) (0.8 std) in the primary effluent, 7.0 ($\sim 1.1 \times 10^7$ viruses/L) (0.6 std) for the membrane influent and 1.9 log units ($\sim 7.8 \times 10^1$ viruses/L) (0.5 std) for the final effluent.

Our findings are consistent (within 10%) with past full-scale conventional and MBR WWTPs studies (Kitajima et al., 2009; Laverick et al., 2004; Nordgren et al., 2009; da Silva et al., 2007) showing an average 72% NV detection in both influent and effluent samples as compared to 63% of our samples. It was observed in the current study that NV2 was present in the influent for all eight sampling events over a period of eight months at an average concentration of 7.7 log units (5.1×10^7 viruses/L) from the winter through summer months. Interestingly, NV1 was not detected in any of the thirty-two samples over the course of eight months. However, it has been previously stated (Haramoto et al., 2005) that a seasonal pattern for the detection of NV1 and NV2 may exist throughout the year with NV1 predominately in the summer months and NV2 usually detected at higher concentration during the winter months. Also, Nordgren (2009) reported a significant difference in NV2 concentration during the winter months compared to other months. However, in our study NV2 was detected in 8/8 influent (average 4 – 6 log units), 8/8 primary effluent (2 – 8 log units), 4/8 membrane influent (5 – 6 log units) and 0/8 effluent samples. The results here indicate the highest and lowest concentration of NV2 was in January at 8.6 log units (4.0×10^8 viruses/L) and 5.9 log units in July (1.2×10^5 viruses/L), respectively. However, the samples for February, March, April (#1 and #2) and August averaged 5.9 log units (7.8×10^5 viruses/L), suggesting no significant ($p\text{-value} > 0.05$) seasonal variation was found in the concentration of NV2 in the influent samples.

Enteric Virus Removal by MBR

The removal values determined throughout this study clearly indicate that the MBR system is capable of achieving an average HAdV removal of 5.2 log unit (0.8 std) as compared to 5.1 (0.9 std) and 4.1 (0.4 std) for EV and NV2, respectively for removal by the MBR.

Interestingly, in the current study NV2 was only found in 4/8 membrane influent samples as compared to 08/08 in the influent samples. We observed almost complete removal (> 5.0 log units) of NV2 during primary settling in half of the samples or an average 1.0 log unit for the positive membrane influent samples. In comparison, these results are not consistent with those reported by Katayama (2008) where NV1 and NV2 were routinely detected (92% and 89% of samples, respectively) in the final effluent. Suggesting that during the wastewater treatment process in their study, the conventional activated sludge process was unable to remove a significant amount of NV. In the current study the MBR treatment process was able to almost completely remove NV2 during treatment (> 5.0 log units), indicating that at high concentrations of suspended solids the majority of NV may be able to attach to the particles and be removed through physical processes in primary settling before reaching secondary biological treatment. More sampling and analysis is needed to evaluate and confirm the observed trends.

The NV removal results observed in the current study are consistent with those reported by da Silva (2007), where a full-scale MBR WWTP in France was able to achieve up to 5.5 and 5.2 logs reduction of NV1 and NV2, respectively. In that study, NV1 was detected in 73% of the influents and only 18% of the effluent samples and NV2 in 100% and 0% of the influent and effluent samples, respectively. However, the assay used had a detection limit of 5.0×10^3 and 2.0×10^2 viruses/L for NV1 and NV2, respectively. As a result of a higher detection limit NV1

could still be present in the effluent at a concentration of 500 viruses/L and NV2 at 200 viruses/L. During the current study we used the NV1 assay published by da Silva (2007). However, we were able to optimize the assay to a sensitivity of 10 viruses/rxn; depending on the sample volume is approximately 10 – 20 viruses/L for the volume ranges we sampled.

The most interesting result observed during the current study was the overall removal of NV2 in the January sample. It was observed that the combined unit treatment processes achieved a 7.4 log reduction, approximately 2 log units higher removal than the average removal observed in the current study and reported by da Silva (2007). The differences observed between these studies could be attributed to the high concentration of NV2 that was detected in our influent sample and the lack of detection in the final membrane effluent. These results are compared to the detection of NV2 up to 6.8 log units (6.0×10^6 viruses/L) in their effluent samples whereas NV2 was not detected in our effluent samples (0/8). Our results indicate that a full-scale MBR system is able to attain at least a 4.1 log (7.4 log unit was the maximum removal observed) reduction for NV2 through the membrane alone and 5.0 log reduction for the entire treatment process.

Several past studies (Zheng et al., 2006; Lv et al., 2006; Zheng et al., 2007; Ahn et al., 2001; Hu et al., 2003; Oota et al., 2005; Poyatos et al., 2007; Shang et al., 2005; Tam et al., 2007; Ueda et al., 2002; Zhang et al., 2007; Ottoson et al., 2006) have also determined the removal of both viruses and bacteriophage through bench and pilot scale experiments. Ottoson (2006), observed removal values for EV and NV of 0.5-1.8 and 1.0-1.1 log units, respectively in a pilot scale study. However, they were able to achieve a removal of up to 3.1 and 3.8 log units for coliphage and F-specific phage, respectively. Nevertheless, in the current study we determined the average removal values for EV and NV of 5.1 and 4.1 log units, respectively. We

have shown in a full-scale system, EV and NV removal was approximately 3.9 and 3.0 log unit higher as compared to lab and bench scale systems, respectively. It is plausible that the difference between our study and Ottoson (2006), is due to the fact that we sampled an average 69 L (maximum, 91 L) for the membrane influent and 428 L (maximum, 696 L) for membrane effluent samples (Table 2-1), as compared to only 1 L grab samples (for both influent and effluent samples) in their study. The reduced sample volumes used in their study could have significantly underestimated the actual concentrations. Sampling higher volumes of water, especially in waters where the viral concentrations are expected to be lower has been suggested (USEPA, 2001; Sobsey et al., 1980; Polaczyk et al., 2007).

In a study by Hu (2003), a 3.5-4.4 log reduction in MS-2 coliphage was achieved during a bench-scale study using MBR and reverse osmosis. It was observed with a scanning electron microscope that gaps were present in the pores of the membranes that allowed the MS-2 coliphage to pass through. Even though it is expected to observe higher removal efficiency in a system that contains both ultrafiltration and reverse osmosis it was demonstrated by Hu (2003) that some viruses may be able to pass through. During our study a much higher log reduction was observed for both EV and NV over the eight month sampling period without the use of reverse osmosis. The MBR used at the TCWWTP is a hollow-fiber ultrafiltration membrane with a nominal pore size of 0.04 μm and absolute pore size of 0.1 μm which is similar to the pore size reported by Hu (2003) (>0.1 μm). From our results, we concluded that EV and NV concentrations are significantly reduced ranging from 1.9 – 7.4 log units (average 5.1 (0.9 std) and 3.9 (1.1 std) log units, respectively) in wastewater treatment when a MBR system is in use (Figure 2-4). However, when comparing the data for the removal between the influent and membrane effluent samples, a removal of approximately 5.0 log units was achieved for NV2 as compared to 3.1 and

3.6 for HAdV and EV, respectively (Figure 2-5). This could be attributed to the removal efficiency observed in the primary settling effluent for NV2.

Different removal efficiency by MBR treatment among viruses

It is not fully understood why HAdV was removed more efficiently as compared to both EV and NV during the membrane process. It was expected that HAdV would be removed at an increased log unit since this virus is approximately twice the size of both NV and EV. Furthermore, the concentration of HAdV in the influent was higher on average as compared to both EV and NV. It was assumed that due to the influent concentration, a higher removal might be achieved within our detection limits. Conversely, we observed a much higher removal (5.0 log units) of NV as compared to HAdV and EV (3.9 and 3.6 log units, respectively) for the entire treatment process. It is plausible that the adsorptive behavior depends on each individual virus, and differs even between each serotype (Gerba, 1984). The outer protective layer of each virus is composed of various protein polypeptides containing amino acids, upon ionization the viral capsid takes on an electrical charge. As previously stated (Gerba, 1984), depending on the virus being studied, the acquired surface charge and hydrophobicity plays a significant role on the interaction between particles. This could possibly explain why the removal of HAdV, EV and NV are not consistent. More research is needed to determine the reasons why different log removal values were observed using MBR treatment.

Association of viruses with solid particles

Sorption to organic matter and particles during secondary biological treatment reduces virus concentration before reaching the disinfection process. Virus removal and sorption within biological treatment are often dependent on several factors, including isoelectric point, hydrophobicity, temperature, pH, suspended solids, hydraulic retention time and type and strain of EV (Gerba, 1984). To date, human enteric virus sorption in full-scale wastewater treatment has not been thoroughly researched. During the current study, it was observed that EV and HAdV (Kuo et al., 2010) were detected in every influent (08/08 for each virus) and almost every membrane effluent sample (08/08 and 05/08, respectively). However, NV2 was detected in all eight influent samples but was not detected in the membrane effluent. Suggesting NV sorption to organic particles could be higher compared to the other viruses prior to entering the membrane influent. This could be explained by the surface charge resulting in a stronger attraction to the sludge particles but more research is needed to explain this observation. In four of the eight membrane influent samples, the results concluded that NV2 was below detection limit in the membrane effluent suggesting almost complete removal. In contrast, as previously reported (da Silvia et al., 2007), GI and GII were both detected in the effluent on two different samples. They concluded a MBR is not an absolute barrier for restricting virus passage. However, in the current study it was shown that NV2 was unable to pass through the membrane within our detection limit. In addition, HAdV and EV were routinely detected in both membrane influent and effluent samples (16/16 and 16/16, respectively). Since there is limited data available on the removal of NV by membrane technology, more sampling would have to be analyzed to further explain this occurrence.

Our results indicate that both HAdV and EV are associated more with the settled sludge as compared to the filtered supernatant during biological treatment for each individual sampling event. Both HAdV and EV were detected in an average 99.8% and 97.1% of the membrane influent as settled sludge (Figure 2-6). This means that <1% of HAdV and <3% of EV concentration contributes to the viral concentration in the membrane influent as supernatant for viruses that did not settle out. Interestingly, during the January sample the EV concentration in the settled sludge reduced to approximately 77% of the viral load into the membrane. This may indicate that during the winter months, EV attachment to flocs could be reduced as compared to warmer months but further study is required to confirm and explain the observation. However, it is also plausible that various concentrations of different types and strains of EV could have been present in the wastewater matrix during the winter sampling months (mainly January and February). This type of occurrence was previously observed (Gerba, 1984) where EV adsorption to natural solids appeared to be dependent on both type and specific strain. However, further analysis would have to be conducted on our samples to compare the different species of both HAdV and EV present throughout the current study. Nevertheless, it was concluded from the data that no significant difference ($p\text{-value} > 0.05$) was observed between HAdV and EV sorption concentrations over the eight month sampling period.

Conclusion

- In this study, average removal values for EV and NV were 5.1 (4.1-6.8, range) and 3.9 (3.5-4.8, range) log units, respectively by the MBR process.
- EV and NV2 were removed at approximately 3.6 (1.9-4.6, range) and 4.7 (4.6-5.1, range) log units throughout the entire treatment process.
- Average EV and NV load concentrations (viruses/L) for the entire WWTP were reduced from 5.6 to 1.9 log units and 7.7 to at least 1.3 log units, respectively.
- After the membrane influent samples were allowed to settle, 99.8% and 97.1% of HAdV and EV concentration was associated with the settled solids showing a high affinity for the suspended solids.
- No seasonal variation of EV concentration or overall removal was observed during the current study

Tables and Figures

Table 2-1. List of the eight different sampling events and the volumes sampled at each point during this study. The Mem Inf consists of two volumes (Membrane Influent as Supernatant / Membrane Influent as Activated Sludge (total volume)).

Sampling Date	Volume Sampled (L)			
	Influent	Pri-Eff	Mem In	Mem Eff
January	16	30	42 / 91	379
February	17	8	29 / 85	338
March	24	42	47 / 67	438
April #1	14	45	37 / 77	193
April #2	16	51	47 / 67	696
May	13	21	86 / 27	574
July	17	14	39 / 74	170
August	14	17	51 / 63	632

Table 2-2. Cloning primers used for the creation of the standard curves. Degenerate base code: B = C, G or T; D = A, G or T; H = A, C, or T; M = A or C; N = A, C, G or T; R = A or G; Y = C or T.

		Sequence (5' to 3')	Reference
EV	Forward	CCCAGTAGCACTATGAAAGTTGCGAG	Dierseen et al., 2007
	Reverse	GGCTAAGTGGTATAAATCCAACAAAGAGGT	
NV1	Forward 1	ATHGAACGYCAAATYTTCTGGAC	da Silva et al., 2007
	Forward 2	ATHGAAAGACAAATCTACTGGAC	
	Forward 3	ATHGARAGRCARCTNTGGTGGAC	
	Reverse	CCAACCCARCCATTRTACA	
NV2	Forward 1	GGHCCMBMDTTYTACAGCAA	Kageyama et al., 2003
	Forward 2	GGHCCMBMDTTYTACAAGAA	
	Forward 3	GGHCCMBMDTTYTACARNAA	
	Reverse	CCRCCNGCATRHCCRTRTACAT	

Table 2-3. Primers, probes and real-time PCR conditions used for the standard curves and molecular detection assays

<i>Virus Type</i>	<i>Gene Region</i>	<i>Primers/Probes</i>	<i>Sequence (5' to 3')</i>	<i>Reaction Condition (temp, time)</i>	<i>Reference</i>
HAdV40/41	Hexon	Forward	ACCCACGATGTAACCACAGAC	95, 10s -denaturation	Xagorarakis et al., 2007 Modified from Jiang et al., 2005
		Reverse-1	ACTTTGTAAGAGTAGGCGGTTTC	60, 30s -annealing	
		Reverse-2	CACTTTGTAAGAATAAGCGGTGTC	72, 12s -extension	
		Probe	CGACKGGCAGCAAKCGCAGCGT		
HEV	5' - Untranscribed Region	Forward	ACATGGTGTGAAGAGTCTATTGAGCT	95, 15s -denaturation	Dierseen et al., 2007
		Reverse	CCAAAGTAGTCGGTTCCGC	60, 60s -annealing	
		Probe	TCCGGCCCCTGAATGCGGCTAAT		
NV (G1)	5' - Untranscribed Region	Forward	CGCTGGATGCGNTTCCAT	95, 15s -denaturation	da Silva et al., 2007
		Reverse	CCTTAGACGCCATCATCATTTAC	60, 60s -annealing	
		Probe	TGGACAGGAGAYCGCRATCT		
NV (G2)	-----	Forward	CARGARBCNATGTTYAGRTGGATGAG	95, 15s -denaturation	Kageyama et al., 2003
		Reverse	TCGACGCCATCTTCATTCACA	56, 60s -annealing	
		Probe	TGGGAGGGCGATCGCAATCT		

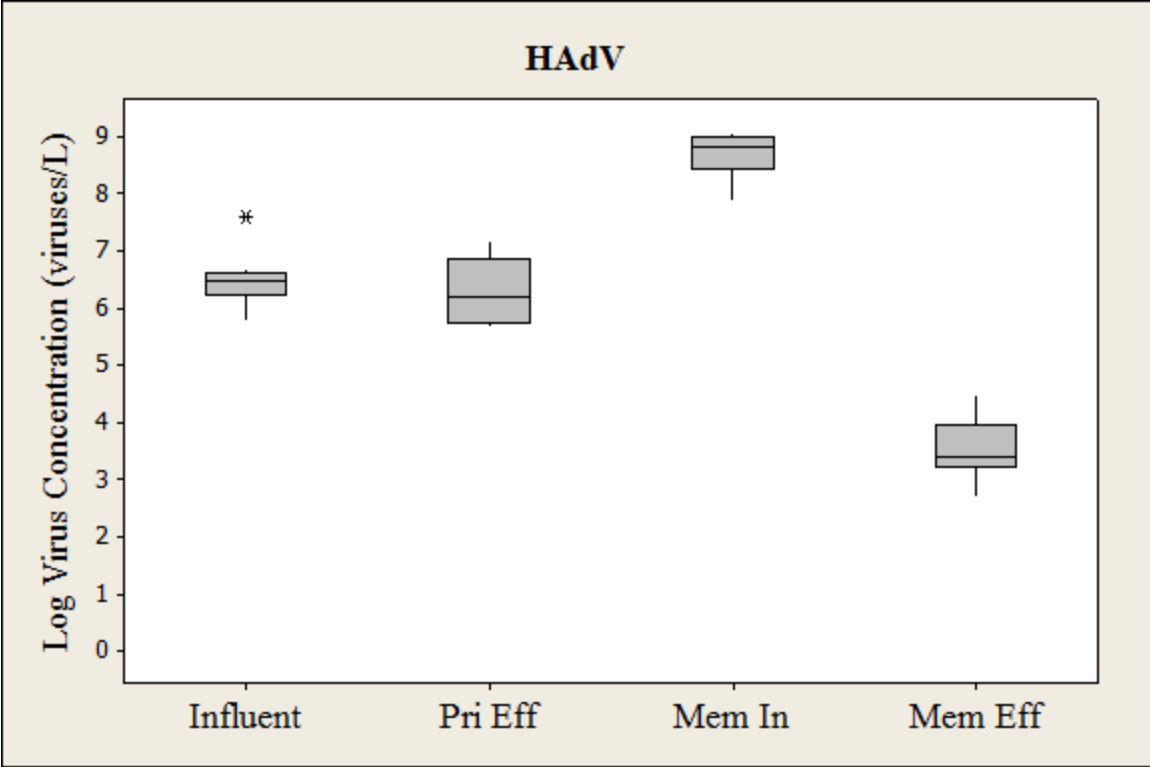


Figure 2-1. Overall HAdV distribution within the Influent (n=8), Pri Eff - Primary Effluent (n=8), Mem In - Membrane Influent (n=8) and Mem Eff - Membrane Effluent (n=8) for all eight sampling events.

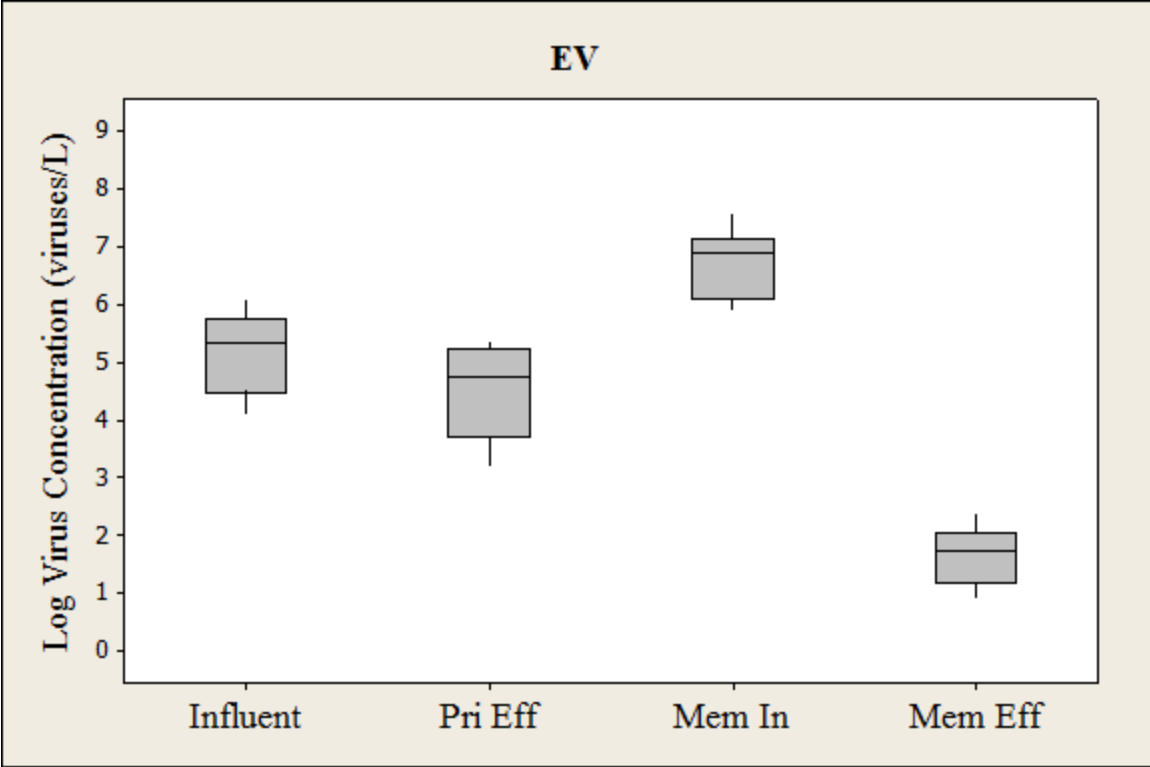


Figure 2-2. Overall EV distribution within the Influent (n=8), Pri Eff - Primary Effluent (n=8), Mem In - Membrane Influent (n=8) and Mem Eff - Membrane Effluent (n=8) for all eight sampling events.

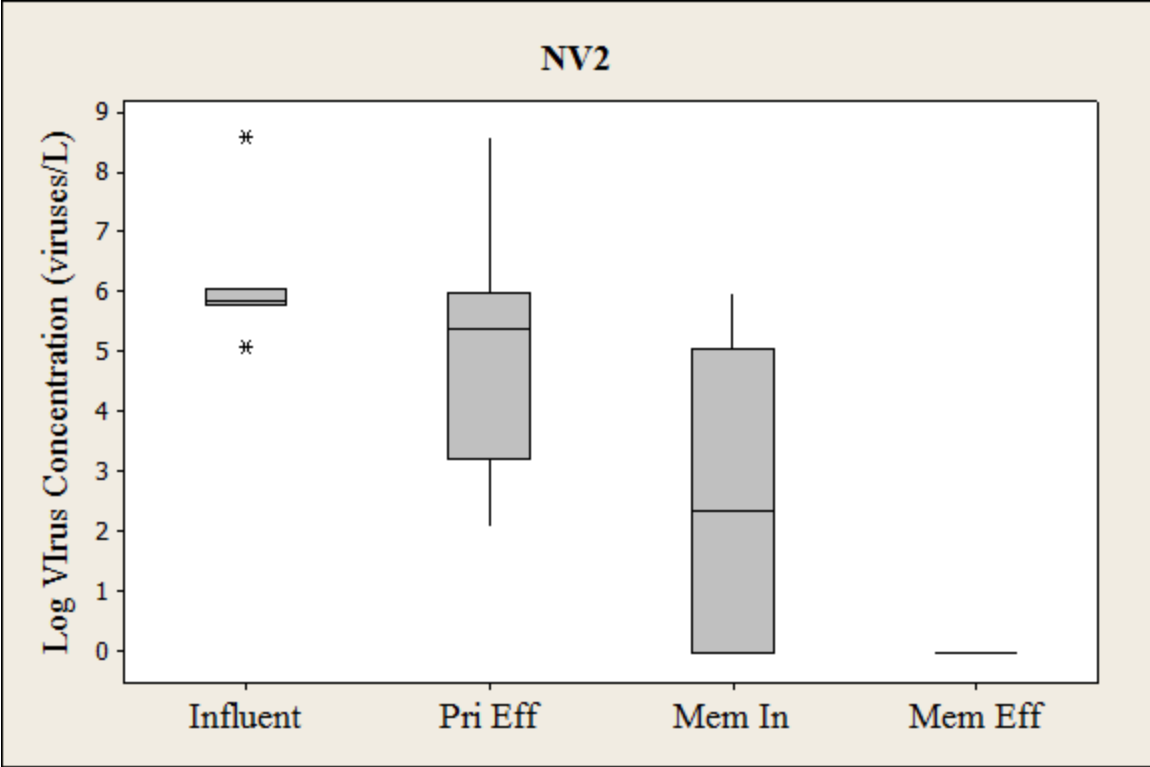


Figure 2-3. Overall NV2 distribution within the Influent (n=8), Pri Eff - Primary Effluent (n=8), Mem In - Membrane Influent (n=8) and Mem Eff - Membrane Effluent (n=8) for all eight sampling events.

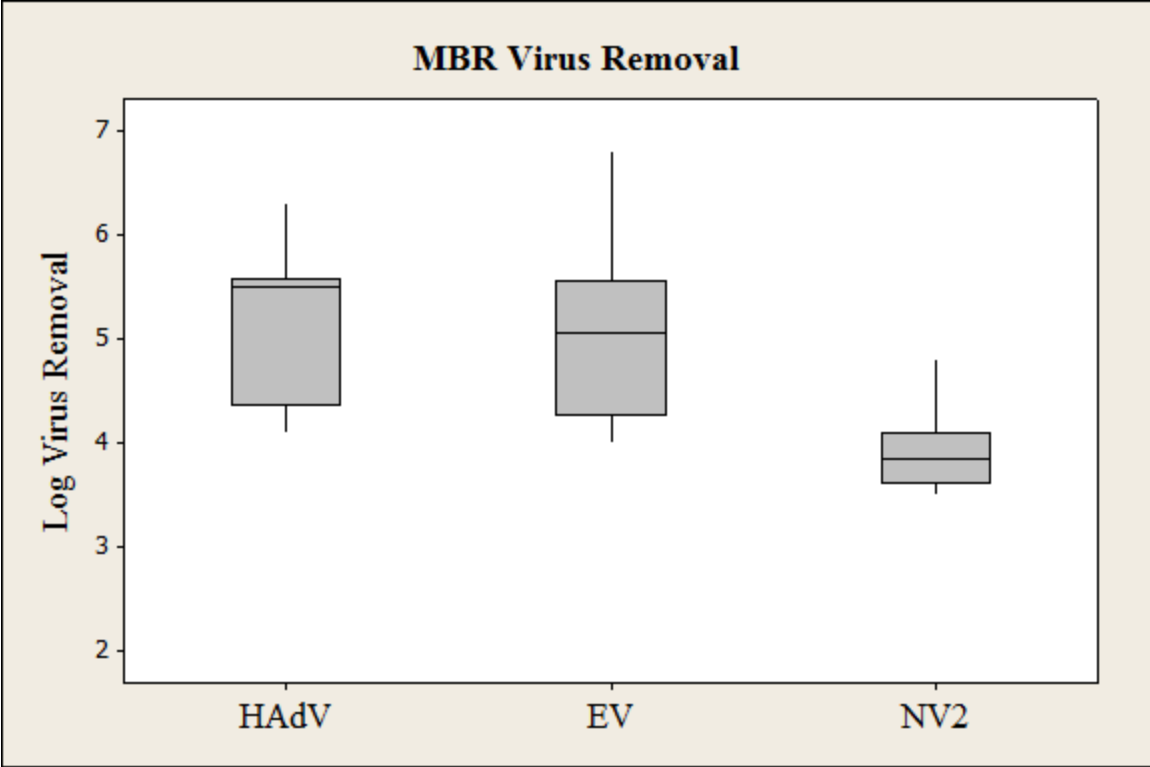


Figure 2-4. Virus removal by the MBR (membrane influent and effluent). HAdV (n=8), EV (n=8) and NV2 (n=8).

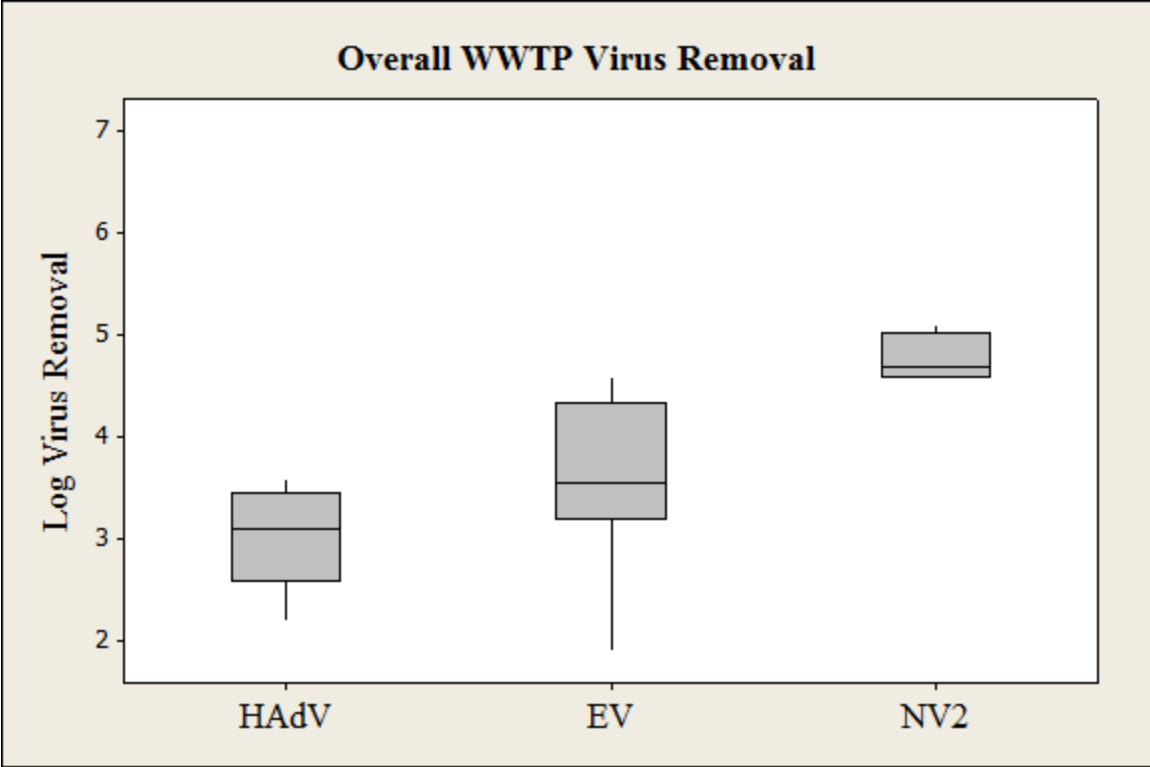


Figure 2-5. Virus removal between the influent and MBR effluent. HAdV (n=8), EV (n=8) and NV2 (n=8).

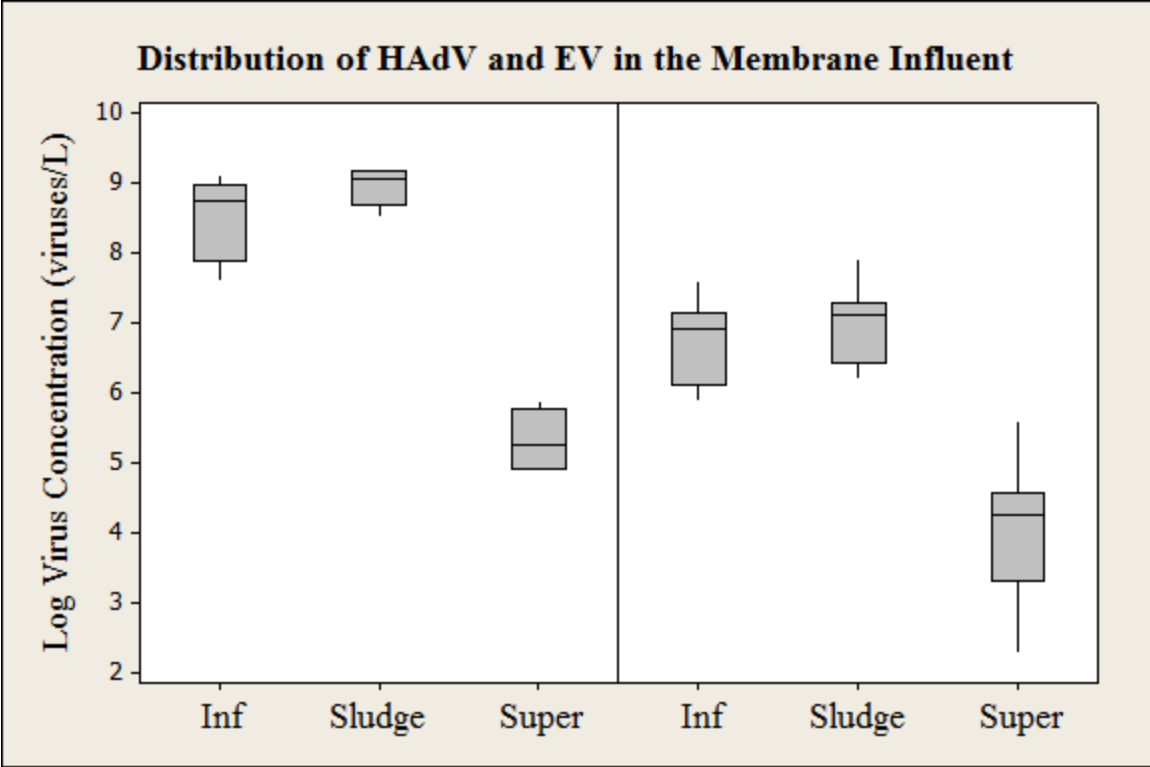


Figure 2-6. Overall distribution of HAdV (left side) and EV (right side) for the entire eight month sampling period. Inf-Membrane Influent (n=8), Sludge - Settled Sludge (n=8) and Super - Filtered Supernatant (n=8) for each HAdV and EV.

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CHAPTER 3

RELEASE OF INFECTIOUS HUMAN ENTERIC VIRUSES BY FULL-SCALE WASTEWATER UTILITIES

Abstract

Since no regulations regarding the release of viruses after wastewater treatment are presently in-place in the United States, infectious human enteric viruses are introduced daily into the environment through the discharge of treated water and the digested sludge (biosolids). In this study, a total of 30 wastewater and 6 biosolids samples were analyzed over five different months (May-September 2008-2009) from five separate full-scale wastewater treatment plants (WWTPs) in Michigan using real-time PCR and cell culture assays. Samples were collected from four different locations at each WWTP (influent, pre-disinfection, post-disinfection and biosolids) using the 1MDS electropositive cartridge filter. Adenovirus (HAdV), enterovirus (EV) and norovirus genogroup II (NV2) were detected in 100%, 67% and 10%, respectively of the wastewater samples using real-time PCR. Cytopathic effect (CPE) was present in 100% of the cell culture samples for influent, pre and post disinfection and biosolids with an average concentration of 2.2×10^4 , 5.9×10^1 , 6.2×10^0 and 2.9×10^7 MPN/100L, respectively. A significant log reduction (2.3 - 4.5) in infectious viruses throughout the wastewater treatment process was observed. Based on real-time PCR data, MBR treatment was able to achieve an additional 2 and 0.5 log reduction of HAdV and EV, respectively. A significant difference (p -value < 0.05) was observed for HAdV but not for EV (p -value > 0.05) removal in MBR as compared to conventional treatment. This study has demonstrated the release of infectious enteric viruses in the final effluent and biosolids of wastewater treatment into the environment.

Introduction

Human enteric viruses are currently listed on the United States Environmental Protection Agency Contaminant Candidate List (USEPA CCL) as emerging contaminants. To this date, no regulations have been implemented into the monitoring of wastewater viral concentration before being discharged into a natural water body. Human Adenovirus (HAdV), Human Enterovirus (EV), Norovirus genogroups 1, 2 and 4, (NV1) and (NV2), and Hepatitis-A (HAV) are some of the enteric viruses of concern (Gerba et al., 2002; Haramoto et al., 2007; Kittigul et al., 2006). These viruses have been related to several waterborne diseases, such as acute gastroenteritis, conjunctivitis and respiratory illness in both developed and developing countries world-wide. There are several routes whereby the public can become infected, including direct contact (fecal-oral route or dermal contact) and food borne illness and contamination (Godfree et al., 2005). Furthermore, human norovirus emerged as the leading cause of non-bacterial gastroenteritis world-wide, accounting for 94% of confirmed cases (Gerba et al., 2002).

A large number of enteric viruses are excreted in human feces and urine, which makes wastewater one of the most concentrated sources of these viruses. During the peak of an infection, it has been reported that enteric viruses are often detected in feces at elevated levels averaging 10^{11} viruses/gram (Rose et al., 1996). Often, hospital wastewater is sent directly to the WWTPs without any pre-treatment, which increases the possibility of elevating levels of pathogenic viruses to be removed. Due to their low infectious dose, survival in environmental waters and their detrimental effect on human health, enteric viruses pose a considerable threat to the public (Castignolles et al., 1998; Xagorarakis et al., 2007). Excreted enteric viruses are continually being detected in wastewater, rivers, recreational water, sea water, groundwater and

municipal drinking water (Borchardt et al., 2003; Chapron et al., 2000; Chen et al., 2008; De Paula et al., 2007; Gersberg et al., 2006; Haramoto et al., 2009).

Virus removal from wastewater continues to receive attention due to the epidemiological significance of viruses as waterborne pathogens and because of the high diversity that is excreted in human waste (Rose et al., 1996). According to the literature, the concentrations of active and inactive HAdV, EV and NV detected in untreated and treated wastewater are approximately $10^4 - 10^9$ and $10^2 - 10^7$ viruses/L, respectively (Bofill-Mas et al., 2006; Carducci et al., 2008; Fong et al., 2010; He et al., 2005, Kuo et al., 2010; Laverick et al., 2004; Rodriguez et al., 2008). To ensure the safety of the public, inactivation of viruses is usually achieved through certain disinfection process before being discharged, often chlorine and ultraviolet (UV) treatment. Previous bench-scale studies (Rodriguez et al., 2008; Simonet et al., 2006) have determined the inactivation of viruses from the influent and final effluent of approximately 1-4 log units and 0.1 – 1.2 log units for the chlorination and UV disinfection unit processes alone.

In addition to the final effluent, a considerable amount of sludge is generated in primary and secondary settling tanks during the treatment process. Secondary biological treatment processes use active biomass to reduce contaminants in the incoming water. As a result of the biological process, secondary sludge is generated and then treated by mesophilic anaerobic digestion (MAD), lime stabilization, composting or simply dewatering. Depending on the level of treatment, biosolids are often considered class A or B and depending on the particular crop and its intended use, class A or class B biosolids are applied on land. In the US, approximately 5 million tons of dry biosolids is generated annually and 60% is used for agricultural land application to provide additional nutrients for crops. Class B biosolids are the most commonly

produced in the United States by MAD (Gerba et al., 2002; Viau et al., 2009). It has been stated that a variable fraction, as high as 50% of the enteric virus present in the raw sewage, may be associated with the solids (Payment et al., 1986), suggesting that the concentration of viruses in biosolids can be higher than in wastewater. Several studies have reported the occurrence of enteric viruses in biosolids after the digestion process (Chapron et al., 2000; Monpoeho et al., 2004; Bofill-Mas et al., 2006; Gallagher et al., 2007; Guzman et al., 2007; Viau and Peccia 2008; Wong et al., 2010).

Past studies (Bofill-Mas et al., 2006; Katayama et al., 2008; Villar et al., 2007, Rodriguez et al., 2008; Laverick et al 2004; Carducci et al., 2008; da Silvia et al., 2007) have determined the concentration of DNA/RNA viruses using real-time PCR in addition to viral infectivity in wastewater treatment (Aulicino et al., 1995; Gantzer et al., 1998; Petrinca et al., 2009, Rodriguez et al 2008; Sedmak et al., 2005). However to our knowledge, no studies have looked at the overall release of enteric viruses from a full-scale wastewater treatment through the final effluent and biosolids using both real-time PCR and cell culture methods. As a result, it is unclear whether exposure to final effluent or biosolids poses a greater risk of contamination to the public. In the current study, we analyzed a total of 30 wastewater and 6 biosolids samples over five different months (May-September) from five separate full-scale WWTPs to determine the release of human enteric viruses into the environment. The results in this study provide important information on the overall release of both infectious and non-infectious enteric viruses into the environment after treatment.

The objectives of this study were to (i) determine the concentration of human enteric viruses within the wastewater treatment process using real-time PCR data (ii) determine the release of infectious viruses found in the final effluent and biosolids samples, (iii) compare virus removal

efficiency between a MBR and conventional wastewater treatment process using real-time PCR data and (iv) compare the effectiveness of two different disinfection processes for virus inactivation. Four different sampling points (raw, pre-disinfection, post-disinfection and biosolids) were chosen to determine how viruses are removed and inactivated during treatment. The main viruses in this study are Human Adenovirus F40 and F41 (HAdV), Human Enterovirus (EV), Norovirus Genogroup 1 (NV1), Norovirus Genogroup 2 (NV2) and Hepatitis A (HAV).

Methods and Materials

Wastewater Treatment Plants

Five different wastewater treatment plants (WWTPs) in Michigan's Lower Peninsula were sampled from 07/17/2008 – 09/24/2009 in duplicate during separate sampling events. Four different locations were sampled from, including the influent (raw sewage), pre-disinfection (after secondary biological treatment), post-disinfection (final effluent) and biosolids. Table 3-1 lists the characteristics of each WWTP that was sampled from during this study. The WWTPs used in this study had different configurations of biological unit processes and disinfection methods. The 5 WWTPs varied with their biological treatment processes, which consists of a standard activated-sludge, activated-sludge with a membrane bioreactor, oxidative ditch and rotating biological contactors and using chlorination (Cl) and ultraviolet (UV) disinfection methods. All the WWTPs in this study had different average daily inflows.

Wastewater and biosolids sampling

The Zeta Plus 1MDS used in this study is an electropositive 10 inch (25.4cm) cartridge filter made of charged modified glass and cellulose that contains approximately 4.2 ft² (39cm²) of media. 30 wastewater samples were collected during 10 different sampling events onsite following the procedure explained in the USEPA Manual of Methods for Virology (USEPA 2001). Approximately 20 L of influent, 375 L of pre-disinfection and 410 L of post-disinfection (final effluent) were sampled at a rate of about 11-12 L/min (3 gal/min). Biosolids samples were collected at three of the five different WWTPs (Table 3-1). 2 L grab samples of each of the anaerobically digested samples were collected from the post digestion holding tanks and then transferred or shipped on ice overnight. The dewatered samples were collected from the exiting conveyor belt in the loading bay. All samples collected were then stored on ice and transported to the Water Quality Engineering Laboratory at Michigan State University. Upon arrival, samples were placed in a 4°C cooler before processing.

Virus elution process for filters and biosolids

All wastewater samples collected were eluted 12-24 hours after initial sampling according to the Concentration and Processing of Waterborne Viruses by Positive Charge 1MDS Cartridge Filters and Organic Flocculation (USEPA 2001). The biosolids virus elution and concentration were performed according to the ASTM-4994. Briefly, 10% beef extract was added to 25g biosolids and stirred for 30 minutes to elute the viruses. The solids were then spun down by centrifugation and the supernatant was kept for further concentration. The supernatant was flocculated by adjusting the pH to 3.5 and spun again to form a pellet. The pellet was then dissolved in

phosphate buffer saline (PBS) and 0.22- μ m filtered. The final eluents of both wastewater and biosolids were kept in a -80°C freezer for further analysis.

Nucleic acid extraction

Following the viral elution process, eluent from each filter and biosolids samples were extracted. Viral samples were extracted using the MagNa Pure Compact System automatic machine (Roche Applied Sciences, Indianapolis, IN). The extraction kits used were the MagNA Pure Compact Nucleic Acid Isolation Kit-Large Volume. The program used required 1000 μ L of sample to be extracted and concentrated for a final volume of 100 μ L. Immediately following the completion of the extraction all samples were placed in a -80°C freezer to preserve the integrity of the RNA molecule. Following extraction the quantity of viral nucleic acid extracts from all samples were checked using the NanoDrop Spectrophotometer (NanoDrop[®] ND-1000, Wilmington, DE).

Real-time PCR standard curves, sequencing and detection limit

The standard curves for sample quantification of HAdV, EV, NV1, NV2 and HAV were created using stock cultures of HAdV 40 (VR-930), EV Coxsackie virus B5 (ATCC VR-1036AS/MK), HAV HM175 (ATCC VR-1402) and NV2 stool samples were supplied by the Ingham County Health Department following a confirmed outbreak at Michigan State University. All standard curve assays performed used the LightCycler[®] 1.5 Instrument (Roche Applied Sciences, Indianapolis, IN). Briefly, the PCR amplicons from HAdV, EV, NV2 and HAV from pure culture and stool sample extracts were cloned into a plasmid vector (i.e., pCR[®] 4-TOPO[®]) which follows the one-shot chemical transformation described in the manufacturer instructions (TOPO

TA Cloning[®] Kit for Sequencing, Invitrogen, Carlsbad, CA). The plasmids carrying the cloned HAdV, EV, NV1, NV2 and HAV were purified using Wizard[®] Plus SV Minipreps DNA Purification System (Promega, Madison, WI) and sent for sequencing at the Research Technology Support Facility at Michigan State University. All target gene sequences were compared with those published in the National Center for Biotechnology Information (NCBI) database by using the program of Basic Local Alignment Search Tool (BLAST). The concentrations were determined by using the NanoDrop spectrophotometer and then the samples were serially diluted 10-fold ($10^1 - 10^8$ viruses/reaction) and used for creating the standard curves for all target viruses. A two-step (reverse transcription and then real-time PCR separately) approach standard curve was generated for EV, NV1, NV2 and HAV. The R-squared values for each standard curve for HAdV, EV, NV1, NV2 and HAV are 0.995, 0.996, 0.995, 0.999 and 0.996, respectively. All standard curve reactions were run in triplicate and the detection limit for EV and NV2, is 10 viruses/reaction and 100 viruses/reaction for HAdV, HAV and NV1.

Quantitative real-time PCR assays

The crossing point (Cp) value for each PCR reaction was automatically determined by the LightCycler[®] Software 4.0 and used to calculate the overall viral concentration. The primer and probe sequences and reaction conditions used in this study are summarized in Table 3-2. Briefly, all real-time PCR reaction mixes included 10 μ L of 2X LightCycler 480 TaqMan Master Mix and the appropriate volume of primers and probes for each assay used (HAdV, EV, NV1, NV2 and HAV) are as previously described (Dierssen et al., 2007; Jothikumar et al., 2005; Kageyama et al., 2003; da Silva et al., 2007; Xagorarakis et al., 2007). The real-time PCR running program

(all thermocycles were performed at a temperature transition rate of 20°C/s) was 95°C for 15 min; followed by different cycles of denaturation, annealing, extension and a final cooling step.

Each reverse transcription reaction mix for EV, NV and HAV included 2.5 µL of 10 µM reverse primer, 1 µL of reverse transcriptase (Promega Corporation, Madison, WI), 4 µL of 5X transcriptase reaction buffer, 20U of protector Rnase inhibitor, and 2 µL of 10 mM deoxynucleotide (Roche Applied Sciences, Indianapolis, IN). Reaction conditions for all three RNA viruses were the same; initial incubation at 55°C for 30 minutes followed by 85°C for 5 minutes to inactivate the enzyme. All samples were run in triplicate and included a negative control reaction (PCR-grade H₂O without template) and a positive control reaction for all viruses.

Inhibition control

To determine if inhibition occurred during viral analysis, the methods previously explained (Viau et al., 2009; Rajal et al., 2007) were used. Bovine Enterovirus (BEV) was chosen as the virus to spike all samples to determine if inhibition was present, BEV was quantified following the methods previously published (Jimenez-Clavero et al., 2005). Prior to the inhibition check, all samples were initially analyzed for BEV using real-time PCR. Next, all extracted samples and molecular grade H₂O were spiked with a final concentration of 10⁵ viruses/rxn of BEV.

Following the analysis, the Cp values of the extracted water and wastewater samples were compared to see if inhibition was present in the samples. If the Cp values of both the spiked water and wastewater samples were within an acceptable level (5%), then inhibition did not affect our analysis.

Calculations of enteric virus concentration.

All real-time PCR assays were converted from viruses/reaction to viruses/L, using the following equations:

$$\frac{\text{Viruses}}{L} = \frac{\frac{\text{Viruses}}{\text{rxn}} \times \frac{1 \text{ rxn}}{5 \mu\text{L}} \times 100\mu\text{L} \times \frac{1}{1000\mu\text{L}} \times 30,000\mu\text{L}}{\text{Volume}} \quad (3 - 1)$$

$$\frac{\text{Viruses}}{\text{dry gram}} = \frac{\frac{\text{Viruses}}{\text{rxn}} \times \frac{1 \text{ rxn}}{5 \mu\text{L}} \times 100\mu\text{L} \times \frac{1}{25 \text{ grams}}}{\% \text{ Solids}} \quad (3 - 2)$$

The 5 μL is the amount of sample per reaction tube, the 1000 and 100 μL is the amount of sample extracted and the volume of the extract, respectively. The 30,000 μL is the amount of concentrated eluent after the final filtration through a 0.22 μm syringe filter (Millipore, Billerica, MA) and dividing by the volume of water sampled. In equation 3-2, the 25 grams was the weight of biosolids that were concentrated.

Biosolids Conversion

To express the biosolids concentration volumetrically, the conversion from viruses/dry gram to viruses/L is as follows:

$$\% \text{ solids} = \frac{\text{dry grams}}{100 \text{ ml}}$$
$$\frac{\text{Viruses}}{L} = \frac{\text{Viruses}}{\text{dry grams}} \times \frac{\text{dry grams}}{100 \text{ ml}} \times \frac{10}{10} \quad (3 - 3)$$

Cell culture

In total 15 different wastewater (from all-sampling points (influent, pre and post-disinfection)) and 3 different biosolids samples were analyzed for virus infectivity and were cultured using BGM cell line (passage #157). In addition, A549 cell line (passage #126) was used for the post-disinfection samples to determine the final active virus concentration being released. A total of 200 BGM and 50 A549 flasks were used for the final analysis. All samples followed the USEPA Total Culturable Virus Assay detailed in the “Information Collection Rule”. Positive and negative flasks were inoculated to act as controls to ensure the quality of the cells. Cytopathic effect (CPE) of each flask was then recorded and the mean viral concentrations of the samples were estimated by the free most-probable-number (MPN) software downloaded from (<http://www.i2workout.com/mcuriale/mpn/index.html>). The results for the BGM cell lines are expressed as MPN/100L. The confirmation flasks were then stored at -80°C for ICC-PCR assay.

Log Removal

Overall log removal achieved by the MBR and conventional WWTPs was calculated using equation 3:

$$\text{Log Removal} = \log_{10} \left(\frac{\text{Influent Concentration}}{\text{Effluent Concentration}} \right) \quad (3 - 4)$$

For pre and post-secondary treatment samples that were below the detection limit, the log removal values were calculated by using the detection limit of the individual assays. This indicates that certain removal values may be greater than reported. However, this will allow for conservative calculations when this value is needed for comparisons.

Statistical analysis

Log removal values for each WWTP were analyzed using t-test in Microsoft Excel ® using an alpha value (α -value of 0.05), showing a 95% confidence interval.

Results

Inhibition control

BEV was not initially detected in the 30 wastewater (00/30) and 6 biosolids (00/06) samples. All 36 samples were then spiked with 10^5 viruses/rxn of BEV following extraction including a PCR grade- H_2O . The Cp values for the WWTPs and biosolids samples had an average Cp value 26.57 (std 0.14) and the H_2O samples had an average Cp value 26.67 (std 0.08) were within 2% of each other. This indicates that any inhibition that may be present in the extracted samples was not able to suppress the detection of the viruses in this study. This was concluded based on the average Cp values for all samples spiked with BEV and a *p-value* > 0.05.

Quantification of human enteric viruses in WWTPs

HAdV - 10 different sampling events were analyzed at each sampling point (influent, pre- and post-disinfection). Figure 3-1 shows the average concentration of *HAdV* from the two different sampling events at each WWTP. *HAdV* was detected in all 30 samples, 10/10 influent and 20/20 for both the pre- and post-disinfection samples from all 5 WWTPs. WWTP 1 had the highest levels detected in all plants sampled. The concentration in the influent, pre and post-disinfections samples were between $2.2 \times 10^7 - 6.7 \times 10^8$ (average 3.5×10^8), $3.6 \times 10^2 - 1.5 \times 10^4$ (average

7.6×10^3) and $1.8 \times 10^2 - 2 \times 10^3$ (average 1.1×10^3) genomic equivalent copies/L (viruses/L), respectively. WWTPs 2, 3 and 5 showed similar results of approximately $1.0 \times 10^5 - 5.0 \times 10^6$ (average 1.3×10^6), $4.5 \times 10^2 - 4.5 \times 10^4$ (average 1.2×10^4) and $1.1 \times 10^1 - 2.3 \times 10^3$ (average 5.8×10^3) viruses/L for influent, pre-disinfection and post-disinfection, respectively. For each sample event, WWTP 4 had the lowest average concentration of approximately 8.3×10^4 , 2.6×10^2 and 1.0×10^2 viruses/L in the influent, pre and post-disinfection, respectively. It is possible that the lower concentration could be due to the average daily flow into the plant, which receives the lowest of all 5 plants sampled (Table 3-1). WWTP 4 is located within a rural city; where most residential homes use individual septic tanks instead of the city sewer system.

EV - 10 different sampling events were analyzed for *EV* at each sampling point. Figure 3-2 shows the average concentration for the two sampling events at each WWTP. In total 10/10 influent, 04/10 pre-disinfection and 06/10 post-disinfection samples were positive for *EV*. The influent *EV* concentrations ranged from $3.0 \times 10^2 - 1.1 \times 10^6$ viruses/L (average 2.1×10^5 viruses/L). The highest and lowest concentrations were observed in WWTP 1 and 5, respectively, but WWTPs 2, 3, and 4 had similar concentrations ranging between $10^4 - 10^5$ viruses/L. The concentration detected in the pre-disinfection samples ranged from below detection limit (BDL) - 2.6×10^3 viruses/L, with an average of 2.9×10^2 viruses/L. However, *EV* was detected in 06/10 post-disinfection (compared to 04/10 in pre-disinfection) samples with concentrations between BDL - 9.4×10^2 viruses/L and an average of 1.6×10^2 viruses/L. Some of the samples that were positive for *EV* at the pre and post disinfection points fell within the detection range.

NV1 and NV2 - NV1 was not detected in the 5 WWTPs sampled (00/30). However, 03/30 samples were positive for NV2. Three of ten influent samples from WWTPs 1 and 2 were positive for NV2 with concentrations ranging from 5.2×10^4 to 1.1×10^6 (average 4.3×10^5) viruses/L. However, NV2 was not detected in any of the pre or post-disinfection samples. It is possible that the activated sludge and the MBR biological processes might be able to remove NV2 below our detection limit for the assay used. Since NV2 was only detected in 3 samples it is difficult to determine the removal efficiency for each WWTP.

HAV - HAV was not detected in the 30 samples analyzed.

Quantification of human enteric viruses in biosolids

Figure 3-3 shows the comparison of the average log concentration values quantified with real-time PCR for the influent, pre and post disinfection and biosolids samples for HAdV and EV (WWTPs 1, 2 and 5). The average HAdV log concentrations are 6.3, 3.9, 3.2 and 7.4 viruses/L and EV was approximately 5.2, 2.0, 1.8 and 5.8 for the influent, pre and post-disinfection and biosolids, respectively. Neither HAdV nor EV was detected in the biosolids at WWTP 1. Since it is possible that these viruses were present at low concentrations (below our detection limit), half the detection limit value was used in the calculation (Table 3-3). Interestingly, both NV1 and NV2 were detected in the biosolids samples at all three WWTPs but were not detected in the influent, pre or post-disinfection wastewater samples. NV1 was detected in 06/06 biosolids samples at each WWTP (WWTPs 1, 2 and 5) at an average log concentration of 5.2, 5.6 and 7.3 log units, respectively. NV2 was detected in 06/06 biosolids samples at 5.2, 6.3 and 7.7 log units,

at WWTPs 1, 2 and 5 respectively. HAV was not detected in any of the biosolids samples analyzed.

Infectivity of viruses

CPE was detected in all five WWTPs ($n=15$) at each of the three sampling locations with BGM cell line, indicating the presence of infectious viruses in all types of samples. For WWTP 1, the influent, pre and post-disinfection concentration of infectious viruses are 500, 0.02 and 0.017 MPN/L, respectively. Both WWTPs 3 and 4 showed similar concentrations of 230 and 360 MPN/L for the influent, 0.13 and 0.014 MPN/L for the pre-disinfection and 0.014 MPN/L for both post-disinfection samples, respectively. Figure 4 shows the overall average log concentration of infectious viruses for all 5 WWTPs monitored in this study in the influent, pre and post disinfection samples.

Figure 3-5 shows the results for the three different WWTPs with the biosolids data showing the overall release of infectious viruses from wastewater treatment in both the final effluent and biosolids. An average infectious virus log concentration of 3.9, 1.7, 0.5 and 7.4 MPN/100L was detected in the influent, pre and post-disinfection and biosolids samples from WWTP 1, 2 and 5, respectively. Table 3-3 shows both the real-time PCR and A549 cell culture results for HAdV and EV. The ICC-PCR results for A549 are presented as presence or absence due to the difficulty with determining CPE within this particular cell line. As the table shows, HAdV was detected in the post-disinfection samples at $1.1 \times 10^1 - 2.9 \times 10^4$ viruses/L and in the biosolids from $3.8 \times 10^4 - 4.7 \times 10^9$ viruses/L. According to the data, WWTP 2 did not show CPE for either HAdV or EV for A549, but CPE was detected with BGM. This could have occurred due to the low concentration detected in BGM (0.53 MPN/L).

Removal of infectious viruses

From the cell culture results, it was determined that an overall removal of infectious viruses from all WWTPs for the influent and pre-disinfection (after biological treatment) was approximately 4.4, 1.2, 3.2, 4.5 and 1.6 log units for WWTPs 1, 2, 3, 4 and 5, respectively. It was observed that WWTPs 1, 3 and 4 which use MBR, activated sludge and an oxidative ditch, respectively in addition to UV disinfection were able to achieve comparable removal values of 4.4, 3.2 and 4.5 log units, respectively. In addition, WWTPs 2 (activated sludge) and 5 (RBCs) use Cl disinfection which had comparable removal values of 1.2 and 1.6 log units for infectious viruses. More sampling events would have to be conducted to further analyze the differences in overall removal of infectious viruses after biological treatment.

Inactivation of infectious viruses using UV and Cl

In total 5 WWTPs were sampled during this study, WWTPs 1, 3 and 4 use UV and WWTPs 2 and 5 use Cl for disinfection prior to discharge. According to Figure 3-6, the average log infectious virus concentration for WWTPs 1, 3 and 4 for the pre and post disinfection samples was approximately 0.28 and 0.15 log units and for WWTPs 2 and 5, 2.02 and 1.06 log units. It was observed that the overall average inactivation of infectious viruses for UV and Cl was about 0.13 and 0.96 log units.

MBR and conventional WWTP

In total, 8 samples from the TCWWTP (MBR treatment) (Kuo et al., 2010) and 8 samples from the current study (conventional treatment) were used to compare the log removal values for both

HAdV and EV using real-time PCR data (due to insufficient data, NV was not included in the comparison). An average influent concentration of HAdV at 6.3 (5.5 - 6.4 range, with an outlier at 7.9) and 5.6 (4.3 - 6.7 range) log units was achieved in MBR and conventional treatment, respectively. However, the effluent concentration for each plant (MBR and conventional treatment) was 2.2 and 3.5, resulting in an overall removal of 4.1 and 2.2 log units, respectively (Figure 3-7). Therefore, indicating that MBR treatment was able to achieve an extra 2 log reduction as compared to conventional treatment. There was a significant difference ($p\text{-value} < 0.05$) between the two types of secondary treatments log removal values.

EV was detected at an average 5.4 (range 4.1-6.1) and 4.8 (range 4.5-5.5) log units for the influent and 1.7 and 1.5 log units for the post-secondary treatment for the MBR and conventional treatment, respectively. According to Figure 3-7, the MBR process achieved an average 3.6 log reduction (range 1.9-4.6). However, conventional treatment achieved an average 2.9 log reduction and the range of removal was 1.3 - 4.3 log units. EV only achieved a removal of 3.6 and 2.9 log units for MBR and conventional treatment, respectively. The t-test results for EV, showed a $p\text{-value}$ of 0.08 indicating no significant difference. However, since the $p\text{-value}$ was close to 0.05, more samples would have to be analyzed to determine if no significant difference continues to exist for EV removal in MBR and conventional wastewater treatment.

Discussion

Release of viruses by WWTPs

The main objective of wastewater treatment is to minimize human risk of exposure to chemical and biological contaminants in the environment through the final effluent and biosolids. To this

date there are no requirements on the level of human enteric viruses that are allowed to be released after wastewater treatment. The results of this study provide conclusive evidence of the levels of infectious viruses, in addition to HAdV, EV and NV total genomic loads that are being released in the effluent by five different WWTPs and biosolids in three WWTPs using real-time PCR and cell culture.

During the current study, the presence of HAdV was detected in 100% of the samples analyzed with real-time PCR. These results are consistent with past studies (Bofill-Mas et al., 2006; Carducci et al 2008; Katayama et al., 2008; Pusch et al., 2005; Rodriguez et al., 2009) who have reported the presence of HAdV between 55 – 100% of samples with an average of 88%. In addition to HAdV, EV was detected between 65 – 89% with an average of 76% of past studies (Pusch et al., 2005; Rodriguez et al., 2008; Katayama et al., 2008) compared to 67% in this study. Furthermore, we observed a range in EV concentration of $10^2 - 10^6$ and $10^1 - 10^4$ viruses/L in the influent and final effluent in 20/30 samples analyzed, respectively.

The presence of NV was detected in an average of 72% in past studies (Kitajima et al., 2009; Laverick et al., 2004; Nordgren et al., 2009; da Silva et al., 2009) but was only detected in 10% of our samples (NV1 was not detected). Here, NV2 was only detected in 03/10 influent ($10^4 - 10^6$ viruses/L) and 00/10 effluent samples. It was assumed that almost complete removal of NV2 was observed in our samples. Interestingly, NV2 was detected in the July and August samples for WWTP 1 and 2 at an average concentration of 4.3×10^5 viruses/L. This might suggest that during our sampling events, NV was not as prominent as observed in other studies but NV2 was present in three different samples during the summer months. However, NV1 was not detected in the summer sampling months but NV2 was detected approximately 10^6 viruses/L, concluding no seasonal variation among genogroups was observed.

Based on our cell culture data we were able to determine that an average concentration of 2.0×10^4 MPN/100L enter (raw sewage) the three WWTPs (WWTPs 1, 2 and 5), 1.6×10^1 MPN/100L are discharged as final effluent and 6.0×10^8 MPN/100L are retained in the biosolids. It is assumed that there is some virus removal in primary sedimentation (0.1 – 1.0 log units) as previously reported (Nordgren et al., 2009); however samples were not collected at this particular location. We observed an overall removal of infectious viruses between 2.3 – 4.5 (average 3.6) log units from influent to final effluent. These results are comparable to previous full-scale studies (Aulicino et al., 1995; Sedmak et al., 2005; Petrinca et al 2009) using cell culture assay reporting removals between 0 – 4.0 log units.

CPE was detected in 100% of our influent and effluent samples as compared to past studies (Aulicino et al., 1995; Rodriguez et al., 2008; Sedmak et al., 2005) averaging 88% and 45% CPE in the influent and effluent samples, respectively. Furthermore, the reported concentration of infectious viruses in the above studies fluctuated between 1-4 and 0-3 log units in the influent and final effluent, respectively. This shows inconsistencies with determining the concentration of infectious viruses before and after treatment. Proper monitoring of infectious viruses is dependent on consistent detection to ensure safe levels of viral activity. In the current study, the influent (2.8 – 4.8 log units) and effluent (0.1 – 1.6 log units) concentrations only fluctuated by 2 and 1.5 log units, respectively. It is plausible that the low sample volumes used in the above studies often ranged from 0.1-5 and 1-20 L grab samples for the influent and final effluent, respectively. In the current situation, a significant increased volume (20 L, 375 L and 410 L of influent, pre-disinfection and post disinfection, respectively) was sampled at each WWTP. It has been suggested that for adequate virus quantification, sample volumes of 2-20 and

300-2000 L of raw sewage and treated waters should be collected. This will increase the chance of virus recovery from source waters with a low concentration of viruses (Sobsey et al., 1980).

In total 6 different biosolids samples were analyzed for both infectious and non-infectious enteric viruses from three different WWTPs (Table 3-1). The average real-time PCR concentration for HAdV and EV was approximately $10^7 - 10^8$ viruses/L. In addition, we detected an average 3.4 log units of infectious viruses in our 6 samples as compared to previously published (Guzman et al., 2007; Monpoeho et al., 2004) values of 0.4 – 1.6 log units. The differences in concentrations found in this study could be the various detention times for the biosolids between WWTPs. Depending on the duration of time the biosolids are held and allowed to accumulate additional infectious viruses could account for the approximate 2 log increase.

Interestingly, NV1 and NV2 were both detected in 06/06 biosolids samples at an average concentration of 6.5×10^6 and 6.3×10^7 viruses/L, respectively but NV1 was not detected in any of the wastewater samples and NV2 was only detected in 03/30 samples. This could also be due the longer retention times as compared to the instantaneous sampling events taken at each WWTP. In addition, WWTP 2 may hold the sludge for an extended period of time before dewatering as compared to the anaerobic digestion process. It was assumed that NV was present in the wastewater prior to our sampling events and accumulated in the biosolids over time.

According to Figure 3-3, the average real-time PCR concentration of HAdV and EV in the biosolids is 4.2 and 4.0 log units higher, respectively as compared to the final effluent. This is consistent with a previous study (Kuo et al., 2010) where HAdV particles were often associated with the settled sludge (average 10^9 viruses/L) as compared to the supernatant (average 10^6 viruses/L). More samples would have to be analyzed to determine the sorption behavior of

HAdV and EV on organic matter to determine the extent virus association with the settled sludge or the supernatant.

Inactivation of infectious viruses between UV and Chlorine

In this study, WWTPs using UV disinfection achieved an average removal and inactivation of 4.4 log units of infectious viruses as compared to 2.4 log units for the WWTPs using Cl between the influent and final effluent samples. However, the average log reduction of infectious viruses between the pre and post disinfection processes was only 0.13 and 0.96 log units for UV and Cl, respectively. Our results indicate that chlorination was only able to achieve 0.83 log unit higher inactivation of viruses as compared to UV. This suggests that the given configuration of unit processes in the WWTPs sampled from are able to achieve a higher inactivation and removal of viruses as opposed to just the disinfection process. As shown in Figure 3-6, WWTPs using UV were able to achieve a more consistent final effluent inactive virus concentration but no significant difference ($p\text{-value} > 0.05$) was observed between UV and Cl. Similar results were previously reported (Rodriguez et al., 2008) where it was determined that the inactivation of infectious viruses between pre and post-disinfection samples in a full-scale WWTP of approximately 0.3 – 1.3 log units. It was reported that CPE in 03/37 (8%) WWTP samples on BGM cell lines ranging from 1.48-1.63 MPN/L were detected. During their study, only 1L grab samples were analyzed for infectious virus concentration for all sampling points. It is possible that due to insufficient sample volumes the actual concentration present in the final effluent could be underestimated. As opposed to our study where we sampled volumes of 20 L, 375 L and 410 L in the influent, pre-disinfection and post-disinfection, respectively and CPE was observed in 15/15 samples (05/05 effluent) with concentrations ranging from 0.1 – 500 MPN/L.

In addition to using BGM, this study also analyzed the final effluent (post-disinfection) for the five WWTPs using A549 cell line. HAdV was only positive for WWTPs 1 (UV disinfection) and 5 (Cl disinfection) and negative for EV for all WWTPs using ICC-PCR for A549. It has been stated (Rodriguez et al., 2008) that some level of specificity for certain cell lines for different viruses exist. This suggests that multiple cell lines can maximize the chances of detecting overall infectivity in treated wastewater samples for various species of viruses. Unfortunately, current methods for the detection of infectious viruses are often time consuming, even for a single cell line. More research is needed to develop a detection method that is able to determine multiple viral infectivity levels more readily.

Comparing the removal of HAdV and EV in MBR and conventional WWTPs

HAdV removal results observed in our conventional treatment samples agree with previous studies (Carducci et al., 2008, Haramoto et al., 2007) where an average 1.0 – 3.0 log reduction was reported over the sampling period. However, in the current study average removal efficiencies in conventional wastewater treatment of approximately 2.0 log units lower for HAdV was calculated as compared to MBR treatment with real-time PCR data. The higher removal observed can be explained by the components of an MBR system. Enteric virus removal in a MBR system is subjected to three processes, the membrane, the active biomass in the reactor and the attached growth biofilm (Shang et al., 2005). The combination of these components may result in a higher removal as compared to conventional treatment, which only offers active biomass and attachment to sludge floc particles. From our observations, it is also plausible due to the larger average size (80-110 nm) of HAdV, affinity to solids and surface properties; physical treatment (membranes) processes are able to achieve higher log removal values as compared to

other enteric viruses. Further suggesting that due to the smaller size of EV as compared to HAdV, there may be an increased chance of EV passage into the permeate.

Interestingly, as shown in Figure 3-7, the average removal of EV through MBR treatment was similar to what we observed (average of 0.5 log removal increase with the MBR) during conventional treatment. Our findings are consistent with a previous study (Katayama et al., 2008) in conventional treatment process that reported an average EV log influent and effluent concentration of 4.2 and 1.2 log units, indicating an average removal of 2.6 log units. It can be suggested from our data that EV might sorb to sludge flocs more readily during secondary biological treatment giving similar removal values between conventional and MBR processes. In this case, once the viruses are attached to the flocs they will often settle out of the liquid phase in the settling tank during conventional treatment or simply be removed through the membranes during MBR treatment. Furthermore, virus removal and sorption within biological treatment are often dependent on several factors, including temperature, pH, suspended solids, hydraulic retention time and type and strain of EV (Gerba, 1984). However, in the current study we did not differentiate the specific type and strain of EV so it is uncertain if this factor was involved. Further studies are needed to determine how the surface characteristics vary between species and serotypes of other enteric viruses which may help explain viral attachment to particles in both MBR and conventional wastewater treatment.

We have shown in the current study an average 3.9 log reduction over a period of seven months with little fluctuation in the data. Based on our results, we can conclude that HAdV is removed more effectively with the addition of the membrane filtration as compared to conventional treatment. When comparing the MBR and conventional treatment data, a significant difference ($p\text{-value} < 0.05$) was observed for HAdV. In addition, we did not show a

significant difference ($p\text{-value} > 0.05$) in overall HAdV removal between the three different types of conventional treatment processes. It can be concluded that because of the additional physical barrier provided by the MBR system a much higher removal of HAdV was achieved as compared to EV.

Conclusions

- This study has demonstrated the release of both infectious and non-infectious enteric viruses into the environment via the final effluent and biosolids as evaluated by real-time PCR and cell culture assays.
- The results from this study suggest the need for a comparative quantitative human health risk assessment for the final effluent and biosolids released through wastewater treatment to ensure the safety of the public.
- It was observed that there is a significant log reduction (2.3 - 4.5) in infectious viruses throughout the wastewater treatment process before being discharged into natural waterways.
- It was observed that the reduction in infectious viruses treated with UV or Cl can range from 0.1 – 1.2 log units as indicated by cell culture data.
- Based on real-time PCR data, we concluded a MBR system is able to achieve approximately 2 log higher reduction of HAdV (average 4.1 log units) as compared with conventional wastewater treatment (average 2.2 log units). However, similar EV log removal values (3.6 for MBR and 2.9 for conventional) were observed between the two types of treatment processes.

Acknowledgements

We would like to thank the wastewater utilities personnel for their assistance during this study.

Tables and Figures

Table 3-1. Characteristics of the different WWTPs used in this study

WWTP	Wastewater Treatment Process (Biological Treatment)	Average Flow (MGD)	Capacity (MGD)	Disinfection	Sludge Treatment	Sludge Production	Disposal of Biosolids
1	MBR	4.0	17.0	UV	MAD	4500	Land Application
2	Activated Sludge	12.5	19.0	Chlorine	Dewatering	15955	Landfill
3	Activated Sludge	17.0	20.0	UV	Lime Stabilization	---*	Land Application
4	Oxidation Ditch	0.2	0.4	UV	Gravity Thickening	---*	Land Application
5	Rotating Biological Contactors	0.8	2.2	Chlorine	MAD	1369	Land Application
* - No biosolids were collected from these utilities							
MAD - Mesophilic Anaerobic Digestion							

Table 3-2. List of enteric virus primer and probes, gene regions, reaction conditions and references used in this study

<i>Virus Type</i>	<i>Gene Region</i>	<i>Primers/Probes</i>	<i>Sequence (5' to 3')</i>	<i>Reaction Condition (temp, time)</i>	<i>Reference</i>
HAdV	Hexon	Forward	ACCCACGATGTAACCACAGAC	95, 10s -denaturation	Xagorarakis et al., 2007 Modified from Jiang et al., 2005
		Reverse-1	ACTTTGTAAGAGTAGGCGGTTTC	60, 30s -annealing	
		Reverse-2	CACTTTGTAAGAATAAGCGGTGTC	72, 12s -extension	
		Probe	CGACKGGCACGAAKCGCAGCGT		
EV	5' - Untranscribed Region	Forward	ACATGGTGTGAAGAGTCTATTGAGCT	95, 15s -denaturation	Dierseen et al., 2007
		Reverse	CCAAAGTAGTCGGTTCGCGC	60, 60s -annealing	
		Probe	TCCGGCCCCTGAATGCGGCTAAT		
NV (G1)	5' - Untranscribed Region	Forward	CGCTGGATGCGNTTCCAT	95, 15s -denaturation	da Silva et al., 2007
		Reverse	CCTTAGACGCCATCATCATTTAC	60, 60s -annealing	
		Probe	TGGACAGGAGAYCGCRATCT		
NV (G2)	-----	Forward	CARGARBCNATGTTYAGRTGGATGAG	95, 15s -denaturation	Kageyama et al., 2003
		Reverse	TCGACGCCATCTTCATTACACA	56, 60s -annealing	
		Probe	TGGGAGGGCGATCGCAATCT		
HAV	ORF1-ORF2	Forward	GGTAGGCTACGGGTGAAAC	95, 10s -denaturation	Jothikumar et al., 2005
		Reverse	AACAACACCAATATCCGC	55, 20s -annealing	
		Probe	CTTAGGCTAATACTTCTATGAAGAGATGC	72, 15s -extension	

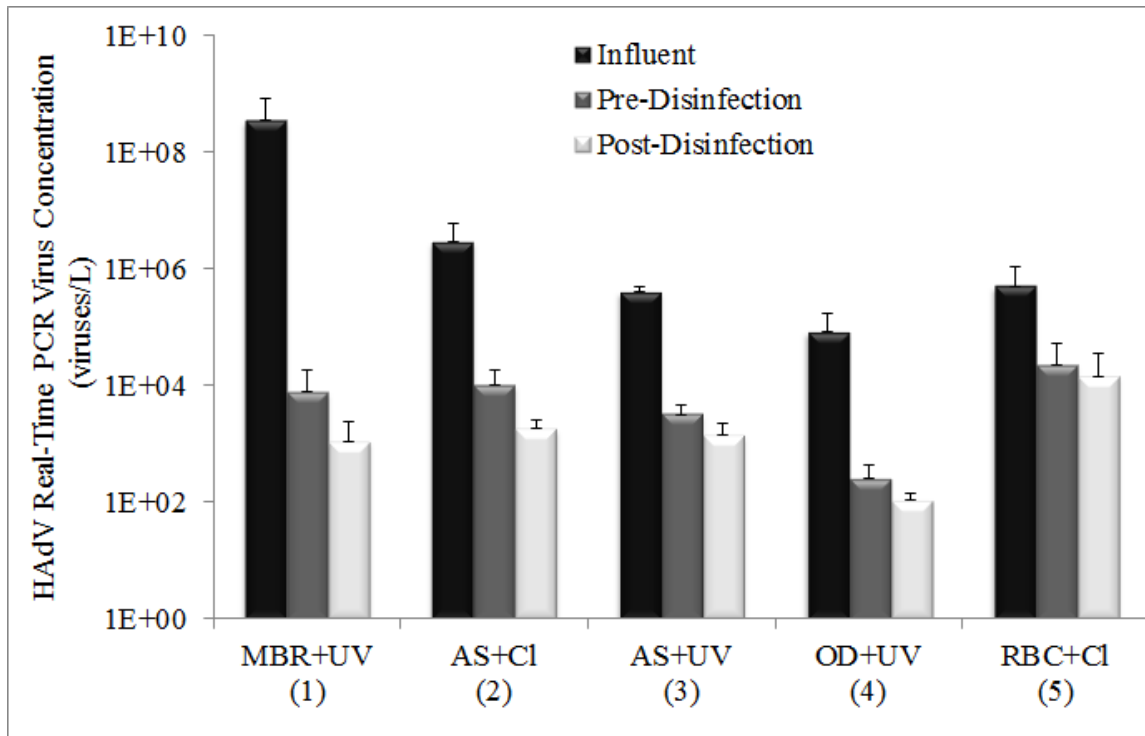


Figure 3-1. Average (n=2) HAdV real-time PCR virus concentration detected at the five different WWTPs at each sampling point and their respective biological and disinfection processes.

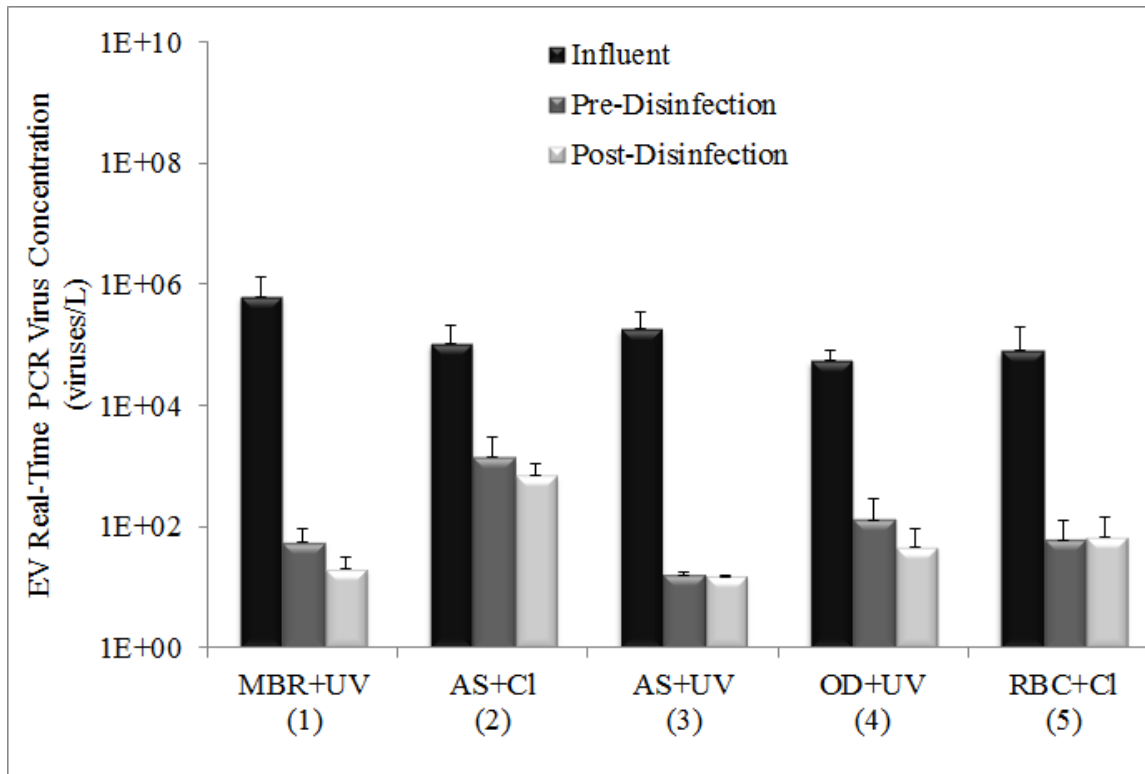


Figure 3-2. Average (n=2) EV real-time PCR virus concentration detected at the five different WWTPs at each sampling point and their respective biological and disinfection processes. All samples are expressed as viruses/L. The detection limit was used for the pre

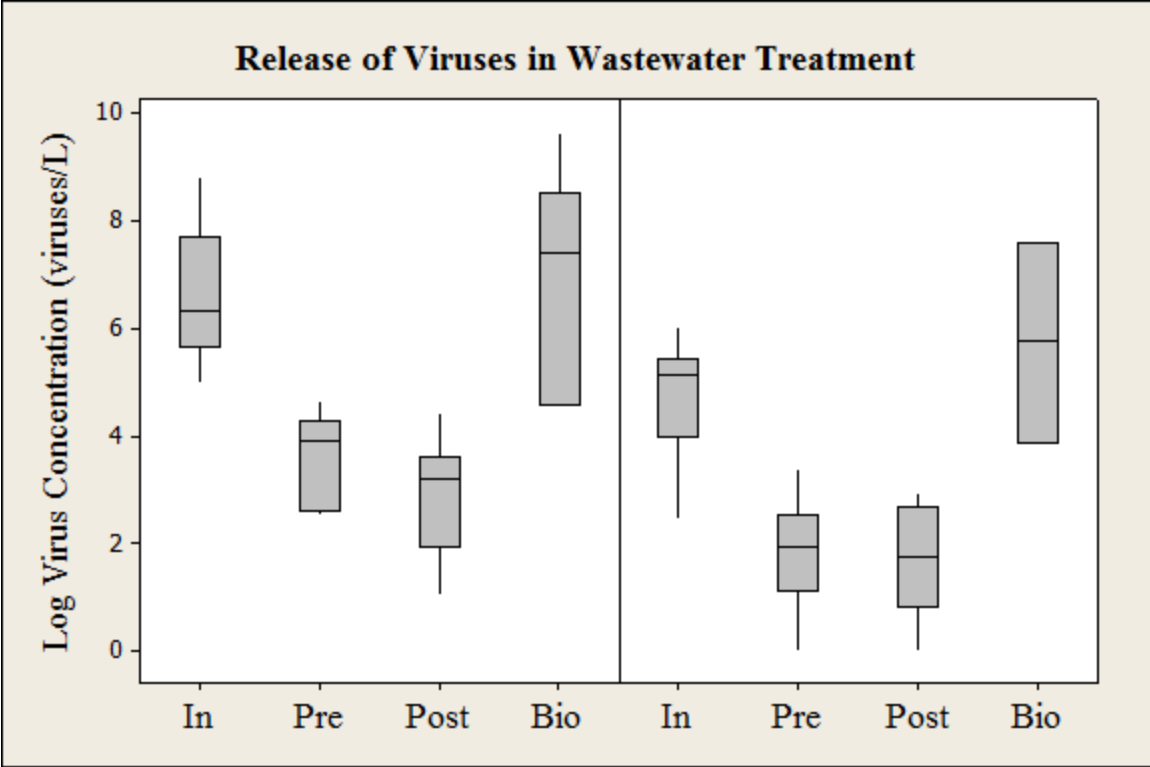


Figure 3-3. Real-time PCR data showing the distribution of HAdV (left side) and EV (right side) in WWTPs 1, 2 and 5. The values were compiled for the influent (n=6), pre disinfection (n=6), post-disinfection (n=6) and biosolids (n=6).

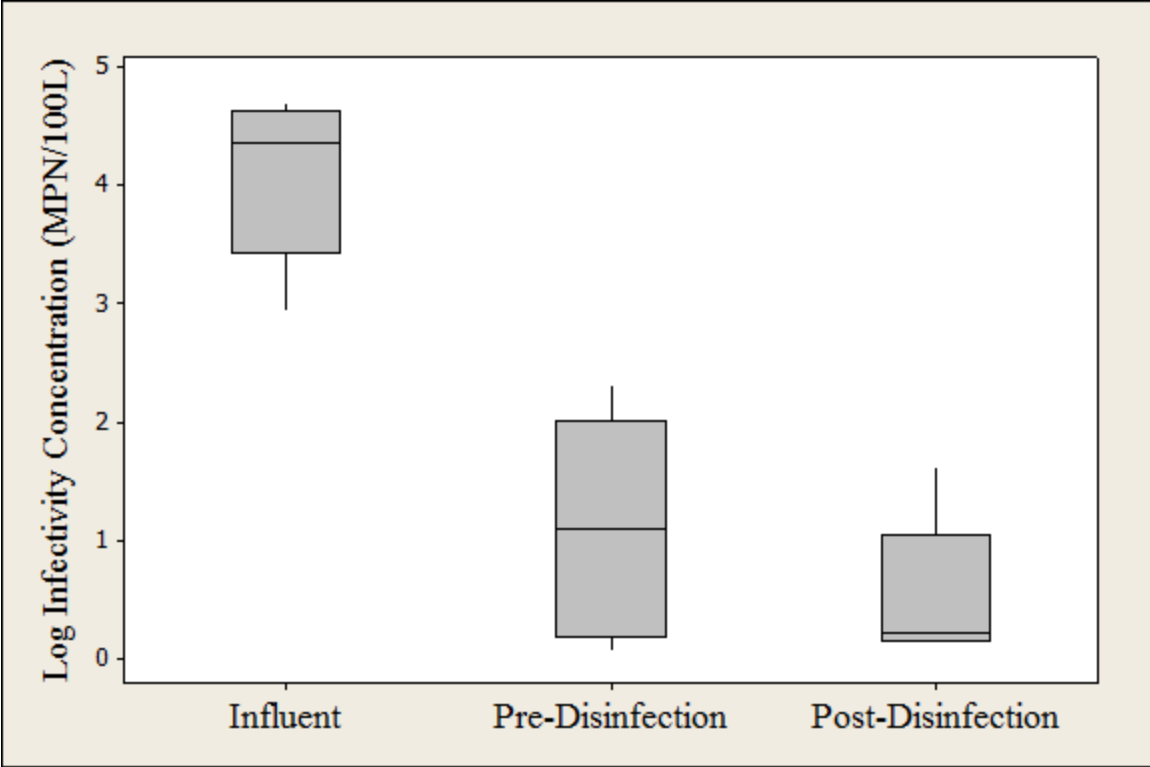


Figure 3-4. Virus infectivity distribution using BGM cell line throughout the three different sampling points from all 5 WWTPs (n=5 for Influent, Pre and Post Disinfection). The values are expressed as MPN/100L.

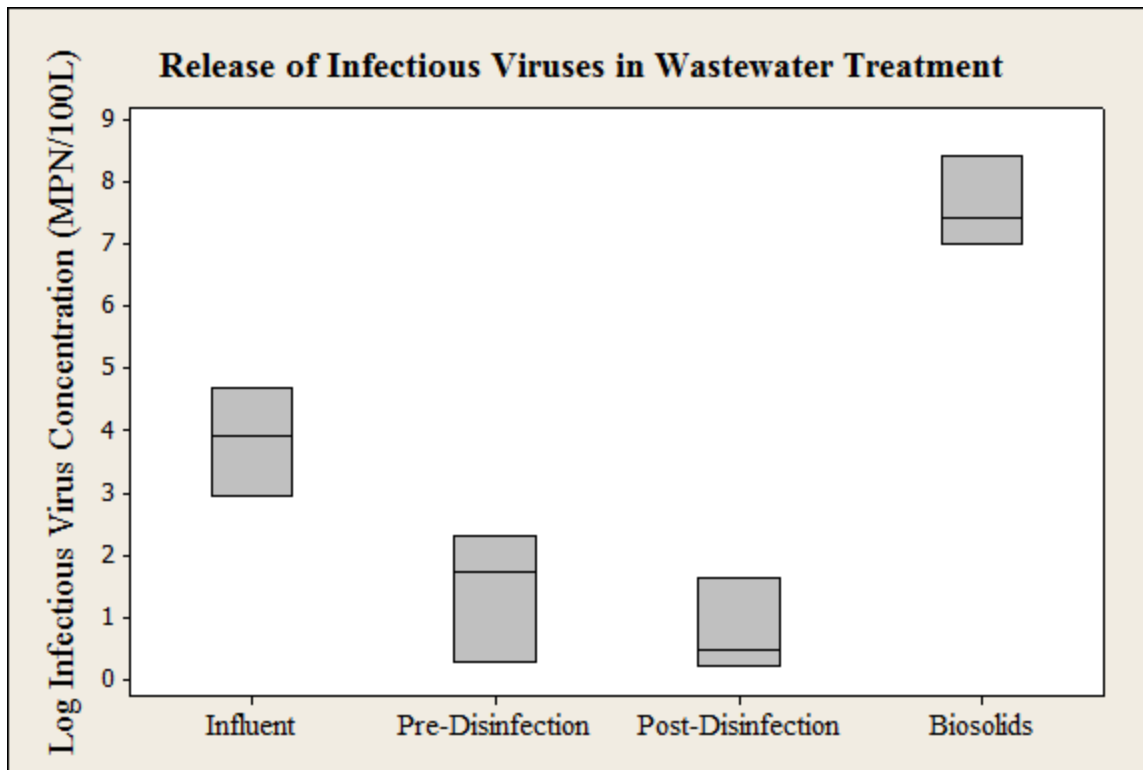


Figure 3-5. BGM cell culture data showing the concentration of viruses being released into the environment from WWTPs 1, 2 and 5. All results are expressed as MPN/100L (n=3 for influent, pre and post-disinfection and biosolids).

Table 3-3. Real-time PCR and A549 cell culture results for WWTPs 1, 2 and 5. The samples represent both the final effluent and biosolids infectivity that are released into the environment. ± - presence versus absence for CPE and ND – not detected. Values are e

<i>WWTP</i>	<i>Sample</i>	<i>qPCR(viruses/L)</i>		<i>ICC-PCR (A549)</i>	
		<i>HAdV</i>	<i>EV</i>	<i>HAdV</i>	<i>EV</i>
<i>1</i>	Post-Disinfection	2.0×10^3	2.9×10^1	+	-
		1.8×10^2	1.2×10^1	+	-
	Biosolids	* 3.8×10^4	* 6.8×10^3	-	-
		* 4.2×10^4	* 8.1×10^3	-	-
<i>2</i>	Post-Disinfection	1.3×10^3	9.4×10^2	-	-
		2.3×10^3	4.1×10^2	-	-
	Biosolids	7.9×10^7	6.7×10^7	+	+
		1.5×10^8	7.4×10^7	+	-
<i>5</i>	Post-Disinfection	1.1×10^1	1.2×10^2	+	-
		2.9×10^4	ND	+	-
	Biosolids	5.1×10^6	9.2×10^6	+	-
		4.7×10^9	3.8×10^4	+	-

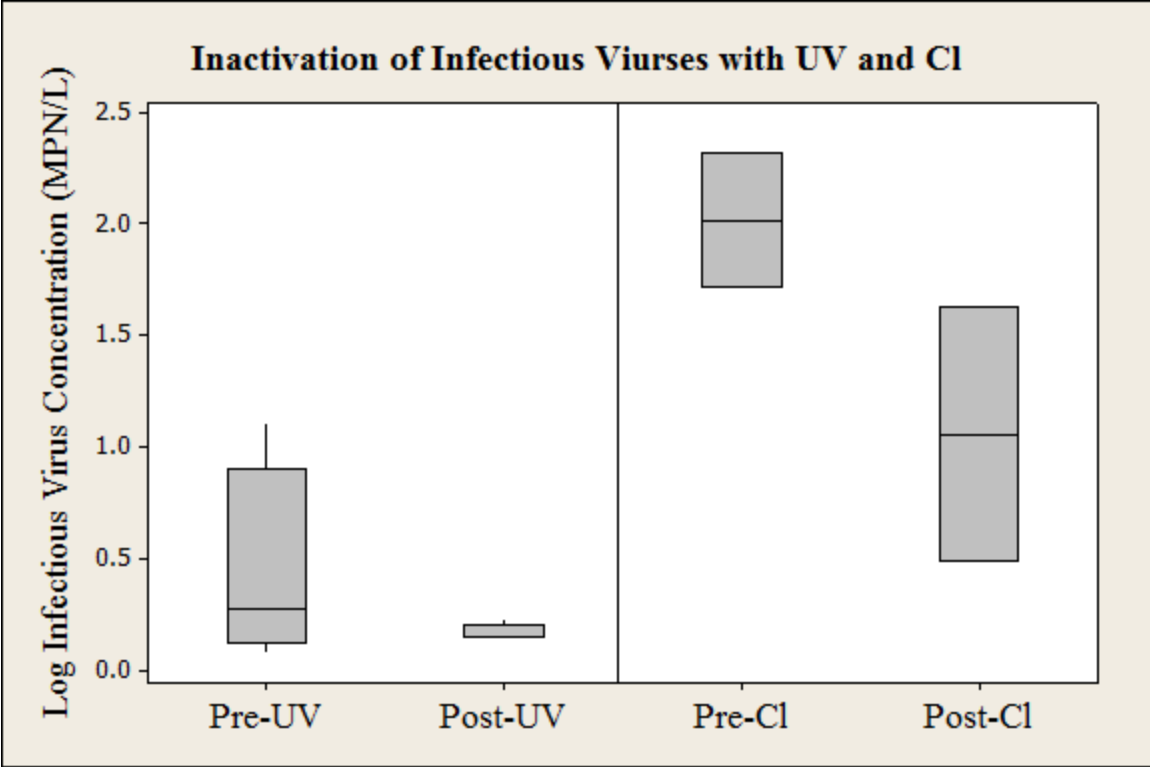


Figure 3-6. Infectious virus reduction between WWTPs using UV and Cl disinfection. Pre and Post-Disinfection UV (n=4), Pre and Post-Disinfection Cl (n=2).

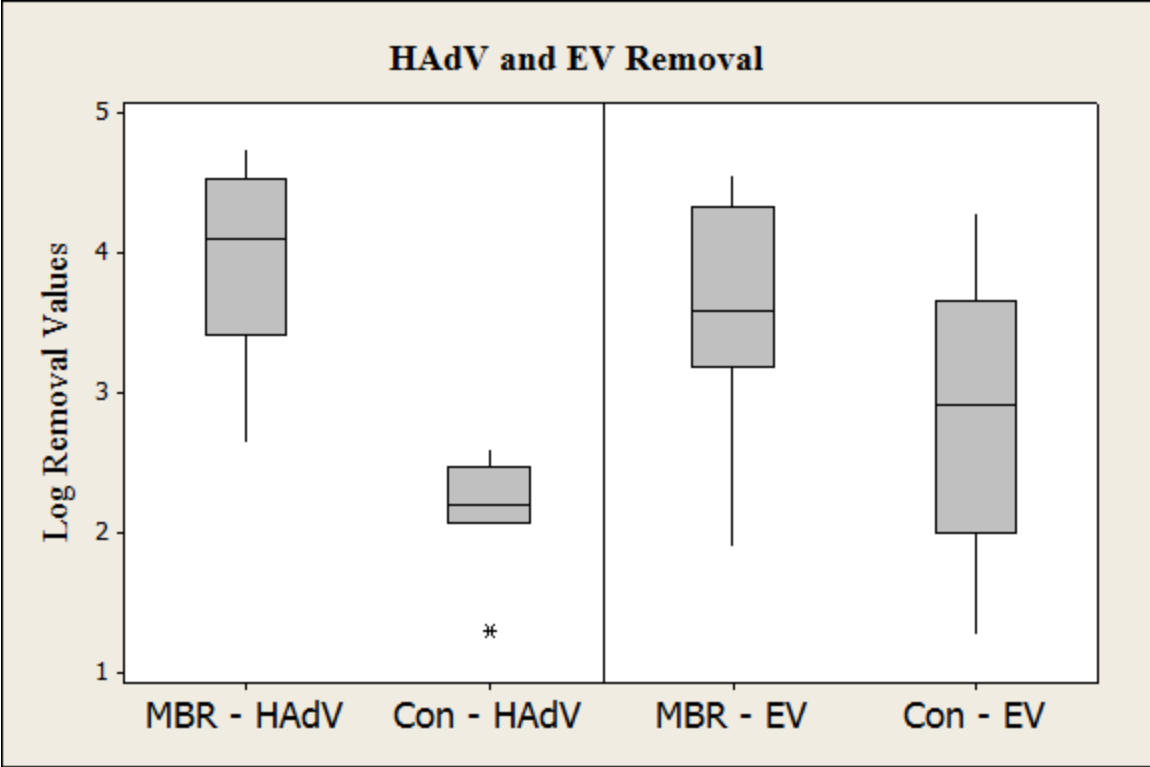


Figure 3-7. Comparison of HAdV and EV log removal values using real-time PCR data for MBR and conventional wastewater treatment. (MBR removal n=8, Con removal n=8).

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CHAPTER 4

CONCENTRATION OF ENTERIC VIRUSES FROM WASTEWATER USING NANOCERAM ELECTROPOSITIVE FILTERS

Abstract

Aims: Compare the NanoCeram and 1MDS electropositive cartridge filters for the detection and quantification of total and infectious viruses using real-time PCR and cell culture.

Methods and Results: 18 NanoCeram and 18 1MDS filters were used at four separate full-scale wastewater treatment utilities at the influent and pre and post-disinfection processes for the infectivity and quantification of Human Adenovirus F40/41 (HAdV) and Human Enterovirus (EV). The average HAdV concentration for the NanoCeram and 1MDS filters was 2.5×10^5 and 4.6×10^5 in the influent samples, 1.7×10^3 and 9.4×10^3 for the pre-disinfection and 8.8×10^2 to 5.6×10^3 viruses/L for the post-disinfection samples, respectively. The average EV concentration using the NanoCeram and 1MDS was 1.7×10^5 and 1.1×10^5 in the influent, 1.1×10^3 and 3.0×10^1 in pre-disinfection, and 1.1×10^2 and 8.9×10^1 viruses/L in the post-disinfection samples. Based on the cell culture results, the average log value of infectious viruses for the NanoCeram and 1MDS at the influent, pre and post-disinfection samples were 3.69 and 4.14, 1.02 and 1.42, and 0.11 and 0.32 MPN/100L, respectively.

Conclusions: No significant differences ($p\text{-value} > 0.05$) were detected between the filters for the detection of HAdV using real-time PCR and infectious viruses.

Significance and Impact of the Study: During this study, it was demonstrated that the NanoCeram filter is just as reliable as the 1MDS filter for the detection of both infectious and non-infectious viruses, but at a much lower cost per unit filter.

Introduction

Human enteric viruses are one of the most common pathogenic groups associated with several clinical symptoms such as gastroenteritis, conjunctivitis, and respiratory diseases (Swenson *et al.* 2003) and have been related to several waterborne diseases in both developed and developing countries world-wide. Currently, human enteric viruses have been placed on the United States Environmental Protection Agency (USEPA) Contaminant Candidate List as emerging contaminants. High concentrations of enteric viruses are excreted in human feces and urine making wastewater one of the most concentrated sources of these viruses (Castignolles *et al.* 1998; Ottoson *et al.* 2006). It has been stated that sampling high volumes of water (5 – 700 L) has a much greater chance of detecting viruses, especially when concentrations are expected to be low (Polaczyk *et al.* 2007; Sobsey *et al.* 1980; USEPA 2001).

The technique most commonly used to concentrate viruses from water and wastewater samples is the adsorption-elution microporous filters method, which are also known as VIRADEL. The filters for VIRADEL could be electropositive, electronegative, and glass wool. According to Maier *et al.* (2000), negatively charged filters can handle the most turbid water samples and are shown to be useful to concentrate viruses from large volumes of tap water and surface water. However, adjustment of the cationic salt concentration and pH is needed before sample processing. Electropositive filters can also handle large volumes of sample and are suitable for sampling tap water, sewage, and seawater using either a 5” or 10” (height) cartridge. Contaminants in the water are retained based on the charge of the particle and not by pore size. Most viruses naturally carry a net negative charge in the environment. Electropositive filters are a more convenient to use for field sampling since neither cationic salt nor acidic/neutral pH preconditioning is needed.

Currently, there are a few different types of electropositive cartridge filters used for water sampling. The most widely used is the 1MDS (Cuno®) which is recommended by the United States Environmental Protection Agency (USEPA) for sampling and recovery of human enteric viruses from various water sources. Unfortunately, this filter is not the most cost efficient way (\$260.00/filter at the time of this study) of sampling and monitoring viral activity. However, in recent years another electropositive cartridge filter was introduced by Argonide Corporation, the NanoCeram® SOE Series (NanoCeram) which at the time of this study was only \$50.00/filter. This may be an alternative for routine monitoring of enteric viruses from various water matrices.

The focus of this study is to compare the performance of two different electropositive cartridge filters (NanoCeram® SOE Series and 1MDS) used for sampling, quantification and infectivity determination of human enteric viruses. Three different points; raw (influent), pre-disinfection and post-disinfection (final effluent) were sampled within the treatment process at four separate wastewater treatment plants (WWTPs). The viruses being detected are Human Adenovirus F40 and F41 (HAdV) and Human Enterovirus (EV).

Methods and Materials

Four different WWTPs in Michigan's Lower Peninsula (Table 4-1) were sampled from 07/01/2009 – 09/24/2009. Three different locations were sampled and included, influent (raw sewage), pre-disinfection (after secondary biological treatment) and post-disinfection (final effluent) with both NanoCeram and 1MDS filters. In total 18 NanoCeram and 18 1MDS filters were used in this study.

The NanoCeram filter is made from a blend cellulose infused with nano alumina fibers and microglass fibers, is 10 inches (25.4cm) in height and a filter surface area of 3.1 ft² (29cm²). The nano alumina fibers are approximately 0.3µm long and 2nm in diameter and are electro adhesively grafted to microglass fiber, which has a zeta potential of ~+40 mV at pH 7. The Zeta Plus 1MDS used in this study is an electropositive 10 inch (25.4cm) cartridge filter made of charged modified glass and cellulose that contains approximately 4.2 ft² (39cm²) of media.

All 36 wastewater samples (Table 4-1) were collected during 6 different sampling events onsite following the procedure explained in the USEPA Manual of Methods for Virology, Chapter 14 (USEPA 2001). The manual was followed identically with no deviation for either the NanoCeram or 1MDS filters. Approximately 20 L of influent, 375 L of pre-disinfection and 410 L of post-disinfection were sampled using both NanoCeram and 1MDS filters at a rate of about 11-12 L/min (3 gal/min). The sampling apparatus was disinfected in between filters by submerging both the intake and outlet tubes for sampling in 0.17% hypochlorite solution and running the apparatus for 20 minutes. During the 20 minutes of disinfection, the filter housing was also in complete contact with the hypochlorite solution. Afterwards the apparatus was dechlorinated using 2% sodium thiosulfate solution. All samples collected were stored on ice and transported to the Michigan State University Water Quality Engineering Laboratory in East Lansing, MI. Upon arrival, samples were placed in a 4°C cooler for 12-24 hours before processing.

All samples collected were eluted according to the Concentration and Processing of Waterborne Viruses by Positive Charge 1MDS Cartridge Filters and Organic Flocculation (USEPA 2001). Following the processing, all eluents were stored at -80°C until further analysis.

Viral samples were extracted using the MagNa Pure Compact System automatic machine (Roche Applied Sciences, Indianapolis, IN). The extraction kits used were the MagNA Pure Compact Nucleic Acid Isolation Kit-Large Volume. The program used required 1000µL of sample to be extracted and concentrated for a final volume of 100µL. Immediately following the completion of the extraction all samples were placed in a -80°C freezer to preserve the integrity of the RNA molecule. Following extraction the quantity of viral nucleic acid extracts from all samples were checked using the NanoDrop Spectrophotometer (NanoDrop[®] ND-1000, Wilmington, DE).

The standard curves for the molecular detection of HAdV and EV were created using stock cultures of HAdV 40 (VR-930) and Coxsackie virus B5 (ATCC VR-1036AS/MK). All standard curve assays used the LightCycler[®] 1.5 Instrument (Roche Applied Sciences, Indianapolis, IN). The standard curves methods used in the current study followed those as previously published (Kuo *et al.* 2010; Dierssen *et al.* 2007). The concentrations were determined by using the NanoDrop spectrophotometer and then the samples were serial diluted 10-fold ($10^1 - 10^8$ viruses/reaction) and used for creating the standard curves for all target viruses. A two-step (reverse transcription and then real-time PCR separately) approach standard curve was generated for EV. All standard curve reactions were run in triplicate and the detection limit for EV is 10 viruses/reaction and 100 viruses/reaction for HAdV.

The crossing point (Cp) value for each PCR reaction was automatically determined by the LightCycler[®] Software 4.0 and used to calculate the overall viral concentration. The primer and probe sequences and reaction conditions used in this study are summarized in Table 4-2. Briefly, all real-time PCR reaction mixes included 10 µL of 2X LightCycler 480 TaqMan Master

Mix and the appropriate volume of primers and probes for each assay (Table 4-2). The real-time PCR running program (all thermocycles were performed at a temperature transition rate of 20°C/s) was 95°C for 15 min; followed by different cycles of denaturation, annealing, extension and a final cooling step.

Each reverse transcription reaction mix included 2.5 µL of 10 µM reverse primer, 1 µL of reverse transcriptase (Promega Corporation, Madison, WI), 4 µL of 5X transcriptor reaction buffer (Roche Applied Sciences, Indianapolis, IN), 20U of protector Rnase inhibitor (Roche Applied Sciences, Indianapolis, IN), and 2 µL of 10 mM deoxynucleotide (Roche). The reaction conditions for all three RNA viruses were the same; the reaction mix was incubated at 55 °C for 30 minutes and then 85 °C for 5 minutes to inactivate the enzyme. All samples were run in triplicate and included a negative control reaction (PCR-grade H₂O without template) and a positive control reaction for all viruses. All samples run through real-time PCR assays were then quantified from viruses/reaction to viruses/L using the method describe from our previous study (Kuo *et al.* 2010).

In total 12 NanoCeram and 12 1MDS filters (one sampling event from each WWTP) were analyzed for virus infectivity. Viruses from all-sampling points (influent, pre and post-disinfection) were cultured using BGM cell line (passage #157). In total 175 BGM flasks were used for the final analysis. All samples followed the USEPA Total Culturable Virus Assay (USEPA 2001).

For statistical analysis, T-test were run in Microsoft Excel ® using an alpha value (α -value of 0.05), showing a 95% confidence interval for both real-time PCR and cell culture data.

Results

A total of 18 NanoCeram and 18 1MDS filters were used during 6 different sampling events at each sampling point. The performance of these filters was compared based on both real-time PCR and cell culture data. HAdV was detected in 36/36 samples. The average HAdV concentration (Figure 4-1) for the NanoCeram and 1MDS filters for the influent samples were 2.5×10^5 and 4.6×10^5 viruses/L, respectively. Average pre-disinfection concentration was 1.7×10^3 and 9.4×10^3 viruses/L and post-disinfection concentration was 8.8×10^2 to 5.6×10^3 viruses/L for the NanoCeram and 1MDS filters, respectively. No significant difference (*p-value* > 0.05) was observed for the overall quantification of HAdV at all sampling points using both filters.

EV was detected in 19/36 samples analyzed for both NanoCeram and 1MDS filters. Figure 2 displays the average concentrations for EV using the NanoCeram and 1MDS for the influent (1.7×10^5 and 1.1×10^5 viruses/L, respectively) pre-disinfection (1.1×10^3 and 3.0×10^1 viruses/L, respectively) and post-disinfection (1.1×10^2 and 8.9×10^1 viruses/L, respectively).

Figure 4-3 shows the average concentration of infectious viruses that were detected using BGM cell lines at the three different sampling locations. As the figure shows, the NanoCeram and the 1MDS resulted in a very comparable detection of infectious viruses over the three separate sampling points. The average value for the NanoCeram and 1MDS at the influent, pre and post-disinfection samples were 3.69 and 4.14, 1.02 and 1.42, and 0.11 and 0.32 log MPN/100L, respectively. According to our T-test results, no significant difference (*p-value* > 0.05) was observed for both the NanoCeram and 1MDS filters for the detection of infectious viruses at the influent, pre and post-disinfection sampling locations.

Discussion

The use of an electropositive cartridge filter allows for sampling of high volumes of water and wastewater for viral analysis. Another technique that can be used to sample high volumes of water is glass wool filtration. However, glass wool filters are not commercially available and requires both a washing step and self-packing into housings with a modified hand-press for each filter (Lambertini *et al.* 2008) prior to sampling. Electropositive filters are able to sample 1000's of liters of water (depending on the source water) (USEPA 2001) and are capable of recovering approximately 30 ml of eluent. However, this method is often expensive resulting in a limited number of sampling events. The percent recoveries were previously reported for the 1MDS at approximately 30-60% (Sobsey *et al.* 1980; Polaczyk *et al.* 2007) and 22-77% (Karim *et al.* 2009) for the NanoCeram filters.

Another commonly used technique often used in the field, only concentrate a few liters of environmental water, which may not be sufficient in routine monitoring. When using this method, small volumes (< 1 L) of water are filtered using a 90-mm type HA (negatively charged) 0.45 μ m pore size filter adding salts on the surface of the filter (Haramoto *et al.* 2009) and this method yields various percent recoveries ranging from < 1 - 100 % (Fong *et al.* 2010), which can greatly underestimate the concentration of viruses being released. It is apparent that this may cause uncertainties when trying to determine the concentration of infectious viruses at locations where the public may be at risk of exposure.

In this study, we compared the overall performance of the NanoCeram and 1MDS 10'' (height) electropositive cartridge filter for virus detection, quantification and infectivity. After comparing the results from 18 NanoCeram and 18 1MDS filters, we have concluded that no significant difference in the performance or detection of HAdV (*p-value* > 0.05) between the two

filters was observed. For EV, our results did show a $p\text{-value} < 0.05$ for EV with our real-time PCR data indicating a significant difference for this particular virus. Interestingly, EV was detected with real-time PCR in 3/6 of the pre-disinfection samples using the NanoCeram filters, whereas EV was not detected (0/6) with the 1MDS filter. EV was only detected in 2/6 post-disinfection samples for both NanoCeram and 1MDS filters and not in similar sampling events. It is plausible that this inconsistent detection with real-time PCR could account for the significant difference.

A previous study (Karim *et al.* 2009) concluded that for EV and norovirus detection in natural waters, the NanoCeram cartridge filter can be used as an alternative to the 1MDS. During their study, EV was continually detected between both the 1MDS and NanoCeram filters using both spiked river and tap water samples. We have concluded that further studies with repeated sampling events would be beneficial in order to conclude the performance of EV detection in wastewater using real-time PCR.

According to our cell culture results, it was concluded that no significant difference ($p\text{-value} > 0.05$) was found between the two filters. Regardless of the significant difference detected for EV using real-time PCR data, we did not observe a significant difference in the detection of infectious viruses. It is crucial during routine monitoring of wastewater treatment to be able to detect both infectious and non-infectious viruses especially at the discharge point where the public has a chance of exposure from either direct or secondary contact. In the current study, the NanoCeram filter was able to detect low concentration of infectious viruses in conjunction with the USEPA approved 1MDS filter. Our conclusion is consistent with Karim (2009), that the NanoCeram filters can be used as an alternative in concentrating environmental samples instead of the 1MDS in order to achieve similar and reliable results.

Conclusions

Our filter comparison study is unique in the fact that we compared the ability of each filter to capture both non-infectious and infectious viruses in full-scale wastewater using both real-time PCR and cell culture technique. In addition, we used the 10 inch NanoCeram filter as compared to the 5 inch filter used in the previous study (Karim *et al.* 2009). The 10 inch filter was used because of the high amount of total and suspended solids throughout the wastewater process. During the current study, it is apparent that the detection, quantification and cell culture results for both filters were comparable. We have determined from our study that for routine monitoring of infectious enteric viruses the NanoCeram performed similar to the 1MDS but costing 1/5 the price (\$260.00 for 1MDS compared to \$50.00 for the NanoCeram per filter).

Acknowledgements

We would like to thank the wastewater treatment plant personnel for their assistance during this study.

Tables and Figures

Table 4-1. List of the WWTPs sampled from

WWTP	Location	Wastewater Treatment Process (Biological Treatment)	Average Flow (MGD)	Capacity (MGD)	Disinfection	# of Samples Collected
1	East Lansing, MI	Activated Sludge	12.5	19.0	Chlorine	3/3
2	Lansing, MI	Activated Sludge	17.0	20.0	UV	6/6
3	Imlay City, MI	Oxidation Ditch	0.2	0.4	UV	3/3
4	Romeo, MI	Rotating Biological Contactors	0.8	2.2	Chlorine	6/6

Table 4-2. List of enteric virus primer and probes, gene regions, reaction conditions and references used in this study

<i>Virus Type</i>	<i>Gene Region</i>	<i>Primers/Probes</i>	<i>Sequence (5' to 3')</i>	<i>Reaction Condition (temp, time)</i>	<i>Reference</i>
HAdV	Hexon	Forward	ACCCACGATGTAACCACAGAC	95, 10s -denaturation	Xagorarakis et al., 2007 Modified from Jiang et al., 2005
		Reverse-1	ACTTTGTAAGAGTAGGCGGTTTC	60, 30s -annealing	
		Reverse-2	CACTTTGTAAGAATAAGCGGTGTC	72, 12s -extension	
		Probe	CGACKGGCACGAACKCGCAGCGT		
EV	5' - Untranscribed Region	Forward	ACATGGTGTGAAGAGTCTATTGAGCT	95, 15s -denaturation	Dierseen et al., 2007
		Reverse	CCAAAGTAGTCGGTCCGC	60, 60s -annealing	
		Probe	TCCGGCCCCTGAATGCGGCTAAT		

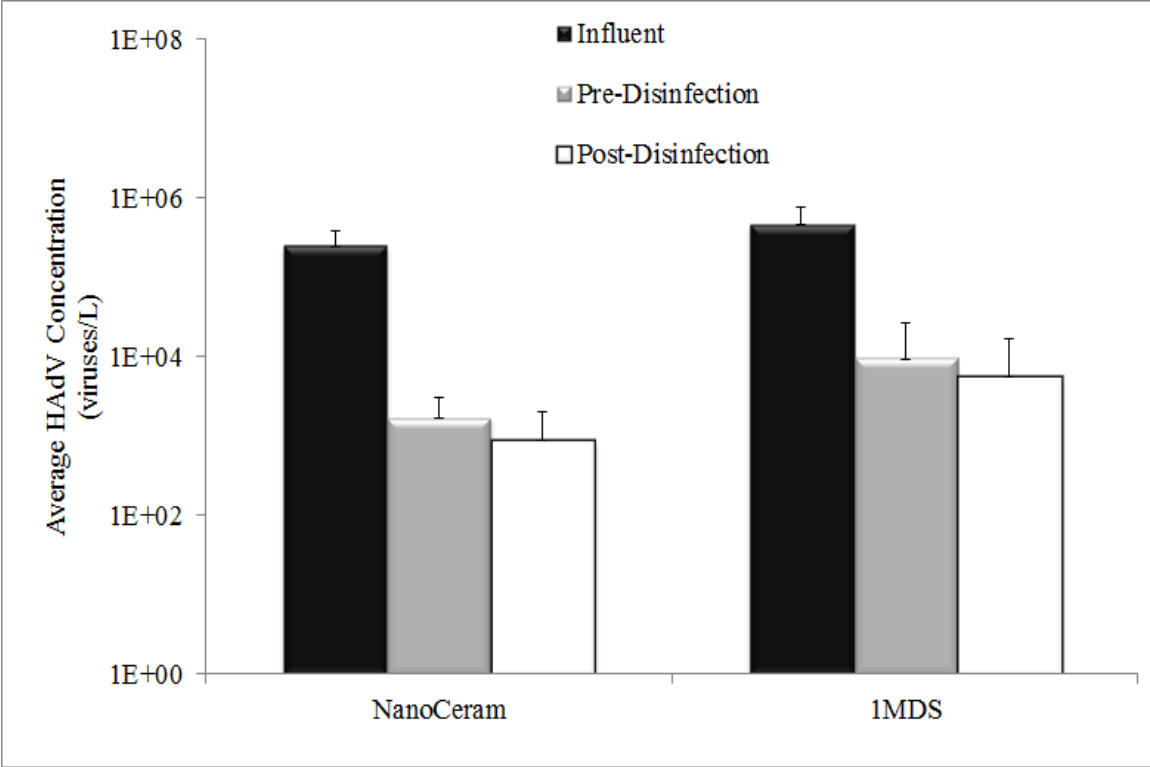


Figure 4-1. Comparison in average real-time PCR concentration of HAdV for the influent, pre and post disinfection between the NanoCeram and 1MDS cartridge filters for all sampling events. Error bars were used to show the standard deviation within the data. Sampl

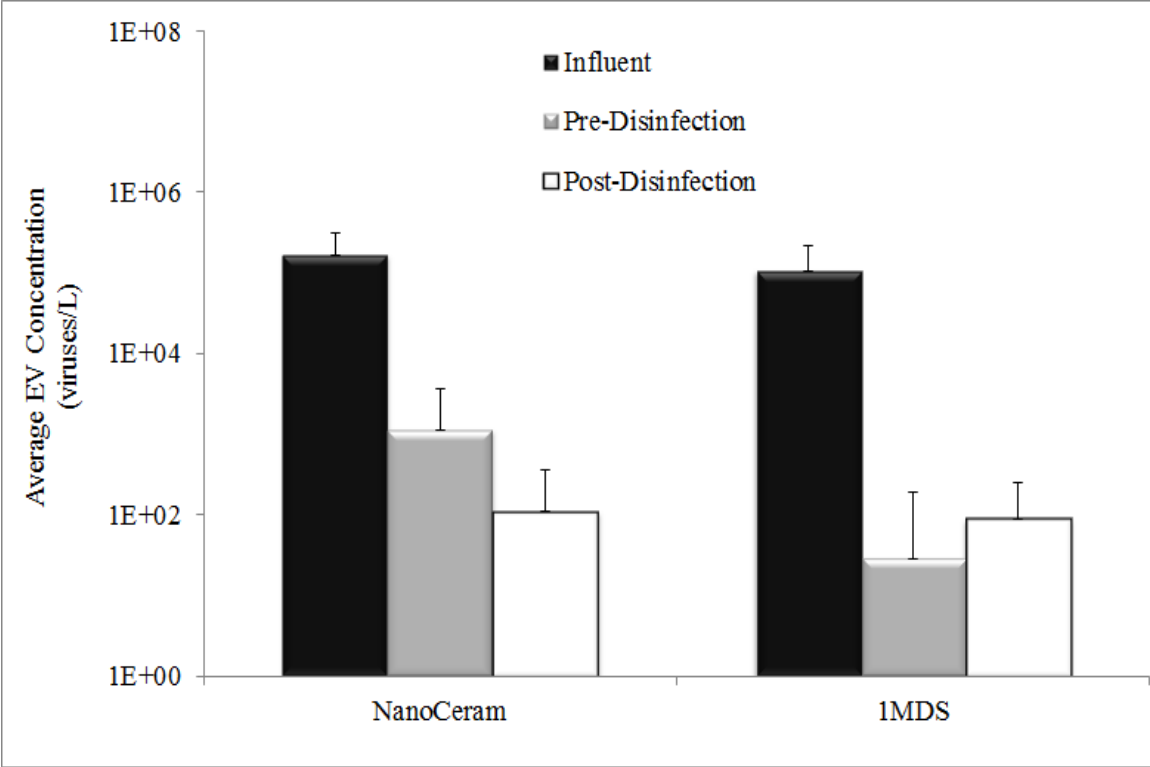


Figure 4-2. Comparison in average real-time PCR concentration of EV for the influent, pre and post disinfection between the NanoCeram and 1MDS cartridge filters. Error bars were used to show the standard deviation within the data. Sample size was n=6 for influent

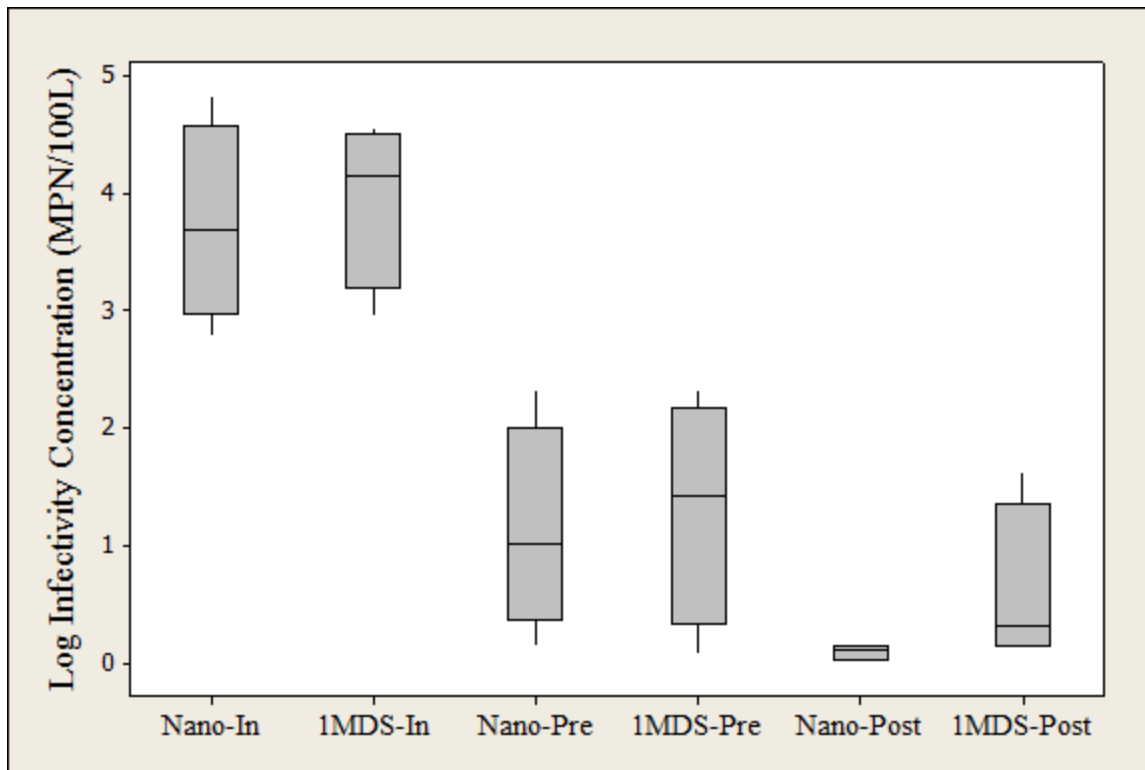


Figure 4-3. Comparison of virus infectivity using BGM cell line between the NanoCeram and 1MDS filters for influent, pre and post disinfection samples. Values are expressed in MPN/100L. Inf. – Influent, Pre – Pre-Disinfection and Post – Post-Disinfection.

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CHAPTER 5

CONCLUDING REMARKS

The fate, occurrence, quantification and removal of emerging biological contaminants were studied throughout the duration of this work. Presently, the USEPA does not require the monitoring of human enteric viruses being discharged in the final effluent or in the sludge that is generated following secondary biological treatment. It was determined during this study that high levels of infectious viruses are routinely introduced daily into the environment through the final effluent discharge, and both land applied biosolids or landfilled sludge. As shown in Chapter 2, MBR treatment was able to achieve approximately 2 log value higher removal of HAdV as compared to three different conventional treatment methods (activated sludge, rotating biological contactors and oxidation ditch). However, MBR treatment was only able to achieve an additional 0.5 log value removal of EV as compared to conventional treatment. As shown in this study, advanced wastewater treatment is able to provide a higher quality effluent while lowering the overall footprint required for treatment. It was also shown in Chapter 2 that both HAdV and EV were consistently detected in the settled sludge (99.8% and 97.1%, respectively) as compared to the supernatant entering the MBR. This clearly shows that a majority of HAdV and EV entering secondary biological treatment sorb to the flocs and settle out in the sludge and then are transferred to the digesters before being released into the environment either through land application or disposal in landfills.

This was further researched in Chapter 3, where infectivity analysis was conducted at four separate locations throughout the treatment process (influent, pre and post disinfection and biosolids). It was shown in detail that the removal and disinfection process together is not sufficient enough to restrict the passage of infectious viruses into the environment. However, due to the complexity and amount of time required for infectivity analysis, only one sampling event at each WWTP was analyzed. Future work could focus on multiple cell lines, samples and additional sampling points to offer a better understanding of the removal and inactivation within the overall treatment process.

Furthermore, as explained in Chapter 2, the detection and removal of enteric viruses was only accomplished using real-time PCR without culture methods. Further studies are required to determine the overall removal of infectious viruses through advanced wastewater treatment as compared to conventional.

In addition, future research is required to determine where the greatest health risks are associated with the release of infectious viruses either through the final effluent or contact of the land applied biosolids. More extensive experiments are required at various distances downstream of the discharge in the receiving water body. This is to determine whether or not diffusion, dispersion and other mechanisms minimize the exposure of direct or secondary contact of the public.

This extended research can be conducted using the electropositive filter as explained in Chapter 4. By using the NanoCeram and opposed to the 1MDS filter more samples can be collected at a fraction of the cost with no difference in the detection of enteric viruses. Furthermore, it is necessary to include several sediment samples in efforts

to determine if sorption of the viruses plays a role in overall accumulation which could also present a major health risk. Additionally, as shown in Chapter 3, an elevated concentration of infectious viruses are often associated with Class B biosolids. Further research may also determine if aerosolization of biosolids during the land application is capable of producing a risk to those who live within certain proximity of the intended agricultural field.

As engineers, accurate mass-balances in wastewater utilities will allow us to quantitatively predict human health risks related to viruses. Ultimately, a better understanding of the health associated risk to the public is necessary to lower the overall number of individuals who come into contact with infectious viruses.