

VIRUSES IN WATER AND WASTEWATER AND THEIR SIGNIFICANCE TO PUBLIC
HEALTH

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ABSTRACT

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Viruses are responsible for millions of disease cases and deaths each year worldwide. Water-related viruses are of particular concern to environmental engineers, especially with regards to wastewater. Wastewater can be a valuable tool in the investigation of viral disease. This dissertation seeks to study the presence, quantification, and diversity of viruses in wastewater in the application of various methodologies for the protection of human, animal, and environmental health.

The first study proposes a One-Health approach for the identification and prevention of water-related viral disease outbreaks. One-Health posits that human, animal, and environmental health are all innately interrelated. The proposed methodology is a three-step approach that calls for the identification of critical water-related exposure pathways for viruses, the design of surveillance systems to observe these pathways, and the implementation of interventions to block viral transmission along these pathways.

The second study proposes a methodology for the use of wastewater as an epidemiological tool to predict and identify viral disease outbreaks. Wastewater can be considered a cumulative sample of the serviced population, and quantifying the concentration of a particular virus in wastewater indicates the number of disease cases in the serviced community. Important considerations, such as population normalization, shedding rates, and correlation with clinical data are discussed. Application of this methodology has the potential to identify outbreaks before disease cases are clinically reported. The third study is an application of the

proposed approach in the state of Michigan from the first two chapters. The goal of this study was to identify factors that are predictive of viral disease. The identification of these factors can inform public health officials on the most effective ways of preventing future viral outbreaks.

The fourth study investigates viral diversity and abundance in wastewater and surface water from Kampala, Uganda. Samples were taken at the influent and effluent of a wastewater treatment plant, as well as surrounding surface waters. Four human viruses were quantified using qPCR, and next-generation sequencing was performed to assess viral diversity. It was found that wastewater effluent had an impact on surrounding surface waters, and that there were temporal fluctuations in the concentrations of human viruses, indicating the potential for wastewater to be used as an epidemiological tool.

The fifth study investigated different wastewater treatment barriers and their effects on wastewater effluent, which can impact environmental health upon release. This study analyzed viral diversity of wastewater effluent samples from membrane bioreactor treatment plants in Michigan and France. Diversity analysis indicated *Herpesvirales* was the most abundant order of potentially pathogenic human DNA viruses in all utilities, and other potentially pathogenic human viruses detected include *Adenoviridae*, *Parvoviridae*, and *Polyomaviridae*. The choice of treatment process (MBR versus activated sludge) had no measurable impact on effluent DNA viral diversity, while the type of disinfection had an impact on the viral diversity present in the effluent.

In summary, these studies illustrate the importance of water and wastewater as a critical reservoir for viral disease. Treatment of these water resources is a vital responsibility of environmental engineers. Moreover, water and wastewater surveillance can prove a valuable tool in the early detection of viral outbreaks protection of public health.

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KEY TO ABBREVIATIONS

5-HIAA	5-hydroxyindoleacetic acid
AdV	Adenovirus
CAFO	Concentrated animal feeding operation
CCL	Contaminant Candidate List
CDC	Centers for Disease Control
cDNA	Complementary deoxyribonucleic acid
CSO	Combined sewer overflow
DNA	Deoxyribonucleic acid
EPA	Environmental Protection Agency
EV	Enterovirus
GI	Gastrointestinal
HAV	Hepatitis A virus
HPV	Human papillomavirus
MBR	Membrane bioreactor
MCL	Maximum contaminant level
MDARD	Michigan Department of Agriculture and Rural Development
MDEQ	Michigan Department of Environmental Quality
MDHHS	Michigan Department of Health and Human Services
MDSS	Michigan Disease Surveillance System
NAHSS	National Animal Health Surveillance System
NGS	Next-generation sequencing

NORS	National Outbreak Reporting System
OLS	Ordinary least squares
PCA	Principal component analysis
PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
RT-qPCR	Real-time quantitative polymerase chain reaction
RTSF	Research Technology Support Facility
RV	Rotavirus
SoND	Summary of Notifiable Diseases
SSO	Sanitary sewer overflow
USDA	United States Department of Agriculture
USGS	United States Geological Survey
UV	Ultraviolet
WHO	World Health Organization
WWSL	Wastewater storage lagoon
WWTP	Wastewater treatment plant

Chapter 1:

Introduction

The protection and management of water is a critical duty in the practice of environmental engineering. Wastewater treatment is a key component of this practice, as wastewater is an important reservoir for pathogens. The removal of pathogens from wastewater is of utmost importance, due to the burden of viral disease around the globe. It is estimated that between 1.5 and 12 million people die of waterborne disease each year [1,2]. Foremost among these waterborne pathogens are viruses, which are potentially the most hazardous pathogens found in wastewater [3,4]. Viruses are of particular concern due to their low infectious dose, ability to mutate, inability to be treated by antibiotics, resistance to disinfection, small size that facilitates environmental transport, and high survivability in water and solids. Multiple viruses are included on the EPA contaminant candidate list, which require treatment of wastewater to meet disinfection standards.

The concept of One-Health is a relatively novel approach to the solving of global health challenges. According to the One Health Commission, the concept is defined as “the collaborative effort of multiple disciplines – working locally, nationally, and globally – to attain optimal health for people, animals and our environment” [5]. The crucial aspect of One-Health is that all aspects of health – human health, animal health, and environmental health – are all innately interrelated. Water and wastewater provide a vital cross-section of these three aspects, making them ideal for the application of the One-Health concept. Moreover, due the aforementioned properties of viruses, in addition to the fact that viruses do not replicate outside a host, it is valuable to approach the study of viruses and viral disease from the One-Health perspective.

Wastewater can therefore be utilized in this approach. Raw wastewater influent can serve as reservoir for the investigation of viruses, especially as influent can be considered to be a population sample from the serviced community. Many kinds of human and animal viruses have been detected in wastewater influent, both with conventional and molecular techniques [6–9] and with next-generation sequencing techniques [10–12]. Treated wastewater effluent, meanwhile, is often released into nearby surface waters, thereby potentially impacting environmental health. Moreover, many viruses, including human viruses, have been detected in this effluent, heightening its importance [13–15].

Consequently, the surveillance of wastewater is of critical importance for the protection of both water resources and public health. This dissertation seeks to examine viruses under the One-Health framework, particularly with regards to wastewater. The following chapters are included in this dissertation.

Chapter 2: This chapter (submitted as: Evan O’Brien and Irene Xagorarakis. A Water-Focused One-Health Approach for Early Detection and Prevention of Viral Outbreaks) develops a methodology to investigate viral disease on a local and regional level using the One-Health perspective. It first provides a review of the global burden of human and animal viral disease and discusses the ways in which water plays a role in the transport and transmission of viruses. It then proposes a three-pronged approach for the prediction and prevention of viral disease: identification of critical pathways, design of surveillance systems, and implementation of intervention strategies.

Chapter 3: This chapter (published as: Evan O’Brien and Irene Xagorarakis. Wastewater-Based-Epidemiology for Early Detection of Viral Outbreaks. Invited book chapter in: Springer Series Title: Women in Engineering and Science; Volume title: Water Quality: Investigations by

Prominent Female Engineers, Editor: Deborah O'Bannon) proposes a methodology for the use of wastewater as an epidemiological tool to identify and predict viral disease outbreaks. It begins with an overview of waterborne and non-waterborne viruses and the viability of wastewater as a surveillance medium for viruses. It then discusses the proposed methodology, including sampling, quantification of viruses, and estimation of population disease cases.

Chapter 4: This chapter (submitted as: Evan O'Brien and Irene Xagorarakis. A Proposed Water-Based Surveillance Approach for Identification and Early Detection of Human and Livestock Viral Outbreaks in Michigan) applies the One-Health methodology to the state of Michigan to identify potential predictors of viral disease and recommend surveillance and intervention strategies. Data, including public health data, land use data, agricultural data, and climatic data, were collected and utilized in exploratory data analysis and subsequent statistical analysis to identify variables that were likely to be predictive of different human viruses. These relationships informed the types of surveillance and intervention strategies recommended for each particular virus.

Chapter 5: This chapter (published as: Evan O'Brien, Joyce Nakyzze, Huiyun Wu, Noah Kiwanuka, William Cunningham, John B. Kaneene, and Irene Xagorarakis. Viral Diversity and Abundance in Polluted Waters in Kampala, Uganda. *Water Research*. 127: 41-49) describes an experiment investigating samples from a wastewater treatment plant and surrounding surface waters in Kampala, Uganda. Wastewater influent, effluent, and surface waters were sampled and four human viruses were quantified with qPCR. Next-generation sequencing and metagenomic analyses were also performed to assess viral diversity of the samples.

The objectives were: (1) to quantify the abundance of four human viruses in surface water and wastewater in Kampala, Uganda, (2) to characterize the viral diversity of these water

samples, and (3) to establish preliminary data that could indicate the possibility of using these methods in future wastewater-based epidemiology studies to identify early signals of and predict future viral disease outbreaks.

Chapter 6: This chapter (published as: Evan O'Brien, Mariya Munir, Terence Marsh, Marc Heran, Geoffroy Lesage, Volodymyr V. Tarabara, Irene Xagorarakis. Diversity of DNA viruses in effluents of membrane bioreactors in Traverse City, MI (USA) and La Grande Motte (France). *Water Research*. 111: 338-345) describes an experiment investigating samples from wastewater treatment plants in Michigan and France. Wastewater effluent was sampled at two membrane bioreactor treatment plants in Traverse City, MI and La Grande Motte, France and at a conventional activated sludge plant in East Lansing, MI. Next-generation sequencing and metagenomic analyses were performed to assess the viral diversity of the effluent samples and to compare the differences between the two treatment types. The impact of disinfection techniques on viral diversity was also analyzed.

The objectives of this study were: (1) to investigate the diversity of human DNA viruses detected in effluents of MBR WWTPs equipped with membranes of different pore sizes, (2) to assess the diversity of DNA bacteriophages in MBR WWTP effluents, (3) to compare the diversity of DNA viruses in MBR WWTP effluents with that in a conventional WWTP effluent, and (4) to investigate the impact of disinfection on DNA virus diversity in WWTP effluent.

Chapter 7: This chapter concludes the dissertation by summarizing the major findings and recommending future work.

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Chapter 2:

A Water-Focused One-Health Approach for Early Detection and Prevention of Viral Outbreaks

This chapter has been submitted to the Journal of One Health as: Evan O'Brien and Irene Xagorarakis. A Water-Focused One-Health Approach for Early Detection and Prevention of Viral Outbreaks.

2.1. Abstract

Despite consistent efforts to protect public health there is still a heavy burden of viral disease, both in the United States and abroad. In addition to conventional medical treatment, there is a need for a holistic approach for early detection and prevention of viral outbreaks at a population level. One-Health is a relatively new integrative approach to the solving of global health challenges. A key component to the One-Health approach is the notion that human health, animal health, and environmental health are all innately interrelated. One-Health interventions, initiated by veterinary doctors, have proven to be effective in controlling outbreaks, but thus far the applications focus on zoonotic viruses transmitted from animals to humans. Environmental engineers and environmental scientists hold a critical role in the further development of One-Health approaches that include water-related transport and transmission of human, animal, and zoonotic viruses. This paper proposes a greater One-Health based framework that involves water-related pathways. The first step in the proposed framework is the identification of critical exposure pathways of viruses in the water environment. Identification of critical pathways informs the second and third steps, which include water-based surveillance systems for early detection at a population level and implementation of intervention approaches to block the critical pathways of exposure.

2.2. One-Health and Viral Disease

The burden of viral disease is a global concern. Due to their unique properties, viruses have a particular relevance when analyzing the interaction among humans, animals, and the environment. Viruses are small compared to other pathogens, facilitating transport in the environment. Moreover, their resistance to disinfection and ability to survive for prolonged periods in water and solids make their transmission from the environment to suitable hosts likely. This is compounded by their low infectious dose, inability to be treated by antibiotics, and their proclivity for adaptive mutation. Additionally, viruses do not replicate outside their host cells, therefore detection in environmental samples can be directly related to the human or animal population that excreted these viruses.

Figure 2.1 summarizes viral exposure pathways and the relevance of the One-Health approach. One-Health is a relatively new approach to the solving of global health challenges. Formally put forth by the One Health Commission in 2007, the concept is defined as “the collaborative effort of multiple disciplines – working locally, nationally, and globally – to attain optimal health for people, animals and our environment [1].” Consequently, a key component to the One-Health approach is the notion that human health, animal health, and environmental health are all innately interrelated. The quality and well-being of one group can directly and indirectly impact the quality of the other two groups. By taking all three aspects of health into account, solutions can be generated that not only address the health problems of a specific group but mitigate the source of those problems as well.

Much of the current work using this methodology is focused upon the exposure pathway between humans and animals, while the water-related exposure pathway has not been thoroughly investigated from a One-Health perspective. The purpose of this paper is to explore water-related

exposure pathways as they relate to human, animal, and environmental health, and to develop a framework with which to apply the One-Health methodology for early detection and management of water-related viral outbreaks.

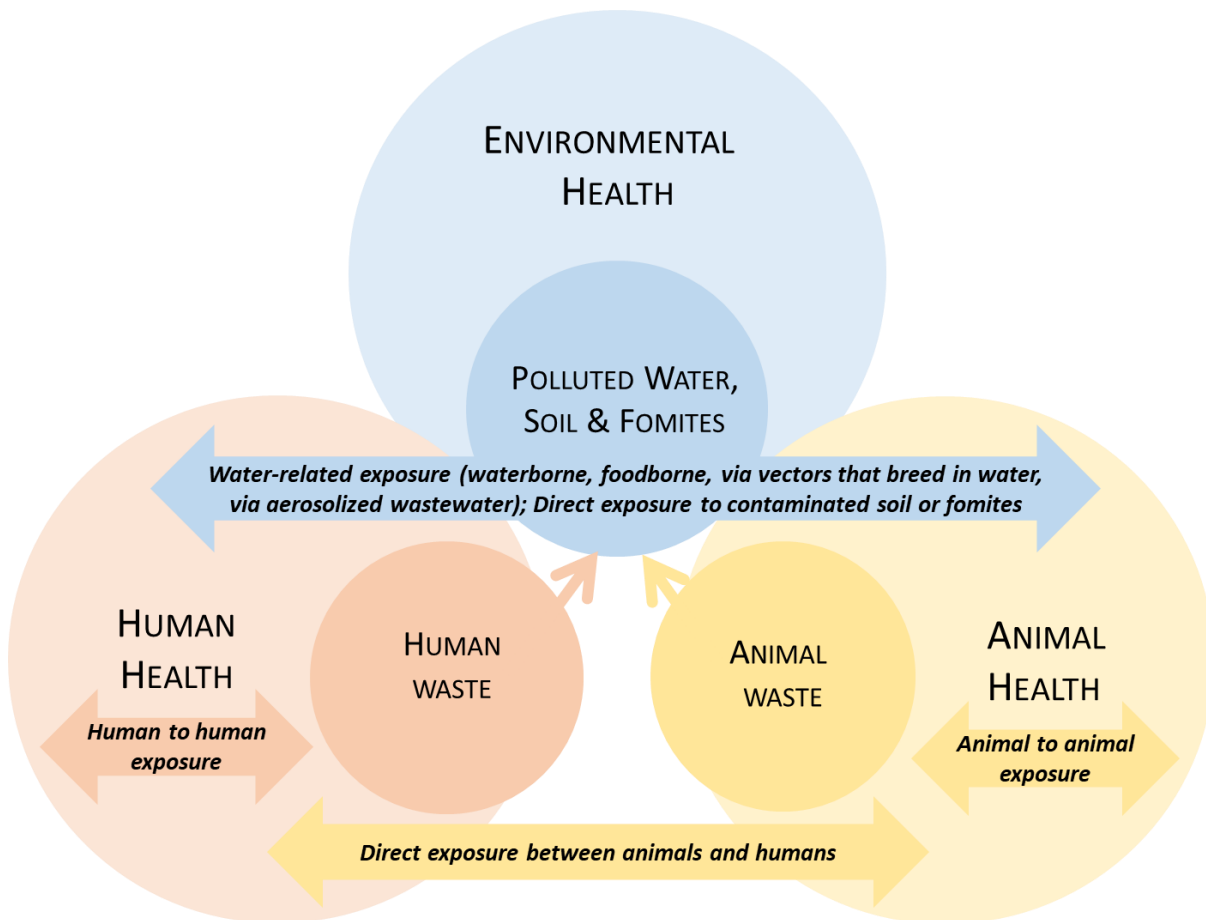


Figure 2.1: Schematic representing the relevance of One-Health to viral disease.

2.3. Burden of Viral Disease

Communicable disease is one of the leading causes of death worldwide. Lower respiratory infections were responsible for 3.0 million deaths in 2016 according to the World Health Organization (WHO), and diarrheal infections contributed to another 1.4 million deaths in the same year [2]. Viral diseases contribute to these categories; influenza, coronavirus, and

adenovirus are all considered lower respiratory infections, and viruses such as rotavirus can cause diarrheal disease. Viral disease outbreaks occur often, with WHO reporting outbreaks of influenza, coronavirus, hepatitis E, yellow fever, Ebola virus, Zika virus, poliovirus, dengue fever, and chikungunya in 2017 alone, located in countries all over the world such as Brazil, Chad, China, France, Italy, Saudi Arabia, and Sri Lanka [3].

WHO gathers surveillance statistics for specific viruses and estimates between 290,000 to 650,000 annual deaths from influenza, greater than previous estimates [4]. Data from February 2018 indicated that the disease burden of influenza was highest in north and east Africa, South America, and Europe [5]. Data from the WHO Mortality Database shows over 100,000 deaths from viral hepatitis since 2012 throughout the world [6]. Outbreaks of gastrointestinal disease are also common around the world. Rotavirus, for example, is associated with high rates of pediatric mortality; rotavirus infection was found to be responsible for approximately 453,000 pediatric deaths in 2008 worldwide, accounting for 5% of all deaths in children younger than five years [7]. Viral disease also disproportionately impacts poorer communities around the world. The aforementioned rotavirus study determined that over half of the pediatric rotavirus deaths worldwide occurred in just five developing nations (Democratic Republic of the Congo, Ethiopia, India, Nigeria, and Pakistan) [7]. Academic studies assessing global disease burden also report substantial burden due to viral disease. One study investigating global foodborne disease burden reported approximately 684 million disease cases and 212,000 deaths due to norovirus globally for the year 2010, the largest for any pathogen studied [8]. The same study found hepatitis A virus responsible for approximately 47 million illnesses and 94,000 deaths in 2010 [8].

Beyond diseases arising from direct infection, there are other secondary diseases associated with viruses, such as cervical cancer, which is strongly associated with papillomavirus [9]. Other viruses have also been linked to increased incidences of heart disease [10,11] and kidney disease [12], particularly in immunocompromised patients. Additionally, it is thought that the true impact of viral disease is underestimated. Many disease outbreaks are reported to be caused by agents of unknown etiology, and some of these outbreaks are suspected to be viral in origin [13]. A One-Health approach could assist in discovering the origin of these disease outbreaks.

In the United States, the Centers for Disease Control (CDC) publish surveillance statistics regarding the rate and occurrence of disease for a number of human viruses, including influenza [14], adenovirus [15], hepatitis A virus [16], rotavirus [17], and West Nile virus [18]. Annual summaries of these surveillance statistics are published in various forms from the CDC. The Summary of Notifiable Diseases (SoND) is an annual report containing information on those diseases for which “regular, frequent, and timely information regarding individual cases is considered necessary for the prevention and control of the disease or condition”, a list of which is updated regularly by the CDC. Viruses reported in the SoND include hepatitis A virus, West Nile virus, and Dengue virus [19]. The CDC also maintains the National Outbreak Reporting System (NORS), which includes information on the number of disease cases and outbreaks for a number of infectious agents, including norovirus, rotavirus, and sapovirus. Influenza statistics are reported most frequently by the CDC via published FluView Weekly Influenza Surveillance Reports, documenting the number of cases of influenza and influenza-like illnesses in the United States.

Each of these sources includes both temporal and geographic data regarding disease cases. This allows for the analysis of viral disease trends on both a monthly and spatial basis. Figure 2.2 presents the number of disease cases by month for influenza A as reported by FluView, West Nile virus and hepatitis A virus as reported by SoND, and norovirus, sapovirus, and rotavirus as reported by NORS from 2012 to 2016 [19–25]. Each of the six viruses exhibit different times of year in which disease cases are more prevalent. Insect-related viruses such as West Nile virus are more common in the warmer months from July to September. Meanwhile, the waterborne viruses (norovirus, sapovirus, rotavirus, and hepatitis A virus) all exhibit different trends. Perhaps most notable is the distinction between norovirus, which is most common in the winter from January to March, and sapovirus, which is most common in autumn from September to November. Norovirus and sapovirus are closely related, both being members of the *Caliciviridae* family, yet they have strikingly different seasonal infection trends. Hepatitis A virus, on the other hand, does not show significant variation throughout the year. Rather, rates of infection are relatively constant from one month to the next.

In addition to temporal variations, virus outbreaks also exhibit spatial variations, with certain areas being more commonly affected than others. The aforementioned CDC sources also publish information regarding the disease cases for each individual state. Figure 2.3 presents heatmaps of disease cases relative to state population for the six viruses mentioned above. West Nile virus is more prevalent in the plains states of the central United States, while norovirus is most common in the Midwest and New England. Moreover, there is no significant spatial differentiation for hepatitis A virus from one region to another, mimicking its temporal trends. Rotavirus and sapovirus, meanwhile, tend to be concentrated in specific states, suggesting that outbreaks are the most common drivers of occurrence of these diseases. It is important to note,

however, that these statistics are only a measure of reported cases, and that the actual incidence of viral disease could be significantly higher than the reported statistics indicate. For example, the CDC estimates that the rates of hepatitis A virus are approximately twice as high as reported incidence rates indicate [16].

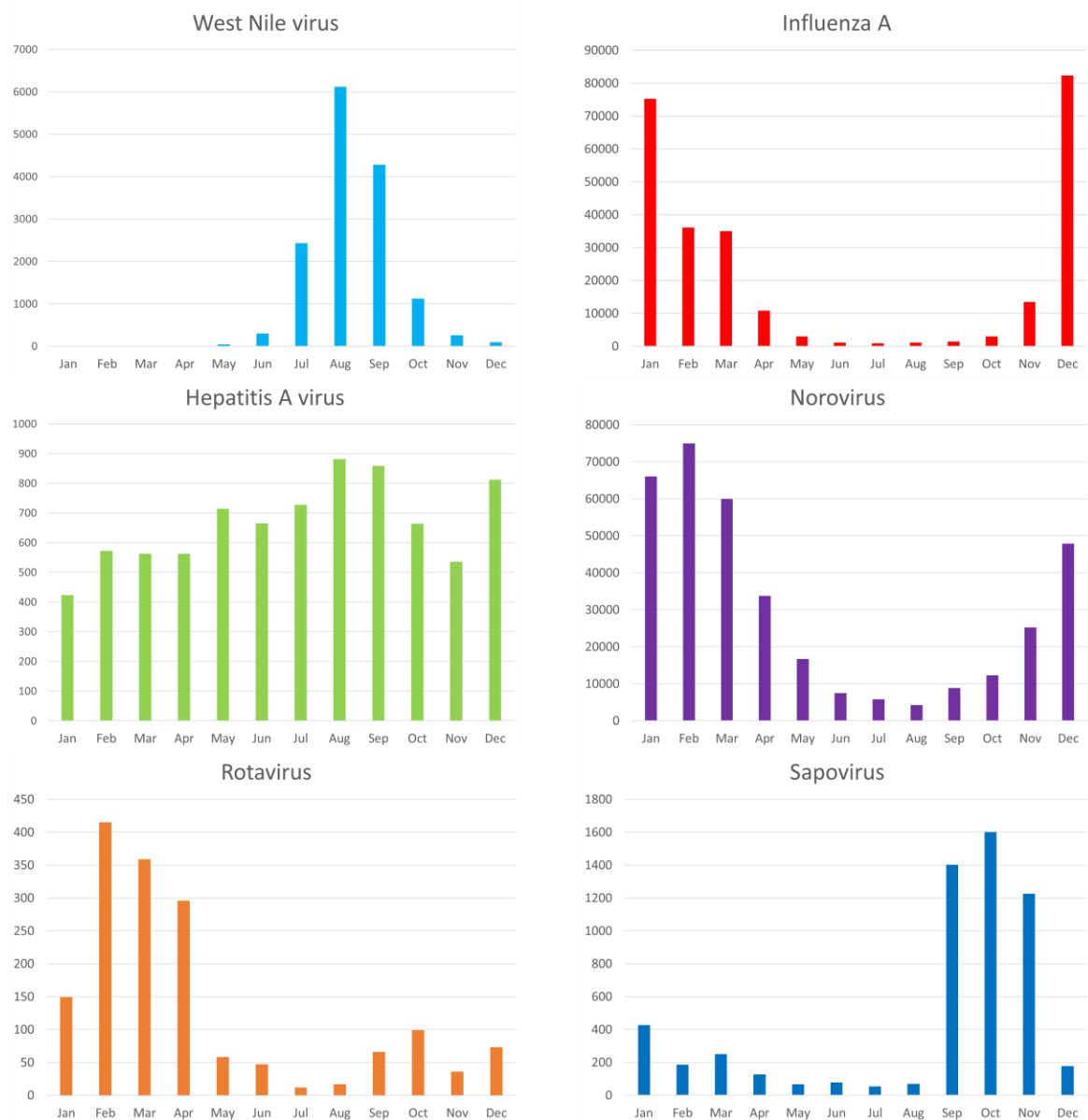


Figure 2.2: Disease cases by month as reported by SoND (West Nile virus, Hepatitis A virus) NORS (norovirus, sapovirus, rotavirus) and FluView (influenza A) for 2012-2016 [19–25]. Data summarized by the authors.

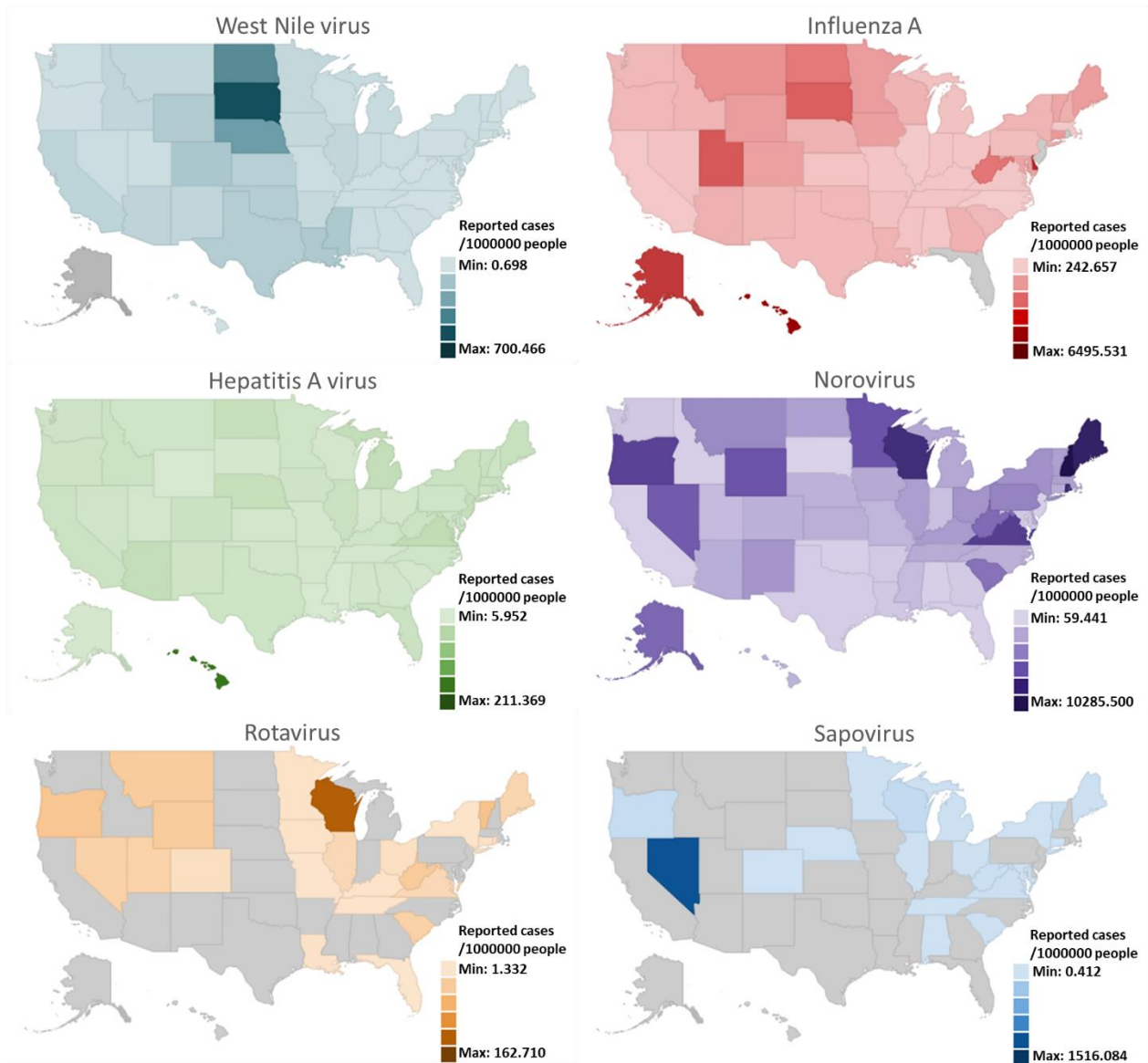


Figure 2.3: Heatmaps of disease cases relative to population in the United States for 2012-2016 as reported by the CDC [19–25]. Data summarized by the authors.

2.4. Viruses of Concern

Viruses that infect humans can be both specific to humans and zoonotic in nature. Human viruses are categorized as those that exclusively infect humans and are transmissible from the environment to humans or from human to human. Zoonotic viruses, meanwhile, are defined as

viruses “which are naturally transmitted between vertebrate animals and man” [26]. Zoonotic viruses can also be further split into direct and indirect categories. Direct zoonoses involve infection via direct contact between humans and animals, such as skin contact, a bite, or ingestion of tissue. Indirect zoonoses, meanwhile, require a vector or vehicle for transmission of the virus between humans and animals [26].

Viruses can also be divided to categories based on their water-related transmission potential. This classification was put forth by Bradley (1977), splitting water-related infections into four main categories: water-washed infections (diseases arising from poor hygiene) and water-based infections (infections from worm parasites that spend their life cycle in an aquatic environment), as well as waterborne infections and infections with water-related insect vectors, the latter two designations being most relevant when discussing water-related viruses [26–28]. The foremost category is that of waterborne viruses, in which a virus is present in water and infection occurs via ingestion of the contaminated water source. Waterborne viruses will often enter the water source due to fecal contamination, making waste and wastewater management a critical pathway for tracking the spread of viral waterborne disease. The second important category of water-related viruses are those with water-related insect vectors [26]. This includes viruses transmitted by insects that breed in water, such as mosquitos, which carry numerous significant human viruses, such as Zika virus and West Nile virus. In areas where primary water sources may be infested with these insect vectors, this is critical pathway for the spread of viral disease. Finally, another potential transmission pathway for water-related disease is the aerosolization of contaminated water [26,29], in which viruses capable of respiratory transmission are inhaled following aerosolization.

With these categories of zoonotic and water-related viruses in place, there is potential for crossover amongst them; some viruses may be both zoonotic and water-related. In a report on waterborne zoonoses in 2004, WHO put forth criteria for determining if a pathogen meets these qualifications: the pathogen must spend part of its life cycle within animal species, it is probable the pathogen will have a life stage that will enter water, and transmission of the pathogen between humans and animals must be through a water-related route. If a virus meets all of these, it can be classified as a zoonotic water-related virus.

Table 2.1 lists several human viruses of concern (including all viruses included in SoND) and classifies them according to the aforementioned categories. As mentioned above, a primary exposure pathway to viral disease for humans is wastewater. The ability to detect viruses in wastewater is therefore critical for investigation via One-Health, and this information is also summarized in Table 2.1. As noted in the table, several of these viruses fall under multiple categories, being both water-related and zoonotic. For instance, enteroviruses, hepatitis E virus, and rotaviruses are all classified as waterborne viruses, and each of them has been reported to have potential zoonotic properties as well, as cases of these viruses have been observed in animals [26]. Additionally, a number of viruses are zoonotic due to their transmission between mosquitos and humans, such as West Nile virus, Zika virus, and Dengue virus [30–32]. Zoonotic diseases comprise approximately 64% of all human pathogens, with viruses accounting for 5% of pathogens [33]. Zoonoses are also responsible for 26% of the disease burden in low-income countries, whereas they only account for 0.7% in wealthier nations [34].

Table 2.1: Categorization of notifiable, waterborne, water-related, and potentially water-related human viruses of concern [26,30–32,35–51]. ^aIncluding California serogroup, eastern equine encephalitis, Powassan, St. Louis encephalitis, and western equine encephalitis viruses.

^bIncluding Lassa, Lujo, Guanarito, Junin, Machupo, and Sabia viruses.

Virus	Transmission Route				Detected in Wastewater or Human Excrement
	Human-to-Human Transmission	Zoonotic	Waterborne	Water-Related	
Adenoviruses	✓		✓		✓
Astroviruses	✓		✓		✓
Enteroviruses	✓	✓	✓		✓
Hepatitis A virus	✓		✓		✓
Hepatitis E virus	✓	✓	✓		✓
Noroviruses	✓		✓		✓
Rotaviruses	✓	✓	✓		✓
Aichi virus	✓			✓	✓
Polyomaviruses	✓			✓	✓
Salivirus	✓			✓	✓
Sapovirus	✓			✓	✓
Torque Teno virus	✓			✓	✓
Dengue virus		✓		✓	✓
West Nile virus		✓		✓	✓
Zika virus		✓		✓	✓
Yellow fever virus		✓		✓	✓
Chikungunya virus		✓		✓	
Rift Valley fever virus		✓		✓	
Coronaviruses	✓	✓			✓
Ebola virus	✓	✓			✓
Influenza	✓	✓			✓
Herpesvirus	✓				✓
Papillomavirus	✓				✓
Parechovirus	✓				✓
Arboviral diseases ^a		✓			
Hepatitis B virus	✓				
Hepatitis C virus	✓				
HIV	✓				
Rabies		✓			
Rubella	✓				
Smallpox	✓				
Varicella	✓				
Crimean-Congo hemorrhagic fever virus	✓	✓			

Table 2.1 (cont'd).

Marburg virus	✓	✓			
Arenaviruses ^b	✓	✓			

Regarding animal viruses of concern, the U.S. Department of Agriculture (USDA) issues annual reports of the domestic status of reportable diseases put forth by the World Organization for Animal Health (OIE) [52]. Table 2.2 summarizes livestock viral diseases that were reported as present in the United States by the USDA. Many of the same viral families that affect humans are represented in this list, including *Coronaviridae*, *Flaviviridae*, *Herpesviridae*, *Orthomyxoviridae*, and *Reoviridae*. A few of the reportable viral animal diseases are also considered zoonotic, which are of even greater significance to human health.

Table 2.2: Summary of viral OIE reportable diseases (2016) for livestock [52–62].

Disease	Virus	Viral Family	Animals Affected	Zoonotic	Water-Related	Detected in Animal Waste
Aujeszky's disease	Suid herpesvirus 1	<i>Herpesviridae</i>	Swine	✓		✓
Avian infectious bronchitis	Avian IB virus	<i>Coronaviridae</i>	Birds			✓
Avian infectious laryngotracheitis	Gallid herpesvirus 1	<i>Herpesviridae</i>	Birds			✓
Avian influenza	Influenza A virus	<i>Orthomyxoviridae</i>	Birds, Mammals	✓		✓
Bluetongue	Bluetongue virus	<i>Reoviridae</i>	Ruminants		✓	
Bovine viral diarrhea	BVD virus 1	<i>Flaviviridae</i>	Cattle			✓
Caprine arthritis/encephalitis	CAE virus	<i>Retroviridae</i>	Goats			
Eastern equine encephalitis	EEE virus	<i>Togaviridae</i>	Equines	✓		
Epizootic hemorrhagic disease	EHD virus	<i>Reoviridae</i>	Ruminants		✓	
Equine herpesvirus 1	EHV-1	<i>Herpesviridae</i>	Equines	✓		
Equine infectious anemia	EIA virus	<i>Retroviridae</i>	Equines			
Equine influenza	Influenza A virus	<i>Orthomyxoviridae</i>	Equines			✓
Equine viral arteritis	Equine arteritis virus	<i>Arteriviridae</i>	Equines			

Table 2.2 (cont'd).

Infectious bovine rhinotracheitis	Bovine herpesvirus 1	<i>Herpesviridae</i>	Cattle			✓
Infectious bursal disease	IBD virus	<i>Birnaviridae</i>	Birds			
Maedi-visna	Visna virus	<i>Retroviridae</i>	Sheep			✓
Myxomatosis	Myxoma virus	<i>Poxviridae</i>	Rabbits			
Newcastle disease	Avian avulavirus 1	<i>Paramyxoviridae</i>	Birds	✓		✓
Porcine reproductive and respiratory syndrome	PRRS virus	<i>Arteriviridae</i>	Swine			
Rabies	Lyssaviruses	<i>Rhabdoviridae</i>	Mammals	✓		
Transmissible gastroenteritis	TGE coronavirus	<i>Coronaviridae</i>	Swine			✓
Turkey rhinotracheitis	Avian metapneumovirus	<i>Paramyxoviridae</i>	Birds			
West Nile fever	West Nile virus	<i>Flaviviridae</i>	Mammals, birds	✓		✓

The USDA also collects and maintains disease data for domesticated, agricultural, and wild animals, primarily via the National Animal Health Surveillance System (NAHSS). A number of viruses are investigated via NAHSS for various animals, including influenza A virus in swine [63], and herpesvirus and West Nile virus in horses [64,65]. Annual reports regarding cases of equine West Nile virus in domesticated horses are published via NAHSS, making them a useful comparison to reported human cases of West Nile virus [65]. In addition to domesticated animals, wild animals are also an important consideration, as wildlife has been shown to be a source of disease to both livestock and humans [61].

2.5. One-Health Methodology: Current Status

The One-Health approach has recently been applied to combat zoonotic viral disease, spearheaded by the veterinary community. Viral infection in humans can be prevented with the use of vaccinations, a common practice for a number of viruses for which vaccines have been developed, including influenza [66], poliovirus [67], and rotavirus [17,68]. Vaccines are known

to lessen the disease burden in a population both by preventing infection and promoting herd immunity [69].

In cases for which the pathway to human infection has an intermediary animal vector between the host organism and humans, the vaccination of the intermediary animal could also prove vital. This was performed in one of the most successful One-Health implementations to date, for a Hendra virus outbreak in Australia. Hendra virus is one of several zoonotic viruses, including Nipah virus, Tioman virus, and lyssavirus, that originate in bats. It was determined that Hendra virus is first transmitted from bats to horses before being transmitted to humans, and no direct infection between bats and humans was observed. Therefore, a Hendra virus vaccine was developed for horses in order to eliminate the transmission route to humans [70].

On different occasions the strategy is to eliminate or lessen the disease vector responsible for transmission of the disease to humans; for example, mosquito control strategies can be utilized to lessen the burden of West Nile virus, Zika virus, and other viruses for which mosquitos are the primary transmission vector. Chemical methods of mosquito control (such as insecticides, insect growth regulators, and sprays) are commonly used, though these methods could also have an adverse effect on the health of humans, animals, and the surrounding environment, which are of important consideration to the One-Health approach. More “eco-friendly” options are also recently in use, such as sterile insect techniques and plant-based non-harmful mosquitocidals [71]. A One-Health approach has been shown in the past to be effective at reducing costs and improving efficiency in the mitigation of Rift Valley fever virus to improve public health [72].

Smaller-scale policy changes that focus on particular viruses could also help to curb the spread of viral disease. For example, after it was determined that bats were the host species and

civets the transmittance vector species for SARS coronavirus, affected areas (such as China) enacted bans on civet trading and the mixing of bats with other species in local markets [70]. This shows that policies can be created to require the use of interventions to block exposure pathways for particular viruses. Public education is also a useful approach, utilized with outbreaks of Nipah virus in Malaysia and Bangladesh, in which people were encouraged to avoid direct contact with bats and taking preventative measures to minimize the chances of viral transmission [70].

2.6. One-Health Methodology: Proposed Approach for Water-Related Viruses

Still, these One- Health success stories have focused on zoonotic viruses, concentrating on how animal health relates to human health. Because viruses can be transmitted in a variety of exposure pathways, environmental health is also of vital importance. While environmental considerations have begun to be considered on a more local scale [73], these aspects have still not been as thoroughly investigated using the One-Health approach. Environmental engineers and scientists therefore have a critical role in the application of the One-Health methodology.

In the application of the One-Health concept to combat water-related viral disease, a three-tiered approach is appropriate (Figure 2.4). The first step is to identify critical pathways of exposure. The goal of this step is to identify and prioritize environmental virus reservoirs and critical exposure pathways that facilitate transmission and transport of viral disease amongst humans and animals. The second step is to design surveillance of the critical environmental reservoirs and pathways. The goal of this step is to identify critical times and critical locations for the onset of water-related viral outbreaks. The final step is to design intervention approaches. The goal of this step is design barriers to interrupt critical pathways at critical times and locations.

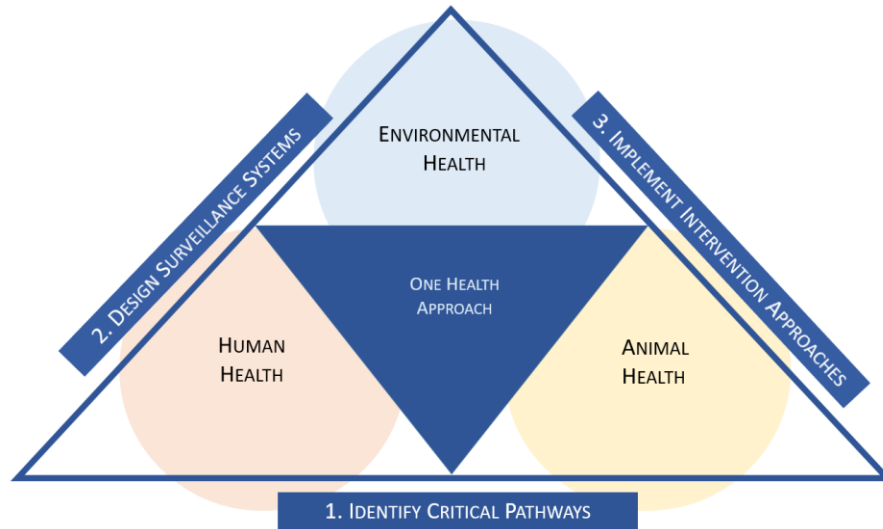


Figure 2.4: Concept map of the proposed One Health framework.

2.6.1. Identification of Critical Pathways

To determine critical pathways of exposure the following should be identified: 1) potential sources/reservoirs of viruses in environmental, human and animal systems; 2) natural processes that affect transport within and between systems; 3) human behaviors that affect exposure to viruses, such as management practices for water, wastewater, agricultural waste, human disease determinants, and animal disease determinants. The goal of this step is to prioritize the most critical reservoirs and exposure pathways for viruses. Figure 2.5 presents an example of water-related pathways that include urban and rural areas in the US.

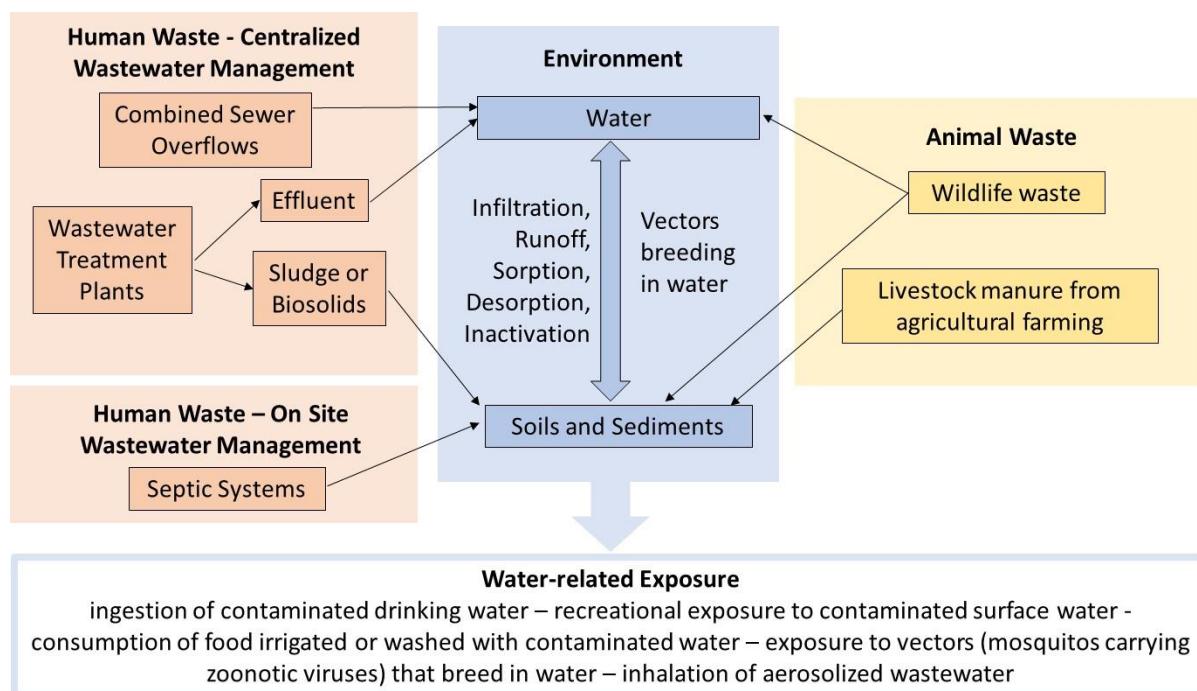


Figure 2.5: Example of water-related pathways in the United States.

There are several potential pathways by which humans are exposed to waterborne or water-related viruses. The foremost among them is the ingestion of contaminated water. Surface water, treated or untreated, may be used as a drinking water source, and there are a number of pathways for viral contamination of surface water. In urban areas, combined sewer overflows during high rainfall events can introduce untreated wastewater into surface water bodies. In impoverished areas, people often dispose untreated wastewater into surface water bodies that are used elsewhere for drinking water, for example downstream of a river. Even treated wastewater effluent, which is often released into surface water, can contain detectable concentrations of human viruses [74]. Treated or untreated livestock waste and wildlife waste is also washed off the land during precipitation events and can be carried into surface water bodies via runoff, and animal wastes have been shown to be a potential exposure pathway of disease to humans [75]. In addition to ingestion of water, recreational exposure to contaminated surface water, for example

in public swimming pools or on public beaches, can be a pathway of viral infection via surface water [76,77]. Groundwater and/or aquifers are also used as drinking water sources around the world, and various pathways exist for the contamination of these sources. For example, in rural areas which dispose of wastewater in private septic systems, the leakage of these septic tanks may allow for the leaching of contaminated wastewater into a groundwater source [78,79]. Another pathway for exposure to waterborne disease is the consumption of food that has been contaminated during the agricultural process. This could be due to irrigation using contaminated water as well as uptake from contaminated soil or sediment. Biosolids (treated wastewater sludge) are often used as an agricultural soil amendment, and viruses are known to have been detected in these biosolids [13,80]. Manure or livestock is a possible source of contamination, whether it is used as a fertilizer or transported in the agricultural environment [81]. Wildlife waste could also contaminate soils and sediments, just as it can contaminate surface water [82].

Data collection is crucial to attain preliminary information for the identification of critical pathways for viral transmission. Numerous governmental agencies publish data regarding clinical cases of disease both spatially and temporally. These data can be collected and analyzed to obtain an understanding of the distribution of disease outbreaks in a region. Animal disease data can also be collected and analyzed in this manner, but there is a need for an integrated human-animal disease surveillance to assess zoonotic disease occurrence [83]. Additionally, appropriate data that may indicate correlations with spatial and temporal patterns may be compiled and analyzed. Numerous factors may contribute to the likelihood of infectious disease in certain areas or time periods, including but not limited to hydrological patterns (e.g. precipitation) [84], land use [85], human/livestock/wildlife population density [86], and others. Potential pathways can be prioritized to determine which are most relevant to the region being

studied. A system of weight factors could be developed to perform prioritization quantitatively with the use of a statistical model. For example, the degree of regulation of wastewater, storm-water, and livestock waste could impact the importance placed upon the potential pathways associated to the impact of waste disposal systems.

2.6.2. Design of Surveillance Systems

The second step in the framework is to design surveillance systems of the critical environmental reservoirs and pathways that will allow for early detection of outbreaks. The objective is to identify critical times and critical locations for the onset of viral outbreaks. This can be achieved by monitoring viral disease indicators (such as concentrations of viruses or other indicators) in critical reservoirs identified in the previous step. The approach includes environmental sampling (such as polluted wastewater from a particular population), as well as clinical samples from infected people, livestock and wildlife. Regular monitoring of critical reservoirs will identify peaks in viral concentrations or indicators that in turn can be related to early signals of disease outbreaks.

Because wastewater is an important reservoir and transmission pathway for viruses, it is a medium that can be seriously considered as a point of surveillance. Figure 2.6 presents an example of potential surveillance systems that could be implemented. Urban communities offer a convenient point of sampling at the influent of a wastewater treatment plant. Untreated wastewater can be considered as a population sample for the serviced community. Wastewater can therefore be used as an epidemiological tool to help identify potential viral outbreaks.

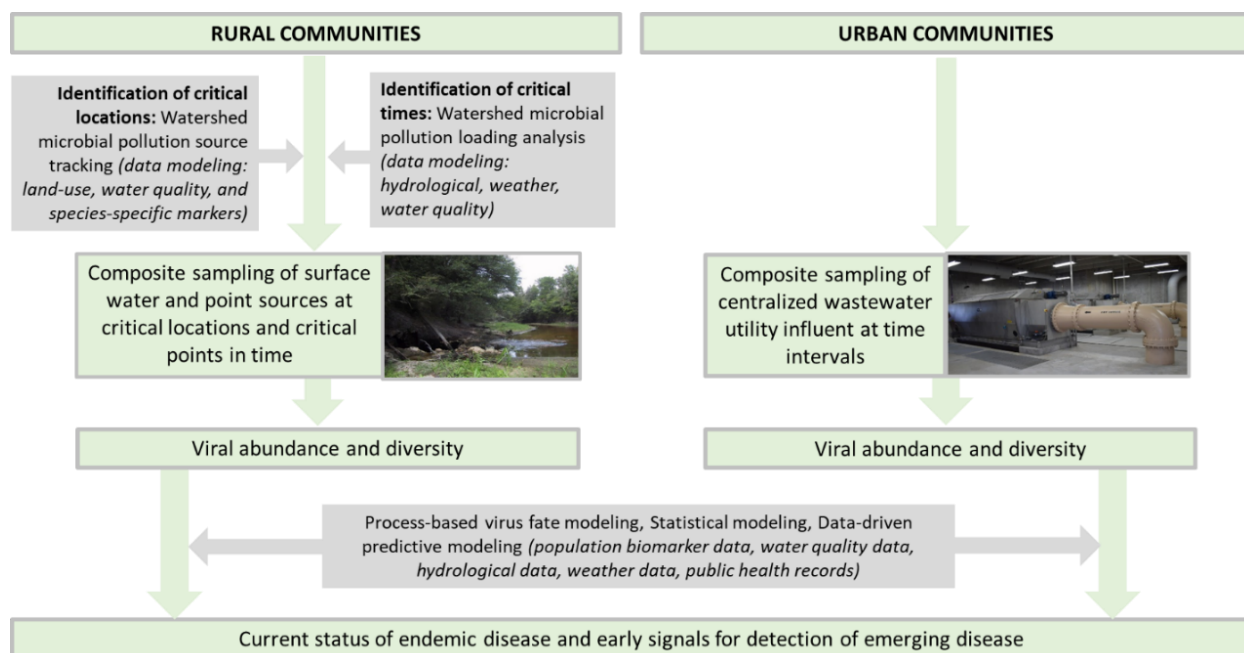


Figure 2.6: Proposed surveillance system in the Great Lakes Region. Notes: sampling and characterizing community wastewater, livestock manure, and wildlife waste represents a snapshot of the status of community human and animal health.

The goal of wastewater-based epidemiology is to sample community wastewater, or polluted water, and identify spikes in concentrations of excreted viruses. This method can determine that an outbreak may be taking place before clinical cases are reported. Already employed in Europe to quantify illicit drug use in a population [87,88], wastewater-based epidemiology can also be applied to quantify the approximate concentrations of viruses in the population serviced by a wastewater treatment plant. Urban wastewater treatment plants that serve metropolitan areas sometimes have several interceptors at which wastewater is collected. Sampling at each interceptor and mapping each interceptor to the specific neighborhoods it serves can facilitate virus occurrence data collection representative of each serviced area of the city. Should viral concentrations be observed to be higher in one interceptor than the rest, the corresponding serviced area would therefore be of greater concern for a potential viral outbreak. Sampling in rural areas is more complex and necessitates the determination of where in the

environment to sample, which can be based upon watershed modeling and microbial source tracking.

Several factors are of importance to attain reliable data when using wastewater-based epidemiology. Normalization of population is vital to ensure that a significant increase in viral concentration in a wastewater sample does not correspond to an increase in population in the serviced area. This can be performed with the quantification of biomarkers in the wastewater sample, substances that are naturally excreted by humans in constant quantities. Other factors that need to be considered include shedding rate (the number of viruses excreted by infected humans) and natural degradation (the rate at which viruses degrade in the wastewater environment). Comparison and correlation with clinical data in the surrounding community is also a valuable tool to confirm the credibility of the methodology.

While wastewater-based epidemiology can primarily be utilized for the examination of waterborne viruses, it has the potential to be applied to non-waterborne viruses as well, provided those viruses can be detected in wastewater or human waste. As shown in Table 2.1, numerous viruses not typically classified as waterborne meet these criteria, such as influenza, coronavirus, herpesvirus, dengue virus, and Zika virus. In addition to the surveillance of wastewater, it is also prudent to perform surveillance of agricultural livestock and other domesticated animals. Other critical pathways identified in step one (see Figure 2.5) could fall under this methodology as well.

The central premise of the proposed surveillance approach is that community fecal pollution represents a snapshot of the status of public health or livestock health. Traditional human and livestock disease detection and management systems are based on diagnostic analyses of clinical samples. However, these systems fail to detect early warnings of public

health threats at a wide population level and fail to predict outbreaks in a timely manner.

Wastewater analysis, manure analysis, or polluted water analysis is equivalent to obtaining and analyzing a community-based urine and fecal sample of the representative sub-watershed.

Monitoring temporal changes in pathogen concentration and diversity excreted in a sub-watershed allows early detection of outbreaks (critical moments for the onset of an outbreak). In addition, carefully designed spatial sampling will allow detection of locations where an outbreak may begin to develop and spread (critical locations for the onset of an outbreak). Modeling the fate of pathogens, including shedding rates, transport, growth and inactivation processes in the environmental, are critical for the effectiveness of the proposed method.

2.6.3. Intervention Approaches

The third and final step is to design intervention approaches. The goal is to design barriers to interrupt critical pathways at critical times and locations. These interventions may include: (1) sustainable engineering technologies for human and animal water/wastewater/waste management, (2) medical and veterinary interventions to manage infections, and (3) education of local communities and governance to modify human behavior, current practices and policy based on critical pathways and relationships between environmental health, human health and animal health.

The foremost intervention for waterborne viruses is that of drinking water and wastewater treatment facilities. Both types of treatment plants provide the most immediate barrier between a drinking water source and consumption and utilize several unit processes, such as filtration and disinfection, to ensure the removal of pathogens, including viruses, from water. Both types of treatment plants have been shown to be effective at reducing the concentrations of human viruses from influent to effluent, but it has also been shown that wastewater treatment plants may release

viruses in effluent [13,74,89–94]. There also exist interventions to prevent non-point-source pollution of water sources. One such intervention is that of watershed protection plans set by the states. Similarly, stormwater management is implemented in several states based on multiple strategies [95–98].

Numerous policy measures in the United States and abroad can be considered examples of the permanent implementation of interventions. Since the Safe Drinking Water Act in 1974, a number of additional policies have been put into effect to strengthen water quality and prevent disease. The EPA sets Maximum Contaminant Levels (MCLs) for several contaminants, including viruses; drinking water treatment facilities are required to attain a 4-log reduction in viral concentration to meet the MCL for viruses [99]. Another example, the Groundwater Rule, was put into effect in 2006 and requires the regular surveillance of groundwater sources that are used for drinking water to ensure that MCLs for pathogens are met [100]. There still exists a need, however, for the regulation of animal waste products, especially in rural areas in which animal waste is determined to be a critical pathway for viral transport.

The modification of human behavior is also imperative to minimize the transmittance of viral disease along pathways in which interventions cannot be performed for reasons of cost, capability, or convenience. The primary method of altering behavior is education. This applies to the education of medical professionals, both doctors and veterinarians, and environmental professionals in One-Health approaches. It is also critical to educate the public, to prevent situations in which people are leaving themselves vulnerable to transmission of disease. Especially in impoverished, high-risk areas, robust measures should be taken to educate the public on the concept of the critical pathways of transmission of viral disease.

2.7. Conclusions

Viral transmission involves complex systems that include interactions between humans, animals and the environment. Understanding the interactions between the involved human, animal and environmental systems, and the processes within each of the systems, is critical for efficient prevention and minimization of viral outbreaks. These systems vary in both spatial and temporal scales. For example, urban systems are different than rural systems, and wet weather is different than dry weather with regards to the potential for viral transmission. The most important step in the process of understanding water-related transmission is the identification of critical viral reservoirs and critical transport pathways in a certain environment at a certain time.

Much of the One-Health based approaches to manage viral disease that have been utilized thus far have been responsive in order to control an existing outbreak. Identification and surveillance of critical pathways of potential exposure can allow early detection of outbreaks at a population level, which is a critical first step for prevention. While it involves the interconnectivity of human, animal, and environmental health, One-Health has still only primarily been embraced by the veterinary community. More deliberate efforts should be made to encourage environmental professionals to analyze the issues of viral disease through a One-Health lens. Only through the extensive participation of all related field stakeholders can One-Health truly reach its potential to mitigate viral disease.

Competing Interests

The authors declare that they have no competing interests.

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Chapter 3:

Wastewater-Based-Epidemiology for Early Detection of Viral Outbreaks

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3.1. Introduction

The immense global burden of infectious disease outbreaks and the need to establish prediction and prevention systems has been recognized by the World Health Organization (WHO), the National Institutes of Health, the United States Agency of International Development, the Bill and Melinda Gates Foundation, and by the international scientific community. Despite multiple efforts, this infectious burden is still increasing. For example, it has been reported that between 1.5 and 12 million people die each year from waterborne diseases [1,2] and diarrheal diseases are listed within the top 15 leading causes of death worldwide [3]. Rapid population growth, climate change, natural disasters, immigration, globalization, and the corresponding sanitation and waste management challenges are expected to intensify the problem in the years to come.

Most infectious disease outbreaks in the U.S. have been related to microbial agents [4–7]. In the vast majority of cases, the infectious agents have not been identified. However, the Environmental Protection Agency (EPA) suggests that most outbreaks of unidentified etiology are caused by viruses [8]. Viruses have been cited as potentially the most important and hazardous pathogens found in wastewater [9] and are included in the EPA contaminant candidate list. Viruses can lead to serious health outcomes, especially for children, the elderly and immunocompromised individuals, and are of great concern because of their low infectious dose,

ability to mutate, inability to be treated by antibiotics, resistance to disinfection, small size that facilitates environmental transport, and high survivability in water and solids.

Infectious outbreaks can cause uncontrollable negative effects especially in dense urban areas. Traditional disease detection and management systems are based on diagnostic analyses of clinical samples. However, these systems fail to detect early warnings of public health threats at a wide population level and fail to predict outbreaks in a timely manner. Classic epidemiology observes disease outbreaks based on clinical symptoms and infection status but does not have the ability to predict “critical locations” and “critical moments” for viral disease onset. Recent research efforts in developing optimized detection systems focus on rapid methods for analyzing blood samples, but this approach assumes that patients are examined at a clinical setting after the outbreak has been established and recognized.

The central premise of the proposed approach is that community wastewater represents a snapshot of the status of public health. Wastewater analysis is equivalent to obtaining and analyzing a community-based urine and fecal sample. Monitoring temporal changes in virus concentration and diversity excreted in community wastewater, in combination with monitoring metabolites and biomarkers for population adjustments, allows early detection of outbreaks (critical moments for the onset of an outbreak). In addition, carefully designed spatial sampling will allow detection of locations where an outbreak may begin to develop and spread (critical locations for the onset of an outbreak).

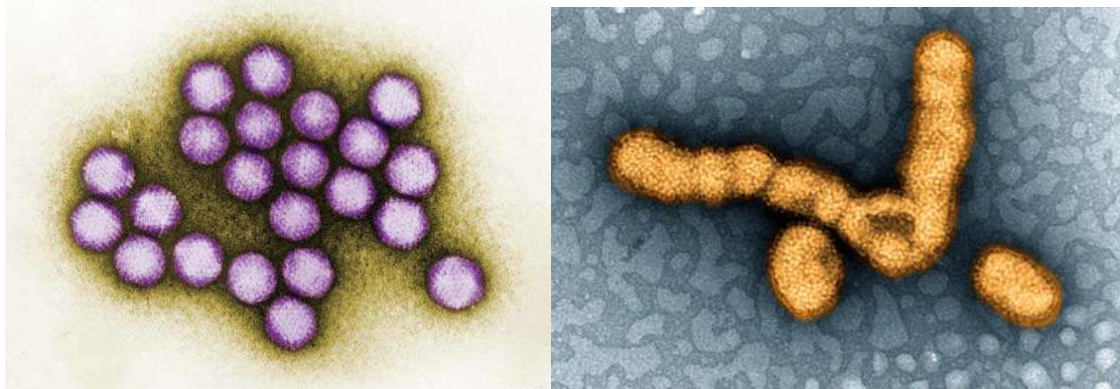


Figure 3.1: Photomicrograph of adenovirus particles (left) and influenza virus particles (right). Adenovirus image from Dr. G. William Gary, Jr./CDC, influenza image from National Institute of Allergy and Infectious Diseases.

3.2. Background

Similar detection systems have been used for the investigation of illicit drugs in various locations around the world [10–12]. The approach was first theorized in 2001 [10] and first implemented and reported for several illicit drugs in 2005 where the method was termed sewage epidemiology [11]. The methodology considers raw untreated wastewater as a reservoir of human excretion products; among these products are the parent compounds and metabolites of illicit drugs. If these excretion products are stable in wastewater as they travel through the sewage system, then the measured concentration from a wastewater treatment plant (WWTP) could correspond to the amount excreted by the serviced population. Table 3.1 presents a summary of prior studies utilizing the wastewater-based-epidemiology methods to assess levels of various substances in a population.

Any substance that is excreted by humans and is stable (or has known kinetic pathways) in wastewater can be back-calculated into an initial source concentration. An important step in the application of wastewater-based-epidemiology is the estimation of the contributing

population and its sampled wastewater. Both census and biomarker data can be used in this approach to estimate the number of individuals that contribute to the wastewater sample.

Table 3.1: Summary of substances investigated via wastewater-based-epidemiology.

Substance	Country	References
Alcohol	Norway	[13]
Amphetamines	Australia, Belgium, Italy, Spain, South Korea, United Kingdom, United States	[14]
Cocaine	Australia, Belgium, Germany, Ireland, Italy, Spain, United Kingdom, United States	[14]
Counterfeit Medicine	Netherlands	[15]
Opiates	Germany, Italy, Spain, South Korea	[16]
Tobacco	Italy	[17]

3.3. Occurrence of Viruses in Wastewater

Waterborne viruses comprise a significant component of wastewater microbiota and are known to be responsible for disease outbreaks. A critical characteristic of viruses is that they do not grow outside the host cells. Therefore, viral concentrations in the wastewater stream will represent the concentrations excreted by the corresponding human population. Table 3.2 summarizes studies that detected waterborne and non-waterborne viruses in wastewater and human excrement.

3.3.1. Waterborne viruses

There are several groups of commonly detected and studied waterborne viruses, including adenoviruses, astroviruses, enteroviruses, hepatitis A and E viruses, noroviruses, and rotaviruses. Adenoviruses are known to cause gastroenteritis and respiratory disease [18] and have been linked to outbreaks of disease [19,20]. Adenoviruses are a commonly studied group of viruses in water. They are commonly detected in raw wastewater [21–36] and have been cited as among the most significantly abundant human viruses in wastewater [24,27,28,33,37]. Adenoviruses have also been detected in human excrement of infected persons, including both feces and urine [38–

47]. Studies have found the concentration of adenovirus in stool of infected persons to range from 10^2 to 10^{11} copies per gram with an average concentration in the range of 10^5 to 10^6 copies per gram of stool [39,41,42,46] as quantified by qPCR.

Astroviruses are a group of RNA viruses that have been linked to outbreaks of gastroenteritis [19,48]. They have been cited as one of the more important viruses associated with gastroenteritis [49] but they have not been as commonly studied in wastewater compared to other groups of human enteric viruses. Nonetheless, they have been detected using standard PCR in wastewater in prior studies [24,29,50,51]. They have also been detected in clinical samples of human excrement of infected people [43,44,47,52–54], making them a viable candidate for wastewater epidemiology. While qPCR has been used as a detection method for astroviruses in human feces [44,47,54], and for quantification purposes in wastewater [51], no cited studies have reported quantitative values for astroviruses in human excrement.

Enteroviruses comprise several types of human enteric viruses, including polioviruses, coxsackieviruses, and echoviruses [55,56]. Enteroviruses can cause an array of afflictions depending on type, including the common cold, meningitis, and poliomyelitis [57] and have been linked to outbreaks of these diseases [19]. Enteroviruses have been detected via PCR in raw wastewater by numerous studies [25,26,28,29,31,33,34,58,59], as well as detected in human feces [43,53,60–63]. qPCR has not as yet been extensively employed to quantify enteroviruses in stool samples, though one study determined the enterovirus load to be in the range of 1.4×10^4 to 6.6×10^9 copies per gram of stool [60].

Two species of hepatitis viruses, hepatitis A virus and hepatitis E virus, are considered to be waterborne viruses. Hepatitis is a liver disease that can cause numerous afflictions, including fever, nausea, and jaundice [64]. Hepatitis A virus has been linked to disease outbreaks [65], and

it has been suggested that even low levels of viral water pollution can produce infection [66]. Hepatitis A virus is often detected via PCR in raw wastewater [29,30,58,67,68] and several studies have also detected the virus in human stool samples [69–72]. Like enteroviruses, there has not been significant investigation into the quantification of hepatitis A virus in stool, though one study reported values in the range of 3.6×10^5 to 5.6×10^9 copies per gram of stool [70].

Hepatitis E virus, meanwhile, has only recently begun to become a pathogen of interest compared to other waterborne human viruses [73]. Like hepatitis A, hepatitis E virus can cause liver disease with many of the same symptoms; in fact, hepatitis E is not clinically distinguishable from other types of viral hepatitis infection [74]. While not investigated to the extent of other human enteric viruses, hepatitis E virus has been detected via PCR in raw wastewater [21,34,75]. There have also been studies that have detected hepatitis E virus in human stool samples [76–78]. One such study also used RT-qPCR to quantify the concentration of hepatitis E virus in stool and reported values in the range of 10^1 to 10^6 copies per μL of stool [77].

Noroviruses, also known as Norwalk-like viruses, are a genus of viruses within the *Caliciviridae* family. They are one of the more significant gastroenteritis-causing viral agents, considered to be a leading cause of the disease [79–81] and are commonly associated with disease outbreaks [19,82,83]. Noroviruses are one of the more commonly investigated and detected viruses in wastewater [24–26,28–30,32,33,36,59,60,84,85]. A number of studies have also investigated the presence of noroviruses in human feces [43,47,53,54,79,86–88]. One such cited study reported quantification values for norovirus in stool following qPCR, in the range of 9.7×10^5 to 1.1×10^{12} copies per gram, with a mean value of approximately 10^{11} copies per gram [87].

Rotaviruses are another primary cause of gastroenteritis with symptoms including diarrhea, vomiting, and fever, in accordance with other enteric viruses [89]. They are commonly detected via PCR in raw wastewater [29–31,36,50,58,59,90–92] and are commonly investigated and detected in human feces [43–45,47,53,93–95]. Like other waterborne viruses, though, only a handful of studies on rotaviruses have used qPCR as a detection tool, and none reported quantification values in terms of a number of copies.

In addition to the commonly investigated waterborne viruses described above, there are other human viruses that are commonly detected in wastewater and human stool but not as frequently studied, such as Aichi virus, polyomaviruses, salivirus, sapovirus, and torque teno virus. Aichi virus is a member of the *Picornaviridae* family, the same family as enteroviruses, and is believed to cause gastroenteritis [96]. Salivirus, another member of the *Picornaviridae* family, is also associated with gastroenteritis, as well as acute flaccid paralysis [97]. Sapovirus, like norovirus, is a member of the *Caliciviridae* family, and like its relative is a common cause of gastroenteritis [98]. Polyomaviruses are associated with a variety of diseases in humans, including nephropathy, progressive multifocal leukoencephalopathy, and Merkel cell carcinoma [99]. Torque teno virus is commonly detected in humans, but the clinical consequences of infection are unclear [100]. These viruses are included in Table 3.2.

3.3.2. Non-waterborne viruses

Non-waterborne viruses have also been detected in wastewater or human excrement (included in Table 3.2). While it is logical to investigate the applicability of waterborne viruses to wastewater-based epidemiology, it is also important to note the potential for other categories of viruses to fit into this methodology.

There exists a category of water-related viruses that are transmitted via insects (like mosquitos) that breed in water, such as Zika virus, West Nile virus, Rift Valley Fever virus, Yellow Fever virus, Dengue virus, and Chikungunya virus, in addition to confirmed waterborne viruses. These viruses also fall into the category of zoonotic viruses, which are viruses that can be transmitted between humans and animals. Other zoonotic viruses include avian influenza virus, SARS Coronavirus, Menangle virus, Tioman virus, Hendra virus, Australian Bat lyssavirus, Nipah virus, and Hantavirus. Specific animal species of concern that are vectors for these zoonotic viruses include avian species, bats, rodents, and mosquitos. While these zoonotic viruses are not classified as waterborne, they are associated with potential waterborne transmission, such as exposure to aerosolized wastewater, which can occur when wastewater undergoes turbulence, such as in flush toilets, converging sewer pipes, and aeration basins [101,102] as well as irrigation and land application systems.

It has been shown that coronaviruses have been detected in wastewater [103] and SARS coronaviruses have been detected in stool and urine samples. Furthermore, detection in both human stool and urine [104–106] as well as wastewater [107] has been reported for influenza. Detection in urine has been reported for the mosquito-associated Zika virus [108], West Nile virus [109,110], Dengue virus [111,112], and yellow fever virus [113]. These observations indicate that the concept of wastewater-based-epidemiology could be applied to a wide range of viruses beyond the confirmed waterborne viruses.

Table 3.2: Summary of human viruses detected in wastewater or human excrement.

Note: The primary method of laboratory detection is the studies presented in Table 3.2 is polymerase chain reaction (PCR), as well as real-time quantitative PCR (qPCR). PCR uses specific primers to replicate target sequences of nucleic acids; designing a primer to replicate a specific sequence in a given viral genome allows for the detection of that particular virus. qPCR can also determine the concentration of a virus in a sample by quantifying the number of copies of the target sequence.

Virus	Detected in Excrement	Detected in Wastewater	Reported Concentrations in Wastewater (copies/L)	Refs.
Adenoviruses	Yes	Yes	6.0×10^2 - 1.7×10^8	[21–36]
Astroviruses	Yes	Yes	4.0×10^4 - 4.1×10^7	[24,29,43,44,47,50–54]
Enteroviruses	Yes	Yes	6.9×10^2 - 4.7×10^6	[24–26,28,29,31,33,34,36,43,53,58–63]
Hepatitis A virus	Yes	Yes	4.3×10^3 - 8.9×10^5	[29,30,58,67–72]
Hepatitis E virus	Yes	Yes	7.8×10^4	[21,34,75–78]
Noroviruses	Yes	Yes	4.9×10^3 - 9.3×10^6	[24,24–26,28–30,32,33,43,47,53,54,59,60,79,84–88]
Rotaviruses	Yes	Yes	1.8×10^3 - 8.7×10^5	[29–31,36,43–45,47,50,53,58,59,90–95]
Aichi virus	Yes	Yes	9.7×10^4 - 2.0×10^6	[36,96,114]
Polyomaviruses	Yes	Yes	8.3×10^1 - 5.7×10^8	[24,35,36,85,99,115,116]
Salivirus	Yes	Yes	3.7×10^5 - 9.7×10^6	[97,114,117]
Sapovirus	Yes	Yes	1.0×10^5 - 5.1×10^5	[24,36,98,118]
Torque teno virus	Yes	Yes	4.0×10^4 - 5.0×10^5	[23,24,35,119]
Coronaviruses	Yes	Yes		[120,121]
Influenza	Yes	Yes		[104–107,122]
Dengue virus	Yes			[111–113,123]
West Nile virus	Yes			[109,110,124]
Zika virus	Yes			[108,125]
Yellow fever virus	Yes			[126,127]

3.4. Variations of Viruses in Wastewater

The quantity of human enteric viruses in wastewater has been shown to have seasonal variation, indicating that infection resulting from these viruses is more prevalent at certain times of the year. A study conducted in Japan by Katayama et al. 2008 found that norovirus concentrations in wastewater were highest during the months of November through April [26], while enterovirus and adenovirus concentrations were largely consistent throughout the year. A nine-year study in Milwaukee, Wisconsin by Sedmak et al., 2005 found that concentrations

reoviruses, enteroviruses, and adenoviruses were highest during the months of July through December. This study also analyzed clinical specimens of enterovirus isolates and found the incidence of clinical enterovirus infection corresponded to the concentration of these viruses in wastewater during the same time periods [31]. Another study in Beijing, China by Li et al. 2011 found that rotavirus concentrations were highest during the months of November through March [90], and that these findings also corresponded with clinical rotavirus data reported in China [128].

Additionally, variation in viral concentration in wastewater can occur on a smaller timescale. For example, tourist locations could experience higher wastewater loads, and consequently higher viral concentrations, on weekends where there is an influx of population. For example, Xagorarakis's research group conducted a study which observed an increase in adenovirus concentration in wastewater following the July 4th holiday in Traverse City, MI, a popular vacation destination [27]. Likewise, urban centers may experience higher loads during the day on weekdays, while people are at work. Accounting for these population changes would be vital for understanding when viral outbreaks occur.

Wastewater has been used in the past as a tool to investigate viruses for other purposes as well, such as spatial surveillance and evaluation of immunization efficacy. Two particular studies were able to use wastewater to observe the spatial variation of particular viral strains; Bofill-Mas et al. observed that particular strains of polyomavirus were endemic to specific regions, while Clemente-Cesares et al. detected Hepatitis E virus in areas previously considered non-endemic for the virus [129,130]. Lago et al. 2003 investigated the efficacy of a poliovirus (a type of enterovirus) immunization campaign in Havana, Cuba by quantifying concentrations of the virus in wastewater [61]. Poliovirus was detected in 100% of wastewater samples prior to the start of

the immunization campaign and dropped to a 0% detection rate in wastewater 15 weeks after the campaign, indicating the usefulness of wastewater surveillance. A study by Carducci et al. 2006 investigated the relationship between wastewater samples and clinical samples and found that the same viral strains could sometimes be detected between the two sets of samples [131].

3.5. Proposed Methodology

Waterborne and non-waterborne viruses have been detected in wastewater, variations of concentrations in time have been observed, and virus presence in wastewater has on occasion been correlated with occurrence of clinical disease. However, wastewater-based epidemiology methods have not yet been applied to assess and predict viral disease outbreaks in a systematic way. Wastewater-based epidemiology has the potential to predict “critical locations” and “critical moments” for viral disease onset. Designing spatial and temporal sampling appropriate to the area of concern, as well as modeling the fate of viruses are critical for the effectiveness of the proposed method. This methodology is summarized in Figure 3.2. In the following sections, critical factors for implementation are discussed.

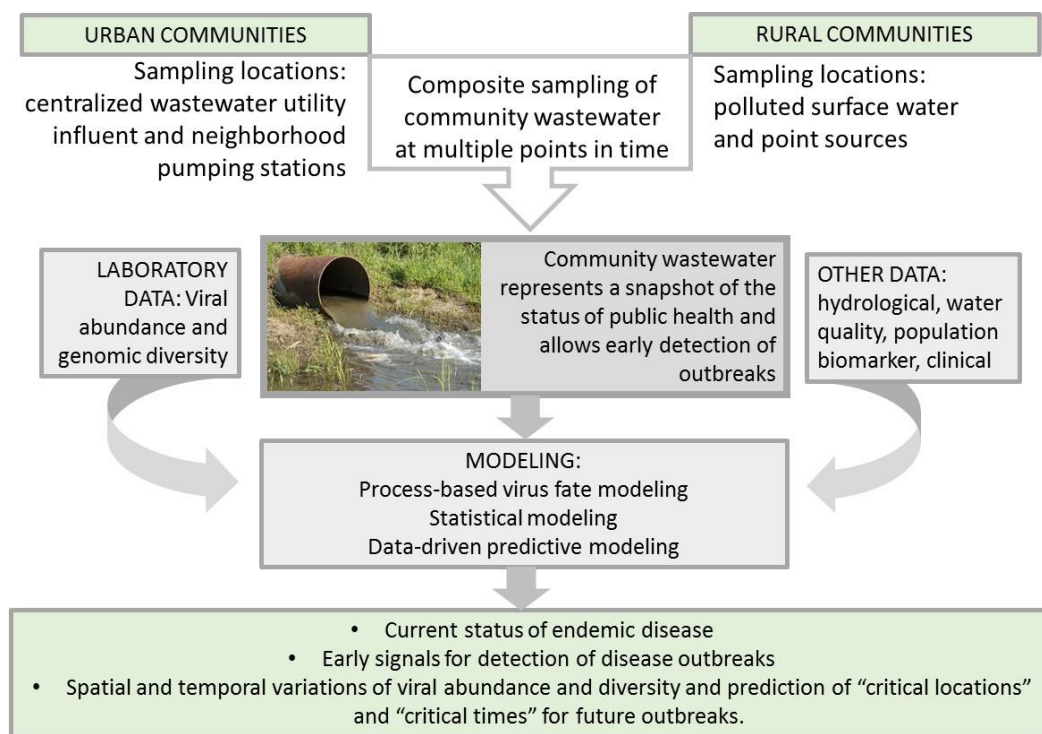


Figure 3.2: Summary of the proposed wastewater-based epidemiology methodology.

3.5.1. Sampling in Urban and Rural Locations

The most critical parameter for the effective application of wastewater-based epidemiology is the selection of a surveillance program, including spatial and temporal sampling. Considerations must be made in the differences between urban and rural wastewater systems. Urban sewage systems offer a convenient confluence of wastewater in the serviced population, as all wastewater will ultimately flow to a WWTP, providing a sampling point representing the entire community. Additionally, localized sampling can be performed in specific neighborhoods where access points are available. By surveying both the combined wastewater at the treatment plant and the localized samples from neighborhoods, viral outbreaks can be traced to a more specific location and the urban areas of concern can be identified. Xagorarakis's research group is currently conducting an NSF-funded study of this nature in the city of Detroit, sampling at

several interceptors at the Detroit wastewater treatment plant, as well as sampling from sewer lines in residential areas throughout the city.

More rural or underdeveloped areas that do not have sewage collection systems pose sampling problems. In these areas, wastewater is often disposed in open space, latrines, or septic tanks. As a result, for wastewater-based epidemiology sampling to be effectively applied to these areas, disposal, fate, and transport of wastewater in the environment must be taken into account. Watershed modeling would therefore become an integral component of the wastewater-based epidemiology methodology for rural locations. In a study performed by Xagorarakis's research group, preliminary investigation into the wastewater epidemiology methodology was conducted [132]. Samples were collected from a wastewater treatment plant and surrounding surface waters in Kampala, Uganda. Three sampling events were conducted in two-week intervals. Four human viruses (adenovirus, enterovirus, hepatitis A virus, and rotavirus) were quantified at each sampling location via qPCR. Concentrations of each virus at each location from each sampling event were compared to one another to determine if significant differences could be observed from one sampling event to the next. Results indicated that statistically significant differences in viral concentration were observed for the measured viruses at several sampling locations.

The selection of the sampling times and locations is of paramount importance to the methodology, regardless of whether sampling takes place in urban or rural areas. Sampling should be based upon expected critical pathways of viral transport and transmission. These critical pathways include environmental reservoirs for viruses and the timing and locations where viruses are most easily transported and transmitted between humans and the environment. By determining sampling times and locations based upon critical pathways, "critical locations" and

“critical moments” – areas and times most impactful to the spread of viral disease – would be most readily and effectively identified.

3.5.2. Quantification of viruses

Quantitative data of viruses of concern, such as those obtained with qPCR are critical for the proposed methodology, as peaks in viral concentrations will indicate potential onset of disease outbreaks. While detection in human excrement or raw wastewater has not been reported for all viruses, it is possible that they have simply not been investigated in this context, as detection of viruses via conventional methods (cell culture, PCR, qPCR) is specific to the virus being investigated. Thus, while qPCR is important to detect and quantify common waterborne viruses, next-generation sequencing and metagenomic methods could also be performed to screen for the presence of other viruses. If genomic sequences of viruses of concern are found, then quantification with qPCR can follow.

Metagenomic methods have been applied to investigate viruses in wastewater and have been found to produce more conservative results of viral detection compared to conventional methods; viruses detected with metagenomic methods are typically also detected with conventional methods, whereas viruses detected via qPCR may not be detected with metagenomic methods. These metagenomic methods, however, can detect the presence of viruses not commonly quantified using qPCR [37,133–136]. Xagorarakis’s research group’s studies have used metagenomic methods to identify human viruses of potential concern in wastewater. The first of these studies, conducted with samples from both Michigan and France, detected a comparatively high number of metagenomic hits for human herpesviruses and also detected human parvovirus and human polyomavirus in wastewater effluent [37]. Their other study, conducted in Uganda, detected human astroviruses, papillomaviruses, as well as a BLAST

hit for Ebola virus [132]. While more research is still required to attain more robust genomic information and comparison databases, metagenomic methods can still be a useful tool for the identification of potential viruses that can then be monitored with qPCR methods. Table 3.3 presents a summary of studies that have used metagenomic methods to detect human viruses in wastewater and human excrement.

Table 3.3. Summary of studies using metagenomic methods to detect viral sequences in wastewater and human excrement.

Note: The following sequences have been confirmed via PCR for the listed study. Bibby and Peccia 2013: adenovirus, enterovirus, prechovirus (131); Cantalupo et al. 2011: adenovirus, polyomavirus, salivirius (134); O'Brien et al. 2017a: adenovirus (37); O'Brien et al. 2017b: adenovirus, enterovirus, rotavirus (130).

Detected In	Virus	Ref.
Wastewater	Adenovirus, enterovirus, polyomavirus, papillomavirus	[135]
	Adenovirus, Aichi virus, coronavirus, herpesvirus, torque teno virus	[137]
	Adenovirus, Aichi virus, astrovirus, coronavirus, enterovirus, herpesvirus, papillomavirus, parechovirus, parvovirus, rotavirus, salivirius, sapovirus, torque teno virus	[133]
	Adenovirus, Aichi virus, astrovirus, norovirus, papillomavirus, parechovirus, polyomavirus, salivirius, sapovirus	[136]
	Adenovirus, herpesvirus, parvovirus, polyomavirus	[37]
	Adenovirus, astrovirus, Ebola virus, enterovirus, papillomavirus, rotavirus, torque teno virus	[132]
Human Excrement	Adenovirus, astrovirus, enterovirus, norovirus, parvovirus, rotavirus, torque teno virus	[138]
	Adenovirus, Aichi virus, enterovirus, parechovirus, rotavirus	[139]

3.5.3. Population normalization

Population normalization is also a critical factor for the application of wastewater-based epidemiology. Proper quantification of biomarkers in wastewater would allow for an appropriate estimation of serviced population via statistical modeling, which would provide context to measured viral concentrations and ensure that differences in viral concentration could not be attributed to changes in population. When observed viral concentrations are significantly high relative to the estimated population, a viral outbreak could be indicated.

Quantification of biomarkers (substances naturally excreted by humans) in wastewater can be used as a method of estimating population in an area. Governmental census information

has been found to underestimate the population of a community compared to estimation using biomarkers [140], and certain substances detected in wastewater have been shown to correlate with census data [141]. Several substances have been proposed and investigated as population biomarkers (Table 3.4), including creatinine [142], cholesterol, coprostanol [143], nicotine [144], cortisol, androstenedione, and the serotonin metabolite 5-hydroxyindoleacetic acid (5-HIAA) [145]. Nutrients such as nitrogen, phosphorus, and oxygen demand [12] as well as ammonium [146] have also been proposed as population biomarkers, but these may more adequately reflect human activity and industry footprint rather than population [145,147,148].

Table 3.4. Summary of biomarkers proposed for population adjustment.

Biomarker	Description	Excreted In:	References
5-HIAA	Metabolite of serotonin	Urine	[145]
Ammonium	Form of ammonia found in water	Urine	[146]
Androstenedione	Sex hormone precursor	Urine	[149]
Atenolol	Drug (beta blocker) used to treat hypertension	Urine	[140]
Cholesterol	Lipid molecule, key component of cell membranes	Feces	[143]
Coprostanol	Metabolite of cholesterol	Feces	[143]
Cortisol	Steroid hormone produced by adrenal glands	Urine	[150]
Cotinine	Metabolite of nicotine	Urine	[145]
Creatinine	Metabolite of creatine phosphate in muscle	Urine	[142]
Nicotine	Stimulant found in tobacco	Urine	[144]
Nutrients (N, P, BOD)	Water quality parameters	n/a	[12]

3.5.4. Estimation of shedding rates

The shedding rate (the rate with which viruses are released from the body in excrement) for each waterborne virus group encompasses a wide range, from 10^2 copies per gram at minimum to 10^{12} copies per gram at maximum. This variability is summarized for selected viruses in Table 3.5. For example, mean concentration values of adenoviruses in excrement ranged from 10^4 to 10^6 depending on the study and whether the virus is excreted in stool or urine, indicating a wide data variance [39,41]. Many factors can impact the shedding rate of viruses in

excrement, including viremia (the presence of the virus in the bloodstream) [40,87,151]. The duration of the presentation of a particular disease can also impact the shedding rate [105,121].

Table 3.5: Summary of reported shedding rates for viruses.

Virus	Range of Shedding Rate, copies/g stool	References
Adenoviruses	$1.0 \times 10^2 - 1.0 \times 10^{11}$	[39,41,42,46]
Enteroviruses	$1.4 \times 10^4 - 6.6 \times 10^9$	[60]
Hepatitis A virus	$3.6 \times 10^5 - 1.0 \times 10^{11}$	[70]
Hepatitis E virus	$1.0 \times 10^1 - 1.0 \times 10^6$	[77]
Noroviruses	$1.1 \times 10^5 - 1.1 \times 10^{12}$	[87]
Sapoviruses	$1.3 \times 10^5 - 2.5 \times 10^{11}$	[98,118]

3.5.5. Transport of Viruses in the Environment

Waterborne viruses survive well in water, but all viruses are susceptible to natural degradation determined by factors such as temperature, exposure to UV light, and the microbial community [152,153]. The kinetic decay rate of a virus would thereby be primarily dependent not only on the characteristics of the individual virus but also environmental conditions within the sewage system, which could vary from location to location. Moreover, the fate of viruses may be different between wastewater systems in urban areas which typically use enclosed underground sewer pipes and rural areas which may utilize septic tanks, catchments, and the open environment. Viruses can also adsorb to or be enveloped by particulate matter in wastewater which would lead to confounding factors in measurement of these viruses.

3.5.6. Correlation with public health records and unidentified clinical data

Comparison with clinical data is another key component of these methods. Correlations between measured viral concentrations in wastewater and reported clinical cases of disease could be established, strengthening the proposed methodology. The establishment of these correlations can serve as a validation for a prediction model that accounts for the factors discussed above,

providing evidence for the notion that changes of viral concentrations in wastewater will indicate changes in viral disease cases in humans. Moreover, were preventative public health measures implemented once an outbreak is identified, tracking clinical data could provide a quantifiable indicator of the efficacy of preventative measures.

3.6. Conclusions

Infectious viral outbreaks can cause uncontrollable negative effects especially in densely populated areas. Early detection is critical for effective management and prevention of outbreaks. Recent research efforts in developing optimized detection systems often focus on rapid methods for analyzing blood or excrement samples, however, these approaches require that individuals are examined in clinical settings, typically after an outbreak has been established. Wastewater-based-epidemiology is a promising methodology for early detection of viral outbreaks at a population level. Analyzing wastewater is equivalent to obtaining and analyzing a community excrement sample. In the determination of whether an outbreak is imminent or already in progress, quantifying viral concentration in raw wastewater is a crucial first step in this process. Waterborne viruses appear to be prime candidates, as they are detectable and quantifiable in both wastewater and human excrement. Non-waterborne viruses have been shown to be detected in human excrement, and some have been reported to be detected in wastewater. Wastewater-based epidemiology therefore has the potential to expand beyond waterborne viruses.

Routine monitoring for temporal changes in virus concentration and diversity in community wastewater, in combination with monitoring metabolites and biomarkers for population adjustments, allows early detection of outbreaks (critical moments for the onset of an outbreak). In addition, carefully designed spatial sampling of wastewater will allow detection of locations where an outbreak may begin to develop and spread (critical locations for the onset of

an outbreak). Considerations in sampling locations must be taken with regards to the area of investigation, as urban and rural areas may have differences in the respective wastewater systems that can affect viral transport in the water environment. Moreover, to obtain an accurate estimation of disease cases in a population, other factors must be considered such as viral shedding rates, environmental transport and degradation rates, and correlation with reported clinical disease data. Ultimately, there is great opportunity for the use of wastewater-based-epidemiology to investigate viral outbreaks within a community. Comprehensive application of the various factors discussed above is crucial for the full potential of this methodology to be realized. Further research could clarify many of these issues and allow for the full development and application of this new epidemiological technique for studying, identifying, and predicting viral outbreaks.

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Chapter 4:

A Proposed Water-Based Surveillance Approach for Identification and Early Detection of Human and Livestock Viral Outbreaks in Michigan

This chapter is in preparation and will be submitted to the Journal of One Health.

4.1. Abstract

The environmental aspect of the One Health framework has not been investigated thoroughly, but it has great potential in the identification, early detection, and prevention of viral disease in humans and animals. Water-related viral infections in Michigan are discussed here as an example to demonstrate factors that may be considered when designing studies to identify critical pathways and surveillance systems for identification and early detection of viral disease. Michigan is a state with large urban centers as well as a sizeable rural and agricultural population. This study discusses spatial and temporal distribution of selected human viral disease in Michigan for the year 2017, it explores relationships with land use and precipitation and it proposes a surveillance system for identification and early detection of human and livestock viral disease outbreaks. The proposed system is based on sampling human wastewater, animal waste, and polluted surface water.

4.2. Introduction

Viral disease has been demonstrated to impact human, animal, and environmental health in the state of Michigan. Numerous human outbreaks due to multiple viral agents have been reported. These outbreaks include coronavirus in Lenawee County in 1966 [1], norovirus in Macomb County in 1979 [2] and in Ottawa County in 2008 [3], hepatitis A virus in Calhoun and Saginaw Counties in 1997 [4], and West Nile virus in Kent County in 2002 [5]. Michigan has also been in the midst of an outbreak of hepatitis A virus since 2016 [6]. Illustrated in these

examples is both the variety of human viral diseases that have impacted the state as well as that different areas of the state are subject to outbreaks.

The Michigan Department of Health and Human Services (MDHHS) maintains the Michigan Disease Surveillance System (MDSS) which publishes weekly disease reports on a number of communicable diseases [7]. Data taken from these MDSS reports show an increase in viral disease over the past five years, as shown in Figure 4.1. For this paper, gastrointestinal (GI) illnesses, influenza-like illnesses, hepatitis A illnesses, and norovirus illnesses are selected. A large percentage of GI illnesses and influenza-like illnesses are expected to be of viral origin and all hepatitis and norovirus illnesses are of viral origin. These diseases have been selected since they have different exposure pathways. Influenza illnesses may be zoonotic but are not waterborne. Hepatitis A illnesses are waterborne but are not zoonotic. Norovirus is commonly foodborne, it may be waterborne, and it is not zoonotic. GI illnesses may be both waterborne and zoonotic.

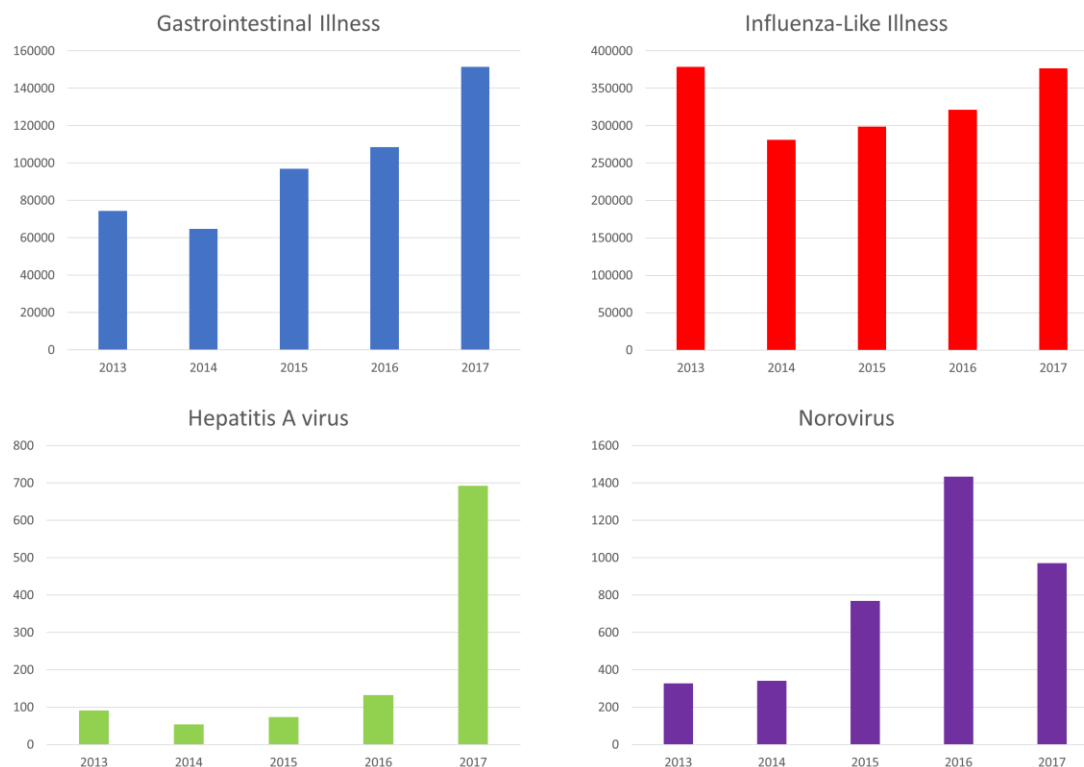


Figure 4.1: Reported cases in Michigan over the past five years for gastrointestinal illnesses, influenza-like illnesses, Hepatitis A virus, and norovirus as reported by MDSS [7]. Note: MDSS is a continually active system and reported numbers in the MDSS weekly reports are not final.

Viral disease outbreaks have also affected animals in Michigan, including viral diarrhea in cattle [8], eastern equine encephalitis virus in deer [9], and an outbreak of a novel calicivirus in rabbits [10]. According to the USDA report on death loss in U.S. cattle and calves (2015), 31.8% of non-predator cattle deaths and 42.3% of non-predator calf deaths were due to digestive or respiratory causes. These figures are amplified in the state of Michigan; the percentages are 37.8% and 66.3% respectively, equating to approximately 9027 cattle deaths and 27926 calf deaths in the state [11]. While the report does not specify the etiological nature of the deaths, a portion of these illnesses are due to viral causes, illustrating the potential burden of viral disease on animals. The Michigan Department of Agriculture & Rural Development (MDARD) also publishes annual statistics on reportable animal diseases. The MDARD report from 2017

includes many viral animal disease cases, including 373 cases of bovine leukemia virus, 160 cases of caprine arthritis encephalitis, 17 cases of porcine reproductive and respiratory syndrome virus, 7 cases of swine enteric coronavirus, 9 cases of canine influenza, 7 cases of eastern equine encephalitis, 10 cases of equine herpesvirus, and 15 cases of West Nile virus in equines [12].

Moreover, viruses have been detected in Michigan environmental samples. Human viruses (such as adenovirus) have been detected in the effluent of the East Lansing [13] and Traverse City [14] wastewater treatment plants, which is released into surrounding surface waters. Adenovirus and other human viruses have also been detected at public recreational beaches in Michigan, leading to beach closures [15,16].

The goal of this paper is to propose a water-based surveillance system for the identification and early detection of human and animal viral disease in Michigan. The system is based on monitoring surface water and wastewater at critical times and locations. The premise of the proposed water-based surveillance system is that polluted water and wastewater is a medium that can be considered as a point of surveillance since it is an important reservoir for viruses. In addition to waterborne viruses, the proposed system is applicable to non-water related viruses such as influenza, since they can be detected in human and animal excrement. To identify the critical locations and times at which water sampling should be conducted, spatial and temporal distribution of viral disease in Michigan is investigated. In addition, parameters that may correlate with the observed distribution of disease are evaluated, such as land-use and precipitation.

4.3. Methods

4.3.1. Data Collection

Disease data was collected from weekly MDSS reports for 2017 from MDHHS, which reports a number of cases for each disease for each county for both the current week and year-to-date (note: MDSS is a continually active system and reported numbers in the MDSS weekly reports are not final) [7]. Year-to-date values were chosen as the values utilized in this data analysis as they were found to be more comprehensive compared to current week values; it is suspected this is because some cases for given weeks would not be reported until after those weeks' reports were published, thus they would only be reflected in the year-to-date values.

To adequately compare counties to one another, population data for each county was collected from U.S. Census data [17]. Population data was used to calculate the relative number of disease cases per capita for each county and each week. Weeks which contained days in more than one month were grouped into the month for which there were more days in that week (e.g., the week of 1/29-2/4 was designated as February as it contains four days in February compared to three days in January). The relative numbers of disease cases are expressed as “number of reported disease cases per 1000 people” and are considered the dependent variables for the analysis that follows.

Data for independent variables were collected from various sources. Land use data at the county level was collected from the United States Geological Survey (USGS) Land Cover Data Viewer [18]. Absolute land cover was collected as hectares and relative land cover was also calculated using total land area for each county. County-level agricultural data were collected from the USDA Census of Agriculture [19]. Precipitation information was collected from the

USDA as a 30-year average of monthly precipitation for each county in Michigan; annual values were also reported [20].

4.3.2. Exploratory Data Analysis

After data collection, exploratory data analysis was performed to investigate relationships between independent and dependent variables. Spatial distributions of variables were visualized with the creation of county-level heatmaps. Correlations were performed between variables to obtain correlation coefficients and determine which pairs of variables exhibited relationships with one another. Scatter plots were also created between independent and dependent variables to represent relationships between variables visually.

4.3.3. Statistical Methods

Independent variables determined to have a potential correlation with disease levels were selected and utilized in the development of a statistical model. Spatial regression analysis was performed in R to assess the validity of the independent variables as predictors of the corresponding dependent variables. First, ordinary least squares (OLS) regression was performed to determine whether the collected independent variables were significantly related to the diseases studied. Independent variables were introduced into the OLS model based upon the prior exploratory data analysis; those with the highest correlations with disease levels were interpreted as the most likely predictors and were incorporated first, followed by the next highest correlation, and so on. The regression model was run each time a new variable was introduced. Those that did not exhibit a relationship with 85% confidence (i.e. p -value not <0.15) were omitted from further consideration. This conservative level of confidence has been employed in prior studies performing spatial regression of environmental data [21]. Predictor variable collinearity was assessed using the calculation of variance inflation factor (VIF) scores; it was

ensured that no predictor variable had a VIF score greater than 3.0 [22]. This analysis provided an initial model with which to assess the relationships between variables.

However, OLS regression does not account for spatial autocorrelation in the data, and other regression models that do can be more appropriate in this analysis [21]. The degree of spatial autocorrelation was assessed in R and quantified with Moran's I and Lagrange multiplier diagnostics using k -nearest neighborhoods of different sizes. It was found that values of k greater than 1 provided appropriate results; a value of $k = 5$ was utilized in diagnostic tests to adequately account for spatial autocorrelation. These diagnostic tests found the existence of spatial autocorrelation in this dataset, and determined that a spatial lag model would be more appropriate. The spatial lag regression model was therefore performed in R to adjust the regression coefficients of the selected predictor variables. Akaike information criterion (AIC) values for each of the models were calculated to determine which model was of higher quality.

4.4. Results and Discussion

4.4.1. Spatial and Temporal Distribution of Viral Disease in Michigan

Included in the MDSS reports are disease statistics by county for various viruses, and certain areas of the state are more commonly affected by viral disease than others. Figure 4.2 shows heatmaps for cases of four diseases (GI illnesses, influenza-like illnesses, hepatitis A virus, and norovirus) for each Michigan county. Variation in spatial distribution of diseases can be observed in Michigan, with GI illnesses concentrated in the southwest portion of the state, whereas the eastern portion of the state is most affected by hepatitis A.

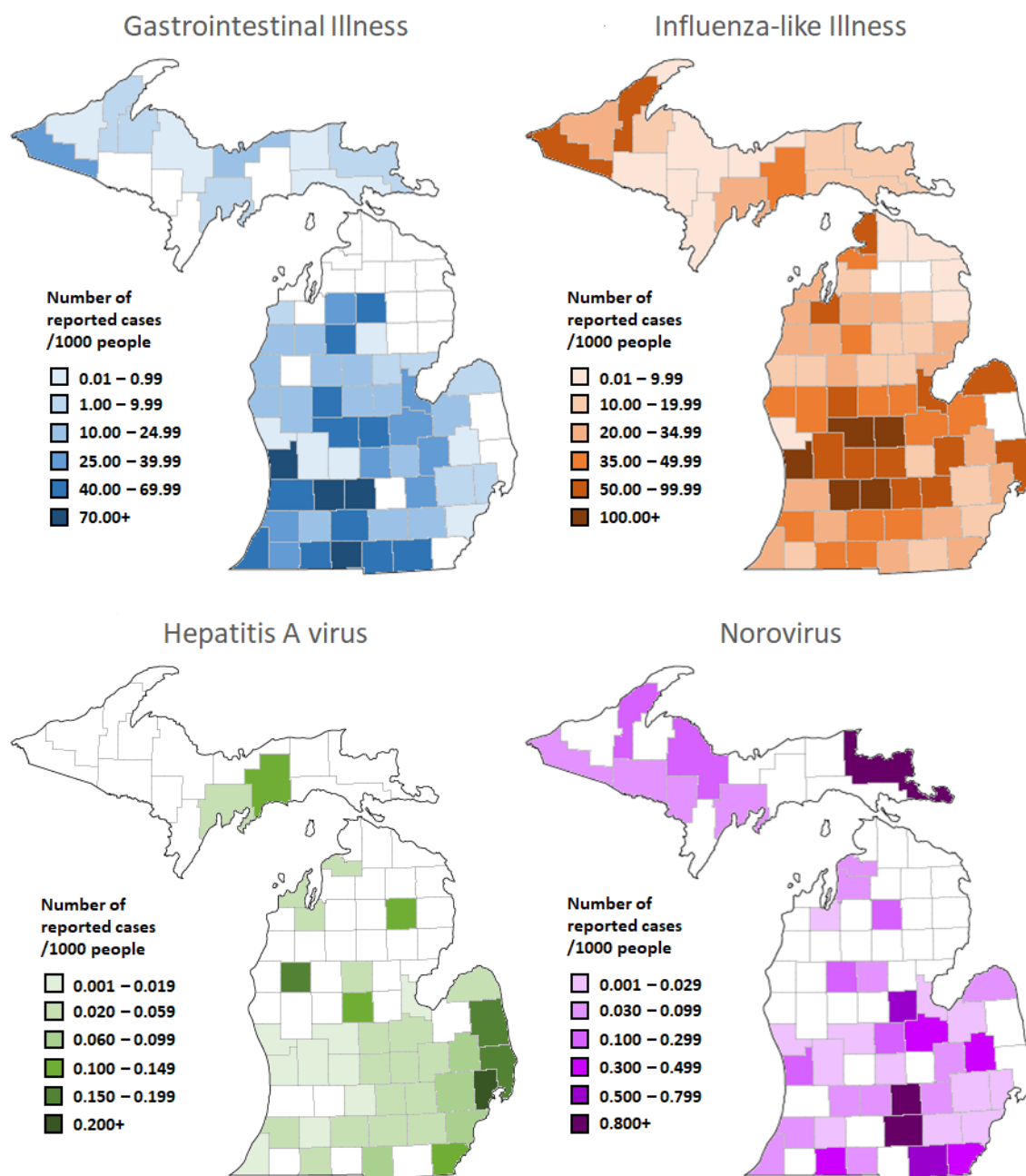


Figure 4.2: Heatmaps of disease cases relative to population for Michigan counties for the year 2017 as reported by MDSS. (number of cases divided by population for county multiplied by 1000) [7]. Maps prepared by the authors. Note: MDSS is a continually active system and reported numbers in the MDSS weekly reports are not final.

Because MDSS issues weekly reports on disease statistics, temporal trends can also be observed for the illnesses in question. Figure 4.3 displays the number of disease cases by month

for the state of Michigan in the year 2017 for gastrointestinal illnesses, influenza-like illnesses, hepatitis A virus, and norovirus. GI illness and influenza norovirus are all more prevalent in the winter and spring months. Hepatitis A virus cases are more common in the latter half of the year, but there is relatively little annual variation as compared to the other diseases in question.

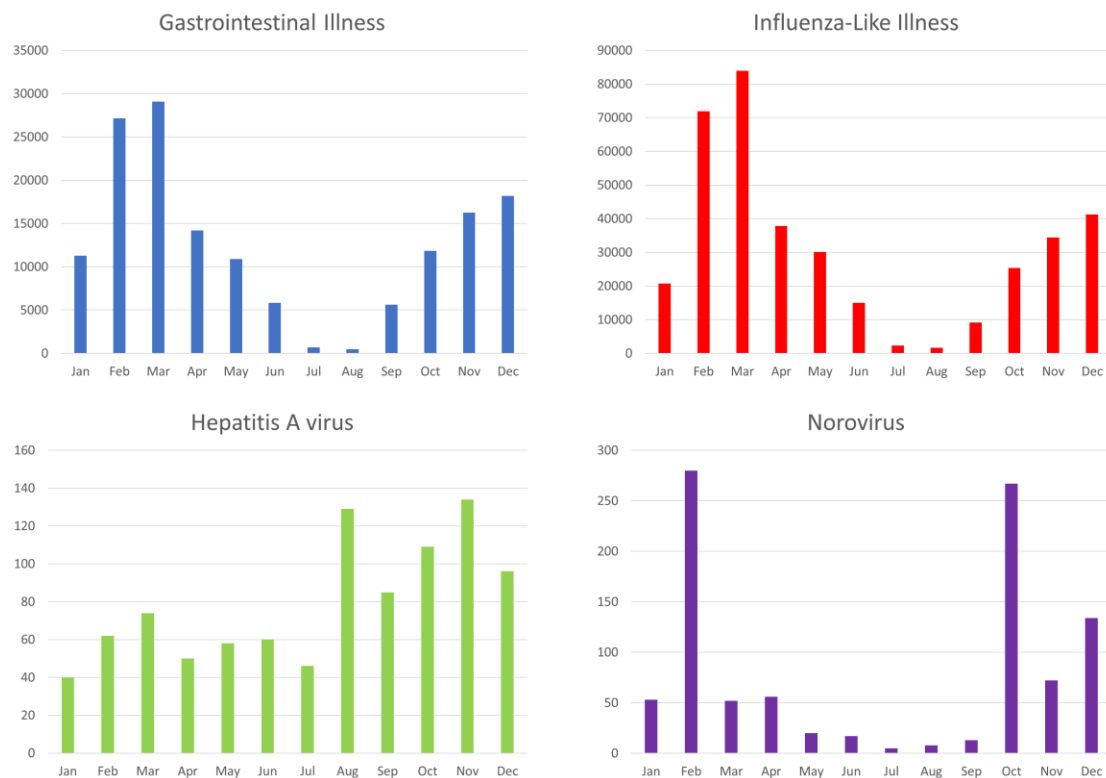


Figure 4.3: Disease cases by month in Michigan for the year 2017 as reported by MDSS [7]. Note: MDSS is a continually active system and reported numbers in the MDSS weekly reports are not final.

4.4.2. Parameters that May Correlate with Spatial Distribution of Viral Disease

It is important to collect data that will inform the identification of factors that may correlate with spatial distribution of viral disease. Some factors have been shown in prior studies to contribute to the likelihood of infectious disease, including land use [23], precipitation [24], and population density [25]. Land use is relevant to determine the environmental state of the area, and can be impactful during runoff events. Precipitation levels inform where these runoff

events may occur. Population and population density can affect the spread of viral disease and can also be used to normalize disease levels from one county to another.

Other factors can be used to further characterize land use, such as agricultural information. Variables such as livestock population or levels of fertilizer application can not only illustrate the level of agricultural activity in an area, but also illustrate the expected quality of nearby surface water after runoff events. In addition to agricultural data, information on surface water quality can also be of use. The Michigan Department of Environmental Quality (MDEQ) reports figures for public beach closures, which occur when surface water contamination is detected during regular screening for pathological indicators. MDEQ also summarizes sanitary sewer overflow (SSO) and combined sewer overflow (CSO) events, which occur when wastewater levels traveling through municipal sewer systems exceed the systems' capacity, resulting in untreated wastewater discharging into nearby surface waters.

The primary spatial factor to consider in this case is land use. For each county in Michigan, correlations are calculated between the number of reported cases of disease (normalized to population) and the types of land use for that respective county as reported by USGS [18]. Table 4.1 presents the calculated correlation coefficients between these two variables.

Table 4.1: Correlation coefficients between disease cases normalized to population for each MI county and relative land cover for different types (hectares of type in county per total hectares in county).

Relative Land Cover	Disease Cases (normalized to population)			
	Influenza-Like Illness	Gastrointestinal Illness	Hepatitis A virus	Norovirus
Forest & Woodland	-0.424	-0.423	-0.321	-0.146
Shrubland & Grassland	-0.015	-0.057	0.217	0.270
Agricultural Vegetation	0.454	0.489	0.183	0.191
Developed & Other Human Use	0.113	0.030	0.441	0.007
Recently Disturbed or Modified	-0.218	-0.199	-0.200	-0.219
Open Water	-0.266	-0.273	-0.181	-0.122

There are some striking values when analyzing these results. The correlations of the diseases with agricultural vegetation presents a prominent contrast; the relationships of influenza-like illnesses and gastrointestinal illnesses with agricultural land use (bolded in the table) are markedly stronger than those of hepatitis A virus and norovirus. This indicates the possibility that agricultural activity may have an impact on the transport of influenza-like illnesses and gastrointestinal illnesses; the notion that agricultural land use can introduce pathogens to surrounding surface waters is supported by the literature [26–29]. This is an expected finding given that some influenza-like illnesses and GI illnesses may be zoonotic, whereas hepatitis A virus and norovirus are not thought to be zoonotic. Similarly, the relationship of developed land with hepatitis A virus is much higher than the relationship this type of land use has with the other three diseases studied. This implies that more heavily populated areas may contribute to the incidence of hepatitis A virus. This relationship exists despite the fact that the number of disease cases for each county was normalized to that county's population, signifying that this relationship does not arise merely from a large number of reported cases in urban areas.

These relationships can be more plainly distinguished with the use of scatter plots. Figure 4.4 displays scatter plots for the correlation between agricultural vegetation and the four diseases investigated. The positive correlation is observable in the first two plots representing gastrointestinal illness and influenza-like illness, especially when contrasted with hepatitis A virus, which shows no relationship between the two variables. The plot for norovirus also reveals what appears to be a somewhat positive relationship between the two variables, with one county (Chippewa) as an outlier. Interestingly, removal of this county's data from the correlation calculation increases the correlation coefficient from 0.191 to 0.315. This indicates that

norovirus may also have a relationship with agricultural land use, though one not as strong as GI illness or influenza-like illness.

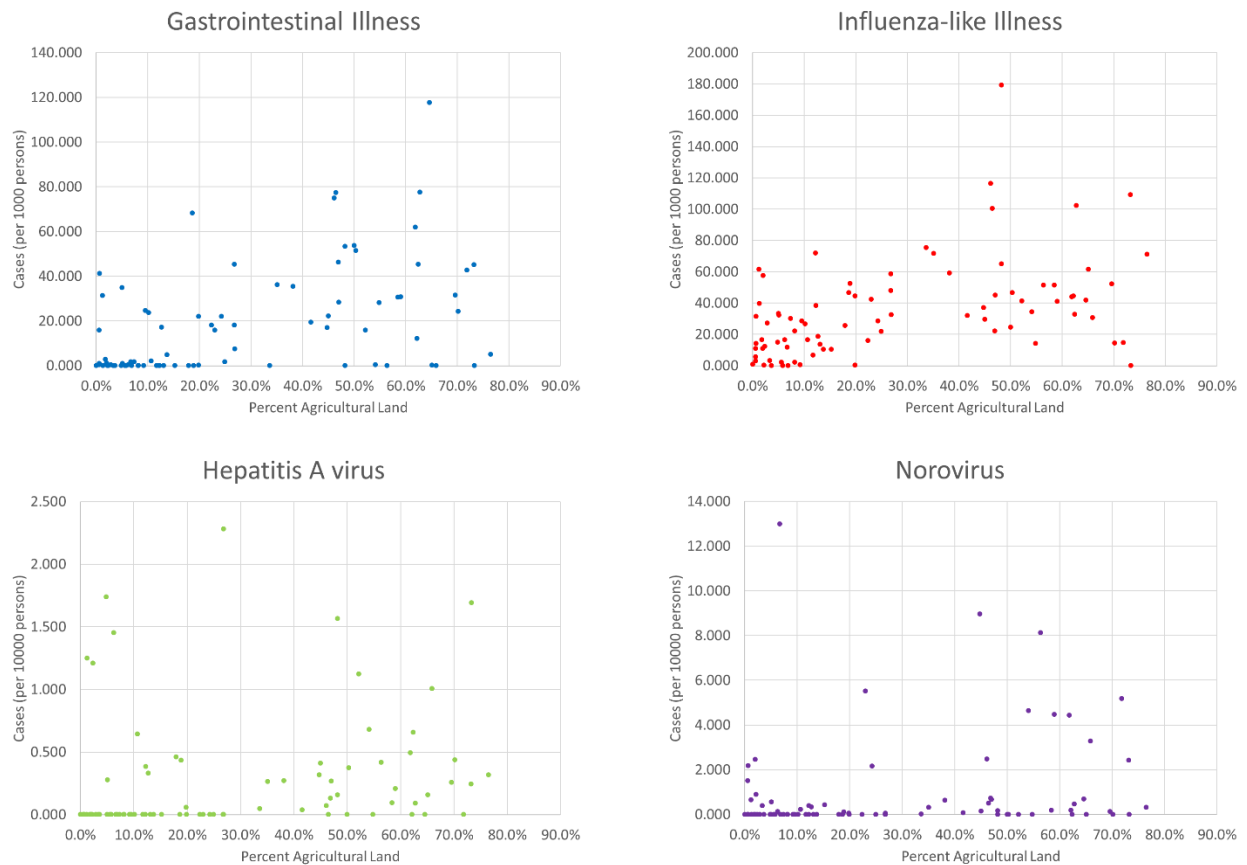


Figure 4.4: Scatter plots displaying correlation between relative agricultural land cover in each county with reported disease cases (normalized to population) in each county for gastrointestinal illness, influenza-like illness, hepatitis A virus, and norovirus.

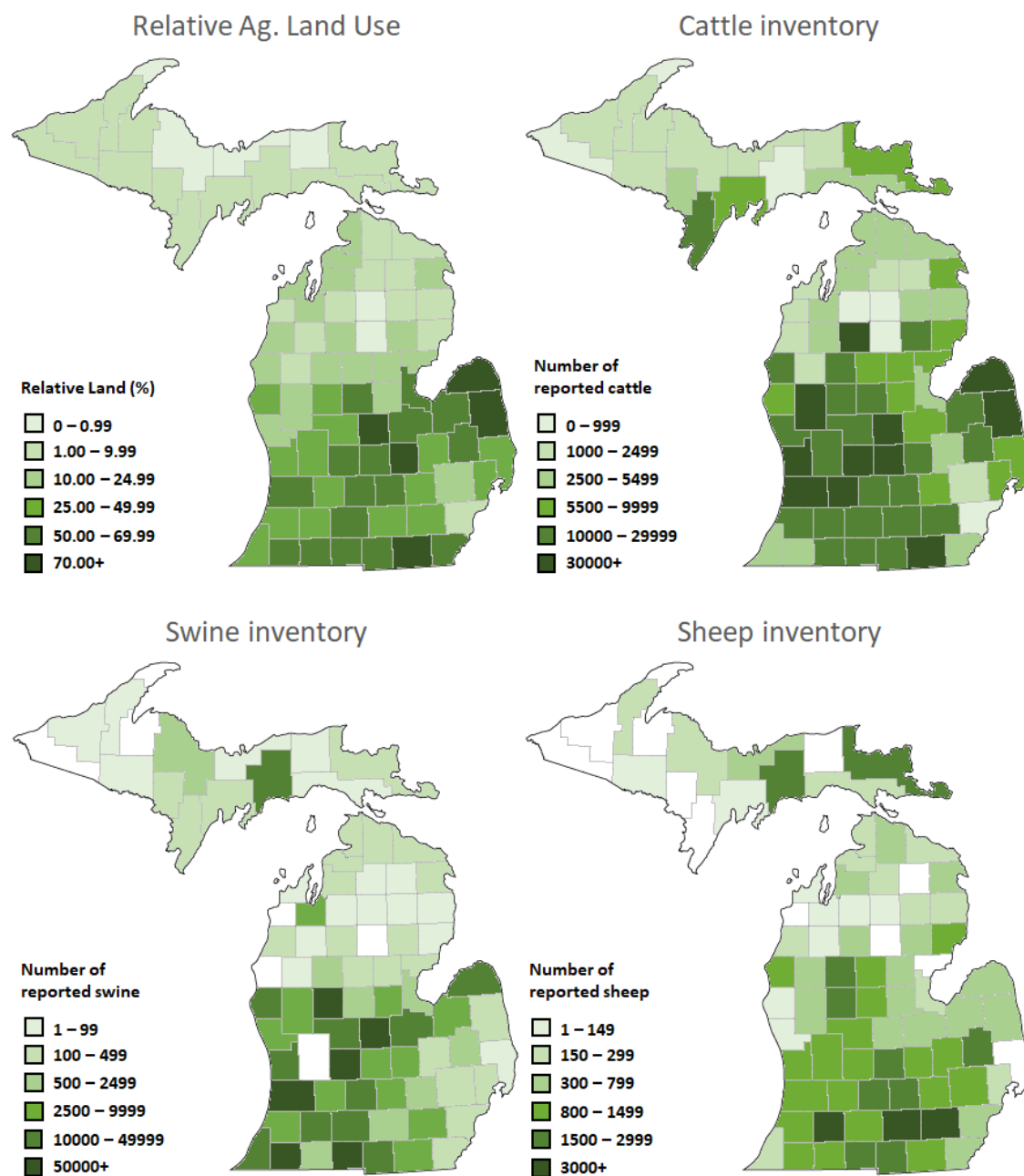


Figure 4.5: Heatmap of agricultural data by county for the state of Michigan as reported by USDA (2012). Top-left: farmland acreage, top-right: cattle inventory, bottom-left: swine inventory, bottom-right: sheep inventory [19]. Maps prepared by the authors.

Agricultural data can assist in determining critical locations, as comparisons can also be made to agricultural trends. Figure 4.5 displays heatmaps of farmland acreage, cattle population,

swine population, and sheep population as reported by the USDA [19]. According to visual examination, the most commonly affected areas of viral disease appear to typically be contained within major watersheds, including the Grand River watershed for influenza-like illnesses. These illnesses also appear to correspond to areas with high cattle populations. With these observations in mind, particular attention could be paid to those factors when determining where to sample in these locations.

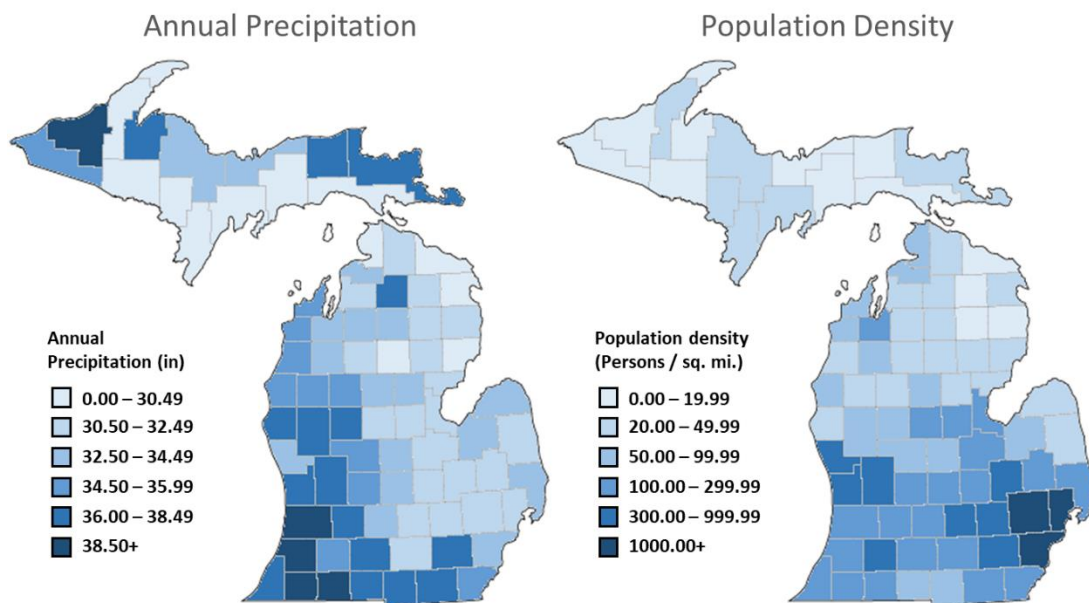


Figure 4.6: Left: average annual precipitation by county for Michigan for the years 1981-2010. Right: Population density in persons per square mile by county for Michigan. Maps prepared by the authors.

Figure 4.6 presents heatmaps of average annual precipitation and population density for each county in Michigan as reported by the Agricultural Applied Climate Information System [20] and U.S. Census Bureau [17] respectively. Visual examination determines that precipitation levels appear highest in the western part of the state, similar to the areas most commonly affected

by GI illness and influenza-like illness. Meanwhile, population density is highest near the Detroit area, which is the most area most affected by hepatitis A virus.

Table 4.2 presents county-level correlations between the four diseases investigated and the aforementioned variables. As is suggested by the heatmaps, precipitation has a high degree of correlation with GI illness, and a slight correlation with influenza-like illness, while showing no substantial relationship with the other two diseases. Meanwhile, population density has a high correlation with hepatitis A virus; this is an understandable result given the established correlation with developed land use. Livestock inventory is also seen to have somewhat high correlations with different diseases, but these correlation coefficients are not as strong as other variables investigated.

Table 4.2: Correlation coefficients between disease cases normalized to population for each MI county and agricultural data, precipitation data, and population density for each MI county.

Parameter	Disease Cases (normalized to population)			
	Influenza-Like Illness	Gastrointestinal Illness	Hepatitis A virus	Norovirus
Cattle inventory	0.351	0.219	0.063	0.009
Hog inventory	0.136	0.359	-0.122	-0.063
Sheep inventory	0.095	0.149	0.016	0.336
Annual Precipitation	0.230	0.474	0.041	0.024
Population Density	0.050	-0.043	0.470	-0.001

4.4.3. Parameters that May Correlate with Temporal Distribution of Viral Disease

The temporal variation of factors such as precipitation and surface water runoff may also help to explain viral disease occurrence. Surface water discharge, such as the flow rates of specific rivers in the state, can also provide valuable information about the status of a watershed over time. Temperature also assists in determining when runoff and first-flush events will occur. The timing of SSO/CSO events can give an idea of the times in which certain areas are most at-risk for pathogen exposure. Similarly, comparison of the timing of manure application with the

timing of runoff events can help to determine the impact of land application of biosolids on environmental water quality.

Comparisons can be made between the temporal distribution of disease cases and these temporal factors. One such comparison can assess the relationship between reported monthly disease cases and monthly precipitation. Accurate county-wide monthly precipitation measurements are not readily available for every county in Michigan during the year 2017, but the Agricultural Applied Climate Information System reports the 30-year average monthly precipitation levels for Michigan counties in addition to annual figures [20]. These precipitation levels in each county can be correlated with reported diseases cases in each county by month, taking the spatial analysis from above and introducing a more granular temporal element. A summary of these correlation coefficients is presented in Table 4.3.

Table 4.3: Summary of correlation coefficients for the relationship between average 30-year precipitation in the county with reported disease cases (normalized to population) in the county for each month.

Month	Influenza-Like Illness	Gastrointestinal Illness	Hepatitis A virus	Norovirus
Jan	0.031	0.084	-0.007	0.245
Feb	0.297	0.317	0.125	-0.064
Mar	0.125	0.356	0.119	0.131
Apr	0.271	0.442	0.164	-0.032
May	0.290	0.532	0.012	-0.073
Jun	-0.017	0.115	0.104	-0.173
Jul	-0.097	0.071	0.020	0.080
Aug	-0.002	0.316	-0.189	-0.104
Sep	0.205	0.083	0.002	0.047
Oct	-0.132	-0.034	-0.285	-0.095
Nov	0.363	0.351	-0.093	-0.041
Dec	0.198	0.210	0.138	-0.011
Annual	0.230	0.474	0.041	0.024

This analysis reveals that the spatial correlations (represented by the annual figures) fluctuate at different points throughout the year. For example, as mentioned, GI illnesses have a

correlation of 0.474 with annual precipitation on the spatial level, but this relationship is strongest in the month of May, when it reaches a correlation coefficient of 0.532. Moreover, the correlation coefficients between GI disease and precipitation increase in magnitude from February to May. This finding is interesting because the spring months are the times in which land application of fertilizers and manure are most common, as it is the beginning of the growing season. This relationship with precipitation, combined with the aforementioned relationship between GI disease and agricultural land use, strengthens the possibility that agricultural runoff could be a critical pathway for GI diseases in Michigan. Also of note are the increases in correlation in the months of August and November for GI illnesses, and September and November for influenza-like illnesses. These values indicate that these months, in addition to the aforementioned spring months, could be critical times at which runoff is a critical pathway for these illnesses.

Other independent variables that have not been collected could be utilized as data becomes available. In addition to spatial and temporal distribution of publicly available human disease data, livestock and wildlife disease data would be very useful. However, governmental agencies do not collect or provide such data to the same detail as human disease data.

4.4.4. Spatial Regression Modeling

Exploratory data analysis as displayed above is vital in this process to generate hypotheses concerning potential critical pathways of viral disease, and a full application of this methodology would require more robust statistical techniques to confirm these relationships. Other statistical methods would further strengthen the determination of critical pathways in a full implementation of this methodology. The development of a statistical model to test the data of the collected independent variables against the observed disease cases could more readily

determine the most influential independent variables. Spatial regression can also help to determine whether there is any spatial autocorrelation observed in the data, and regression models can be employed that account for such autocorrelation.

Based on exploratory data analysis, relative agricultural land use and annual precipitation were determined to be independent variables most of interest for GI illness and influenza-like illness. The OLS regression model performed showed that the initial inferences drawn from exploratory data analysis were appropriate, as all other variables (other types of land use, livestock information) did not meet the threshold of confidence (p-value not <0.15) for further consideration. A summary of the results is listed in Table 4.4. As shown, both variables display a relationship with GI illness with a high degree of confidence ($p < 0.001$). Of the other diseases, none were related to precipitation with a high degree of confidence, and while all three meet the threshold for consideration ($p\text{-value} < 0.15$), influenza-like illness was found to be related to agricultural land use with a higher degree of confidence ($p\text{-value} < 0.001$). Additionally, the regression coefficients for agricultural land use for both hepatitis A virus and norovirus were much smaller than those for GI illness and influenza-like illness, suggesting that the relationship is not nearly as strong as with the two latter diseases.

Table 4.4: Summary of OLS regression results from R for each disease investigated.

Disease	Independent variable	Regression coefficient	Standard error	Test statistic	P-value
Gastrointestinal Illness	Agricultural	35.5565	9.665	3.679	0.000423
	Precipitation	2.6549	0.7713	3.442	0.000921
Influenza-like Illness	Agricultural	53.7628	13.4585	3.995	0.000143
	Precipitation	0.6955	1.074	0.648	0.519096
Hepatitis A Virus	Agricultural	0.046447	0.022812	2.036	0.045
	Precipitation	-0.0023	0.00182	-1.262	0.211
Norovirus	Agricultural	0.189612	0.106758	1.776	0.0795
	Precipitation	-0.00365	0.00852	-0.429	0.6691

Because OLS regression does not account for spatial autocorrelation, Moran's I and Lagrange multiplier diagnostic tests were performed for the GI regression model to assess the degree of spatial autocorrelation. It was determined from these diagnostic tests that spatial autocorrelation exists among the data and a spatial lag model would be more appropriate. The spatial lag model adjusted the regression coefficients for the GI model to 28.87805 for agricultural land use (P-value = 0.005703) and 2.22179 for precipitation (P-value = 0.003851). The regression coefficients for the spatial lag model are less than those for the OLS model, as the spatial lag model accounts for spatial autocorrelation, lessening the influence of the predictor variables. The AIC values for each of the models also determined that the spatial lag model was of higher quality than the OLS model, validating the use of spatial regression.

This analysis is one rudimentary example of the types of statistical techniques that can be employed to assess the relationships between collected disease data and other independent variables. Moreover, the variables assembled in these analyses is a non-exhaustive list of the potential factors that can impact viral disease. As more data becomes available, more relationships of interest may be observed in exploratory data analysis, and new predictor variables could be incorporated in the above spatial regression analysis. Additionally, this regression analysis only accounts for spatial interactions between variables, and exploratory data analysis revealed that precipitation is more strongly related to GI illness in certain months of the year. Temporal data could therefore also be incorporated into future regression analyses to further pinpoint the critical times and locations for GI illness.

Additionally, disease data such as the data used in this analysis also contains many zero values (counties and times at which no cases were reported). In this case, zero-inflated linear regression techniques, such as the zero-inflated Poisson model could be of use. Furthermore,

more robust reporting of clinical data would be valuable in this analysis. One potential reason for the inconclusive relationships between norovirus and the investigated independent variables could be that norovirus is not as widely reported as GI illnesses or influenza-like illnesses, making it more difficult to observe correlations between variables. More data collection for other independent variables could also be valuable, as while none of the independent variables used in this study were significantly related to norovirus, there may be other variables that would be found to be predictive of the disease.

4.4.5. Proposed Surveillance System

Ultimately, the goal is to develop a system of prioritization for each county in Michigan per month. Analysis of the collected spatial and temporal data can determine the factors that are most critical in specific places and at specific times. This can lead to the determination of which locations and time periods are of the greatest concern for each of the four diseases investigated. This will, in turn, lead to higher levels of preparedness to combat viral disease outbreaks, as these critical times and locations can be surveyed before the disease develops. If increased concentrations of human viruses are observed, action can be taken to introduce barriers and interventions depending on the critical pathways that have been found to impact the viral outbreaks in question. Examples based on the statistical analyses performed are shown below.

To summarize, there are two potential findings from the above analyses. Gastrointestinal illnesses may be related to agricultural land use and precipitation, and this relationship with precipitation is strongest in the springtime, although GI illnesses are most common in the winter months. Meanwhile, hepatitis A virus appears to be most closely related with developed land use, and is more common in the later months of the year in autumn and winter. With these considerations in mind, sampling times and locations can be more strategically determined based

on watershed monitoring. However, the surveillance approach utilized will vary depending on the type of watershed being studied.

Urban areas offer a convenient point of sampling at the influent of a wastewater treatment plant. Untreated wastewater can be considered as a population sample for the serviced community. Wastewater can therefore be used as an epidemiological tool to help identify potential viral outbreaks. The goal of wastewater-based epidemiology is to sample wastewater and identify spikes in concentrations of excreted viruses. This method can determine that an outbreak may be taking place before clinical cases are reported. Urban wastewater treatment plants that serve metropolitan areas sometimes have several interceptors at which wastewater is collected. Sampling at each interceptor and mapping each interceptor to the specific neighborhoods it serves can facilitate virus occurrence data collection representative of each serviced area of the city. Should viral concentrations be observed to be higher in one interceptor than the rest, the corresponding serviced area would therefore be of greater concern for a potential viral outbreak. Public health officials could then issue a warning to this particular neighborhood serviced by that interceptor, educating the public on the potential disease outbreak. Public health resources could also be devoted to treating afflicted individuals in that neighborhood to prevent the spread of the outbreak.

For example, the Detroit area is an urban environment in southwest Michigan. Suburban Detroit also includes Macomb County, the county for which the highest rates of hepatitis A virus were observed in 2017, and hepatitis A virus was determined to be more closely related with developed land area compared to other types of land use. This area is conducive to the analysis of wastewater as a critical pathway, as urban sewage systems will collect wastewater at the influent of the treatment plant. As rates of hepatitis A virus were highest in the latter months of

the year, sampling should therefore take place in the months of August to December at the wastewater treatment plant servicing the greater Detroit area. The Detroit wastewater treatment plant also has three interceptors servicing different parts of the metropolitan area. All three interceptors could be sampled, informing which regions of the city are experiencing the highest rates of viral disease.

4.4.5.1. Surveillance of the Grand River Watershed

Surveillance systems in mixed-use watersheds that include rural areas are more complex. While sampling of wastewater can still be useful for these watersheds, other techniques such as watershed modeling and microbial source tracking can help in the determination of sampling points. For the purposes of this example, a mixed-use watershed in Michigan is discussed. The Grand River watershed is a large watershed encompassing Grand Rapids, Lansing, and surrounding agricultural areas, where the highest occurrence of GI and influenza disease was observed in 2017 (Figure 4.2). This section describes an example of proposed water-based surveillance system in the Grand River watershed, which aims for the identification and early detection of human and livestock viral disease.

Figure 4.7 displays the location of the Grand River watershed in Michigan. The Grand River watershed includes the Grand River as well as many smaller tributaries and empties into Lake Michigan. Because the Grand River watershed contains the counties for which the highest rates of GI illness and influenza-like illness were observed in 2017 (as seen in Figure 4.2), focus can be placed upon the critical pathways related to GI illness observed in the previous section. A watershed-based surveillance system will identify occurrence of and relationships between human and livestock viral disease. The proposed locations for sample collection are discussed below. Analysis of samples will include human and zoonotic viral species identification.

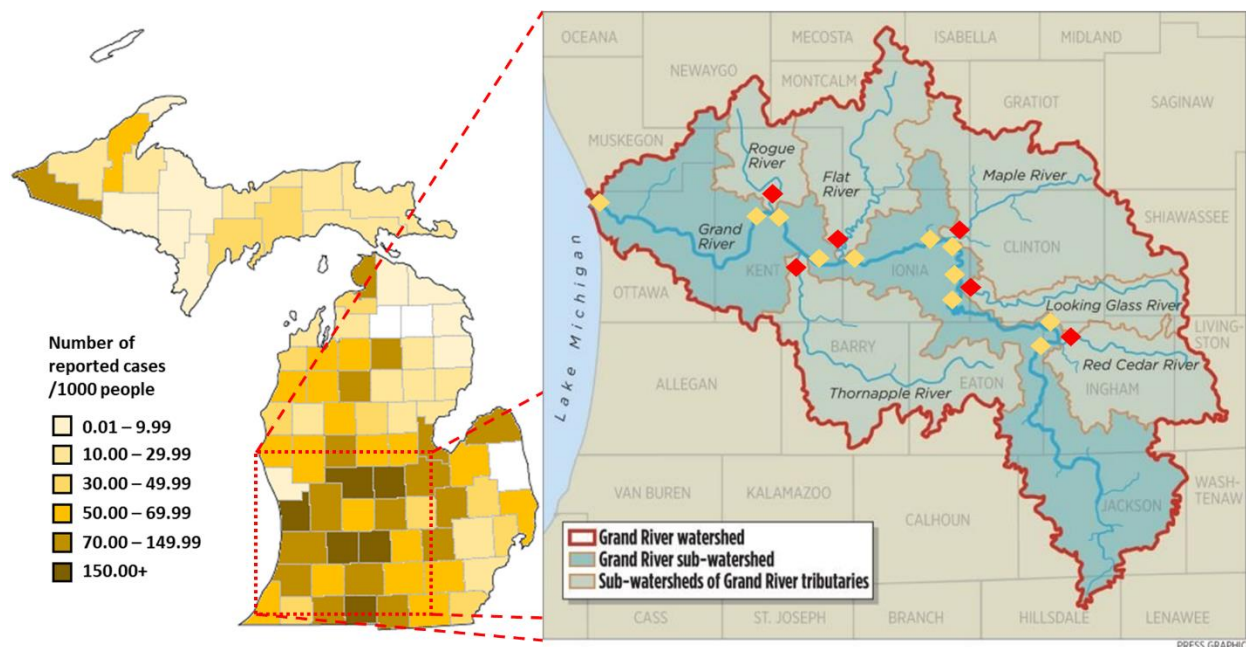


Figure 4.7: Location of Grand River watershed in relation to GI illnesses + influenza-like illnesses in Michigan. Red diamonds indicate tributary sampling points. Yellow diamonds indicate Grand River sampling points. Additional sampling points will include: raw and treated wastewater at all municipal wastewater utilities in the area, manure application sites, and wildlife waste.

In this case, agricultural runoff may be a critical factor in the transport of GI illness. Therefore, sampling locations and times should be chosen based on where and when agricultural runoff could be most impactful. To capture the entire watershed, sampling should take place at the discharge site of the watershed. Sampling could also take place at the discharge sites of sub-watersheds, which would capture the effect of these sub-watersheds and could most specifically trace the source of any observed viral contamination. These sites are also shown in Figure 4.7, indicating sampling points for both the Grand River and its tributaries. Wastewater treatment plants can still be valuable sampling points in mixed-use watersheds as well. The Michigan Water Environment Association maintains a list of wastewater facilities in the state of Michigan, and Table 4.5 summarizes the facilities located within the Grand River watershed [30].

Table 4.5: List of wastewater treatment plants located within the Grand River watershed county [30,31]. WWTP: Wastewater treatment plant. WWSL: Wastewater storage lagoon.

County	Facility Designated Names	Number of CAFOs
Barry	City of Hastings WWTP	2 dairy
Clinton	Bingham Township WWTP, City of St. Johns Wastewater Treatment Facility, Elsie WWSL, Southern Clinton County Clean Water Facility, Village of Fowler, Village of Ovid WWSL	10 dairy, 1 beef, 1 swine
Eaton	City of Eaton Rapids WWTP, Dimondale/Windsor WWTP, Grand Ledge WWTP, Potterville WWTP, Sunfield WWSL, Vermontville WWTP	7 swine, 4 dairy
Gratiot	Perrinton WWSL, Village of Ashley	
Ingham	City of East Lansing Water Resource Recovery Facility, City of Mason WWTP, Delhi Township WWTP, Lansing Wastewater Treatment, Leslie City Wastewater Plant, Mason Manor WWSL, VFW National Home for Children, Webberville WWSL, Williamston Wastewater Treatment	3 dairy, 1 mixed
Ionia	City of Belding WWTP, Clarksville-Morrison Lake WWTP, Ionia WWTP, Lakewood Wastewater Authority WWTP, Portland WWTP, Village of Muir WWSL, Village of Saranac WWSL	6 swine, 4 poultry, 3 dairy, 1 beef
Jackson	City of Jackson WWTP, Leoni Township WWTP	
Kent	Caledonia WWTP, City of Grand Rapids Water Filtration Plant, City of Grandville Clean Water Plant, City of Wyoming Clean Water Plant, Creekside Estates Mobile Home Park, Grand Rapids Water Resource Recovery Facility, Lowell WWTP, PARCC Side Clean Water Plant, Sparta WWTP, Village of Kent City	3 dairy, 1 swine
Livingston	Fowlerville WWSL, Handy Township WWTP	
Montcalm	Carson City WWSL, Greenville WWTP	3 swine, 2 dairy
Ottawa	Allendale WWTP, Chester Township WWTP, Coopersville WWTP, Crockery Township WWTP, Grand Haven-Spring Lake Sewer Authority, Wright Township-Ottawa County WWSL	1 beef, 1 dairy, 1 poultry, 1 swine
Shiawassee	Perry WWSL	1 dairy

Sampling can also take place at other locations, such as storm drains, agricultural field runoff drains, and areas that have recently experienced combined sewer overflows. Other agricultural data could also be valuable in determining sampling points in rural areas of the watershed, such as concentrated animal feeding operations (CAFOs). The Sierra Club maintains a readily available map of CAFOs throughout the United States, including in Michigan [31]. CAFO locations can help to determine where livestock populations are most abundant, heightening the risk for both animal disease and zoonotic disease.

Sampling times, meanwhile, can be determined by other factors. As observed in the previous section, the relationship between precipitation and GI illness was strongest in the spring

months, coinciding with land application of agricultural fertilizers. Therefore, sampling in spring, from March to May, would be ideal to assess the impact of agricultural runoff on the occurrence of GI illness. This runoff would be at its peak when the flow rate of the Grand River would be highest. Therefore, examining the discharge of the Grand River can aid in determining the most critical times at which to sample. Figure 4.8 displays the discharge of the Grand River for 2017 as reported by USGS. Precipitation levels for Grand Rapids, MI are shown on the same graph. Peaks in discharge are observed during the months of March, April, and May, often after high precipitation events. Sampling events should occur at or soon after these peaks, as this is when runoff will be most impactful. High runoff can also occur in early spring due to changes in temperature leading to large snow melts; sampling should be timed to capture these first flush events.

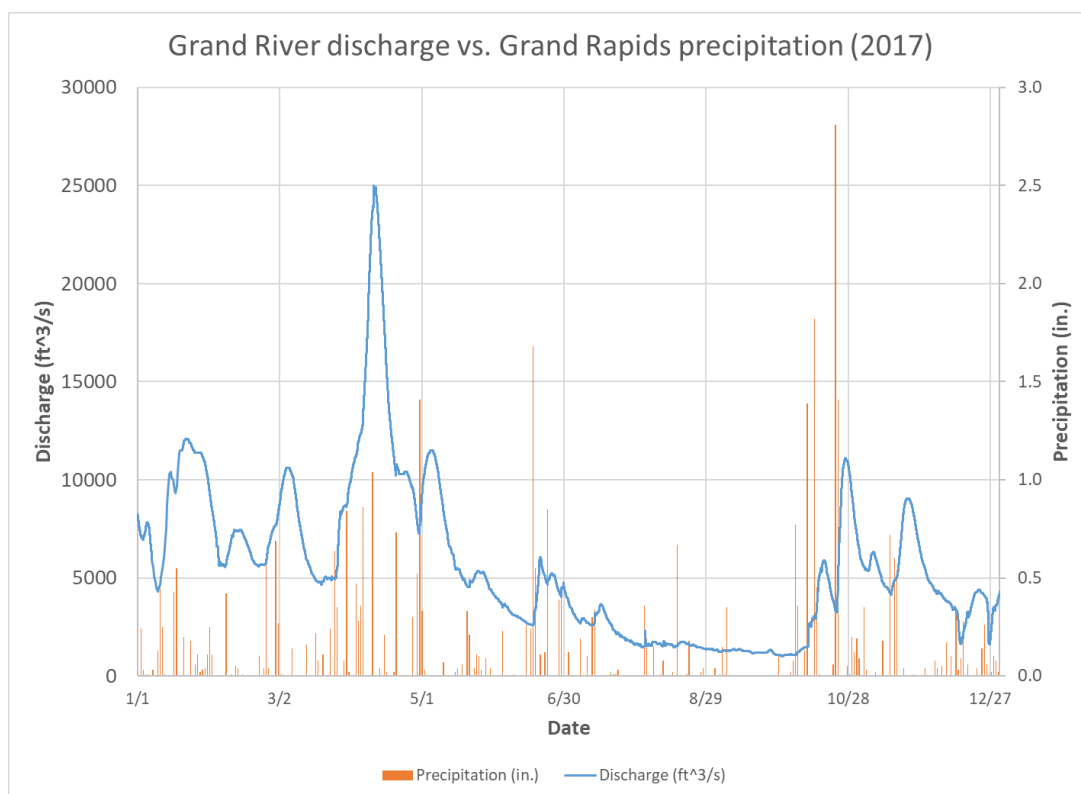


Figure 4.8: Discharge for the Grand River (42°57'52" N, Longitude 85°40'35" W) and precipitation for Grand Rapids, MI as measured by USGS.

Ultimately, prioritization of the most critical locations and times for viral outbreaks can be performed. The aforementioned statistical analysis provides an initial prioritization of which locations to sample, and at which times. Once sampling has occurred, laboratory methods quantitative polymerase chain reaction can be employed to quantify concentrations of viruses in the samples. Because public clinical data is available weekly (temporally) on the county level (spatially), and counties will each have many sampling points and many different times, sampling data must be aggregated in some fashion (e.g. average, median) to obtain an overall concentration for each county and each week. Concentrations can then be analyzed for spatial and temporal trends, leading to the critical times and locations for which viral disease is most prevalent. Further statistical tests, such as the t-test, can determine when and where measured concentrations are significantly different from historical values. Moreover, sampling results can determine whether the initial assumptions that environmental factors correlate with clinical cases of viral disease are appropriate. Should the areas and times that were prioritized from the initial data analysis be found to exhibit the highest viral concentrations, these assumptions would be strengthened and the methodology be validated. If other areas or times were instead found to exhibit the most burdensome concentrations of viruses, other pertinent environmental factors could be investigated and the predictive model could be adjusted accordingly.

Upon validation of this methodology, it could be employed to better protect or prepare communities against the spread of viral disease. For example, in rural areas, the source of a viral outbreak could potentially be traced to animal waste runoff in a particular sub-watershed, in which case regulations could be put in place to prevent animal waste from entering surface water or runoff, and the local population could be educated on the impacts of animal waste on human health. Accordingly, when screening for human viruses in samples, it is important to also screen

for animal viruses, as this could provide information regarding animal health in the area as well as further assess the impact of animal waste on the environment.

4.5. Conclusions

The One Health framework can be readily applied to the investigation of viral disease, and determination of critical environmental factors are an important part of this process. This study shows the existence of significant relationships between clinically reported human viral infections and environmental factors such as land use and precipitation. The identification of these relationships can assist in the determination of the most critical times and locations for which humans and animals are most at risk of viral infection. Once these times and locations are determined, surveillance systems can be implemented and interventions can be introduced to mitigate potential viral outbreaks. While the relevant environmental factors will vary spatially and temporally, these concepts are still relevant regardless of where and when this methodology is implemented. The utilization of the One Health framework in its full capacity can better help to identify, predict, and prevent viral disease outbreaks.

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Chapter 5:

Viral Diversity and Abundance in Polluted Waters in Kampala, Uganda

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5.1. Abstract

Waterborne viruses are a significant cause of human disease, especially in developing countries such as Uganda. A total of 15 virus-selective samples were collected at five sites (Bugolobi Wastewater Treatment Plant (WWTP) influent and effluent, Nakivubo Channel upstream and downstream of the WWTP, and Nakivubo Swamp) in July and August 2016. Quantitative PCR and quantitative RT-PCR was performed to determine the concentrations of four human viruses (adenovirus, enterovirus, rotavirus, and hepatitis A virus) in the samples. Adenovirus (1.53×10^5 - 1.98×10^7 copies/L) and enterovirus (3.17×10^5 - 8.13×10^7 copies/L) were found to have the highest concentrations in the samples compared to rotavirus (5.79×10^1 - 3.77×10^3 copies/L) and hepatitis A virus (9.93×10^2 - 1.11×10^4 copies/L). In addition, next-generation sequencing and metagenomic analyses were performed to assess viral diversity, and several human and vertebrate viruses were detected, including *Herpesvirales*, *Iridoviridae*, *Poxviridae*, *Circoviridae*, *Parvoviridae*, *Bunyaviridae* and others. Effluent from the wastewater treatment plant appears to impact surface water, as samples taken from surface water downstream of the treatment plant had higher viral concentrations than samples taken upstream. Temporal fluctuations in viral abundance and diversity were also observed. Continuous monitoring of wastewater may contribute to assessing viral disease patterns at a population level and provide early warning of potential outbreaks using wastewater-based epidemiology methods.

5.2. Introduction

It has been reported that between 1.5 and 12 million people die each year from waterborne diseases [1,2] and diarrheal diseases are listed within the top 15 leading causes of death worldwide [3]. Rapid population growth, climate change, natural disasters, immigration, globalization, urbanization, and the corresponding sanitation and waste management challenges are expected to intensify the problem in the years to come. In the vast majority of cases, all of the infectious agents have not been identified. However, most outbreaks of unidentified etiology are suggested to be caused by viruses [4]. Viruses have been cited as potentially the most important and hazardous pathogens found in wastewater [5]. Viruses can be responsible for serious health outcomes, especially for children, the elderly and immunocompromised individuals, and are of great concern because of their low infectious dose, ability to mutate, inability to be treated by antibiotics, resistance to disinfection, small size that facilitates environmental transport, and high survivability in water and solids.

This study focuses on Kampala, the capital city of Uganda. Uganda, like any other developing country, still faces challenges in meeting Sustainable Development Goals (SDGs) on improved sanitation as outlined by the United Nations. By 2015, Uganda had not met the SDG target on sanitation with only 29% of the urban population having access to improved sanitation facilities such as flush/pour toilets and ventilated improved pit latrines [6]. Kampala has undergone a 27% increase in population from approximately 1,189,150 in 2002 to 1,516,210 in 2014 [7]. The rapid increase in population is mainly attributed to rural-urban migration in search of better living standards resulting in rapid expansion of impoverished settlements which accommodate more than 50% of the city's population [8]. The settlements face challenges of poor sanitation and hygiene that have exacerbated as more people move into the city [8–11].

The increase in population has also heightened the need for sufficient treatment of wastewater. With approximately 10% of the urban population connected to the sewer system [12], most of the residents in Kampala dispose of the wastewater in open channels and space. This is mainly caused by a lack of financial resources and little space for construction of sewer systems [13], especially among the more impoverished areas of the city [8]. A case study by Kulabako et al., 2010 conducted in Bwaise III (an impoverished area in Kampala) revealed that 37% of the residents dispose of wastewater in open drains whereas 23% use both open drains and open space. The wastewater consequently finds its way into surface water bodies, compromising the quality of such water sources.

Kampala has experienced diarrheal disease outbreaks from cholera, dysentery, and cryptosporidiosis [13–15]. The outbreak or occurrence of such diseases is mainly attributed to unsafe water supplies, poor hygiene and sanitation practices. Numerous prior studies have investigated the quality of water sources in and around Kampala, determining that many of these water sources were contaminated [10,16–20]. In these studies, water quality parameters were correlated with the prevalence of waterborne diseases such as cholera and dysentery [19]. Contamination of drinking water sources posed a health risk to a majority of the city's population [18], and contamination of the water source was primarily due to poor waste disposal [10].

However, these studies focused on investigating bacterial contamination indicators. Waterborne viruses, meanwhile, have been shown in three studies to be responsible for diarrheal disease outbreaks in Kampala, particularly in children, with each study attributing recent diarrheal outbreaks to the presence of rotavirus in stool specimens [21–23]. Diarrhea has been determined to be one of the top causes of death in young children worldwide [24], and rotavirus

has been shown to be responsible for approximately 45% of diarrheal cases in young children in Uganda [25]. Recently, studies have been performed investigating viral contamination of water sources in Kampala [26,27], but these studies focused primarily on surface water and did not investigate wastewater or its impact on the surrounding environment.

Rotavirus (RV), adenovirus (AdV), enterovirus (EV), and hepatitis A virus (HAV) were the human viruses chosen for investigation in this study as they are the most common viruses detected in wastewater [27–31] and are all linked to disease outbreaks around the world [32–34]. Additionally, it has been concluded that adenovirus can serve as a reliable indicator of human pollution [35–37]. Quantitative polymerase chain reaction (qPCR) was used to detect and quantify these viruses as it is rapid, sensitive, reliable, and effective at low viral concentrations [35,38].

In addition, next-generation sequencing and metagenomic analysis has been used to assess viral diversity. Several studies have used these methods to investigate the detection and diversity of viruses in wastewater [39–43]. While metagenomic analyses are presently only able to identify a fraction of viruses present in the environment [39,41], these methods still offer comprehensive characterization of the viruses in a sample, allowing for a wide range of detection and the possibility of identifying viruses previously unknown to be present in a sample.

There is potential to employ wastewater as an epidemiological tool to better identify and predict viral disease outbreaks. This approach has been used to track illicit drug use in various locations around the world, but so far has not been applied to track viral disease outbreaks. The approach was first theorized in 2001 [44] and first implemented and reported in the monitoring of cocaine use in 2005 where the method was termed sewage epidemiology [45]. The methodology considers raw untreated wastewater as a reservoir of human excretion products that

can serve as a sampling point for assessing population health. Environmental surveillance has already proven useful in the efforts to eradicate polio [46–48], and wastewater could prove itself another tool in the efforts to improve public health. Viruses notably do not replicate outside a host, are commonly excreted in human waste, and waterborne viruses are stable in wastewater [31]. Therefore, viruses could be an ideal candidate for the wastewater epidemiology methodology.

The purpose of applying wastewater epidemiology to viruses is to more rapidly determine whether an outbreak is imminent or already in progress within a given population. Such an approach should include frequent sampling and analysis for viral concentrations and biomarkers, for population adjustment. Viral shedding rates and survival in wastewater should also be taken in to account. Attaining baseline concentrations of viruses in wastewater would be a necessary step in the wastewater epidemiology process, as it would establish levels with which sudden large rises in viral concentration could be compared. Continued monitoring of viral abundance could provide useful information for the development of wastewater-based epidemiology methods.

This study seeks to quantify the abundance of four human viruses in surface water and wastewater in Kampala, Uganda, characterize the viral diversity of these water samples, and to establish preliminary data that could indicate the possibility of using these methods in future wastewater-based epidemiology studies to identify early signals of and predict future viral disease outbreaks.

5.3. Materials and Methods

5.3.1. Sample Collection

A total number of 15 samples from five sampling locations were collected in the summer 2016. Samples were collected every other week at a depth of less than 1 m from the five locations in southwest Kampala: Bugolobi Wastewater Treatment Plant (WWTP) influent and effluent, Nakivubo Channel upstream and downstream of the WWTP, and Nakivubo Swamp, as shown in Figure 5.1.

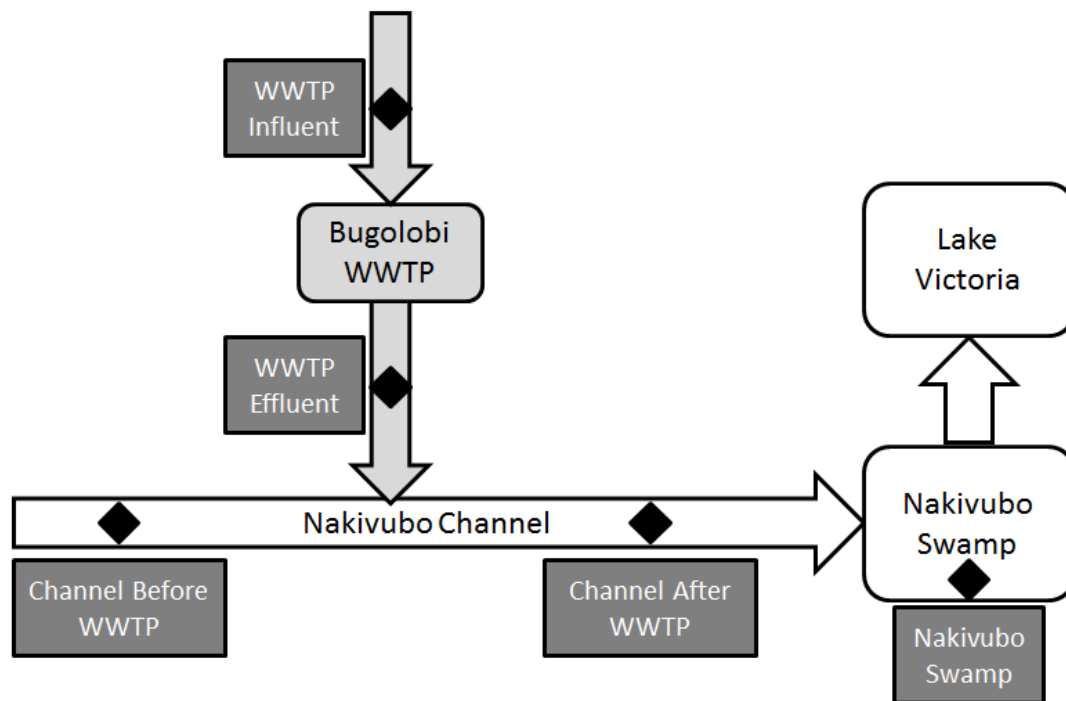


Figure 5.1: Flowchart of sampling locations and surrounding surface waters. Note: Diamond symbols indicate sampling locations.

The Bugolobi WWTP utilizes conventional activated sludge methods to treat wastewater. For each sampling event, water was pumped through a NanoCeram Virus Sampler filter (Argonide Corporation) at a rate of 11 to 12 L/min using a previously described method [42,49,50] shown to be effective in viral recovery from water samples [51]. Water was collected

until the membrane fouled beyond the point at which water would no longer flow through the filter. Table 5.1 summarizes the locations, dates, and volumes for each sampling event. Filters were immediately kept on dry ice and transported to Michigan State University (MSU) in East Lansing, MI for further processing. The filters arrived at the MSU laboratory within 48-72 hours of each sampling event.

Table 5.1: Summary of sampling volumes (L) for each sampling date and location in the study. Note: Sampling volumes and elution volumes were taken into account when calculating qPCR concentrations for viruses.

Sampling Date	Sampling Location				
	Channel Before WWTP	WWTP Influent	WWTP Effluent	Channel After WWTP	Swamp
12 July 2016	30.66	18.17	5.68	26.88	39.37
25 July 2016	10.98	2.65	19.31	9.46	24.23
8 August 2016	23.47	9.08	5.68	18.55	53.37

5.3.2. Sample Processing

All NanoCeram filters used to concentrate the samples were eluted immediately upon receipt according to the standard method [52] which has been shown to be effective [51]. Briefly, a 1.5% w/v beef extract (0.05 M glycine, pH 9.0–9.5) solution was used as the eluent. The filters were submerged for a total of 2 min (two separate 1 min elutions) in filter housings with 1 L of beef extract added to the pressure vessel. After the beef extract was passed through each filter, the pH of the eluate was adjusted to 3.5 ± 0.1 using 1 M HCl and flocculated for 30 min. Further concentration of the solution was performed by two stages of centrifugations for 15 min at 2500 $\times g$ and 4 °C. The supernatant was then decanted and the process was repeated until all the beef extract solution was centrifuged. The accumulated pellets were resuspended using 30 mL of 0.15 M sodium phosphate (pH 9.0–9.5) and mixed until the pellet was mostly dissolved. The pH was then adjusted to 9.0–9.5 using 1 M NaOH. The solution was placed into a 50 mL centrifuge tube

and centrifuged for 10 min at 4 °C at 7000 ×g. The supernatant was poured off into a separate 50 mL centrifuge tube, the pH was adjusted to 7.0–7.5 for stabilization of the virus particles, and the pellet was discarded. The supernatant was loaded into a 60 mL syringe and passed through a 0.22 µm sterilized filter for removal of bacteria, fungi and other contaminants. Samples were completely mixed, placed into 2 mL cryogenic tubes, and stored at –80 °C until further analysis.

5.3.3. Nucleic Acid Extraction

Nucleic acids were extracted from the viral-concentrated samples using the QIAamp Viral RNA Mini Kit (Qiagen) following manufacturer's instructions. This kit allows for the recovery of both viral DNA and RNA. Extracted nucleic acid samples were stored at -20 °C until further analysis. The viral-concentrated eluate samples were also kept –80 °C for future analyses.

5.3.4. qPCR Analyses

Real-time quantitative PCR and real-time quantitative reverse-transcriptase PCR (qRT-PCR) was performed on each sample using a Roche LightCycler 1.5 instrument (Roche Applied Sciences) for the detection of human adenovirus 40/41 (AdV), human enterovirus (EV), human rotavirus (RV), and hepatitis A virus (HAV) using previously described assays [53–56]. Table 5.2 displays primers and probes used for virus quantification. For each of the four assays, the amplification efficiency was >98.0% with a detection limit of 10¹ copies per reaction.

For each sample, a 20-µL PCR mixture was created in triplicate containing 4 µL of 5x LightCycler TaqMan Master Mix, 0.8 µL of 10 µM forward primer (final concentration, 400 nM), 0.4 µL of each 10 µM reverse primer (final concentration, 200 nM), 0.6 µL of 10 µM TaqMan probe (final concentration, 300 nM), 8.8 µL of PCR-grade water, and 5 µL of DNA extract. The real-time PCR program used a denaturation step for 15 min at 95 °C, followed by an

amplification step of 45 cycles at 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 10 s, concluding with a cooling step at 40 °C for 30 s.

Table 5.2: Primer and probe sequences for the qPCR assays used in the study.

Target Virus	Primer and Probe	Sequence (5'-3')	Amplicon Size	Reference
Human adenovirus (40,41)	HAdV-F4041-hex157f	ACCCACGATGTAACCACAGAC	88	Xagorarakis et al. 2007
	HAdV-F40-hex245r	ACTTTGTAAGAGTAGGCGGTTTC		
	HAdV-F41-hex246r	CACCTTTGTAAGAATAAGCGGTGTC		
	HAdV-F4041-hex214rprobe	FAM-CGACKGGCACGAAKCGCAGCGT-TAMRA		
Hepatitis A virus	Forward Primer	GGTAGGCTACGGGTGAAAC	89	Jothikumar et al. 2005
	Reverse Primer	AACAACCTACCAATATCCGC		
	Probe	FAM-CTTAGGCTAATACTTCTATGAAGAGATGC-BBQ1		
Enteroviruses	EQ-1	ACATGGTGTGAAGAGTCTATTGAGCT	141	Dierseen et al. 2007
	EQ-2	CCAAAGTAGTCGGTTCCGC		
	EP	FAM-TCCGGCCCCCTGAATGCGGCTAAT-TAMRA		
Rotavirus	Rota NVP3-F	ACCATCTACACATGACCCTC	86	Pang et al. 2014
	Rota NVP3-R	GGTCACATAACGCCCC		
	Probe	ATGAGCACAATAGTTAAAAGCTAACAACACTGTCAA		

5.3.5. Metagenomic Analyses

5.3.5.1. Next-Generation Sequencing

To allow for sequencing of both DNA and RNA viruses, cDNA synthesis was performed to convert viral genomic RNA into cDNA using previously described methods [40,41]. Samples from the WWTP influent and the Nakivubo Swamp for each of the three sampling events were selected for sequencing, for a total of six samples. Viral nucleic acids were sequenced on an Illumina platform (Illumina HiSeq, Roche Technologies) at the Research Technology Support Facility (RTSF) at MSU. Libraries were prepared using the Illumina TruSeq Nano DNA Library Preparation Kit on a Perkin Elmer Sciclone robot following manufacturer's protocols. Completed

libraries underwent quality control and were quantified using a combination of Qubit dsDNA HS and Caliper LabChipGX HS DNA assays. Libraries were pooled in equimolar amounts for multiplexed sequencing. This pool was quantified using the Kapa Biosystems Illumina Library Quantification qPCR kit. The pool was loaded on one lane of an Illumina HiSeq 4000 flow cell and sequencing performed in a 2x150bp paired end format using HiSeq 4000 SBS reagents. Base calling was done by Illumina Real Time Analysis (RTA) v2.7.6 and output of RTA was demultiplexed and converted to FastQ format with Illumina Bcl2fastq v2.18.0.

5.3.5.2. Sequencing File Processing

The raw sequencing files were assessed for quality control using FastQC [57]. The flexible read trimming tool for Illumina NGS data called Trimmomatic was used for trimming the paired-end raw reads from the Illumina sequencer and removing adapters using ILLUMINACLIP [58]. Trimmomatic was also used to trim the leading 26 base pairs representing the universal primer from cDNA synthesis. The trimmed reads were assembled into contig files in order to reduce the chances of false positive detection using an iterative de Bruijn graph de novo assembler for short reads sequencing data with highly uneven sequencing depth called IDBA-UD using a minimum k-mer length of 40, maximum k-mer length of 120, and an interval of 10 [59]. The assembled contig files used in this study are available on the MG-RAST server under project accession ID mgp80872.

5.3.5.3. BLASTn Analysis

The assembled contig files were BLASTed against the Complete RefSeq Release of Viral and Viroid Sequences (downloaded 16 Jan 2017) from NCBI using BLASTn and a maximum e-value of 10^{-3} , which has been used in prior studies and shown to minimize false positives

[40,60]. BLAST output was parsed and annotated using MEGAN to allow for taxonomic classification of reads.

5.3.6. Statistical and Principal Component Analysis

The Wilcoxon signed-rank test was used to test the significance difference in concentrations of the four tested viruses between the samples taken in the Nakivubo Channel before and after the Bugolobi WWTP as well as the samples taken in the WWTP influent and effluent ($p < 0.05$). This test was also used to assess significance of the differences in concentrations of AdV among the three sampling events ($p < 0.01$). Principal component analysis (PCA) was also performed using the six metagenomic samples to assess sample similarity. Microsoft Excel was used to perform PCA with the Real Statistics Resource Pack software (Release 4.5) [61]. In order to perform PCA, the number of hits for each viral order as determined by MEGAN was converted into a relative abundance percentage for each individual sample. The relative abundances were then used in PCA to calculate the values of the first two principal components, PC1 and PC2. The two principal components were then charted on a scatter plot for the six samples analyzed.

5.4. Results

Average concentrations for the four tested viruses at each of the five sampling locations are summarized in Table 5.3 and boxplots for each virus are shown in Figure 5.2. Across all locations, enterovirus (EV) was found to have the highest concentrations, followed by adenovirus (AdV), then hepatitis A virus (HAV), and finally rotavirus (RV) which had the lowest concentrations as calculated by qPCR. Concentrations were highest at the WWTP influent and there was not a significant decrease from the influent to the effluent of the WWTP according to the Wilcoxon signed-rank test. The Wilcoxon signed-rank test also determined that the higher

concentrations of AdV in Nakivubo Channel after the WWTP were significant compared to concentrations from before the WWTP ($p < 0.05$). Concentrations of EV and RV were also higher in Nakivubo Channel after the WWTP compared to before, but these differences were not found to be statistically significant ($p < 0.05$).

Table 5.3: Average concentrations of viruses at each sampling location (copies/L). Ranges of minimum and maximum detected concentrations are listed in parentheses. ^Only one sample with positive signal.

Human Virus	Location				
	Channel Before WWTP	WWTP Influent	WWTP Effluent	Channel After WWTP	Swamp
Adenovirus	5.45×10^5 (1.53×10^5 - 1.25×10^6)	1.17×10^7 (3.33×10^6 - 1.8×10^7)	9.43×10^6 (1.67×10^6 - 1.98×10^7)	2.08×10^6 (7.02×10^5 - 2.95×10^6)	5.72×10^5 (1.60×10^5 - 1.08×10^6)
Enterovirus	3.09×10^6 (1.73×10^6 - 4.71×10^6)	3.91×10^7 (4.73×10^6 - 8.13×10^7)	1.42×10^7 (1.25×10^6 - 2.12×10^7)	4.15×10^6 (1.10×10^6 - 7.22×10^6)	4.69×10^6 (3.17×10^5 - 1.48×10^7)
Rotavirus	1.16×10^2 (1.08×10^2 - 1.25×10^2)	1.81×10^3 (4.22×10^2 - 3.77×10^3)	5.79×10^1 ^	1.31×10^3 (1.87×10^2 - 3.72×10^3)	1.66×10^2 (6.49×10^1 - 2.99×10^2)
Hepatitis A	7.74×10^3 (5.88×10^3 - 1.03×10^4)	4.26×10^3 (2.01×10^3 - 8.39×10^3)	5.79×10^3 (1.93×10^3 - 8.70×10^3)	6.12×10^3 (1.41×10^3 - 1.11×10^4)	2.73×10^3 (9.93×10^2 - 4.48×10^3)

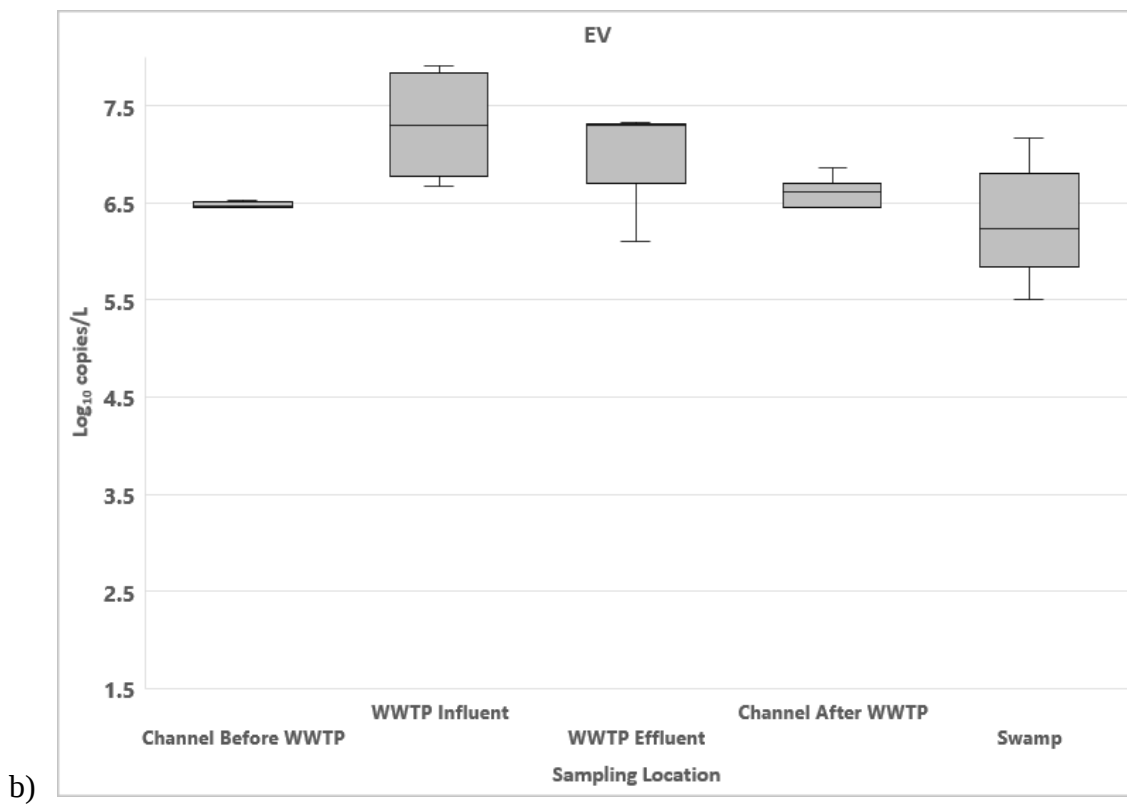
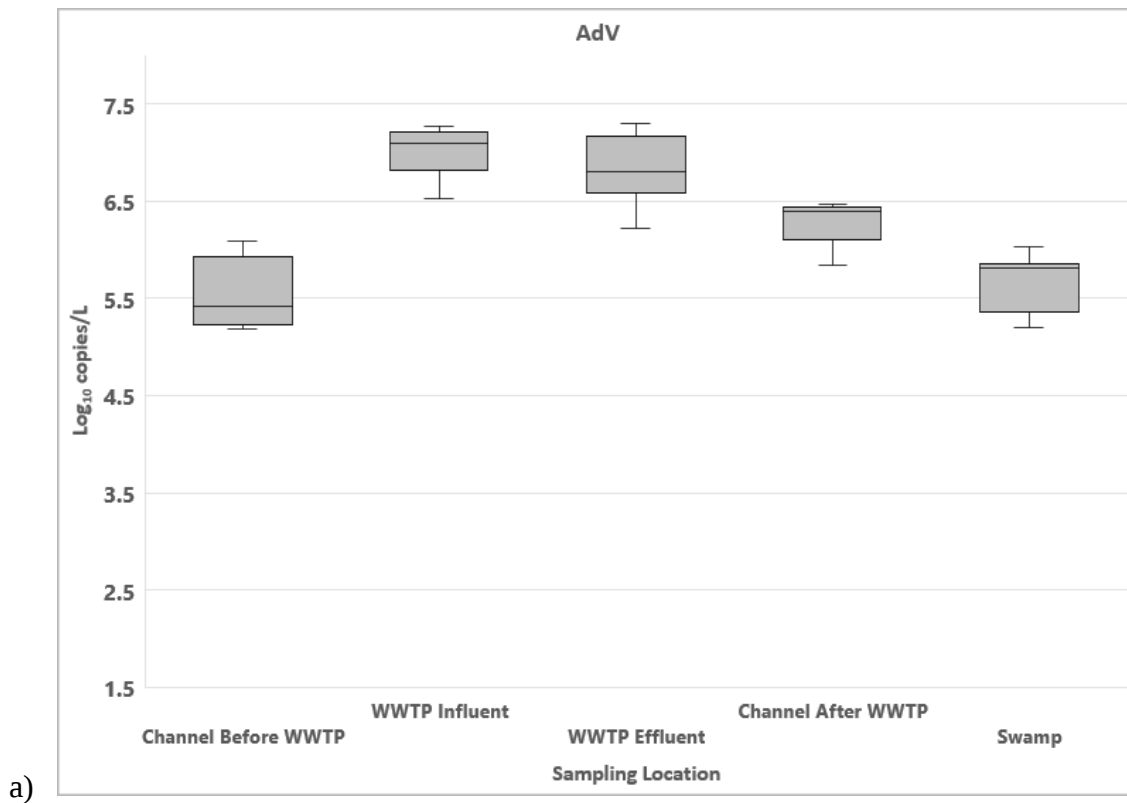


Figure 5.2: Boxplots for detected concentrations of a) adenovirus, b) enterovirus, c) rotavirus and d) hepatitis A virus at each sampling location.

Figure 5.2 (cont'd).

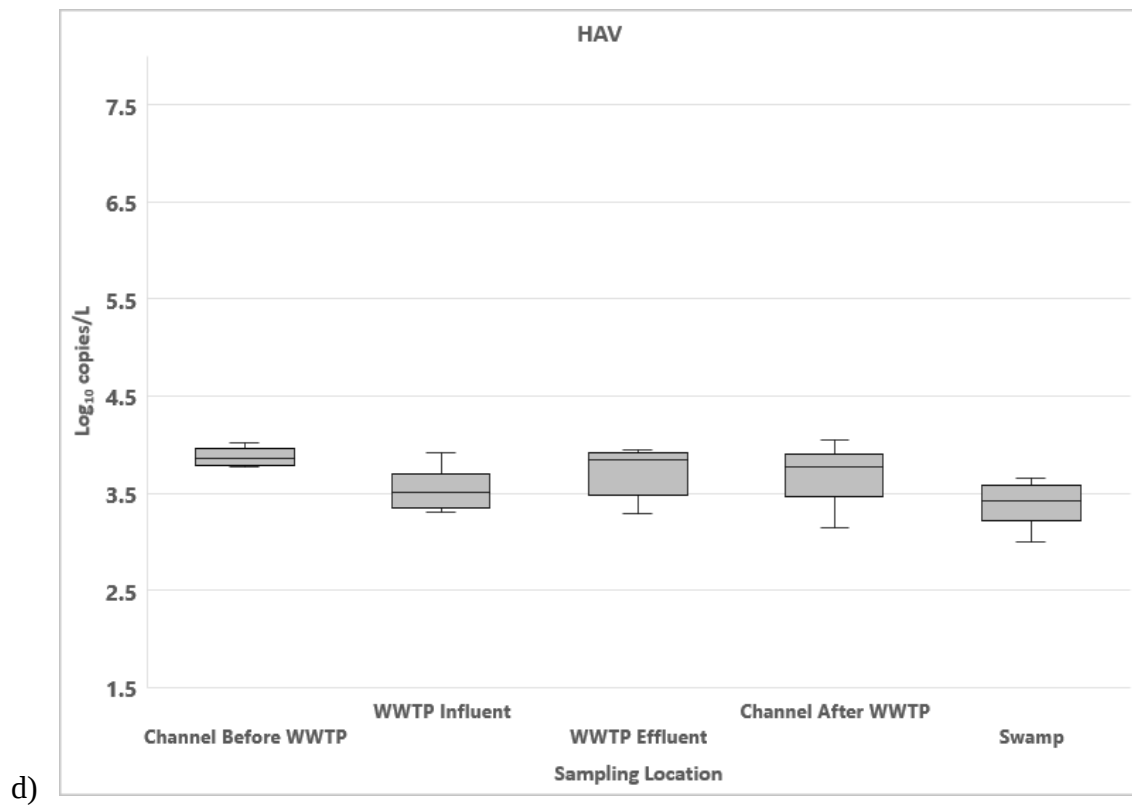
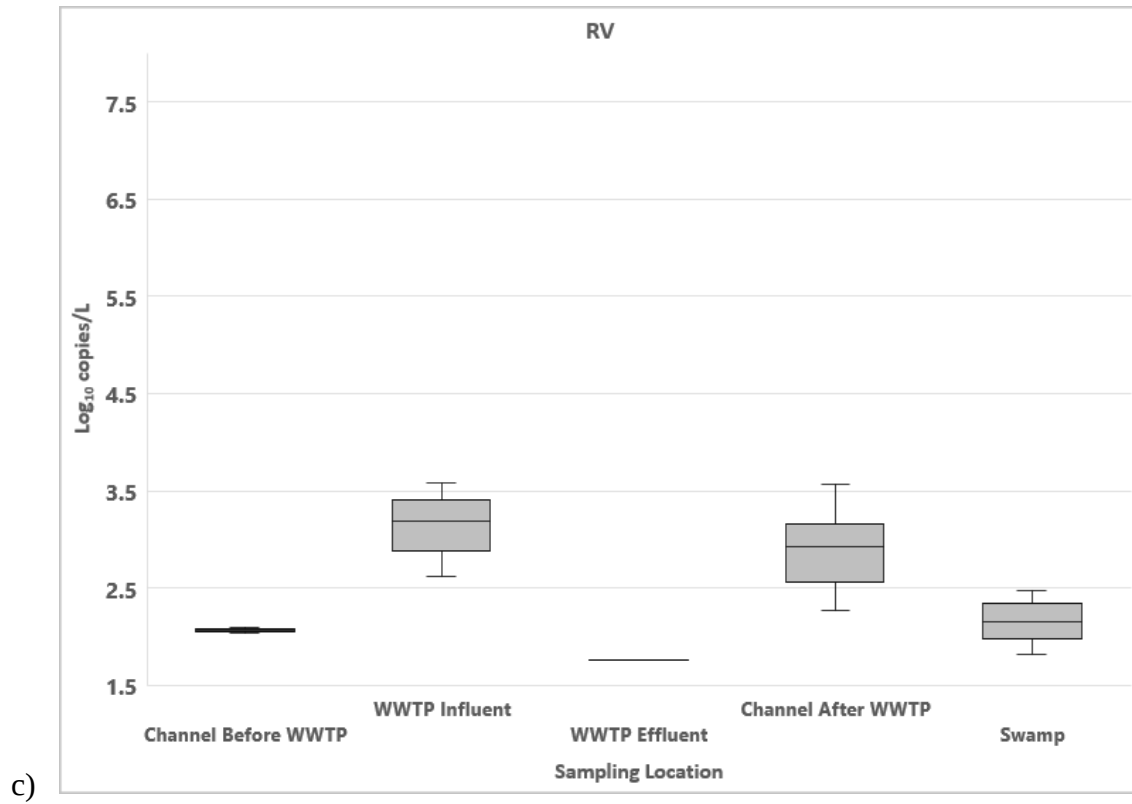


Table 5.4: Number of qPCR samples testing positive for each virus, date, and location. Adenovirus and enterovirus samples were run once in duplicate, rotavirus and hepatitis A virus were run twice in duplicate.

Virus	Sampling Date	Channel Before WWTP	WWTP Influent	WWTP Effluent	Channel After WWTP	Swamp
Adenovirus	12 July	2/2	2/2	2/2	2/2	2/2
	25 July	2/2	2/2	2/2	2/2	2/2
	8 August	2/2	2/2	2/2	2/2	2/2
Enterovirus	12 July	2/2	2/2	2/2	2/2	2/2
	25 July	2/2	0/2	1/2	2/2	2/2
	8 August	2/2	2/2	0/2	0/2	2/2
Rotavirus	12 July	1/4	0/4	0/4	1/4	0/4
	25 July	1/4	1/4	1/4	0/4	2/4
	8 August	0/4	4/4	0/4	4/4	2/4
Hepatitis A	12 July	4/4	2/4	1/4	2/4	3/4
	25 July	3/4	0/4	2/4	0/4	1/4
	8 August	0/4	2/4	3/4	1/4	4/4

Quantitative PCR results were also analyzed for temporal changes among the three sampling events. Table 5.4 shows the occurrence of each virus at each location for each of the three dates on which sampling took place. AdV was detected at all locations on each sampling date, while the other three viruses were detected on certain dates but not others at some locations. For example, RV was most prevalent in the samples from August 8th, while EV was most common in the samples from July 12th. Since AdV was detected in all samples, changes in concentration across the three sampling events were also investigated. Figure 5.3 displays the average concentration for AdV at each location separated by date. Temporal variations in AdV concentration are evident based on these qPCR results; concentrations of AdV were highest in four of the five locations on July 25th, and lowest in all five locations on August 8th. The Wilcoxon signed-rank test determined that the differences in concentrations of AdV among the three sampling dates were statistically significant ($p < 0.01$).

Sequencing data were analyzed using BLAST and MEGAN. A summary of the metagenomics analysis data is shown in Table 5.5. The vast majority of affiliated sequences were

assigned to viruses, with viruses comprising within 89.94% to 99.79% of assigned sequences for each of the six samples. As shown in Figure 5.4, the majority of viral sequences correspond to bacteriophages and invertebrate viruses in each sample. Vertebrate viruses, including those infecting humans, comprise from 1.18% of viral sequences in the August 8th Swamp sample to 5.40% of viral sequences in the July 12th Swamp sample. Table 5.6 displays the number of hits for each vertebrate virus family for each sample.

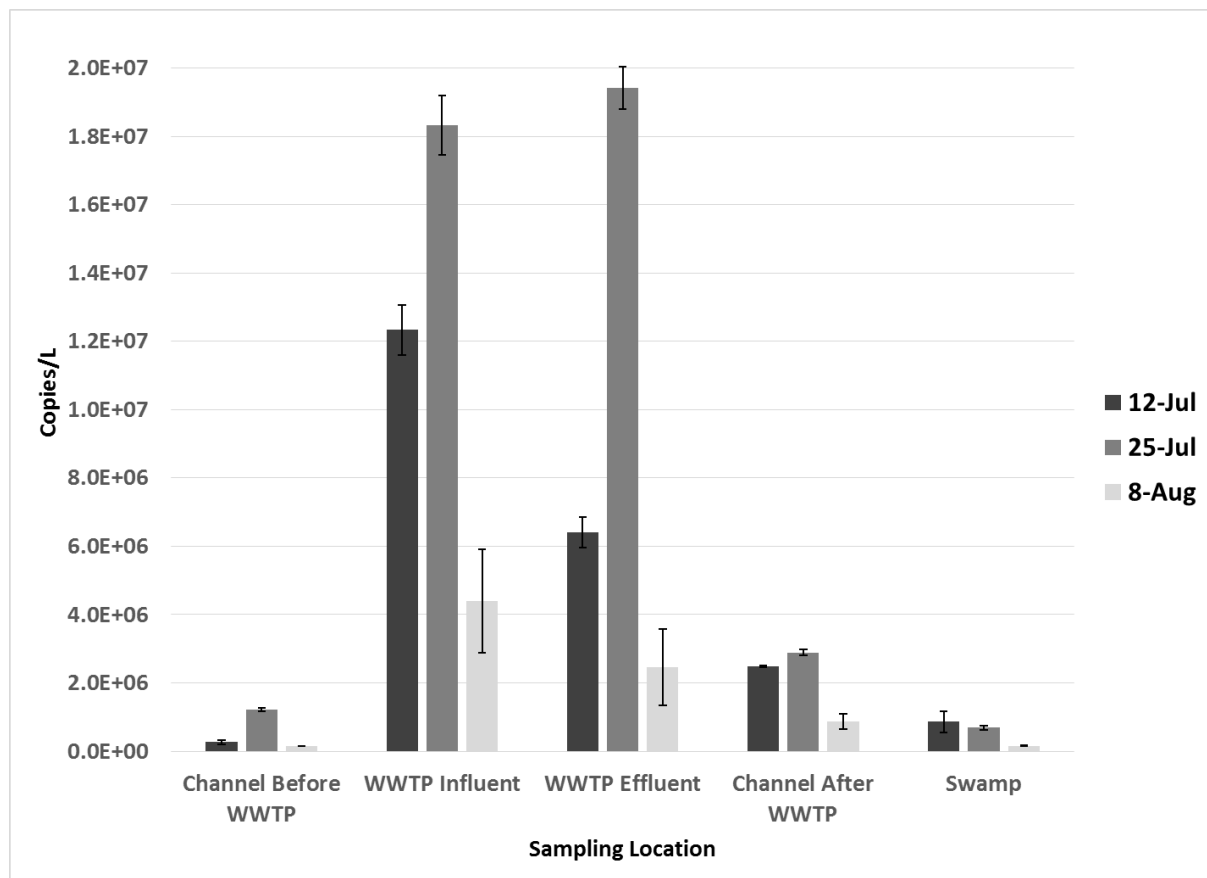


Figure 5.3: Average adenovirus concentration (copies/L) at each sampling location on each sampling date. Error bars represent one standard deviation in each direction.

Table 5.5: Summary of metagenomic analysis statistics. Affiliated sequences refer to the number of sequences that registered a hit for a viral reference genome as determined by BLAST. Unaffiliated sequences did not register a hit for any viral reference genome during BLAST analysis. Affiliated ratio is the percentage of affiliated sequences relative to the number of contigs in the sample.

Metagenome Date	Number of contigs	Affiliated sequences	Unaffiliated sequences	Affiliated ratio
WWTP Influent				
12 July	371741	6203	365538	1.67%
25 July	99741	3063	96678	3.07%
8 August	31489	3479	28010	11.05%
Nakivubo Swamp				
12 July	144446	3685	140761	2.55%
25 July	67860	3821	64039	5.63%
8 August	23166	3731	19435	16.11%

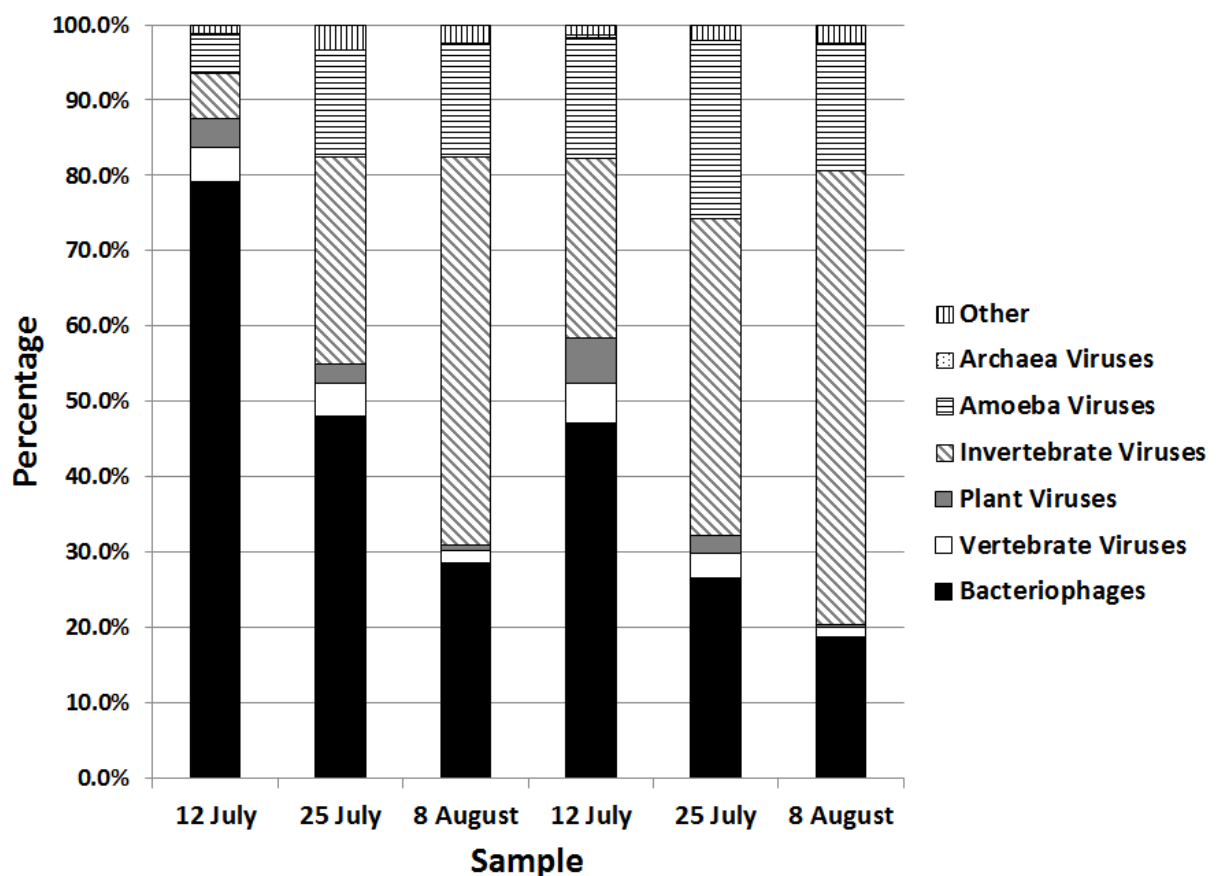


Figure 5.4: Affiliated viral sequences by host type for each sample.

Among the vertebrate viruses detected in the metagenomic samples, a number were human viruses. Three of the viruses analyzed via qPCR (AdV, EV, and RV) were detected with Illumina sequencing, with RV having positive hits in each of the three Influent samples. Human AdV was detected in both the Influent and Swamp samples from July 12th. Human papillomavirus (HPV) was also detected in the two samples from July 12th, as well as Cacipacore virus, a virus of genus Flavivirus, the genus of Zika virus. Other human viruses detected in the samples include astrovirus, picobirnavirus, circovirus, tanapox virus, Torque teno virus, and one hit for Ebola virus.

Table 5.6: Number of hits for vertebrate virus families for each sample.

Human Virus	WWTP Influent			Nakivubo Swamp		
	12 July	25 July	8 August	12 July	25 July	8 August
Adenoviridae	9	1	0	5	1	0
Herpesvirales	15	4	0	4	0	0
Iridoviridae	27	5	2	14	6	2
Papillomaviridae	1	0	0	1	0	0
Polyomaviridae	7	0	0	0	0	0
Poxviridae	62	11	3	47	25	3
Picobirnaviridae	0	4	2	0	0	0
Reoviridae	2	1	4	1	0	0
Retroviridae	0	0	0	1	0	0
Anelloviridae	1	0	0	0	0	0
Circoviridae	60	29	12	34	26	5
Parvoviridae	20	30	2	45	38	6
Bunyaviridae	3	18	32	15	26	24
Orthomyxoviridae	0	1	0	0	0	0
Mononegavirales	6	0	0	0	0	0
Astroviridae	6	1	2	1	1	1
Flaviviridae	1	0	0	1	0	0
Hepeviridae	1	0	0	1	0	0
Nidovirales	4	1	1	4	1	1
Nodaviridae	0	0	0	3	0	0
Picornaviridae	9	8	0	1	0	0
Total	234	114	60	178	124	42

When comparing the six samples against one another, more similarity is observed between samples from each respective sampling date compared to samples from each respective location. Figure 5.5 displays a PCA plot for the six metagenomic samples. As shown in the PCA plot, the first principal component separates the samples by date, and it is the second principal component that separates the samples by location.

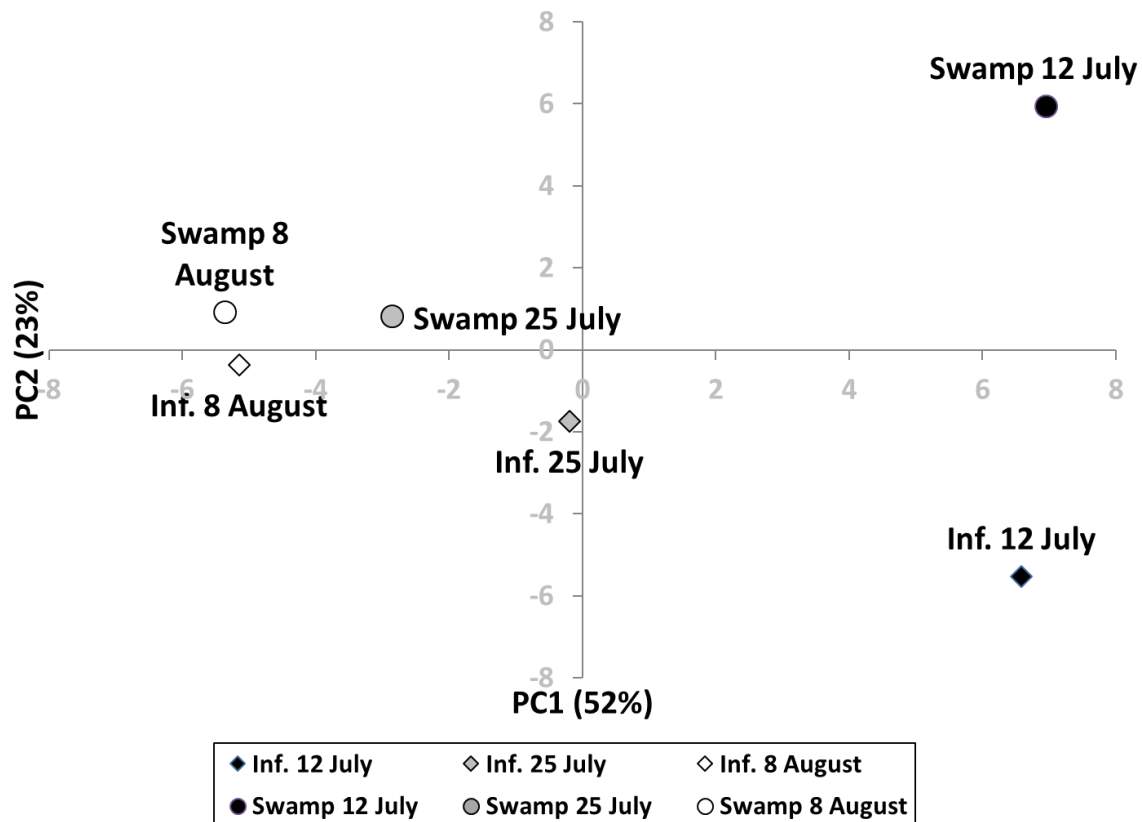


Figure 5.5: Principal component analysis plot for the six metagenomic samples. Relative abundance percentages for each viral order of each metagenome were used to compute the principal components.

5.5. Discussion

All four viruses tested were detected in each of the five sampling locations. With the exception of HAV (which had similar concentrations at each location), the tested viruses followed a similar trend across the five locations. The WWTP influent was found to have the

highest concentration of any location, with a slight reduction (<2 log) in the WWTP effluent. The virus reduction of <2 logs at the WWTP was lower than the log reductions in prior studies from other locations around the world [62,63]. The low virus reduction may be attributed to virus adsorption onto particles that do not settle in the clarifier and not removed effectively during the clarification process. Furthermore, the samples from the Nakivubo Channel after the WWTP had statistically significant higher concentrations of AdV than the samples from the Nakivubo Channel before the WWTP. This suggests that the WWTP effluent is releasing viruses back into the surface waters surrounding the WWTP.

Concentrations were typically further reduced in the samples from the Nakivubo Swamp. Natural wetlands such as the Nakivubo Swamp are capable of reducing viruses in wastewater through exposure to sunlight, microbial interactions, and plant uptake [64,65]. However, persistence of viruses in the wetland is a challenge since viruses are capable of adsorbing onto soil, causing the soil to behave as a reservoir for viruses [66–68].

Effluent from the WWTP is released into the Nakivubo Channel, which empties into the Nakivubo Swamp, ultimately flowing into Lake Victoria, a drinking water source for the area. Therefore, pollution from the WWTP effluent could ultimately affect drinking water quality, hence the necessity for more robust monitoring and improved removal of human pathogenic viruses in the wastewater treatment process.

The large majority of sequences from the metagenomic samples were unaffiliated with any known viral genome, which is consistent with prior studies using these methods that also found significant proportions of unaffiliated sequences [39–43]. Among the sequences affiliated with viral genomes, higher proportions were affiliated with vertebrate hosts compared to prior studies from other locations [40,42,43]. This could suggest that there is a higher viral disease

burden to humans in Kampala compared to other more developed countries around the world, heightening the importance for the implementation of effective wastewater treatment techniques.

AdV, EV, and RV were all among the human viruses detected in the metagenomic samples, in addition to several others that were not investigated via qPCR, including astrovirus, papillomavirus, and even Ebola virus. The fact that other human viruses were assigned approximately the same number of BLAST hits as those viruses investigated via qPCR indicates that the practice of metagenomic methods for diversity analysis can be useful to detect other viruses that may pose a health risk to humans. It is important to note, however, that molecular detection methods such as qPCR and BLAST annotation do not offer information regarding viral infectivity; further investigation would be necessary to assess the health risks associated with the viral populations in these sampling locations.

Results from both qPCR and metagenomic analyses indicate that concentrations and diversity of viruses in wastewater have temporal variation. AdV was shown to have statistically significant differences in concentration from one week to the next at each sampling location, and the other three viruses tested via qPCR were detected in some sampling events but not in others. Results from metagenomic analyses also support the notion that the viral community varies temporally, as samples from different locations during the same sampling event were more similar to one another than samples from the same location during different sampling events as shown by PCA, though it should be acknowledged that PCA was performed with a small sample size of only six samples and therefore the conclusions we can draw from this analysis are limited.

These temporal changes indicate that wastewater can be used as an epidemiological tool to identify and predict disease outbreaks at a population level. The wastewater epidemiology

methodology is founded upon the idea that concentrations of a human excretion product in wastewater can be hind-cast to an initial source concentration. The application of this methodology to viral disease outbreaks is therefore predicated on detecting significant fluctuations over a short timeframe in viral concentration in wastewater, as this could indicate a potential disease outbreak due to the detected virus. This study shows that such significant weekly fluctuations are indeed detectable in wastewater, establishing the viability of the practice of this methodology.

Several steps, though, must be taken in order to implement this methodology. First, accurate baseline concentrations in wastewater for viruses of interest must be established with replicated samples ($N > 3$). This requires regular sampling and quantification via qPCR performed throughout the year to account for seasonal variations. In order to control for variations in serviced population, biomarkers in wastewater should also be quantified. A study of several biomarkers determined 5-HIAA to be a viable biomarker in wastewater for population estimation [69]. Once these data are obtained, the detected concentrations could be compared to clinical data from the surrounding area to determine whether there is a correlation between fluctuations in viral concentration in wastewater and an increase in reported cases of viral human disease. Were a correlation to be established, wastewater would then be an invaluable tool in predicting and identifying viral disease outbreaks.

5.6. Conclusions

This study established the prevalence and concentrations of four waterborne viruses, adenovirus, enterovirus, rotavirus, and hepatitis A virus, in wastewater and surrounding surface waters in Kampala, the capital of Uganda. Additionally, overall and vertebrate viral diversity was assessed. The study provided preliminary data showing that continuous monitoring of

wastewater for viral concentration and diversity can indicate temporal variations that may correlate with changing levels of disease at a population level. These results may be useful in the application of wastewater viral monitoring as an epidemiological tool to better monitor the disease burden of the serviced population and provide indication of early detection of potential viral outbreaks. Further investigation is necessary to establish more statistically robust baseline viral concentrations in these water bodies and to correlate viral concentrations with clinical data to fully implement this methodology.

5.7. Acknowledgments

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Chapter 6:

Diversity of DNA viruses in effluents of membrane bioreactors in Traverse City, MI (USA) and La Grande Motte (France)

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6.1. Abstract

This study assesses diversity of DNA viruses in the effluents of two membrane bioreactor (MBR) wastewater treatment plants (WWTPs): an MBR in the United States and an MBR in France. Viral diversity of these effluents is compared to that of a conventional activated sludge WWTP in the U.S. Diversity analysis indicates *Herpesvirales* to be the most abundant order of potentially pathogenic human DNA viruses in wastewater treated effluent in all utilities. Other potentially pathogenic human viruses detected include *Adenoviridae*, *Parvoviridae*, and *Polyomaviridae*. Bacteriophage order *Caudovirales* comprises the majority of DNA virus sequences in the effluent of all utilities. The choice of treatment process (MBR versus activated sludge reactor) utilized had no impact on effluent DNA viral diversity. In contrast, the type of disinfection applied had an impact on the viral diversity present in the effluent.

6.2. Introduction

Viruses are potentially the most hazardous pathogens among those found in wastewater [1,2]. They are also generally more difficult to detect in environmental samples. A high diversity of human viral pathogens is present in the environment (approximately 200 recognized human viral pathogen species) and is further elevated in samples affected by pollution. Moreover,

additional species are continuously discovered [3]. It has been estimated that 2 to 12 million people die every year from waterborne diseases. While the majority of the outbreaks are caused by unidentified agents, it has been suggested that most agents in question are enteric viruses in groundwater and surface water bodies [4]. Despite recent advances in water and wastewater treatment technology, waterborne diseases still pose a serious threat to public health across the world [1].

The Contaminant Candidate List (CCL) compiled and periodically updated by the U.S. Environmental Protection Agency includes contaminants that are known or anticipated to occur in public water systems, and which may require regulation under the Safe Drinking Water Act. Included on the CCLs are numerous viruses, such as the double-stranded DNA adenoviruses. Double-stranded DNA viruses have been shown to be more resistant to UV disinfection when compared with other virus types [5]. Adenoviruses have been investigated and detected in wastewater in prior studies via conventional methods [6–23] and determined to be among the most abundant human viruses in WWTP effluent [16,24]. It has been concluded that adenoviruses may serve as indicators for general viral contamination [16,25].

In addition to human viral pathogens, bacteriophages may also have a significant impact on the natural water that receives treated effluent from a wastewater treatment facility. It has been shown that bacteriophages are strong regulators of microbial diversity within a WWTP [26]. Bacteriophages can have an effect on the microbial community as well as on eukaryotic members of an ecosystem that rely on bacteria [27], making phages all the more important in maintaining ecological health of the aquatic environment. The majority of bacteriophages contain a DNA genome, making DNA bacteriophages by far the most prevalent group of viruses impacting the microbial community [28].

Both conventional activated sludge (AS) WWTPs and MBRs release pathogenic viruses into surface water [12,13,15]. It has been demonstrated that MBRs can remove several viruses (including adenovirus) at higher efficiencies compared to conventional AS utilities [7,29]; for example, log removal of adenovirus ranged from 1.3 to 2.4 in conventional AS utilities [9,19,30], whereas MBRs accomplished 3.4 to 5.6 log removals [12,13,15]. Though removal of specific viruses in these utilities has been investigated, there has been little application of metagenomics in studying the viral diversity of MBR effluent and how it compares to that of a conventional AS plant.

Next-generation DNA sequencing has recently been applied to study viral metagenomes (viromes) in environmental samples [31–34] as well as at different stages of the wastewater treatment [8,35–39]. These methods have been shown to provide more conservative estimates of viral occurrence compared to the rates detected by qPCR [36]. The advantage of metagenomics is that it allows comprehensive characterization of microbial communities. However, metagenomic methods do not assess infectivity and the sequence annotation is only as reliable and robust as the assembly methods and database used for analysis. Metagenomic analysis is presently only capable of identifying a fraction of the viruses present in the environment [8,35].

Nonetheless, metagenomic methods are an effective tool for analyzing the microbial diversity of environmental samples. Though wastewater has been investigated with metagenomic methods, there has been little use of metagenomics to evaluate the microbial diversity of wastewater effluents, and to the authors' knowledge none comparing different types of wastewater utilities or the impact of disinfection. The specific objectives of this study are: 1) To investigate the diversity of human DNA viruses detected in effluents of MBR WWTPs equipped with membranes of different pore sizes; 2) To assess the diversity of DNA bacteriophages in

MBR WWTP effluents; 3) To compare the diversity of DNA viruses in MBR WWTP effluents with that in a conventional WWTP effluent; and 4) To investigate the impact of disinfection on DNA virus diversity in WWTP effluent.

6.3. Materials and Methods

6.3.1. Sampling locations

Table 6.1: Wastewater treatment plant characteristics.

	EAST LANSING Michigan, USA	TRAVERSE CITY Michigan, USA	LA GRANDE- MOTTE Languedoc- Roussillon-Midi- Pyrénées, France
Wastewater treatment process	Conventional Activated Sludge (CAS)	Membrane Biological Reactor (MBR)	Membrane Biological Reactor (MBR)
Sludge retention time, days	14	7.58	46.5
Capacity, MGD	18.8	17.0	3.2
Average flow, MGD	13.4	8.5	2.6
Disinfection	Hypochlorite	UV	None
Nominal pore size, μm	n/a	0.04	0.45
Sampling dates	Spring 2013	Spring 2013	Summer 2015

The selected MBR WWTPs were facilities located in Traverse City (Michigan, USA) and La Grande Motte (Languedoc-Roussillon-Midi-Pyrénées, France), which are both tourism destinations. Sampling was performed during the warmer seasons when the population of each location is increased due to the large number of vacationers. Effluent samples were collected at three wastewater treatment utilities. In Spring 2013, sampling was performed at the East Lansing WWTP (East Lansing, MI), which is a conventional activated sludge plant employing hypochlorite disinfection, and the Traverse City WWTP (Traverse City, MI), which employs MBR technology with ultrafiltration membranes of 0.04 μm nominal pore size and UV disinfection. A sample was taken from the treated effluent both before disinfection and after

disinfection at each of the Michigan utilities. In summer 2015, sampling was performed at La Grande Motte WWTP (La Grande Motte, France), which is also an MBR plant but employs microfiltration membranes of 0.45 μm nominal pore size and does not have an additional disinfection step. A sample was also taken from the treated effluent at this utility. Main operational parameters for these utilities are summarized in Table 6.1.

6.3.2. Sample collection

Approximately 300 L of sampled treated effluent was passed through a NanoCeram Virus Sampler filter (Argonide Corporation) at a rate of 11 to 12 L/min using a previously described method [15]. Samples from the two Michigan WWTPs were kept on ice and transported to Michigan State University (East Lansing, MI), while samples from France were kept on ice and transported to Université de Montpellier (Montpellier, France) for further processing.

6.3.3. Sample processing

All NanoCeram filters used to concentrate the treated effluent samples were eluted according to the standard method [40] within 24 h of initial sampling. Briefly, a 1.5% w/v beef extract (0.05 M glycine, pH 9.0–9.5) solution was used as the eluent. The filters were submerged for a total of 2 min (two separate 1 min elutions) in filter housings with 1 L of beef extract added to the pressure vessel. After the beef extract was passed through each filter, pH of the 1 L of the eluate was adjusted to 3.5 ± 0.1 using 1 M HCl and flocculated for 30 min. Further concentration of the solution was performed by two stages of centrifugations for 15 min at 2500g and 4 °C. The supernatant was then decanted and the process was repeated until all the beef extract solution was centrifuged. The accumulated pellets were resuspended using 30 mL of 0.15 M sodium phosphate (pH 9.0–9.5) and mixed until the pellet was mostly dissolved. The pH was then adjusted to 9.0–9.5 using 1 M HCl. The solution was placed into a 50 mL centrifuge tube

and centrifuged for 10 min at 4 °C at 7000g. The supernatant was poured off into a separate 50 mL centrifuge tube, the pH was adjusted to 7.0–7.5 for stabilization of the virus particles, and the pellet was discarded. The supernatant was loaded into a 60 mL syringe and passed through a 0.22 µm sterilized filter for removal of bacteria, fungi and other contaminants. All samples were completely mixed and placed into 2 mL cryogenic tubes. Samples from France were shipped on dry ice to Michigan State University, where all samples were stored at –80 °C until further analysis.

6.3.4. Nucleic acid extraction

Viral DNA was extracted using a MagNA Pure Compact Instrument (Roche Applied Science) and a MagNA Pure Compact Nucleic Acid Isolation Kit according to the manufacturer's instructions. A 400 µL sample was loaded in the instrument and yielded an elution volume of 100 µL. DNase treatment is performed by the MagNA Pure Compact prior to extraction. The extracts were stored in a freezer at -20 °C. Following extraction the quantity of viral DNA extracts from all samples were verified for quality control purposes using the NanoDrop spectrophotometer (NanoDrop® ND-1000, Wilmington, DE).

6.3.5. Metagenomic analyses

Viral DNA extracts were sequenced on an Illumina platform (Illumina HiSeq, Roche Technologies) at the Research Technology Support Facility (RTSF) at Michigan State University. DNA-Seq libraries were prepared using the Rubicon Genomics ThruPLEX DNA-seq Kit. After preparation, libraries underwent quality control and were quantified using Qubit dsDNA, Caliper LabChipGX and Kapa Biosystems Library Quantification qPCR kit. The samples were pooled together and the pool was loaded on one lane of an Illumina HiSeq 2500 Rapid Run flow cell (v2) and sequencing was done in a 2x150bp paired end format using

Illumina Rapid SBS reagents. Base calling was performed by Illumina Real Time Analysis (RTA) v1.18.64 and output of RTA was demultiplexed and converted to FastQ format with Illumina Bcl2fastq v1.8.4. The raw sequencing files were then assessed for quality control using FastQC [41]. The flexible read trimming tool for Illumina NGS data called Trimmomatic was used for trimming the paired-end raw reads from the Illumina sequencer and removing adapters using ILLUMINACLIP [42]. The trimmed reads were assembled into contig files so as to reduce the chances of false positive detection using an iterative de Bruijn graph de novo assembler for short reads sequencing data with highly uneven sequencing depth called IDBA-UD using a minimum k-mer length of 40, maximum k-mer length of 120, and an interval of 10 [43].

6.3.6. MetaVir2 analyses

The assembled contig files for all samples were uploaded to the MetaVir2 web server for analysis. MetaVir2 is an online database designed to annotate viral metagenomics sequences (raw reads or assembled contigs) [44]. The MetaVir server provides taxonomic affiliations of the viral sequence reads. Taxonomic composition is computed from a BLAST comparison with the RefSeq complete viral genomes protein sequences database from NCBI (release of 2014-07-10) using BLASTp. Open reading frames (ORFs) are predicted for each contig using MetaGeneAnnotator [45] and are compared to RefSeq through BLASTp, and each predicted translated ORF is affiliated to its best BLAST hit (if any), i.e. to the affiliation of the predicted protein with the highest BLAST score. The number of hits is defined as the occurrences of the input sequence in the database. Best hit ratio is defined as the number of hits for one category divided by total number of hits. Metavir only selects for sequences longer than 300 bp. A maximum E-value cutoff of $1E-5$ was used. Sample metagenomes are publicly available on

MetaVir under the project titles “EastLansing/TraverseCity” (project IDs 3491, 3492, 3534, 3692) and “LaGrandeMotte” (project ID 7215).

6.3.7. Bowtie2 and SAMTools analyses

Following MetaVir analyses, the Bowtie2 and SAMTools modules were used to offer further confirmation of the presence of viruses of interest. Bowtie2 is a module that can align sequencing reads and SAMTools can provide further analyses of the alignment including statistical information regarding how well the two aligned sequences match. Thus, these tools were used to compare existing genomic data of viruses to the raw sequence data of the experimental samples. The Complete RefSeq Release of Viral and Viroid Sequences was downloaded from the NCBI website on 10 December 2015 and used in this analysis. This complete genome was aligned with the paired-end sequencing data of our experimental samples using Bowtie2. SAMTools was then used to provide output data regarding the alignment performed by Bowtie2 in the form of a percentage of coverage of base pairs of the selected genomes as well as statistical and quality control information regarding the alignment.

6.3.8. qPCR analyses

Additional confirmation using real-time quantitative PCR was performed on each sample using a Roche LightCycler 1.5 instrument (Roche Applied Sciences) for the detection of Human Adenovirus 40/41 using a previously described assay [46]. Briefly, for each sample, a 20- μ L PCR mixture was created in triplicate containing 4 μ L of 5x LightCycler TaqMan Master Mix, 0.8 μ L of 10 μ M forward primer (final concentration, 400 nM), 0.4 μ L of each 10 μ M reverse primer (final concentration, 200 nM), 0.6 μ L of 10 μ M TaqMan probe (final concentration, 300 nM), 8.8 μ L of PCR-grade water, and 5 μ L of DNA extract. The real-time PCR program used a

denaturation step for 15 min at 95 °C, followed by an amplification step of 45 cycles at 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 10 s, concluding with a cooling step at 40 °C for 30 s.

6.4. Results

Sequencing data were analyzed using MetaVir2. The number of contigs in the assembled files for all samples was in the range of 76970 to 256064 as computed by MetaVir. A summary of the affiliation of these contig sequences is presented in Table 6.2. A comparison of the ratio of affiliated sequences to unaffiliated sequences is shown in Figure 6.1. The effluent samples before disinfection for the two MBR utilities contained 19.88% (Traverse City WWTP) and 17.36% (La Grande Motte WWTP) affiliated sequences, whereas the effluent sample after disinfection from Traverse City WWTP contained 16.36% affiliated sequences. A similar proportion is seen in the East Lansing conventional WWTP utility, which contained 19.39% affiliated sequences in the effluent sample before disinfection and 11.60% affiliated sequences in the effluent sample after disinfection.

Table 6.2: Metagenome analysis statistics for viral samples (from MetaVir).

Metagenome Name	Number of contigs	Affiliated sequences	Unaffiliated sequences	No. of genes affiliated	No. of genes predicted
East Lansing Wastewater Treatment Plant (EL)					
Effluent Before Disinfection	151994	29479	122515	35856	258135
Effluent After Disinfection	256064	29706	226356	33975	365870
Traverse City Wastewater Treatment Plant (TC)					
Effluent Before Disinfection	151992	30182	121610	36781	258140
Effluent After Disinfection	197517	32316	165201	39649	309715
La Grande Motte Wastewater Treatment Plant (LGM)					
Treated Effluent (No Disinfection)	76970	13364	63606	15072	124682

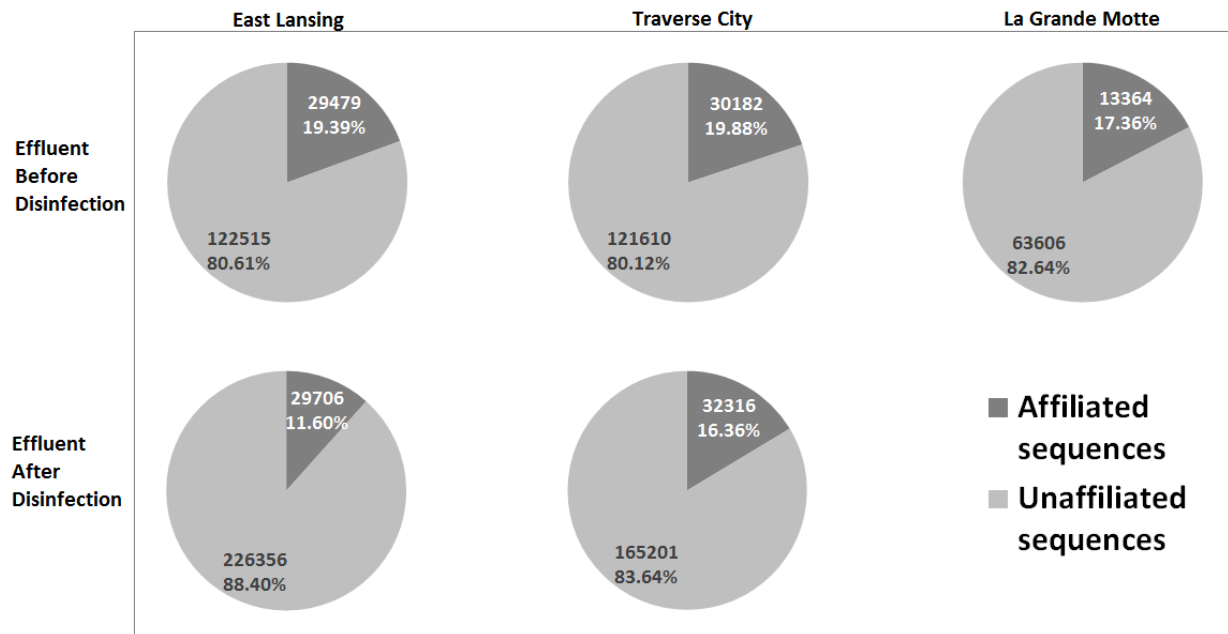


Figure 6.1: Metagenome summary (from MetaVir).

The taxonomic composition of all affiliated sequences for all samples is calculated by MetaVir. The large majority of the viruses detected in this manner are DNA viruses (>96% of affiliated sequences for all samples), which are results consistent with the analysis methods employed in the study targeting DNA viruses. The table in the supplemental information presents the detected DNA viruses (both double-stranded and single-stranded).

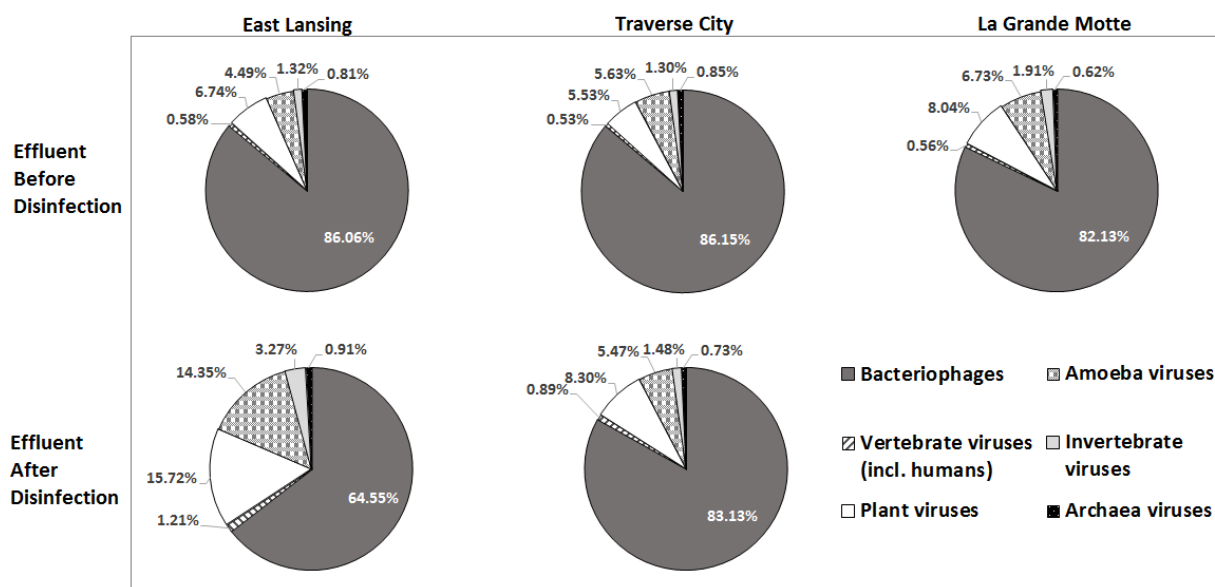


Figure 6.2: Relative abundance (number of affiliated sequences for virus host group divided by total number of affiliated sequences for the sample) for each sample by virus host group (from MetaVir).

Of the detected DNA viruses, the significant majority corresponded to bacteriophages, as shown in Figure 6.2. Similar relative abundances of bacteriophages are observed between the treated effluent samples before disinfection from the MBR utilities (86.15% for Traverse City WWTP and 82.13% for La Grande Motte WWTP of annotated DNA viral sequences), and this bacteriophage abundance is comparable to that observed in the East Lansing conventional WWTP (86.06% of annotated DNA viral sequences). The treated effluent samples after disinfection, however, are different for the two Michigan plants. Traverse City WWTP, which uses UV disinfection, had a bacteriophage relative abundance of 83.13% after the application of disinfection. East Lansing WWTP, which uses hypochlorite disinfection, showed a 64.55% relative abundance of bacteriophage.

Only a small percentage of detected viruses in the MBR samples before disinfection infect vertebrates, including those that infect humans; 0.53% in Traverse City WWTP and 0.56%

in La Grande Motte WWTP of affiliated DNA sequences fall into this category. These numbers are similar to that (0.58%) for the sample from East Lansing WWTP. A smaller relative abundance of vertebrate viruses was present in the effluent before disinfection compared to the relative abundance after disinfection in each case (0.89% in Traverse City WWTP and 1.21% in East Lansing WWTP).

The first table in the supplementary information presents classification of virus families according to the NCBI taxonomy. The first column shows the viral orders detected and each subsequent column shows each sample. The table provides the number of hits for each sample for each viral order. For both MBR samples before disinfection, the order of bacteriophages *Caudovirales* was by far the most abundant; it accounted for 77.62% in Traverse City WWTP and 74.84% in La Grande Motte WWTP of DNA virus sequences. The sample from East Lansing WWTP again proved similar, with *Caudovirales* comprising 77.71% of DNA sequences. The abundance of *Caudovirales* for the after disinfection samples correspond to the relative abundance of bacteriophage; the order comprises 74.52% of sequences in Traverse City WWTP but 60.67% of sequences in East Lansing WWTP.

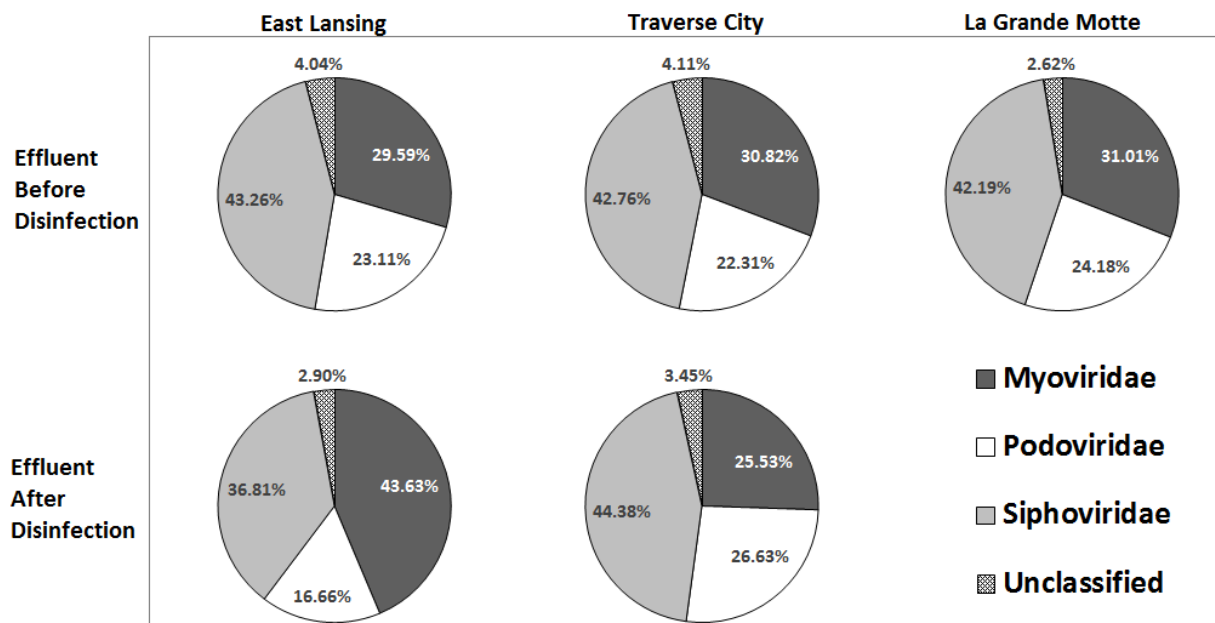


Figure 6.3: Relative abundance of Caudovirales order by family (number of affiliated sequences for each family divided by total number of affiliated Caudovirales sequences) (from MetaVir).

Within the *Caudovirales* order, the family *Siphoviridae* was the most dominant in both MBR effluent samples before disinfection, comprising just over 40% of *Caudovirales* sequences in these samples, as well as in the conventional sample, as shown in Figure 6.3. The other families, *Myoviridae* and *Podoviridae*, also have similar relative abundances across all three effluent samples before disinfection. The most common bacterial hosts within the *Caudovirales* order are *Bacillus*, *Mycobacterium*, and *Pseudomonas*. Bacteria of human concern, including *Vibrio*, *Salmonella*, and *Escherichia*, also show a high number of hits for their respective phages across all samples. The second table in the supplementary information displays the number of hits as reported by MetaVir for each the *Caudovirales* order separated by bacterial host family.

The most abundant order of vertebrate viruses detected in the samples is *Herpesvirales*. The majority of sequences detected in this order belong to the family *Herpesviridae* which is known to infect humans and other mammals. Among the many mammalian herpesviruses detected were many human herpesviruses, including human herpesvirus 1 (herpes simplex virus-

1), human herpesvirus 3 (varicella zoster virus, also known as chickenpox), human herpesvirus 4 (Epstein-Barr virus), and human herpesvirus 8. Other orders of vertebrate viruses detected by MetaVir include the dsDNA virus orders *Adenoviridae*, and *Polyomaviridae*, as well as the ssDNA virus order *Parvoviridae*. To add confidence to the annotation of these pathogenic viruses, the contigs associated with *Herpesvirales* and *Adenoviridae* in each sample were further investigated to assess the annotation of individual genes within each contig using the contig annotations from MetaVir. MetaVir uses a BLAST comparison with the RefSeq complete viral genomes protein sequences database from NCBI (release of 2014-07-10) using BLASTp and a maximum E-value cutoff of 1E-5. In each of the associated contigs, the gene with the best BLAST hit bitscore was a putative protein associated with the respective pathogenic virus (*Herpesvirales* or *Adenoviridae*); these bitscores ranged from 51.6 to 92 with a median of 70.5 for the annotated *Adenoviridae* contigs and 50.8 to 195 with a median of 71.2 for the human *Herpesvirales* contigs.

Quality control analysis from FastQC indicates that the raw sequencing files are of high quality. No sequences were flagged as poor quality in any of the samples. Statistics from Bowtie2 show similar results, with no reads in any sample having been flagged by Bowtie2 as failing quality control.

Results from Bowtie2 showed detection of human herpesvirus in all samples, as well as detection of human adenovirus in the Traverse City WWTP effluent after disinfection. Real-time quantitative PCR was used to assess the presence of human adenovirus 40/41 in all samples; detection mirrored the results as computed by MetaVir. HAdV40/41 was detected in the effluent after disinfection samples for East Lansing and Traverse City WWTPs at a concentration of

7.44*10² and 1.47*10² copies/L, respectively. This corresponds to the samples that returned multiple hits for *Adenoviridae* as reported by MetaVir.

6.5. Discussion

Despite thousands of affiliated sequences corresponding to known virus genomes in our samples, the vast majority of sequences (between 80% and 89%) were unaffiliated with any genome in the MetaVir database. While a large portion of these unaffiliated sequences could be associated with bacteria, as genetic material from organisms aside from viruses may have passed through the sample isolation procedure, a portion of the sequences in the samples may be derived from uncharacterized viral genomes. These findings are consistent with previous studies utilizing next generation sequencing which also found significant proportions of unaffiliated sequences [8,35–37]. Consequently, annotation results are biased by the available sequences in the RefSeq database; this presents a significant hurdle in the use of metagenomic techniques. More robust genomic data and comparison databases are required to truly assess the full diversity of metagenomic samples.

The majority of viruses released in wastewater effluent are bacteriophages, which can affect the microbial community in receiving streams. Bacteriophages have been shown to be important factors in maintaining phage-host systems, with a sufficient concentration of a bacterial host required to begin the production of phage [47]. A heavy influx of phages into a natural water system could potentially disrupt this phage-host system and microbial ecosystem. The figure in the supplementary information shows the most commonly detected bacteriophage hosts (*Psuedomonas*, *Bacillus*, *Mycobacterium*, and *Burkholderia*) for each of the samples. *Bacillus* is widely used as a model organism due to its prevalence in nature, whereas *Pseudomonas* is a group with significant metabolic diversity. All of these groups contain species

known to be pathogenic to humans and have been widely studied, perhaps resulting in high representation in the MetaVir database and high detection rates in the samples.

In addition to bacteriophages, many different virus types exist among the human DNA viruses detected in this study: icosahedral, non-enveloped adenoviruses and polyomaviruses, enveloped herpesviruses, and the much larger enveloped poxviruses. Nonetheless, as shown in Figures 2 and 3, similar relative abundances were observed for all viruses (regardless of structure and type) in each of the wastewater effluents without disinfection. While the numbers of hits as presented by MetaVir are low for human pathogens (including *Adenoviridae*, *Herpesviridae*, *Parvoviridae*, and *Polyomaviridae*), they are nonetheless detected indicating that metagenomic methods can be used to screen for human pathogens in wastewater effluent.

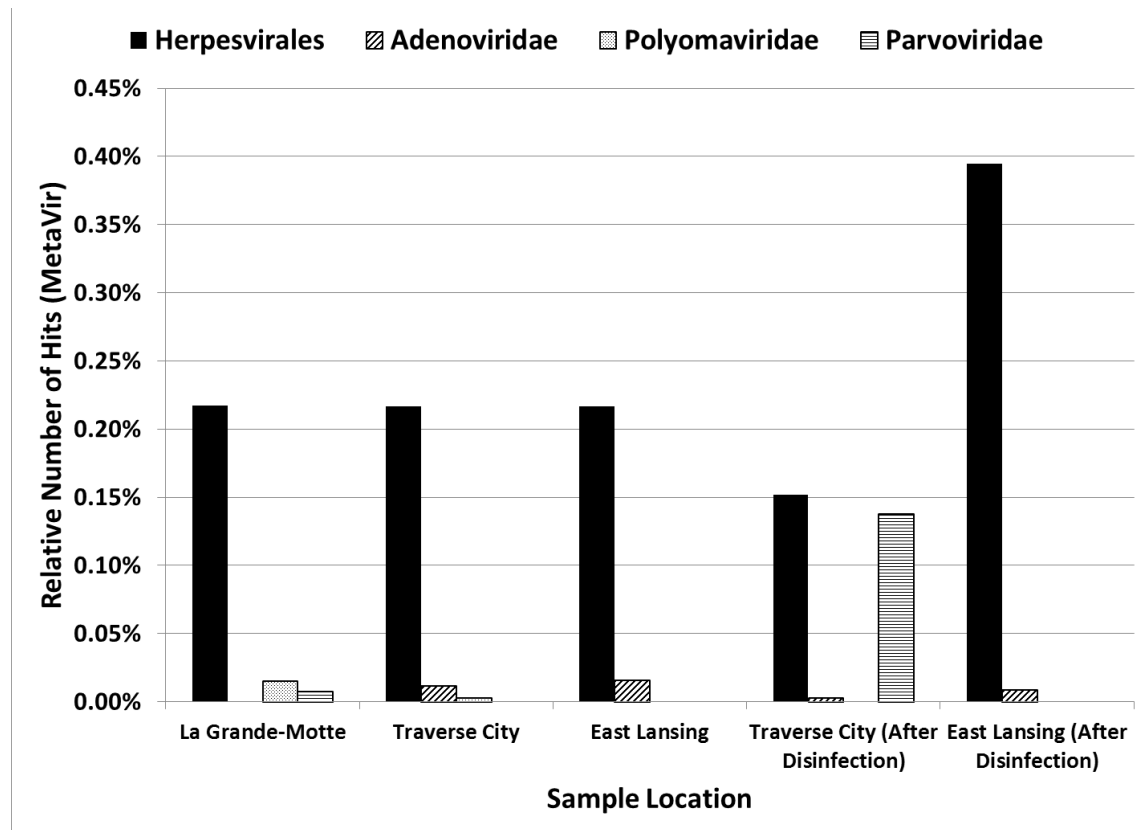


Figure 6.4: Relative number of hits for human viral orders for each sample, measured as the ratio of number of hits for the viral order to the total number of hits in the sample (from MetaVir).

Figure 6.4 describes the abundance of human viruses in each wastewater sample, with *Herpesviridae* being the most commonly detected viral order, significantly more than *Adenoviridae*, suggesting that *Herpesviridae*, like *Adenoviridae*, could also be used as an indicator of viral contamination. However, it should be noted that herpesviruses provide challenges in metagenomic analyses due to the fact that they have been shown to be capable of integrating into host genomes [48], introducing uncertainty into the veracity of the metagenomic detection of herpesviruses in environmental samples. More thorough research should be conducted to assess the presence of herpesviruses in wastewater in order to use herpesviruses as indicators. These results are also consistent with prior metagenomic studies of human pathogens in studies that investigated samples from raw wastewater influent [8,35] or biosolids and sewage sludge [36,38]. Another study that explored all stages of wastewater including effluent [37] reported data only on bacteriophages, the most commonly detected viruses.

This is the first study to compare viral diversity in the effluent of three wastewater utilities with different treatment technologies and disinfection techniques. Because the high cost of sequencing limited the analysis to only one sample per location, quantitative comparison among the samples is difficult. However, striking comparisons can be drawn when analyzing the diversity of the samples. Perhaps the most striking finding from these results is the similarity in diversity between the conventional activated sludge utility (East Lansing) and the two MBR plants (Traverse City WWTP and La Grande Motte WWTP). There is no clear difference among the three facilities in terms of viral diversity in the samples before disinfection. The three effluent samples before disinfection all have practically identical diversity by viral host, and the diversity of the order *Caudovirales* is also very similar among the samples before disinfection.

Although MBRs have been shown to have higher virus removal efficiencies than conventional utilities, the diversity profile in these results indicate that MBRs are not more adept at removing particular kinds of viruses, but rather exhibit relatively equal removal of all DNA virus types. These results hold for both of the MBR WWTPs even though the membranes used at these two facilities have very different nominal pore sizes: 0.04 μm (ultrafiltration) in Traverse City MBR and 0.45 μm (microfiltration) in La Grande Motte MBR. However, the difference in the removal of human adenovirus from model feeds by these two membrane types has been shown to be significant (2.3 log for membranes with 0.04 μm pores and 0.7 logs for membranes with 0.45 μm pores) [49].

It is not until disinfection is performed that a divergence in diversity appears. The disinfection by hypochlorite at EL WWTP greatly reduces the relative abundance of bacteriophages in the effluent, whereas the UV disinfection at the TC WWTP does not affect viral diversity. Analyzing the three *Caudovirales* families, it appears that chlorination reduces the relative abundance of *Siphoviridae* and *Myoviridae*, allowing *Myoviridae* to become the most abundant *Caudovirales* family, whereas UV disinfection affects all families with relative equivalence. These results suggest that from a metagenomic standpoint the diversity of DNA viruses is insensitive to the choice of secondary treatment. Rather, the method of disinfection employed is the treatment process impacting the eventual viral diversity in the wastewater effluent.

Quality control analyses of the samples indicate high sequencing quality. No reads in any sample failed quality control or were flagged as poor quality. Similarly favorable quality control results were generated from the Bowtie2/SAMTools analyses. Nevertheless, it should be noted that due to only having one sample from each location, issues regarding reproducibility should

be acknowledged. While sampling, preparation, and analysis methods were consistent across all samples, further investigation would be necessary to verify the reproducibility of these results.

6.6. Conclusions

Metagenomic analyses were performed using MetaVir2 as a basic tool to determine viral diversity of wastewater effluent in a conventional activated sludge utility and two membrane bioreactor utilities in the United States and France. This study is the first to evaluate diversity of DNA viruses in wastewater effluent using metagenomics and to compare the viral diversity for wastewater utilities of different types and in different locations. The study demonstrates that the majority of viruses released in wastewater effluent are bacteriophages, which can affect the microbial community of receiving streams. The study also reveals that both conventional activated sludge plant and membrane bioreactor utilities have a similar diversity of DNA viruses in their wastewater effluents prior to disinfection. Moreover, the type of disinfection process utilized has an impact in on the diversity of bacteriophages. Further research is required to determine how different disinfection methods impact bacteriophage diversity. This study also demonstrates that potentially human pathogenic DNA viruses are released into the environment via wastewater effluent and the most abundant potential human pathogen observed belongs to the taxonomic order *Herpesvirales*. The observed abundance of herpesviruses in the effluent of treatment utilities prompts further studies to investigate the fate of herpesviruses in wastewater. Other potentially pathogenic human viruses detected in this study include *Adenoviridae*, *Parvoviridae*, and *Polyomaviridae*. Even with the thousands of affiliated sequences in this study, they remain a small fraction compared to the unaffiliated sequences. While metagenomic analysis has progressed significantly, more robust genomic databases are required to fully assess

the biological diversity of a sample. Additionally, while the samples in this study were prepared using consistent methods, there is a significant degree of variation in methods used in published papers making it difficult to draw concrete comparison-based conclusions. To truly unlock the potential of metagenomics analysis, there must be standardization of sample preparation methods, sample analysis, as well as more robust available genomic data to accommodate analysis.

6.7. Acknowledgments

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APPENDIX

S.6.1. qPCR details

The qPCR assay used for analysis was prepared immediately prior to analysis using the methods listed in the reference given [46]. The amplification efficiency of this prepared assay was 98.93% with a detection limit of 101 copies per reaction. No-template controls (NTCs) were included in the analysis and presented no detection signal during the qPCR runs.

S.6.2. Supplementary Tables and Figures

Table S.6.1: Taxonomic viral order-level comparison based on best BLAST hit numbers (max E-value cutoff of 10^{-5}) for contigs (from MetaVir). *No disinfection applied in La Grande-Motte.

Taxonomy	Treated Effluent			Treated Effluent After Disinfection*	
	La Grande-Motte	Traverse City	East Lansing	Traverse City	East Lansing
Viruses	13364	34652	31838	34924	34728
dsDNA viruses, no RNA stage	13006	33487	31022	33875	33480
Caudovirales	10002	26897	24742	26026	21068
Phycodnaviridae	944	1624	1570	2167	4300
Mimiviridae	634	1346	920	1194	3555
Poxviridae	132	175	173	288	580
Ascoviridae	79	61	55	110	322
Iridoviridae	47	168	142	88	237
Baculoviridae	16	66	65	111	222
Marseilleviridae	17	130	0	0	301
Herpesvirales	29	75	69	53	137
Asfarviridae	4	32	42	69	23
Bicaudaviridae	4	18	16	18	45
Lipothrixviridae	0	16	13	13	27
Nudiviridae	3	19	0	0	38
Tectiviridae	1	15	13	27	17
Ligamenvirales	8	20	0	0	31
Nimaviridae	3	9	9	5	5
Rudiviridae	0	4	3	6	4
Plasmaviridae	0	1	1	3	3
Adenoviridae	0	4	5	1	3
Polydnaviridae	0	2	2	3	7
Corticoviridae	0	3	3	3	2
Fuselloviridae	1	1	1	0	1
Polyomaviridae	2	1	0	0	0

Table S.6.1 (cont'd).

viruses	unclassified dsDNA	450	924	1336	1689	1634
phages	unclassified dsDNA	630	1896	1842	2001	949
	ssDNA viruses	56	49	48	156	177
	Inoviridae	48	39	38	78	167
	Microviridae	2	6	6	9	8
	Parvoviridae	1	0	0	48	0
	Circoviridae	0	0	1	4	0
	Geminiviridae	0	0	0	1	0
viruses	unclassified ssDNA	5	4	3	16	2
	unclassified phages	276	957	699	823	754
	unclassified viruses	0	90	1	0	153
	unassigned viruses	8	7	7	5	15
	unclassified virophages	3	7	7	10	2
	unclassified archaeal viruses	1	2	3	2	8

Table S.6.2: Taxonomic comparison based on best BLAST hit numbers (max E-value cutoff of 10^{-5}) for contigs (from MetaVir). Bacteriophages were grouped together by viral host. *No disinfection applied in La Grande-Motte.

Bacteriophage	Treated Effluent			Treated Effluent After Disinfection*	
	La Grande-Motte	Traverse City	East Lansing	Traverse City	East Lansing
Achromobacter phages	6	19	20	41	18
Acinetobacter phages	94	286	290	277	228
Actinoplanes phages	9	32	30	30	10
Acyrtosiphon phages	13	44	42	16	21
Aeromonas phages	81	215	226	189	168
Aggregatibacter phages	9	67	65	73	28
Agrobacterium phages	17	29	30	35	9
Alteromonas phages	24	70	67	55	22
Anabaena phages	19	11	0	0	1
Arthrobacter phages	12	51	50	53	17
Azospirillum phages	76	208	200	254	92
Bacillus phages	1054	2343	1452	1201	3439
Bacteroides phages	7	68	64	54	52
Bdellovibrio phages	45	74	70	91	25
Bordetella phages	130	204	207	287	94
Brochothrix phages	7	49	53	32	62
Brucella phages	76	188	190	260	80

Table S.6.2 (cont'd).

Burkholderia phages	551	1576	1572	1876	1304
Campylobacter phages	12	47	47	32	70
Caulobacter phages	212	369	354	353	271
Celeribacter phages	24	126	123	102	64
Cellulophaga phages	144	996	964	743	364
Clavibacter phages	30	43	53	54	15
Clostridium phages	109	392	395	334	361
Croceibacter phages	20	147	176	117	52
Cronobacter phages	145	531	497	509	569
Cyanobacter phages	87	159	179	190	119
Deftia phages	7	32	41	30	8
Edwardsiella phages	33	100	101	97	36
Endosymbiont phages	14	50	49	46	21
Enterobacteria phages	456	1257	1148	1224	1195
Enterococcus phages	59	137	137	120	54
Erwinia phages	82	433	442	350	353
Escherichia phages	229	482	476	572	435
Flavobacterium phages	7	114	113	106	24
Geobacillus phages	52	182	176	108	68
Gordonia phages	17	35	34	52	10
Haemophilus phages	25	132	140	119	77
Halomonas phages	11	52	51	39	49
Helicobacter phages	15	31	30	34	55
Idiomarinaceae phages	38	0	0	0	0
Iodobacterio phages	10	59	60	69	33
Klebsiella phages	27	120	120	83	69
Lactobacillus phages	71	208	193	181	163
Lactococcus phages	82	187	217	244	315
Liberibacter phages	13	10	9	55	8
Listeria phages	53	199	165	138	64
Listonella phages	2	32	32	39	19
Mesorhizobium phages	36	0	0	0	0
Microbacterium phages	35	40	24	45	66
Microcystis aeruginosa phages	29	94	94	75	119
Mycobacterium phages	807	2148	1873	1907	1179
Myxococcus phages	83	172	165	227	39
Natrialba phages	17	36	37	77	40
Nitrincola phages	21	99	0	0	27
Nocardia phages	16	18	17	26	4
Paenibacillus phages	20	66	28	44	41
Pectobacterium phages	32	102	98	155	45
Pelagibacter phages	234	354	338	616	273

Table S.6.2 (cont'd).

phiJL001 phages	28	64	59	107	32
Planktothrix phages	320	279	309	423	899
Prochlorococcus phages	165	294	282	315	491
Pseudoalteromonas phages	19	105	102	114	42
Pseudomonas phages	768	2660	2322	2632	1453
Psychrobacter phages	52	187	156	132	72
Puniceispirillum phages	76	163	171	199	30
Ralstonia phages	117	301	293	312	232
Rhizobium phages	303	612	589	732	349
Rhodobacter phages	22	34	31	37	18
Rhodococcus phages	104	237	129	141	124
Rhodothermus phages	11	101	99	39	35
Riemerella phages	5	68	64	42	25
Roseobacter phages	83	175	168	208	71
Ruegeria phages	25	0	0	0	0
Salinivibrio phages	16	67	63	105	20
Salmonella phages	246	744	724	789	475
Serratia phages	17	72	63	31	44
Shewanella phages	34	10	10	6	3
Shigella phages	96	147	134	156	227
Sinorhizobium phages	38	141	140	124	85
Sphingomonas phages	8	75	75	47	55
Staphylococcus phages	123	409	412	324	326
Stenotrophomonas phages	56	156	159	193	149
Streptococcus phages	61	166	172	172	128
Streptomyces phages	151	314	311	397	268
Stx2-converting phages	83	173	168	190	447
Synechococcus phages	507	1293	1239	1294	1035
Tetrasphaera phages	22	100	102	60	22
Thalassomonas phages	58	221	219	200	70
Thermoanaerobacterium phages	33	72	66	80	43
Thermus phages	37	167	160	157	165
Vibrio phages	325	1242	1184	1112	630
Xanthomonas phages	74	338	320	300	116
Xylella phages	86	154	153	159	58
Yersinia phages	47	132	135	142	111

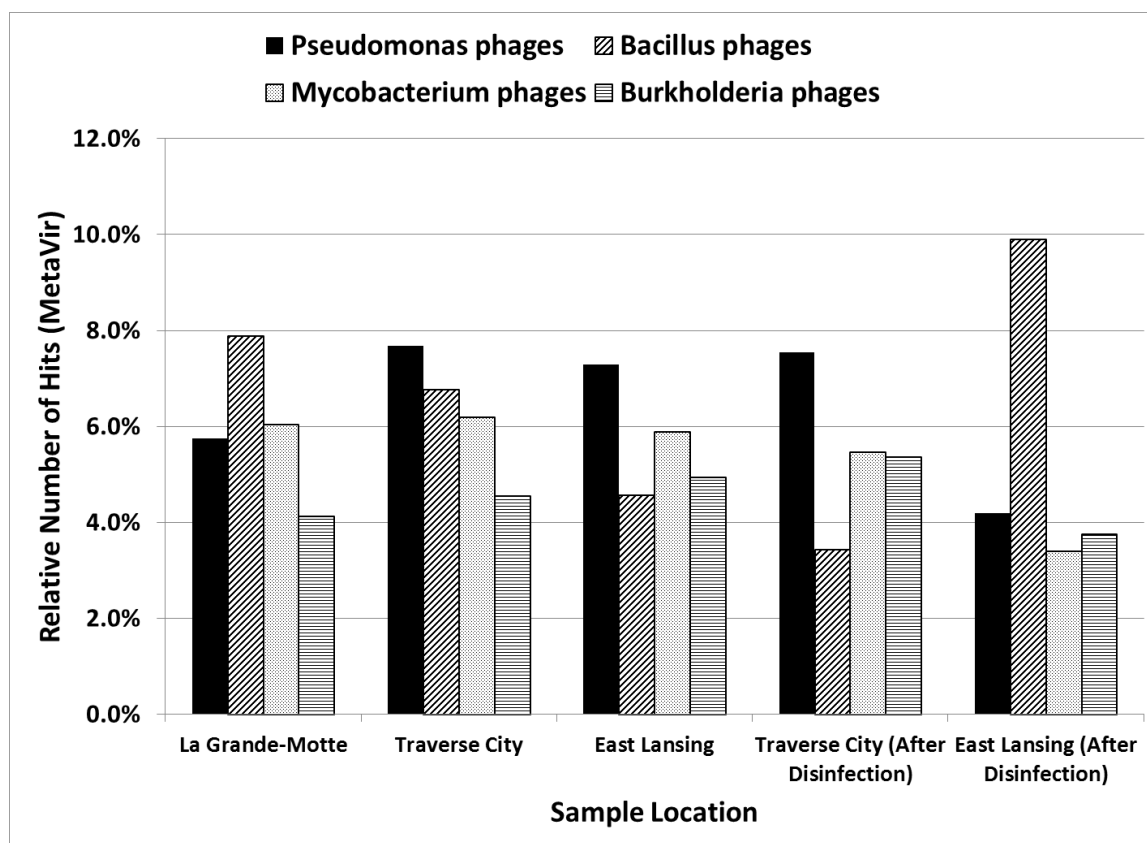


Figure S.6.1: Relative number of hits for the four most prevalent bacteriophage hosts for each sample, measured as the ratio of number of hits for the viral order to the total number of hits in the sample (from MetaVir).

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Chapter 7:

Conclusions

There is great potential in the application of the One-Health approach with regards to viral disease, and environmental engineers play a critical role in this pursuit. Environmental factors are found to be significantly related to clinical reports of viral disease, and the surveillance of wastewater has great promise as an epidemiological tool to assist in the prediction and identification of viral disease outbreaks. The five studies that comprise this dissertation show that while the proposed methodologies are effective, further research is necessary for the full breadth of their impact.

More data is necessary to gain a more comprehensive understanding of the predictors of viral disease. Better and more complete reporting of clinical cases of human and animal viral disease can be of great assistance to this approach, and the exploration of other environmental factors such as agricultural runoff can further illuminate the critical times and locations where viral disease outbreaks are likely to occur. To achieve this, though, more ambitious sampling of surface water and wastewater is necessary. Only with thorough and continuous monitoring of these water resources can significant trends be observed and determined.

Moreover, while these studies show that the wastewater epidemiology methodology has promise, regular surveillance of wastewater influent is necessary to fully implement the methodology. Constant quantification of viral concentrations in tandem with comparison to clinical data can allow for the observation of a relationship between the two. Once the relationship between the occurrence of viruses in wastewater and clinical data has been established, outbreaks can be more quickly identified and public health officials can be better prepared to protect the community.

Furthermore, continued surveillance of wastewater effluent is critical for the protection of environmental health. As One-Health posits, human health is impacted by environmental health, so the protection of one will lead to protection of the other. The role of environmental engineers is therefore not only in the conservation of natural resources, but in the optimization of the human experience as well.