THE Water Research FOUNDATION

PROJECT NO.
4955

Indicator Viruses to Confirm Advanced Physical Treatment

CALIFORNIA Water Boards
STATE WATER RESOURCES CONTROL BOARD
REGIONAL WATER QUALITY CONTROL BOARDS

THE METROPOLITAN WATER DISTRICT OF SOUTHERN CALIFORNIA

Indicator Viruses to Confirm Advanced Physical Treatment

Prepared by:

Walter Q. Betancourt, PhD
University of Arizona

Co-sponsored by:

California State Water Resources Control Board and
Metropolitan Water District of Southern California

2023

The Water Research Foundation (WRF) is the leading research organization advancing the science of all water to meet the evolving needs of its subscribers and the water sector. WRF is a 501(c)(3) nonprofit, educational organization that funds, manages, and publishes research on the technology, operation, and management of drinking water, wastewater, reuse, and stormwater systems—all in pursuit of ensuring water quality and improving water services to the public.

For more information, contact:
The Water Research Foundation

1199 North Fairfax Street, Suite 900
Alexandria, VA 22314-1445
P 571-384-2100

6666 West Quincy Avenue
Denver, Colorado 80235-3098
P 303-347-6100

www.waterrf.org
info@waterrf.org

WRF ISBN: 978-1-60573-651-8
WRF Project Number: 4955

Funding has been provided in full or in part through the Water Research Foundation, California State Water Resources Control Board, and Metropolitan Water District of Southern California (Project #4955). State Water Resources Control Board in California (SWB) Grant and the Advancing Potable Reuse Initiative that leverages the grant. This grant and initiative fund research to advance potable and non-potable reuse in California and across the world.

Acknowledgments

This report has been the coordinated effort of researchers, research staff, plant operators, hydrologist, and engineers from the different facilities participating in this project. The valuable support from graduate, undergraduate students who participated in the multiple activities related to sample collection, processing and analyses is greatly acknowledged: Kaitlyn Chung Maggie Orm, Nemanja Bogunovic, Abbigail Albright, Karen Perez, Diego Salido, Reagan Wagner, Varinia Felix, and Mohamed Alhussaini. The Research Team also acknowledges additional financial support provided by Yokogawa Electric Corporation.

Research Team

Principal Investigator:
Walter Q. Betancourt, PhD
University of Arizona

Project Team:
Bianca Miguel de Souza-Chaves, PhD
University of Arizona

Andrea Achilli, PhD
University of Arizona

Channah Rock, PhD
University of Arizona

Natalie Brassill, MS
University of Arizona

Luisa Ikner, PhD
University of Arizona

Charles Gerba, PhD
University of Arizona

Kelly Bright, PhD
University of Arizona

Patrick Gurian, PhD
Drexel University

Jack Schijven, PhD
Utrecht University, the Netherlands

Christina Morrison, PhD
Southern Nevada Research Institute

Jason Assouline
Carollo Engineers Inc.

Nicola Fontaine
Carollo Engineers Inc.

Margaret Snyder
Tucson Water

WRF Project Subcommittee or Other Contributors

Raul Gonzales, PhD
Hampton Roads Sanitation District

Menu B. Leddy
Essential Environmental & Engineering Systems

Nichole Brinkman, PhD
US Environmental Protection Agency

Matthew D. Hacker
Metropolitan Water District of Southern California

George Di Giovanni, PhD
Metropolitan Water District of Southern California

Cindy Figueroa
California State Water Resources Control Board

WRF Staff

John Albert, MPA
Chief Research Officer

H. Grace Jang, PhD
Research Program Manager

Abstract and Benefits

Advanced treatment processes for potable reuse are indispensable to mitigate microbial and chemical contaminants in recycled water. Viruses are a particular focus of water reuse treatment processes because of their acute health effects, low infectious dose, small size, and resistance to disinfection. Understanding the concentrations of viruses in source waters and specifying the log reduction values required to meet the appropriate risks levels for health protection is fundamental for safe and sustainable water reuse applications.

Virus rejection mechanisms by membrane processes at bench and pilot scales have received a great deal of research, while studies at full scale are scarce, particularly reverse osmosis membranes considered as complete barriers for pathogens with a regulatory credit in the United States limited to 2-$\log_{10}$. Virus reduction by sustainable land-based managed aquifer recharge (MAR) systems such as soil aquifer treatment at full-scale are also scarce. Viruses with minimal public health impact have been extensively used for these purposes.

This work advances the knowledge of virus indicators in water reuse applications by providing a guidance framework for selection of viruses of public health significance that can be implemented for monitoring at field scale in order to confirm advanced physical treatment. Virus indicators of advanced physical treatment are required as part of the regulatory framework on pathogen control and log reduction requirements for implementation of DPR and IPR projects that are protective of public health. This study fulfills three major objectives:

1. **Literature review**. A comprehensive literature review summarizes existing research evaluating pathogen and indicator virus concentration and removal by advanced physical treatment processes.
2. **Virus indicators**. A guidance framework for the selection of endogenous viruses in wastewater that can be used to confirm advanced physical treatment considering the physicochemical properties of the viruses, abundance in raw wastewater, and resilience to treatment processes.
3. **Full-scale data**. Collection of virus data at full-scale to determine source concentrations and log reduction values of selected viruses investigated along with potential online surrogates for exploring correlations with full-scale virus data.

Contents

Tables

Figures

Acronyms and Abbreviations

AMW	Apparent molecular weight
ATP	Adenosine triphosphate
AWWA	American Water Works Association
CDPH	California Department of Public Health
CGMMV	Cucumber green mosaic mottle virus
cMAGs	Circular metagenome assembled genomes
CRESS	Circular replication-associated protein (Rep)-encoding single-stranded (CRESS) DNA viruses
CRISPR-Dx	Clustered regularly interspaced short palindromic repeat diagnostic technologies
dsDNA	Double-stranded deoxyribonucleic acid
dsRNA	Double-stranded ribonucleic acid
GC	Genome copies
GC/L	Genome copies per liter
GI	Genogroup I
GII	Genogroup II
ICC-MS	Integrated cell culture-mass spectrometry
IF	Immunofluorescence
IP	Isoelectric point
JCPyV	JC polyomavirus
kDa	Kilodalton
LRV	Log reduction value
MAR	Managed aquifer recharge
MDa	Megadalton
MFE	Microfiltration effluent
MPN	Most probable number
NF	Nanofiltration
NFP	Nanofiltration permeate
NF-RO	Nanofiltration-reverse osmosis
NRC	National Research Council
NTU	Nephelometric turbidity unit
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PES	Polyethersulfone
PFU	Plaque forming unit
pI	Isoelectric point
PMMoV	Pepper mild mottle virus

PVDF	Polyvinylidene fluoride
rNV-VLPs	Recombinant norovirus-like particles
ROP	Reverse osmosis permeate
SAT	Soil aquifer treatment
SDI	Silt density index
ssDNA	Single-stranded deoxyribonucleic acid
ssRNA	Single-stranded ribonucleic acid
TOC	Total organic carbon
TWDB	Texas Water Development Board
UFP	Ultrafiltration permeate
WCDV-2	Wastewater CRESS DNA virus-2

Executive Summary

This project evaluated fifteen virus indicators to confirm advanced physical treatment at full-scale advanced treatment trains involved in indirect potable reuse. An engineering-scale integrated membrane system was also evaluated under two configurations: ultrafiltration (UF)-reverse osmosis and UF-nanofiltration.

A comprehensive literature review of existing research evaluating pathogen and indicator virus concentration and reductions by advanced physical treatment processes revealed the lack of peer-reviewed publications for media filtration, membrane filtration, and soil aquifer treatment through spreading and injection at full scale.

The viruses investigated in this study represent extremes in the biological size spectrum (15 – 25 to 70 – 90 nm in diameter) with distinctive genome features (i.e., single- and double-stranded DNA [ssDNA, dsDNA] viruses, single-stranded ribonucleic acid [ssRNA], and double-stranded ribonucleic acid [dsRNA] viruses) and documented persistence to chemical and physical disinfectants in wastewater treatment processes. Non-microbial parameters of advanced treatment performance were compared with full-scale virus data in order to explore potential correlations.

Full-scale testing involved water reuse facilities in Arizona, Colorado, and Virginia to represent advanced treatment trains located in three geographical regions of the continental United States: Southwest, West, and Southeast. Advanced treatment trains included membrane-based and carbon-based potable reuse schemes plus an advanced managed aquifer recharge system with soil-aquifer treatment.

The results of this study indicate that virus abundance in source waters upstream of the advanced treatment trains, virus resilience to treatment linked to viral structure, sensitivity of the detection assay, and treatment plants' operational conditions define the outcome of the monitoring framework to determine virus occurrence and reduction in potable water reuse schemes. The latter has been viewed as environmental scenarios of low-level virus contamination. Viruses of public health significance (i.e., enteric viruses) are proposed as the best self-indicators to confirm advanced physical treatment. These results support the recommendations included on the Draft of Anticipated Criteria for Direct Potable Reuse dated August 17, 2021, to adequately protect public health. Under this proposed draft, Norovirus is the selected virus to represent human viruses.

A major contribution of this project is the expansion of information on human enteric viruses and newly recognized virus surrogates within the water reuse infrastructure in the United States. Virus indicators of physical treatment are required as part of the regulatory framework on pathogen control and log reduction criteria for implementation of direct potable reuse (DPR) and indirect potable reuse (IPR) projects that are protective of public health.

ES.1 Key Findings

- Virus abundance and detection frequency upstream of the advanced treatment trains were key players of virus breaking through membrane-based and carbon-based processes for most viruses, however virus resilience to treatment seemed to play a more significant role.
- Virus log reduction credits of advanced treatment technologies may be useful indicators of treatment performance, but redundancy, operational stability of the advanced treatment trains, and continuous monitoring are more important to ensure the safety of recycled water for potable and non-potable reuse applications.
- An exploratory analysis of the correlation between log reduction value (LRV) of full-scale virus data and potential online surrogates favors those methods that measure the rejection of soluble organic contaminants (fluorescence monitoring, TOC, DOC) and indicators of overall microbial activity (ATP), more evident for membrane based advanced treatment trains.
- The development and implementation of more efficient and sensitive methods of virus recovery and detection are fundamental to more clearly understand whether the infrequent detection of low levels of enteric viruses in highly treated recycled waters is a true reflection of low levels of virus occurrence or method deficiencies linked to water matrix effects and/or virus complexity.
- Human enteric viruses are proposed as the best self-indicators to confirm physical treatment. Moreover, these virus indicators should be tailored to the treatment train and continuously re-evaluated.

ES.2 Background and Objectives

Numerous peer-reviewed studies have addressed the capacities of new reuse and treatment technologies for the required reduction of viruses at bench and pilot scale while studies at full scale are scarce.

The main goal of this project was to identify and evaluate potential viral indicator(s) to confirm advanced physical treatment at full-scale potable reuse schemes. Virus structural and biological features, resilience to treatment, wastewater characteristics, treatment technologies, plant capacities, U.S geographic locations, and seasonal effects were considered to evaluate physical reduction by advanced soil-aquifer treatment, membrane-based and carbon-based technologies.

A key objective to achieve this goal was to review and summarize existing research evaluating pathogen and indicator virus concentration and removal by physical treatment processes, including media filtration, membrane filtration, and soil aquifer treatment through spreading and injection. A second key objective was to select and recommend viral indicator or pathogen for use in physical treatment process evaluation, providing reasoning, considering cost, technical requirements, and literature support. A third key objective was to collect full-scale data in order to determine source concentrations and log reduction values of selected viruses investigated in combination with potential online surrogates that may correlate with full-scale virus data.

ES.3 Project Approach

Human enteric viruses and virus surrogates present in wastewater were selected to confirm advanced physical treatment. Virus structural features, including differences in size, virus genome, virus capsid features, and documented resilience to water treatment technologies were the major selection criteria for evaluation at full-scale advanced treatment trains.

Membrane-based and carbon-based treatment trains with multi-step disinfection processes and managed aquifer recharge by soil-aquifer treatment were monitored for the selected virus indicators using adsorption-elution methods for virus recovery and concentration. Digital polymerase chain reaction (PCR) was used for detection and quantification of nuclease-protected virus genomes following quality assurance and quality control criteria. Culturable male-specific and somatic coliphages were used as surrogates of virus infectivity in water.

Non-microbial surrogates of treatment performance that evaluate microbial activity (adenosine triphosphate) rejection efficiencies of ions and removal of soluble contaminants, including fluorescence spectroscopy, size-exclusion chromatography coupled with dissolved organic carbon detection, and UV_{254} absorbance were used to correlate with full-scale virus data.

ES.4 Results

Virus abundance in raw wastewater, virus persistence upstream of the advanced treatment train, and virus structural complexity were useful features of the selected viruses to confirm physical treatment. These features were observed for five human enteric viruses (Norovirus, Human Bocavirus, Adenovirus, Reovirus, Aichi virus) and six virus surrogates (crAssphage, wastewater CRESS DNA virus-2 [WCDV2], Hudisavirus, pepper mild mottle virus [PMMoV], male-specific and somatic coliphages) at full scale treatment.

Infrequent and highly variable detections of viruses were observed in the advanced treatment trains with evidence of virus breakthrough for human enteric viruses of public health significance on both membrane-based and carbon-based advanced treatment processes. Among all viruses monitored in this study, the maximum log reduction was 9-$\log_{10}$, observed for somatic coliphages in the membrane-based treatment train, which is 3-$\log_{10}$ less than the recommended 12-$\log_{10}$ reduction credit required for treated wastewater intended for indirect potable reuse. Human enteric viruses and virus surrogates were detected in advance treated water from reuse facilities fulfilling or not expected treatment performance (e.g., TOC <0.5 mg/L).

Log reductions of up to 6-$\log_{10}$ of human enteric pathogens such as Norovirus by soil-aquifer treatment (SAT) may not ensure appropriate risk levels for protection of public health in potable reuse schemes, which should be the subject of continuous assessment. There were no statistically significant correlations among log reduction credit of viruses and non-microbial surrogates, more likely associated with variability inherently related to ordinary measurements. However, an exploratory analysis of the data generated from this study indicated that for membrane-based treatment processes, methods that assess the rejection/reduction of soluble organic contaminants (fluorescence monitoring, TOC, DOC) and indicators of overall microbial

activity (ATP) may be appropriate candidates for potential on-line indicators of physical treatment.

To completely comprehend virus presence and fate in the water reuse infrastructure, advancements in viral detection methods are essential.

ES.5 Benefits

Understanding virus occurrence and removal by full-scale advanced treatment for potable reuse applications constitutes a high-priority research need to protect human health and to enhance the nation's available water supply alternatives.

The results of this study revealed that multiple biological and structural features of viruses, as well as known or predictive levels of environmental persistence in the extracellular environment were critical to select appropriate viral indicators of advanced treatment performance for safe water reuse projects. The expansion of information on human enteric viruses and potential newly recognized virus surrogates as indicators of physical treatment by advanced water reuse technologies at full scale is fundamental. Virus indicators of physical treatment are required as part of the regulatory framework on pathogen control and log reduction criteria for implementation of DPR and IPR projects that are protective of public health.

This project examines the current knowledge on virus removal by engineered and natural advanced water treatment processes for potable reuse projects. Our knowledge on pathogen occurrence and persistence in the environment has substantially increased with the development of more efficient and sophisticated technologies for pathogen recovery and detection. Site-specific environmental and ecological conditions, the dynamic of human populations that represent the source of pathogenic viruses along with the emergence and evolution of viruses themselves play an important role to understand virus occurrence and physical removal by advanced treatment processes for reuse. Equally important is the application of the best available advanced treatment technologies for the required reduction of pathogenic viruses. All of these factors must be seen as important components of an expanding research agenda to ensure current and future sustainable, reliable and safe water reuse.

ES.6 Related WRF Research

- Advancing Safety and Reliability to Protect Public Health: Identifying Quantitative Reductions of Viral Pathogens and Surrogates for Water Reuse Applications (5126)
- Demonstrating Virus Log Removal Credit for Wastewater Treatment and Reverse Osmosis for Potable Reuse at OCWD (5041)
- Give Membranes the Virus Removal Credit They Deserve, Using Rapid In-Field Molecular-Based Methods (5209)

CHAPTER 1

Virus Reduction by Engineered and Natural Advanced Water Treatment Processes: An Expanding Research Agenda for Safe Water Reuse

1.1 Water Reuse and Regulatory Requirements for Viruses

Advanced treatment processes for potable reuse are indispensable to mitigate microbial and chemical contaminants in recycled water. Viruses are a particular focus of water reuse treatment processes because of their acute health effects, low infectious dose, small size, and resistance to disinfection (Gerba et al., 2017; Gerba et al., 2018). Understanding virus occurrence and removal in full-scale advanced wastewater treatment processes for potable reuse application constitutes a high priority research need to protect human health and to enhance the nation's available water supply alternatives (NRC, 2012). As water sustainability initiatives continue to arise, monitoring viral pathogens and utilizing the most efficient wastewater treatment technologies are necessary to minimize risks (Soller et al., 2017; Soller et al., 2003).

Various guidelines have been suggested for the required reductions of pathogens by the treatment process to ensure minimal risk to the exposed population. The U.S Environmental Protection Agency (EPA) has issued regulatory guidelines that have been adopted by each state in the U.S, for water reuse practices. These guidelines describe treatment technologies, monitoring requirements for recycled water, and setback distances in porous media (EPA, 2012). Under the California Title 22 regulations, disinfected tertiary effluent requires a minimum chlorine CT value of not less than 450 mg-min/L at all times with a modal contact time (time for the highest concentration to pass through the contact chamber) of at least 90 minutes, based on peak dry weather design flow or a 5-$\log_{10}$ virus inactivation if an alternative disinfection process is utilized. In addition, under the Groundwater Replenishments Reuse Project of the state of California and EPA guidelines for water reuse, a 12-$\log_{10}$ reduction of virus is required for treated wastewater intended for indirect potable reuse, i.e., surface water augmentation or groundwater recharge (California Code of Regulations 60320.208 2014) (SBDDW, 2018). The Texas Commission on Environmental Quality has established a minimum baseline target for virus reduction of 8-$\log_{10}$ for the advanced treatment system, not including the wastewater treatment plant (TCEQ, 2022). These reductions are designed to produce recycled water that result in a yearly risk of infection of 1:10,000 or less to persons who may ingest the water, equivalent to 2.7×10^{-7} daily risk of infection (Chaudhry et al., 2017; Gerba et al., 2017; Gerba et al., 2018; Soller et al., 2003). The most recent framework for the direct potable reuse regulation in California requires a 20-$\log_{10}$ reduction value of enteric virus by the treatment train to ensure that the calculated risk of infection does not exceed the daily threshold of 2.7×10^{-7} specified above (SWRCB and DWW, 2021).

The inadequacy of bacteria to indicate viral contamination or viral reduction efficiency by wastewater treatment processes has been widely demonstrated (Francy et al., 2012; Gall et al., 2015; Harwood et al., 2005; Ito et al., 2016; Ottoson et al., 2006; Symonds et al., 2009). To evaluate the incidence, persistence, fate, and transport of human pathogenic viruses in engineered advanced treatment processes, a viral indicator for contamination may be appropriate since monitoring for all human pathogenic viruses is impractical. Bacteriophages of *Escherichia coli* (i.e., coliphages) closely mimic viral pathogen persistence, suggesting they may be adequate sentinels of enteric virus removal (Amarasiri et al., 2017; McMinn et al., 2017). Regulatory authorities in different parts of the world have already considered bacteriophages as indicators of water quality concerning water reclamation (North Carolina Environmental Quality, 2011; Queensland Government, 2005). However, the use of coliphages as indicators in water quality control is not free from controversy (Jofre et al., 2016). Moreover, the failure of measurements of single indicator organism to correlate with pathogens suggests that public health is not adequately protected by simple monitoring schemes based on the detection of a single indicator, particularly at the detection limits routinely employed (Harwood et al., 2005). The appropriateness of a viral indicator of treatment performance requires taking into consideration the dynamic of human populations (e.g., health status, age structure, and standard of living), seasonality of viruses, and types of treatment processes. The incidence of viruses in wastewater depends on human population size, while removal depends on treatment efficiency (Gerba et al., 2017; Schmitz et al., 2016). Therefore, the most effective virus-removal technologies must be implemented during wastewater treatment to minimize the risks associated with viruses in effluent waters for discharge or for reuse purposes (Chaudhry et al., 2017; Gerba et al., 2017; Harwood et al., 2005; Scott et al., 2003; Soller et al., 2003).

Plant viruses, such as PMMoV and CGMMV have shown extreme abundance in wastewater effluent and resistance to treatment (Betancourt et al., 2014; Kato et al., 2018; Kitajima et al., 2014; Morrison et al., 2020; Schmitz et al., 2016; Shirasaki et al., 2017). These viruses have been recently recommended as good process indicators of enteric virus removal by membrane processes (Yasui et al., 2021). However, the rod-shaped capsid of these viruses is unlike that of human enteric viruses, which could potentially make them less relevant as predictors of human virus fate. Moreover, plants viruses have demonstrated resistance to elevated temperatures (thermal inactivation occurs above 80 °C) which render these viruses highly persistent in the environment (Betancourt, 2020). In natural advanced water treatment systems, e.g., soil-aquifer treatment, the selection of the most suitable indicators and surrogates for evaluation of virus attenuation may be tightly associated with site-specific conditions that require thorough understanding for routine monitoring requirements.

This review examines the current knowledge on virus occurrence and reductions by engineered and natural advanced water treatment processes for water reuse systems. Our knowledge on pathogen occurrence and persistence in the environment has substantially increased with the development of more efficient and sophisticated technologies for pathogen recovery and detection. Notwithstanding, site-specific environmental conditions, the dynamic of human populations (source of pathogenic viruses), viral evolution and emergence must be considered in order to understand virus occurrence and physical removal by advanced water reuse treatment processes. Equally important is the application of the best available advanced

treatment technologies for the required reduction of pathogenic viruses. All of these factors must be seen as important components of an expanding research agenda to ensure current and future sustainable, reliable and safe water reuse.

1.2 Viruses in Raw Wastewater as the Source of Recycled Water for Reuse Systems

The frequent occurrence of human viruses in raw wastewater is highly associated with endemic levels of viral diseases in human populations (Corpuz et al., 2020). Knowing the concentrations of these viruses in raw wastewater is critical for the required reduction credits of pathogens for treatment processes that are protective of public health (Gerba et al., 2017; Gerba et al., 2018). Cell culture methods are not capable of detecting all the infectious viruses in wastewater and always underestimate (2- to 100-fold) the true number of infectious viruses present (Gerba and Betancourt, 2019; Rames et al., 2016). Numerous factors are known to control the efficacy of the virus detection assays, including the recovery efficiency of the primary concentration method, cell culture types, particle-to-infectious-unit ratio, and chemical additives in cell culture, cell culture passage number, and sequential passage of a sample in continuous cell lines (Brown et al., 1992; Gerba and Betancourt, 2019; Klasse, 2015). Primary kidney monkey or human primary cell culture are more sensitive for virus detection and have commonly yielded greater numbers in wastewater than continuous cell lines (Sellwood and Dadswell, 1981). However, cell culture assays are capable of detecting a limited number of enteric viruses including enteroviruses, reoviruses and adenoviruses. Recent virus metagenomics studies have indicated that human pathogenic viruses represent a small fraction of the viral community found in untreated wastewater (Aw et al., 2014; Bibby et al., 2019; Bibby and Peccia, 2013; Fierer et al., 2022; Gerba and Betancourt, 2019; Jiang et al., 2022; Ng et al., 2012; Rosario and Breitbart, 2011). These studies have also revealed novel human-associated viruses, both pathogenic and commensal viruses in the human microbiome, that may provide a valuable source to develop viral quality monitoring tools for multiple applications (Bibby et al., 2019). Substantial variations in the relative abundance of virus taxonomic groups in municipal wastewater have been also revealed by viromic studies with some viruses behaving either as transient or actual residents of wastewater treatment plants (Bibby et al., 2019; Bibby and Peccia, 2013; Palermo et al., 2019; Pearson et al., 2016).

Molecular methods cannot directly address the infectivity of waterborne viruses, which is essential to address in order to ensure that the risks in recycled water for potable reuse are minimized (Gerba and Betancourt, 2019). Moreover, the applicability of molecular methods in a diverse range of water matrices requires further refinement and standardization (Girones et al., 2010). Various approaches have been developed to assess infectivity of waterborne enteric viruses using molecular methods, but they are specific to the virus and the mechanism of virus inactivation (Canh et al., 2022; Leifels et al., 2016; Leifels et al., 2019; Randazzo et al., 2018; Wigginton and Kohn, 2012; Wigginton et al., 2012). Pretreatment of viral concentrates with nucleases prior to nucleic acid extraction has been used to digest unprotected nucleic acid in virus metagenomics studies (Ng et al., 2012). The enzymatic treatment enhances the detection and quantification of viral nucleic acid protected from digestion within intact viral capsids. This approach applied for absolute or relative quantification of virus genomes in environmental

samples in combination with cell culture may provide better insights into the number of virus particles present in an equivalent volume of sample that are capable to replicate in cell culture. Currently, there is no universal method that can substitute for cell culture to assess for viral infectivity in humans and animals and therefore quantitative molecular methods such as real-time PCR or the many variations of digital PCR have been widely used for estimating the concentrations of waterborne enteric viruses in untreated and treated wastewater (Chowdhari et al.; Dias et al., 2019; Flannery et al., 2012; Gonzales-Gustavson et al., 2019; Grondahl-Rosado et al., 2014; Haramoto and Otagiri, 2014; Hata et al., 2013; Hellmér et al., 2014; Hewitt et al., 2011; Hornstra et al., 2019b; Ito et al., 2016; Kim et al., 2013; Kitajima et al., 2014; Kiulia et al., 2021; Kobayashi et al., 2017; Kuo et al., 2010). Table 1.1 summarizes studies conducted in the United States showing the greatest levels of infectious waterborne enteric viruses in raw wastewater and secondary treated effluents. Table 1.2 is a selection of studies showing the levels of viruses detected by molecular methods in wastewater.

Table 1-1. Concentrations of Human Culturable Viruses in Raw Wastewater and Secondary Effluents in the United States as Determined by Cell Culture Assay.

Maximum Concentration of Virus Per Liter	Method of Assay	Cell Line	Virus Detected	Location	Period of the Study	Reference
$2.76x10^5$	PFU; 80-100% efficiency	HEL; primary human amnion, HEp_2, Vero	Enterovirus, Adenovirus, Reovirus	California	1974-1977	(Sellwood and Dadswell, 1981)
$2.1x10^5$	IF	Mouse L929	Reovirus	Utah		(Adams et al., 1982)
$3.3x10^3$	MPN/L	BGM, RD, HEp-2, Caco-2	Enterovirus, Adenovirus, Reovirus	Wisconsin	1994-2003	(Sedmak et al., 2005)
$5x10^2$	MPN/L	BGM, A549		Michigan	2008-2009	(Simmons and Xagoraraki, 2011)
$1.3x10^5$	MPN/L	BGM	Enterovirus	California	2019-2021	(Pecson et al., 2022)
$5.1x10^4$		A549	Adenovirus			
$6.5x10^4$		A549	Adenovirus	Texas	2015-2016	(Ryu et al., 2021)
$8x10^2$	PFU/L	HEK-293, A549	Adenovirus	California	2005	(He and Jiang, 2005)
$1.63x10^2$	MPN/L	BGMK, RD	Culturable enteric viruses	Ohio	2008-2010	(Francy et al., 2012)

HEL: human diploid fibroblast, BGM: Buffalo Green Monkey kidney cells, RD: Rhabdomyosarcoma.

Novel approaches such as Integrated Cell Culture-Mass Spectrometry (ICC-MS) and Clustered Regularly Interspaced Short Palindromic Repeat) diagnostic technologies (CRISPR-Dx) have been recently explored for rapid and specific detection of infectious viruses in wastewater as it passes through the different trains of treatment (Liu et al., 2022; Ye et al., 2019), however more advances in these assays are needed. Organoid culture technology has provided new avenues for assessing infectious diseases induced by pathogens (Kim et al., 2022). Organoids are cultured from stem cells capable of forming three-dimensional structures that mimic the components needed to recreate the natural host environment, including multiple cell types,

protein expression, and functions such as absorption, barrier function, and nutrient uptake (Ramírez-Flores and Knoll, 2021). In vitro culture of intestinal epithelia organoids was recently developed for replication of human noroviruses (Costantini et al., 2018; Ettayebi et al., 2016), the leading cause of acute gastroenteritis in the United States, found in high concentrations in wastewater, and with the greatest potential to exceed a 1:10,000 risk of infection (Eftim et al., 2017; Kirby et al., 2014).

Sampling site selection based on the spatial and demographic characteristics of neighborhoods (e.g., population density, poverty levels, household income, and age) are essential to provide better estimates of the distribution and concentrations of viruses in raw wastewater (Haak et al., 2022). In addition, geographical and temporal differences in distributions and concentrations of viral pathogens in raw wastewater are also critical for understanding the molecular epidemiology of waterborne pathogenic viruses and for monitoring the impacts of these factors in the design and operation of sewage networks (Kitajima et al., 2018; Kiulia et al., 2021).

Table 1-2. Maximum Concentrations of Human Enteric Viruses Detected by Molecular Methods in Raw Wastewater and Secondary Effluents in the United States.

Genome Copies Per Liter (maximum)	Virus	Location	Remarks	Reference
$5.10x10^7$ $1.50x10^7$	Norovirus GI, GII Adenovirus	Tucson, AZ	Sample collected the same day; composite sample; 24 % efficiency	(Schmitz et al., 2016)
$1.10x10^6$ $5.20x10^6$	Norovirus GI, GII Adenovirus	Tucson, AZ	Membrane filtration. 105.3% efficiency 73.8% efficiency	(Kitajima et al., 2014)
$6.70x10^8$ $1.10x10^6$ $1.10x10^6$	Adenovirus Enterovirus Norovirus GII	Traverse City, MI	1 MDS method for conc. 30 to 50% efficiency	(Simmons et al., 2011)
$1.20x10^7$	Norovirus GII Norovirus GI	New Orleans, LA	Composite; eff. 87.22% (GI NoV) 96.25% (GII NoV) ultracentrifugation	(Montazeri et al., 2015)
$5.00x10^7$	Norovirus GII	San Diego, CA	Automated magnetic bead-based concentration, 27% efficiency	(Karthikeyan et al., 2021)
$2.01x10^6$	SARS-CoV-2			
$1.55x10^7$	Adenovirus	Tucson, AZ	Membrane filtration followed by centrifugal ultrafiltration	(Schmitz et al., 2016)
$4.90x10^6$ $1.30x10^7$ $2.10x10^6$ $2.00x10^5$ $2.00x10^7$	Enterovirus Adenovirus Norovirus GIA Norovirus GIB Norovirus GII	California	PEG precipitation followed by organic extraction. Eff: 3-180 (PhiX174) 5-162 (MS2)	(Pecson et al., 2022)
$6.03x10^5$	Rotavirus	Virginia	Membrane filtration, eff: 23%	
$1.31x10^5$		California	PEG precipitation eff: 17%	
$3.72x10^6$	Enterovirus	California		
$7.4x10^5$	Adenovirus	California	Composite. Ultracentrifugation	(He and Jiang, 2005)

			followed by chloroform extraction	
$1.07x10^9$		Arizona	Composite. Direct extraction.	Unpublished data
$3.52x10^5$	Enterovirus	New Mexico	Membrane filtration followed by centrifugal ultrafiltration. RT-qPCR	(Delanka-Pedige et al., 2020)

1.3 Virus Reductions in Source Waters for Potable Reuse Projects

Various studies have evaluated the reduction of viruses by different municipal wastewater treatment processes in the U.S. (Francy et al., 2012; Harwood et al., 2005; Hewitt et al., 2011; Kauppinen and Miettinen, 2017; Kitajima et al., 2014; Kuo et al., 2010; Qiu et al., 2015; Qiu et al., 2018; Schmitz et al., 2016; Scott et al., 2003). These studies indicate that full-scale wastewater treatment utilities release infectious and noninfectious viruses in their effluent, with associated health risks dependent on the concentration and receiving water usage (Hewitt et al., 2011; Xagoraraki et al., 2014). The production of recycled water using secondary activated sludge, filtration, and disinfection is not universally effective for the removal of infectious viruses, which can be found at concentrations ranging from 0.3 to 3.3 MPN PFU (plaque forming units)/100 L (Harwood et al., 2005; Scott et al., 2003). Differences in wastewater characteristics, treatment operations, varying filter designs, and disinfection approaches significantly influence the quality of effluents generated by conventional wastewater reclamation processes (Rose et al. 2004). For instance, operation of biological treatment with higher levels of mixed liquor suspended solids (MLSS) and longer mean cell residence time (MCRT) tend to result in increased removal of microbial indicators and pathogens. In addition, prechlorinated shallow sand filters (effective size: 0.6 mm) showed to be more effective than deep bed dual media (anthracite and >1 mm sand) or monomedia (anthracite or sand) for removal of viruses (Rose et al., 2004)

Improving the efficiency of secondary treatment has a significant impact on virus reductions for water reuse applications. For instance, full-scale membrane bioreactor (MBR) plants achieve reductions from 2-$\log_{10}$ to >7-$\log_{10}$ of enteric viruses, including human adenovirus and norovirus while full-scale conventional activated sludge systems only achieve reductions between 2-$\log_{10}$ and 4$\log_{10}$ (Francy et al., 2012; Kuo et al., 2010; Simmons et al., 2011). Studies have also demonstrated that the implementation of an advanced Bardenpho secondary treatment process (intended to enhance nutrient removal), was more proficient at minimizing the incidence of viruses in effluent wastewaters than facilities utilizing activated sludge and trickling filter biotowers (Schmitz et al., 2016).

Studies have shown that inefficiencies in secondary treatment and sedimentation result in a higher microbiological loading on filtration that may impact the effectiveness of filtration and disinfection for reduction of viruses (Scott et al. 2003). In addition, differences in treatment operations, variations in filter designs, and disinfection approaches can produce effluents of varying quality. The effectiveness of full-scale biological treatment, filtration, and disinfection for reductions of enteric viruses and other pathogens was compared in six water reclamation facilities that produce reclaimed water for nonpotable urban applications in the United States (Scott et al., 2003). The relative impacts of loading conditions, process design, and operating

parameters on the removal/inactivation of viruses were evaluated. In the influent, culturable enteric viruses were detected in all samples. Moreover, prechlorinated shallow sand (effective size: 0.6 mm) filters were more effective than deep bed dual media (anthracite and >1 mm sand) or monomedia (anthracite or sand) filters for the reduction of viruses and bacterial indicators (Scott et al., 2003). The study also showed that facilities that had a cloth filter, highest loading rate and lowest range of chlorine contact times produced water with the poorest quality with respect to virus reductions. Nondetectable levels of viruses in treated effluents were achieved in facilities with the longest retention times, deepest filters (dual media), and least amount of ammonia impacting disinfection (70-90 min. contact times with 4-6 mg/L of residual chlorine). The study concluded that the effectiveness of filtration for the reduction and inactivation of viruses and other enteric pathogens could be improved by strategic use of coagulation and prechlorination.

Virus mitigation by coagulation processes in the context of the latest scientific advances in understanding virus sorption and inactivation has been reviewed (Heffron and Mayer, 2016). Chemical coagulation was shown to reduce viruses by 0.5-$\log_{10}$ to 7$\log_{10}$ with a typical reduction of approximately 3-$\log_{10}$. Moreover, chemical coagulation in combination with microfiltration demonstrated up to 8-$\log_{10}$ in pilot-scale studies with a typical reduction of 5-$\log_{10}$ (Heffron and Mayer, 2016). Virion sorption mechanisms and subsequent removal by coagulation processes was shown to be dramatically impacted by electrostatic and van der Waals forces (forces inherently associated with virion electrostatic charge and ionic strength of the aqueous solution) non-electrostatic forces like hydrophobic effect, steric hindrance and the interactions with constituents in the water matrix (e.g., suspended and dissolved solids) (Armanious and Mezzenga, 2022; Armanious et al., 2016b; Heffron and Mayer, 2016).

1.4 Framework for Selecting Indicator Viruses of Advanced Physical Treatment

The organismal virome on Earth has an estimated number of ~10^{28} virus particles (Mushegian, 2020). dsDNA viruses amount to more than a third (38.6%) of all recognized viruses with 38 virus families. These are the most common viruses in the virosphere followed by ssRNA(+) viruses (Fermin, 2018). For instance, recent studies have shown that circular metagenome assembled genomes (cMAGs) of putative viruses from human gut microbiomes are represented by dsDNA genomes of crAss-like phages, which account for nearly 87% of the DNA reads mapped to these cMAGs (Yutin et al., 2021). ssDNA viruses are consistently present in viral communities but outnumbered by dsDNA viruses (Roux et al., 2016). Ambisense virus genome, (ssDNA(+/-)) represent slightly more than 13% of all recognized viruses hosted only by eukaryotes (Fermin, 2018). Recently, viromic studies have been applied to holistically track virus communities entering and leaving wastewater treatment plants. These studies have revealed substantial viral diversity and geographically distinct viral communities, with some viruses groups behaving either as transient or actual residents of wastewater treatment plants (Adriaenssens et al., 2021; Bibby et al., 2019; Bibby and Peccia, 2013; Palermo et al., 2019; Pearson et al., 2016).

Studies have also indicated that single-stranded DNA and RNA viruses are more fragile than double-stranded viruses (Chaitanya, 2019), however capsid and genome types themselves are

not the only determinants of virus complexity and stability outside the host cells. The stability of the viruses outside living host cells results from the interaction of multiple structural and physicochemical properties of the virions. Virus particle stability outside living host cell is attributed to a multiple number of structural determinants, including surface morphology (e.g., icosahedral versus helical), capsid protein concentration and composition, covalent bonds, hydrophobic and electrostatic interactions between capsid units, presence of cementing capsid proteins, capsid-viral nucleic acid interactions (e.g., ssDNA, ssRNA, dsRNA, dsDNA) as well as stabilizing ligands (metal ions) that can entropically stabilize the virion against conformational changes and inhibit genome uncoating, i.e., release of the genome (Mateu, 2013).

Structurally complex icosahedral viruses such as Reovirus, Adenovirus, tailed bacteriophages, and many icosahedral dsDNA phages (e.g., crAssphage) present a larger variety of components in their capsids than structurally simple icosahedral viruses (Parvovirus, Picornavirus, Calicivirus). Fibers, tails, tail tips protruding from the virion capsid or prolate protein shells play architectural and functional roles during the viral cycle protecting the viral genome during infection and conferring resistance to harsh environmental conditions. The capsid of adenoviruses, for instance, has an intricate organization that involves biochemically different hexameric and pentameric capsomers conferring structural stability to the virions while the orthoreovirus (i.e., Reovirus) capsid is triple layered with large turreted structures (San Martín, 2013). In addition to virus structure complexity, It is also known that virus aggregation and interaction with particles in suspensions, other organisms (through endosymbiosis and surface binding), or microbial compounds can enhance the stability of viruses against chemical and physical disinfection processes (Burrell et al.; Folkins et al., 2020; Gerba and Betancourt, 2017; Mateu, 2013; Waldman et al., 2017; Zhang et al., 2022).

The physicochemical properties of the viruses play an important role on virus adsorption to solid-water interfaces, which in turn governs the fate of waterborne viruses in natural and engineered environments (Armanious et al., 2016a). Studies have shown, for instance, that differences in virus structure in combination with environmental factors such as organic matter in water or soil may hinder or enhance virus sorption/removal and inactivation by natural and engineered water treatment processes (Heffron and Mayer, 2016; Schijven and Hassanizadeh, 2000; Schijven and Hassanizadeh, 2002a; Yuan et al., 2008). Organic carbon (OC) is known to play a critical role in the sorption and desorption of human adenovirus, and consequently on its environmental fate and transport (Wong et al., 2013).

The interaction of viruses to solid-water interfaces is largely driven by electrostatic forces and the hydrophobic effect in which the virus surface charge, most often characterized by the isoelectric point (Mi et al., 2020). The surface charge of the virus particle is a function of pH and ionic strength of the aqueous solution (Mi et al., 2020). Differences in the isoelectric point of viruses (p*I*) have been used to compare environmental interactions of different viruses across a range of conditions and experimental methods (Dika et al., 2015; Langlet et al., 2008; Xagoraraki et al., 2014). The isoelectric point of a virus is the pH value at which the virion's net charge is 0 (neutral) (Heffron and Mayer, 2021; Michen and Graule, 2010) and this value provides information about the viral surface charge in an aqueous environment (Scheller et al., 2020) where viruses display different surface charges depending on the pH of the aqueous

solution. At pH levels above the p*I*, the surface is negatively charged in solution; below the p*I*, the surface has a positive charge. Knowledge of the isoelectric points of viruses is important because the solubility and electrical repulsion are lowest at the p*I* and therefore the tendency of viruses to aggregate or precipitate is highest (Heffron and Mayer, 2020). The virus surface charge has been used to evaluate the adhesion forces between viruses and target substrates in order to predict the likelihood of virus attachment to a charged surface. The virus surface charge is also used for the design of filters for the removal of viruses by electrostatic adsorption (Armanious and Mezzenga, 2022). Studies have shown that the thermal stability of adenovirus type 2 is increased in mildly acidic conditions as a result of covalent modifications of viral capsid proteins (Rexroad et al., 2006). In addition, variations in virus symmetry are known to be affected by the ionic content of aqueous solutions as demonstrated for filamentous bacteriophages (Stubbs and Kendall, 2012). This knowledge is beneficial for predicting environmental transport of waterborne viruses and virus fate during physical removal or chemical inactivation processes (Heffron and Mayer, 2020, 2021; Langlet et al., 2008; Mattle et al., 2011; Mayer et al., 2015; Rayfield et al., 2015; Strauss et al., 2017).

Based on the aforementioned information, a selected group of viruses with specific attributes (Table 1.3) are suggested as a guidance framework to select potential indicators to confirm physical treatment for potable reuse projects. Virus abundance in wastewater as well as persistence and susceptibility to inactivation and removal by water and wastewater treatment processes have been well documented (Adriaenssens et al., 2021; Blatchley et al., 2007; Boehm et al., 2019; Cromeans et al., 2014; Mattle and Kohn, 2012; Nwachuku et al., 2005; Rachmadi et al., 2020; Templeton et al., 2008; Torrey et al., 2019; Wigginton and Kohn, 2012). Knowledge of virus abundance in raw wastewater and structural features of known or newly discovered viruses by virus metagenomics studies can be used to develop site-specific viral quality monitoring tools for multiple applications, including the evaluation of physical treatment at full-scale. These criteria may include wastewater characteristics, operational parameters of treatment performance, treatment technologies, plant capacities, geographic locations, and seasonal effects. All these features must be seen as important components of an expanding research agenda to ensure current and future sustainable, reliable and safe water reuse.

Table 1-3. Selected Viruses as Potential Indicators for Evaluation of Advanced Physical Treatment for Potable Reuse Projects.

Virus/Taxonomic Group: Family	Virus Genome [Group][a]	Virion Size nm/[Total Molar Mass, MDa][b]	p*I*[c]	Viral Capsid Symmetry and Stability	Abundance in Raw Wastewater and Stability Outside the Host
Adenovirus Adenoviridae Eukaryotic virus (Human pathogen)	dsDNA linear [I]	(90 – 100) [317-435]	4.5 – 6.75	Icosahedral (12 fibers projecting from the vertices of the icosahedron capsid)	High. Found in recharged water. Also, found in ROP. Most UV-resistant health-related virus
Aichi virus Picornaviridae Eukaryotic virus	ssRNA [IV] linear	(30 – 32) [12-14]	7.2	Icosahedral – surface	High. Also Found in MFE and ROP. Stable at pH 2. Resistant to heat, chlorine, hydrostatic

Virus/Taxonomic Group: Family	Virus Genome [Group][a]	Virion Size nm/[Total Molar Mass, MDa][b]	pI[c]	Viral Capsid Symmetry and Stability	Abundance in Raw Wastewater and Stability Outside the Host
(human pathogen)				depressions like "canyon"	pressure, chloroform, non-ionic detergents and ether (Betancourt et al., 2014; Rivadulla and Romalde, 2020)
CrAssphage sp Unclassified Prokaryotic virus	dsDNA [I]	(75 – 80) [183-223]	Unknown	Icosahedral (spherical with tail structure)	High. Found in recharged water and ROP. Fecal indicator. Highly abundant human-associated virus in the human gut virome and sewage.
CRESS virus Unclassified Eukaryotic virus	ssDNA circular [II]	(15 – 25) [1.5-7]	Unknown	Icosahedral	High. Ubiquitous. New species frequently detected in untreated and treated wastewater. Widely distributed in environmental samples. Resistant to inactivation at pH 3. Virions withstand incubation at 70 °C for 15 min.
Enterovirus Picornavirales Eukaryotic virus (human pathogen)	ssRNA linear [IV]	30 – 32 nm	Varies 8.3, 6.4, 4.5, 6.6	Icosahedral. No projections on virions	High. Genotype-specific resistance to chlorine disinfection (Torii et al., 2022). pH stability (3-9). Thermal stability varies with viruses and enhanced (42-50 C) in the presence of sulfhydryl reducing agents and magnesium cations) as does stabilization by divalent cations. Stability at low or high ionic strength also varies with virus (Zell et al., 2017)
F+ coliphages Leviviridae Inoviridae Prokaryotic virus	ssRNA [IV] linear ssDNA [II] linear	(21 – 30) [4 – 12]	3.9 2.7 – 5.3	Icosahedral (Leviviridae), Helical or filamentous (Inoviridae).	High. F+RNA highly correlated with virus concentrations in raw and treated wastewater. Found in recharged water. Fecal and viral indicator. Resistant to detergents, ether, chloroform pH 3.5
GI-II Norovirus Caliciviridae Eukaryotic virus (Human pathogen)	ssRNA [IV] linear	(27 – 40) [19-28]	5.5 – 6.0	Icosahedral. Capsid with cup-shaped depressions	High. GI Norovirus found in ROP. Stable at pH 3.5. Resistant to inactivation by heat, ether, chloroform, and mild detergents (Vinjé et al., 2019)
Human Bocavirus Parvoviridae Eukaryotic virus (Human pathogen)	ssDNA linear [II]	(23 – 25) [4 – 7]	7.4	Icosahedral. Rugged capsid with elevated protrusions and canyon-like depressions (Luo et al., 2021)	High. Found in MFE. Stable at pH 3–9. Virion withstand incubation at 56 °C for 60 min. Resistant to disinfectants, but sensitive to UV radiation (Cotmore et al., 2019)

Virus/Taxonomic Group: Family	Virus Genome [Group][a]	Virion Size nm/[Total Molar Mass, MDa][b]	p*I*[c]	Viral Capsid Symmetry and Stability	Abundance in Raw Wastewater and Stability Outside the Host
Hudisavirus Unclassified Protist virus	ssDNA [II]	(15 – 25) [1.5 – 7]	Unknown	Icosahedral	Unknown. Viral lineage recently found in human diarrheal disease predicted to infect human protozoan parasites.
Pepper Mild Mottle Virus Virgaviridae Plant virus	ssRNA linear [IV]	(18 x 300-310) [2.5 – 12953]	3.7 – 3.8	Helical (rod-shaped). Four-alpha-helix coat protein core. Helical symmetries of different genera differ	High. Rigid rod-shaped. Physically and chemically stable. Found in recharged water. Also, found in ROP. Index virus. Process indicator. Fecal indicator. Withstand extremes of ionic conditions, temperatures up to 55 °C, pH values between 2 and 9. Thermal inactivation points (10 min) of 90 °C.
Reovirus Reoviridae Eukaryotic virus (Human pathogen)	dsRNA linear [III]	70 – 90 [149-223]	3.9 .	Icosahedral (virion with uneven surface) with double-layered icosahedral protein capsid.	High. Virion infectivity is moderately resistant to heat, organic solvents (ether) and non-ionic detergents. Resistant to chlorine and ozone disinfection. Found in recharged water. Ether and acid-stability (stable at pH 3.5).
Somatic coliphages	ssDNA dsDNA	(21 – 25) [4 – 7] (60 – 111) [94 – 595]	6.6 – 7.3	Icosahedral. very stable, resistant to detergents, ether, chloroform, pH 6.0–9.0 and freezing	High. Found in recharged water. Fecal and viral indicator

a: Baltimore system of virus classification. **b:** (Erickson, 2009) **c:** isoelectric point.

Assuming that viruses with icosahedral capsid structure have a spherical shape, the apparent molecular weight (AMW) for this study was calculated based on virus size as:

$$R_{min} = 0.066 \times AMW^{1/3}$$

where Rmin is the minimum radius of a sphere that could contain a specific mass of protein in nanometer, and AMW is the apparent molecular weight in Dalton. The AMW of Pepper Mild Mottle Virus (PMMoV), a rod-shaped structure virion with a minimum apparent size of 18 nm in diameter and a predominant length of 300-310nm, was calculated considering the minimum apparent size. The minimum radius was calculated as the half size of viruses listed in Table 1.3 Note that proteins/virus have an irregular surface and minimum radius is supposed to be lower than the average size (Souza-Chaves et al., 2022).

1.5 Physical Advanced Treatment Processes

1.5.1 Media Filtration

Media filters are a sanitation technology that use microorganisms that are attached to a high surface area medium to primarily remove soluble organic matter and therefore high pathogen removal rates are not for this type of treatment (Oakley and von Sperling, 2017). Media filtration in nanofiltration (NF) and reverse osmosis (RO) membrane plants has two basic functions: suspended solids removal and improvement of silt density index (SDI) value to less than 3.0. SDI is a key parameter to monitor in reverse osmosis systems and is used to demonstrate the performance of the pretreatment equipment. Common filter media during pretreatment include sand, anthracite, and garnet. Granular media filters are also used in MF-UF installations, although they are less common. Potential benefits of adding granular media filtration upstream of MF-UF include reduced biological and particulate fouling, which can lead to operation at higher flux rates, and reduced time between cleanings (AWWA, 2018).

Granular Activated Carbon (GAC) filtration is a common process for advanced wastewater treatment used extensively in the United States for the removal of micropollutants dissolved contaminants such as pesticides and industrial chemicals. The removal of these organic substances takes place by adsorption and biological processes. GAC uses a random porous structure, containing a broad range of pore sizes ranging from visible cracks and crevices down to molecular dimensions. The porous structure leads to an extremely large amount of adsorption surface area, generally around 73 acre/lb (650 m^2/gram) to 112 acre/lb (1000 m^2/gram) (Fundneider et al., 2021; Newcombe, 2006). The capacity of virus removal by GAC filters has been only evaluated using bacteriophage MS2 at pilot scale with not removal demonstrated by this type of filtration media (Hijnen et al., 2010). More recent studies have made use of GAC as an optimized passive sampling technique to capture SARS-CoV-2 in wastewater, promoting a scalable and convenient alternative for capturing viral pathogens in water (Hayes et al., 2022).

1.5.2 Membrane Processes (MF/UF/RO/NF)

Pressure-driven membrane filtration systems, such as microfiltration (MF), ultrafiltration (UF), nanofiltration (NF) and reverse osmosis (RO), are being increasingly used for multiple reuse applications (aquifer recharge, surface water augmentation, and direct potable reuse) in order to meet water reuse quality requirements (Al-Abri et al., 2019; Singh, 2015; Sun et al., 2015; Van Der Bruggen et al., 2003) The removal of target constituents (nanoparticles and soluble contaminants) from aqueous solution these targets can vary significantly depending on the physicochemical properties of the constituents, membrane type and operational conditions (Warsinger et al., 2018; Yang et al., 2020). Virus rejection mechanisms by membrane technologies have been divided into four major types: mechanical sieving, electrostatic interactions, adsorption retention, and hydrophilic and hydrophobic interactions (Gentile et al., 2018; Goswami and Pugazhenthi, 2020).

MF-UF are used to provide treatment for reverse osmosis and nanofiltration for reuse applications that require high-quality water, e.g., including agricultural reuse, potable reuse via groundwater or surface water augmentation (NRC, 2012). These membranes processes are classified as low pressure (<2 bar) with filtration media pore sizes ranging from 0.1 µm-1 µm for

MF membranes to 0.001 µm-0.1 µm for UF membranes (AWWA, 2018; ElHadidy et al., 2013). Selective filtration or retention of suspended solids and microorganisms greater than their pore size is the main principle of low-pressure membranes (Ferrer et al., 2015a; Warsinger et al., 2018; Yang et al., 2020). Limited reduction of viruses are attributed to MF membranes and virus disinfection credits are rarely awarded. Laboratory-scale drinking water experiments using recombinant norovirus-like particles (rNV-VLPs) and bacteriophages Qß and MS2 demonstrated removal ratios smaller than 0.6$\log_{10}$ (Matsushita et al., 2013).

RO and NF processes, classified as high pressure, are capable of high rejection of most dissolved constituents using a positive hydrostatic pressure gradient to force water through semipermeable membranes that filter out dissolved ions, molecules, and solids (AWWA, 2018). A comprehensive review of the role of polymeric membranes and process components in the treatment of wastewater to potable water quality including the recent advancements and needs in separation processes has been published elsewhere (Warsinger et al., 2018). There are no actual pores for RO-NF processes, however a theoretical pore size of <0.001 µm has been assigned to these types of membranes.

Typically targeted MF-UF filtrate water quality requirements to promote effective NF-RO operation (the California Code of Regulations, Title 22, Water Recycling Criteria, [Title 22]) include the following: SDI (silt density index), <3; suspended solids, undetectable; turbidity, 95th percentile <0.1 NTU; *Giardia*, undetectable; and *Cryptosporidium*, undetectable. For groundwater injection into a potable aquifer in California, the California Department of Public Health (CDPH) drafted regulations for groundwater recharge with reclaimed water requiring an integrated membrane system such as microfiltration or ultrafiltration pretreatment followed by RO treatment to achieve an effluent water quality of <0.5 mg/L total organic carbon (TOC) and <5 mg/L total nitrogen (TN) (CCR, 2015). This type of advanced treatment configuration is also required under the California regulatory framework to achieve log removal credits for viruses. For these applications, integrity monitoring and membrane repair (as needed) are essential for compliance. The California Division of Drinking Water and Environmental Management grants log removal values (LRVs) of viruses corresponding to 0.5-$\log_{10}$ for MF and 2.5-$\log_{10}$ to 4-$\log_{10}$ for UF membranes (AWWA, 2018).

Inadequate reduction of viruses by size-exclusion and ionic diffusion has been attributed to manufacturing imperfections, leaking seals and O-rings, as well as damage during use (Ferrer et al., 2013; Pype et al., 2016a; Vickers et al., 2019). Convective transport through defects is considered the dominant mechanism of leak (Yoon, 2019). Chemical exposure can lead to changes in membrane surface chemistry (i.e., surface charge and hydrophobicity), mechanical strength and pore size all of which can influence the mechanisms of virus rejection by RO membranes either increasing or decreasing the reduction efficiency of specific viruses (Pype et al., 2016a).

Because of these limitations, virus $\log_{10}$ reduction value (LRV) attribution for membrane processes can only be obtained through regular monitoring and testing to ensure membrane integrity. Direct integrity testing is accomplished by pressure decay tests as well as direct spiking of bacteriophage MS2 that are performed off-line at specified intervals (Pype et al.,

2016a; Pype et al., 2016b). Indirect integrity testing is usually performed through on-line monitoring of water conductivity or total organic carbon (TOC) in the permeate stream which is then translated to membrane integrity (US EPA, 2005). However, due to limitations in detection sensitivity of conductivity and TOC, only minimal LRVs can be attributed to membrane processes. RO can receive up to 2 LRVs with regular monitoring in most states, whereas UF can receive up to 1, despite their ability to achieve greater reductions during experiments with spiked bacteriophages (Antony et al., 2016; ElHadidy et al., 2013; ElHadidy et al., 2014; Kitis et al., 2003; Kreissel et al., 2012; Mi et al., 2004; Pierre et al., 2011; Pype et al., 2016a).

The lack of data of virus reduction at full scale integrated membrane systems is likely a symptom of the impracticality of spiking with MS2, which is intensive and require high cost and effort at full scale. Few studies have evaluated the reductions of naturally occurring virus by integrated membrane processes. Previous studies (Ferrer et al., 2013; Otaki et al., 1998) examined the reduction of naturally occurring bacteriophages during pilot-scale UF of surface water and demonstrated LRVs of < 1-$\log_{10}$ to 3-$\log_{10}$. Another study (Lee et al., 2019) found LRVs of Pepper Mild Mottle Virus (PMMoV) ranging from < 1-log10 to 4-$\log_{10}$ during UF of treated effluent with variations in reductions attributed to the extent of membrane damage. More recently, the integrity of RO membranes in pilot-scale operation was evaluated by selecting novel natural indigenous freshwater viruses and bacteriophages that were present abundantly in surface water (Hornstra et al., 2019a). The novel viruses were identified by metagenomics sequencing approaches and their genomes were quantified by real time PCR, demonstrating LRVs of > 7-$\log_{10}$ for an intact, pilot-scale RO membrane. Additional studies (Prado et al., 2019) have evaluated LRVs of human adenoviruses (HAdV) and JC polyomaviruses (JCPyV) across a full-scale advanced treatment scheme that included RO membranes. These studies demonstrated LRVs of 2.65-$\log_{10}$ for HAdV and 2.3-$\log_{10}$ for JCPyV. Table 1.4 summarizes different studies evaluating virus reductions during UF and RO at both pilot and full-scale membrane systems.

Table 1-4. Summary of Studies on Virus Reductions by Membrane Processes.

Membrane	Scale	Material/ Parameters	Solution	Virus	LRV Log_{10}	Reference
UF	Bench	Hollow Fiber PES (0.02 um)	Tap water/DI water; Surface water	MS2	2.5-6	(Kreissel et al., 2012)
UF	Bench	Hollow Fiber PES (0.02 um)	Tap water/DI water; Surface water	phiX174	2.5-4.5	(Kreissel et al., 2012)
UF	Bench	Hollow fiber PVDF UF	Di with different pH	MS2	3.7	(ElHadidy et al., 2013)
UF	Bench	Hollow fiber PVDF UF	Di with different pH	phiX174	3.7 (pH 6.5), 2.5 (pH 9.4)	(ElHadidy et al., 2013)
UF	Bench	Hollow Fiber PVDF UF (2--56 nm)	Surface Water	MS2	3.5-6	(ElHadidy et al., 2014)
UF	Bench	Hollow Fiber PVDF UF (2--56 nm)	Surface Water	phiX174	3-5.9	(ElHadidy et al., 2014)
UF	Bench	Hollow fiber CA (100 KDa)	Tap water	MS2	5.7-6.4	(Pierre et al., 2011)
UF	Bench	Hollow fiber CA (100 KDa)	Tap + NaCl	MS2	5.6-5.7	(Pierre et al., 2011)
UF	Bench	Hollow fiber CA (100 KDa)	DI + 1 or 9 g/L NaCl or PBS	MS2	5-6	(Pierre et al., 2011)
UF	Bench	PA	Tap water	MS2	~2	(Hu et al., 2003)
UF	Bench	PS	Tap water	MS2	~1	(Hu et al., 2003)
UF	Pilot	0.035 uM	DI	MS2	3.0 - 4.0	(Jacangelo et al., 2005)
UF	Pilot	100 KDA	Surface Water	MS2	>7.0	(Jacangelo et al., 1991)
UF	Pilot	100 KDA	Surface Water	MS2	>6.7	(Jacangelo et al., 1991)
UF	Pilot	100 KDA	Surface Water	MS2	>6.5	(Jacangelo et al., 1991)
UF	Pilot	100 KDA	Surface Water	MS2	>7.2	(Jacangelo et al., 1991)
UF	Pilot	Hollow fiber PE (0.1 um)	River water	E. coli K12 indigenous phages	<1 to 2	(Otaki et al., 1998)
UF	Pilot	Hollow fiber PE (0.1 um)	River water	E. coli C indigenous phages	2-3	(Otaki et al., 1998)
UF	Pilot	Hollow fiber PVDF (0.03 um 200 Kda) and PES (100KDa)	River water	MS2	>4	(Boudaud et al., 2012)
UF	Pilot	Hollow fiber PVDF (0.03 um 200 Kda) and PES (100KDa)	River water	QB	>4	(Boudaud et al., 2012)

Membrane	Scale	Material/ Parameters	Solution	Virus	LRV Log_{10}	Reference
UF	Pilot	Hollow fiber PVDF (0.03 um 200 Kda) and PES (100KDa)	River water	GA	1.6	(Boudaud et al., 2012)
UF	Pilot	Hollow fiber PVDF (0.04 um)	River water	Indigenous somatic coliphages	2.8	(Ferrer et al., 2015b)
UF	Pilot	Hollow fiber PVDF (0.04 um)	River water	Indigenous F-specific coliphages	3	(Ferrer et al., 2015b)
UF	Pilot	ND	Reclaimed water	NoVGI, Nov GII	< 1 to > 1 (GI) 1 to >3 (GII) depending on membrane damage	(Lee et al., 2019)
UF	Pilot	ND	Surface Water	GA	3.02	(Ferrer et al., 2015b)
UF	Pilot	ND	Surface Water	MS2	2.81	(Ferrer et al., 2015b)
UF	Pilot	ND	Surface Water	PRD1	>5.0	(Ferrer et al., 2015b)
UF	Pilot	UFG10	Secondary Effluent	MS2	5.3	(Madireddi et al., 1997)
UF	Full	ND	Surface Water	MS2	5.4	(Kruithof et al., 2001)
RO	Bench	CA	Tap water	MS2	~4	(Hu et al., 2003)
RO	Bench	Composite PA	Saline solution	MS2	5-6 (intact membrane)	(Mi et al., 2004)
RO	Bench	ND	Synthetic salt water / filtered effluent	MS2	~4.6 to >6 depending on module set up and membrane age	(Pype et al., 2016a)
RO	Bench	PA	Tap water	MS2	~5	(Hu et al., 2003)
RO	Bench	PA-TFC	DI	MS2	>6.7	(Madireddi et al., 1997)
RO	Bench	PA-TFC	DI	MS2	5.6	(Madireddi et al., 1997)
RO	Bench	PA-TFC	DI	MS2	2.7	(Madireddi et al., 1997)
RO	Bench	Polyamide, new and aged	Saline solution	MS2	>6.3 (new), 2.8-4.1 aged	(Antony et al., 2016)
RO	Bench	RO CA	DI	MS2	>4.9	
RO	Bench	RO CA	DI	MS2	4.6	(Madireddi et al., 1997)
RO	Pilot	ND	Surface Water	Novel viruses, MS2	> 7.0	(Hornstra et al., 2019b)
RO	Pilot	ND	Filtered secondary effluent	MS2	~3 to > 6	(Kitis et al., 2003)

Membrane	Scale	Material/ Parameters	Solution	Virus	LRV Log_{10}	Reference
RO	Pilot	ROSG/Ag4040	Secondary Effluent	MS2	6.7	(Madireddi et al., 1997)
RO	Full	ND	Surface Water	MS2	3.0-4.8	(Kruithof et al., 2001)
RO	Full	ND	Effluent	MS2	4-6 depending on membrane integrity	(Vickers et al., 2019)
Disc filter, biotreatment, MBR, RO, ClO2	Full	ND	Effluent	HAdV	2.65	(Prado et al., 2019)
Disc filter, biotreatment, MBR, RO, ClO3	Full	ND	Effluent	JCPyV	2.3	(Prado et al., 2019)
Disc filter, biotreatment, MBR, RO, ClO4	Full	ND	Effluent	RVA	2.9	(Prado et al., 2019)

1.5.3 Soil Aquifer Treatment: A Natural Advanced Water Treatment Process for Virus Attenuation

Soil-aquifer treatment (SAT) provides a valuable soil-based natural treatment process for the attenuation and/or reduction of chemical and microbial contaminants associated with reclaimed effluents used as source water in sustainable land-based managed aquifer recharge (MAR) systems (Amy and Drewes, 2007). SAT along with bank filtration, represent natural attenuation process of water purification that differ in flow conditions. While SAT involves unsaturated and saturated flow conditions, bank filtration systems are primary saturated flow (Fox and Makam, 2009). SAT and bank filtration can result in significant reductions of microbial pathogens in the recharged source water, and thus constitute important components for indirect potable reuse applications (Asano and Cotruvo, 2004; Bekele et al., 2011; Betancourt et al., 2014). During natural attenuation processes, the physicochemical properties of soils act as an additional treatment barrier where processes such as filtration, adsorption, and even pathogen inactivation can occur during infiltration (Bradford et al., 2017; Lakretz et al., 2017). Recharged groundwater can be used to maintain aquifer capacity in times where groundwater pumping exceeds natural recharge, such as in times of drought, and more importantly for potable and non-potable reuse applications. As potable reuse of wastewater becomes more of a reality in arid regions, it is likely that the incorporation of SAT systems as a multi-barrier treatment approach, prior to advance treatment, will increase.

Treated wastewater effluent used as a source water for recharge operation may contain large concentrations of human enteric viruses, with values as high as 10^4-10^5 genome copies per liter (Gerba et al., 2017). Thus, it is of value to better understand the ability of SAT to remove and/or inactivate waterborne pathogenic viruses present during infiltration since viruses represent the most resilient biological entities in the wastewater environment (Betancourt et al., 2014). Viruses are structurally diverse and also possess different types of chemical composition (i.e.,

arrangement, type, and ratio of atoms in molecules) which can influence virus absorption to solid-water interfaces as well as virus fate and transport in engineered and natural systems (Armanious et al., 2016a). Solid surfaces in wastewater treatment and disinfection processes may include minerals, dissolved and particulate organic matter, as well as skins from vegetables, fruits, and humans. In addition, electrostatic interactions governed by pH and ionic strength of aqueous solutions as well as non-electrostatic interactions (i.e., van der Waals) can favor or hinder virus absorption to solid-water interfaces. Viruses also have great potential for long distance transport through soils and aquifers due to their small size, colloidal properties and environmental persistence (Boehm et al., 2019; Schijven and Hassanizadeh, 2002a; Tesson et al., 2018; Xagoraraki et al., 2014; Yates et al., 1988). Thus, a thorough understanding of the fate of viruses through water reclamation processes is crucial for managing the risks from pathogenic viruses.

Understanding the factors that control virus migration and survival through subsurface formations is critical for proper design and operation of sustainable land-based managed aquifer recharge (MAR) systems (Amy and Drewes, 2007). Multiple factors may affect both survival and transport of viruses in subsurface systems, including microbial activity, soil type, soil properties (e.g., soil particle distribution, clay composition, pH, soil organic content, soil solution composition, and ionic strength), flow velocity, degree of water saturation, and presence of colloids, temperature, and virus type (Schijven and Hassanizadeh, 2000; Yates and Yates, 1988). Notwithstanding, adsorption to soils is the most relevant process of virus attenuation, mostly associated with virus inactivation and irreversible attachment to solid-water interfaces having favorable charge characteristics (Bradford et al., 2017; Schijven and Hassanizadeh, 2000; Yates and Yates, 1988).

Variations in hydrological conditions, unexpected treatment failures due to variable concentrations of pathogens or treatment deficiencies, and site-specific conditions associated with heterogeneities in the subsurface system have been recognized as major limitations for predicting pathogen removal by MAR systems. Consequently, the selection of the most suitable indicators and surrogates for evaluation of virus attenuation by MAR systems may be tightly associated with site-specific conditions that include source water pretreatment, quality, pathogen occurrence frequency, as well as MAR system characteristics (e.g., type of MAR system, geological material, vadose versus saturated conditions, residence time, dynamic hydrological conditions) and the post-treatment process. These and other aspects related to pathogen reduction through MAR processes have been thoroughly addressed in a WRF report (Rauch-Williams, 2022). Recent studies stress the importance of continuous monitoring of the SAT vadose zone's physicochemical conditions for optimal operational performance, since the vadose zone processes play a central role in determining the quality of the water that recharges the aquifer (Elkayam et al., 2015; Turkeltaub et al., 2022). Simulation studies have also clearly demonstrated that changes in hydraulic conditions during floods can affect the efficacy of riverbank filtration to remove viruses. Under these conditions, fluctuations in river water level cause further transportation of higher concentrations of viruses into the riverbank. These studies revealed that a 1-5 m increase in river water level led to a 2-$\log_{10}$ to 4-$\log_{10}$ increase in virus concentration and to up to 30% shorter travel times (Derx et al., 2013).

A comprehensive review of the peer-reviewed literature related to the reduction of viruses in MAR systems was conducted in Google Scholar, Lens.org, and CORE databases and indexers available through the University of Arizona Libraries system. The combination of search criteria included the terms “virus removal” AND aquifer treatment AND (soil aquifer treatment), AND (MAR systems) or “virus removal” AND (bank filtration), “virus occurrence” AND (groundwater). Studies on virus occurrence and reduction in groundwater recharged operations have largely focused on either enteroviruses, commonly found in wastewater, or spiked bacteriophages as virus surrogates (Elkayam et al., 2015; Gerba et al., 1991; Schijven et al., 1999). There are limited studies which examine the use of viruses typically found in wastewater, despite the potential for these viruses to provide better insight to virus removal during full scale SAT (Betancourt et al., 2014).

For soil aquifer treatment and groundwater recharge, there are numerous studies that model virus transport through soil by use of packed-columns, many of which have been applied to understand virus reductions by SAT (Frohnert et al., 2014; Schijven and Hassanizadeh, 2000; Walshe et al., 2010). However, field studies that directly assess reductions of naturally occurring viruses by MAR systems of treated wastewater effluent are scarce. The few existing field studies tend to examine low volumes of groundwater and largely examine culturable human viruses and/or bacteriophages (Hornstra et al., 2018; Schijven and Hassanizadeh, 2002a). Previous studies evaluated the reduction of selected enteric viruses and a potential surrogate for virus reduction at three full-scale managed aquifer recharge (MAR) systems located in different regions of the United States (Arizona, Colorado, and California) that employ different treatment technologies, different recharge operations, and different uses of application after recharge (Betancourt et al., 2014). Samples of source water (i.e., river water receiving treated wastewater, secondary and tertiary treated wastewater) before recharge and recovered groundwater at all three sites were tested for adenoviruses, enteroviruses, Aichi viruses and pepper mild mottle virus (PMMoV) by quantitative polymerase chain reaction (qPCR) assays and integrated cell culture qPCR. PMMoV was the most commonly detected virus in the groundwater samples. Reovirus was detected by ICC-PCR in only one groundwater sample with a subsurface residence time of five days. Residence time played an important role in the reduction efficiency of human enteric viruses (Aichi virus, Enterovirus, Adenovirus, Reovirus). The results of the study suggested that in groundwater with a residence time of greater than 14 days most viruses could be reduced to below the detection limit. PMMoV was suggested as a suitable conservative tracer of enteric virus reduction in managed aquifer treatment systems. Reovirus was considered as a relevant waterborne virus for further research at field –scale operations. The ability to quantify the reduction of human enteric viruses was limited by the concentrations of the particular virus in the infiltrated wastewater, but the study revealed that at least a >2-$\log_{10}$ reduction could be expected with a travel time of >15 days.

A recent study evaluated the transport and reduction of viruses during SAT of tertiary treated effluent. Adenovirus and Enterovirus were the two human enteric viruses evaluated along with PMMoV and crAssphage as virus surrogates due to their relative abundance in tertiary treated effluent used as source water for infiltration. PMMoV and crAssphage were detected in groundwater associated with a set of recharge basins that exhibited shorter wetting/drying

cycles and faster infiltration rates. LRVs for crAssphage and PMMoV at this site ranged from 3.9-$\log_{10}$ to 5.8-$\log_{10}$, respectively. Adenovirus and Enterovirus were not detected in any of the groundwater associated with SAT. The study concluded that wetting/drying cycles and increased infiltration rates favored the detection of PMMoV and crAssphage (Morrison et al., 2020) but not enteric viruses that were reduced at greater than values. Wetting/drying cycles is a parameter that has been investigated for attenuation of trace organic chemicals in MAR systems (Filter et al., 2021).

The discovery of new viruses by next-generation sequencing approaches (Ng et al., 2012; Rosario et al., 2009; Wylie et al., 2012) poses a challenge for natural and engineered water reclamation processes for potable reuse since little is known about the fate and transport of these viruses in the environment. of particular importance, replication-associated protein (Rep)-encoding single-stranded (CRESS) DNA viruses represent an extreme in the biological size spectrum (15 – 22 nm), as they include the smallest capsid-encoding pathogens known to infect eukaryotic organisms (Rosario et al., 2012b). Although there has not been convincing direct causal relation of CRESS viruses to any specific disease, infections may play a role in autoimmunity, indirectly affecting the severity of disease caused by other pathogens (Shulman and Davidson, 2017). CRESS DNA viruses are endemic in human populations (35% - 80%) being detected repeatedly in human samples (stools, serum, and cerebrospinal fluid), both from healthy individuals and from patients with neurological disease, speculating that they will emerge as potential pathogens (Biagini, 2004; La Bella et al., 2020; Malathi and Renuka Devi, 2019).

1.6 Studies That Have Evaluated Biological Biomass by Measuring ATP Concentrations

Direct measurements of Adenosine Triphosphate (ATP) concentrations for rapid and accurate monitoring of the concentration and health of living biomass have been used at numerous full-scale municipal treatment plants for evaluating maximum treatment performance. ATP is an energy-rich compound produced by all metabolically-active cells. For more than four decades, it has been used as a monitoring tool for biomass accumulation during biological wastewater treatment (Patterson et al., 1970). Further, it has been researched as an indicator of microbial quality for various matrices including drinking water, groundwater, surface water, and wastewater effluent (Deininger and Lee, 2001; Hammes et al., 2010). Moreover, ATP monitoring can serve as a screening and routine monitoring tool for detecting total quantity of active microorganisms to reveal the onset of regrowth or changes from baseline conditions (Travis and Tracey, 2016). For instance (Travis and Tracey, 2016), proactive biological monitoring using advanced ATP was able to guide mitigation activities and optimize several design modifications to improve plant operation and product water quality.

Traditionally, heterotrophic plate count (HPC) assays using R2A growth medium have been employed to quantify total numbers of bacteria in aquatic systems. However, HPC assays require up to 7 days for results, and the observed growth represents <1% of the total microbial community present due to the predominance of viable but non-culturable bacteria. Total cell numbers in water samples may also be assessed using the Acridine Orange Direct Count (AODC) method, although differentiation between living and dead bacteria cannot be determined. In

contrast, the Direct Viable Count (DVC) process targets only living cells, but is more time-consuming in its requirement of 24 to 36 hours for assay completion. In comparison, ATP quantification assays present a viable and attractive option as a performance indicator of advanced water treatment due in part to the following factors:

- rapid assay completion times of <10 minutes per sample for near real-time data
- conversion capability of ATP levels to Microbial Equivalents (i.e., total numbers of metabolically-active cells)
- ability for differentiation of extracellular (background) ATP and cellular ATP

TOC and BOD measurements have also proven highly useful in the real-time monitoring of water quality and wastewater treatment processes. However, these parameters serve as indirect measurements of viable bacteria levels within water. The incorporation of ATP quantification as a direct, near real-time biological assay conducted concurrently with on-line measurements of TOC and BOD will better allow for direct comparison of these parameters one another, and to the reduction of viruses during advanced water treatment.

In a recent study, bulk water cell counts, adenosine triphosphate concentrations, and assimilable organic carbon were measured throughout a pilot-scale direct potable reuse facility and three parallel chlorinated simulated distribution systems fed with the pilot's finished water (Miller et al., 2020). The study also investigated the impacts of treatment operations (e.g., membrane cleanings) and perturbations (e.g., incomplete wastewater nitrification) on microbial water quality. Intact cell counts and total adenosine triphosphate concentrations were reduced to near or below method quantification limits (22 cells per mL and 10^{-4} nM, respectively) by reverse osmosis and advanced oxidation. The study demonstrated that the combination of ATP measurements with flow cytometry more completely tracked microbial abundance and viability throughout advanced treatment than either method alone. In addition, measured removal of ambient ATP was a better indicator of pathogen removal by NF/RO than the typically credited removal of conductivity or total organic carbon. However, the study demonstrated that the measurements of intracellular ATP across MF/UF membranes and ozonation were low as compared to removal observed by cell counts. Overall, the results of this study indicated the application of enhanced microbial evaluation tools for monitoring the performance of advanced wastewater treatment processes, but also highlighted the challenges that need to be addressed in order to overcome limitations associated with differences in water treatment matrices.

1.7 Summary of Traditional and Advanced Analytical Methods for Measuring and Concentrating Viruses

It has long been recognized that the best methods to assess the occurrence of viruses in water will be those that are simple, rapid, inexpensive, and consistent. While a number of techniques have been developed and refined, it has proven difficult to achieve the detection of all relevant virus types over the spectrum of water quality matrices that exist in nature and human-constructed facilities (Ikner et al., 2012). Because viruses are generally present in low concentrations in treated wastewater, the concentration of large volumes (100 – 1,000 L) is required to enhance the usefulness of the recovery and detection assays by culture- or molecular-based methods (Cashdollar and Wymer, 2013; Haramoto et al., 2018; Ikner et al.,

2012). The VIRADEL method based on the adsorption of viruses to electro-positively or electro-negatively charged filters has commonly been used for the primary concentration of viruses. Representative recovery efficiencies for each VIRADEL method by water type have been previously described (Gibson and A. Borchardt, 2016). The most commonly used filters for the primary concentration of viruses from large volumes of water are the positively charged Zeta Plus 1MDS and NanoCeram. Both filters are components of the United States Environmental Protection Agency's (USEPA) Method 1615 developed with the goal of providing a standardized approach for the measurement of enterovirus and norovirus occurrence by cell culture and reverse transcription real-time polymerase chain reaction RT-qPCR (Cashdollar et al., 2013). Cartridge-type mixed-cellulose ester filters such as the Opticap XL (pore size 0.5 µm; Merck Millipore, Billerica, MA) have also been used for concentrating viruses from large volumes of water (Hata et al., 2015).

Hollow fiber ultrafiltration that relies on size exclusion for virus concentration has also been used for the concentration of viruses from water, including treated wastewater (Liu et al., 2012). Ultrafiltration membranes are constructed from a variety of polymers such as polysulfone, polyacrylonitrile, and cellulose triacetate and can be used in two different modes: tangential (cross) flow (TF) and direct (dead-end) flow (DE) (Gibson and A. Borchardt, 2016). However, ultrafilters are impractical for field sampling or processing of turbid water.

The use of NanoCeram filters has shown to be practical and relatively efficient for virus recovery and concentration from 1,000 to 2,000 liters of highly treated wastewater (Betancourt et al., 2018; Ikner et al., 2011). Collection of such a large volume of water allows looking at a large range of viruses in an individual sample. For instance, to assess a 6-$\log_{10}$ reduction of viruses, 50 to 100 liters for each virus is required. Overlooked in most studies is that the efficiency of concentration of the virus may vary from one sample to the next. To adjust for this, the efficiency of this large-volume primary virus concentration method needs to be determined using a model virus, including murine norovirus (MNV), a non-polio enterovirus strain, and MS2 bacteriophage. Multiple viral strains have been used for different water matrices as reviewed elsewhere (Haramoto et al., 2018).

Current technologies for monitoring viruses in wastewater have been recently reviewed (Jiang et al., 2022). It Passive sampling techniques (Allan et al., 2021) and tangential flow filtration (Lasareishvili et al., 2021) are technologies that have either not been or less explored for the recovery and concentration of viruses within the water reuse infrastructure. Again, all these features represent important components of an expanding research agenda to ensure current and future sustainable, reliable and safe water reuse.

CHAPTER 2

Methodological Approach

2.1 Advanced Treatment Facilities and Sampling Points

The facilities and treatment trains included in this study are listed in Table 2.1.

Facility A is an advanced water treatment facility in Arizona with a treatment train that generates advanced treated recycled water for indirect potable reuse (i.e., groundwater augmentation). The facility takes tertiary effluent from a conventional water reclamation treatment plant and further treats it through ozonation, membrane ultrafiltration, reverse osmosis and ultraviolet photolysis. The schematic of the treatment train is depicted in Figure 2.1 below including sampling points.

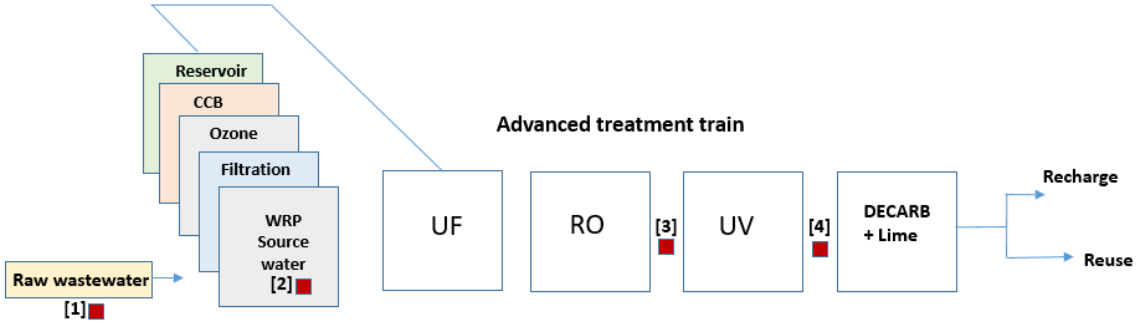


Figure 2-1. Schematic of the Treatment Trains for Indirect Potable Reuse Implemented in Facility A (red squares denote sampling points)**.**

Facility A treats up to 22,000,000 gallons a day of water to drinking water quality standard for groundwater recharge with a recharge operation of 1.7 billion gallons of ultra-purified recycled water annually.

Facility B is a recharge facility that forms part of the reclaimed system infrastructure of The City of Tucson, Arizona. The recharge facility is located in the northern semi-arid reaches of the Sonoran Desert in eastern Pima County, Arizona, adjacent to the Santa Cruz River, where recycled water is stored in the alluvial aquifer. The operational underground storage and recovery facility relies on a series of interconnected recharge basins with a storage capacity of 1.6 M m^3/year, making it the largest constructed underground storage facility in Tucson.

Table 2-1. Facilities and Water Treatment Trains Investigated for Indicator Viruses to Confirm Advanced Physical Treatment.

Facility and Location	Utility Capabilities	Water Source	Treatment Technologies	Treatment Train Investigated
Facility A AZ	Treatment capacity: 22,000,000 gallons a day Operating conditions: Integrated membrane system: MF-RO LG Chem NanoH_20^{TM} Brackish Water RO membranes Three-stage configuration 85% recovery Fluxes: 0.07 gfd/psi (35.24 m s^{-1} KPa^{-1})	Tertiary treated effluent	1) Coarse screen/primary clarification 2) Activated sludge 3) Secondary clarification 4) Cloth disc filtration 5) Ozonation/ chloramination 6) Ultrafiltration 7) Reverse osmosis 8) Ultraviolet photolysis disinfection: UV banks runs @ 17-20 Power (Kilowatts) , intensity >95% UV dose set @ 1.0 log, NDMA target set at 1.0 log (usually above 0.5 log)	**Sampling point 1** – Raw wastewater (RW) **Sampling point 2** – Tertiary effluent (TEFF) **Sampling point 3** – RO permeate (ROP) **Sampling point 4** – UV permeate (UVP)
Facility B AZ	Treatment capacity: 32,000,000 gallons a day (MGD) Operating conditions: HRT: 1.1 ft/day (006A), 0.7 ft/day (008A) Basin area: 2.8 acres (006A) 4.1 acres (008A) Basin Wet/Dry ratio: 0.14 (006A) 0.31 (008A) Average DTG: 83.1 meters	Tertiary treated effluent	1) Screening and grit removal 2) Dissolved air flotation 3) Four modified 5-stage Bardenpho 4) Disk filtration 5) Chlorination 6) Dechlorination 7) Soil aquifer treatment	**Sampling point 1** – Raw wastewater **Sampling point 2** – Tertiary effluent **Sampling point 3** – Extraction well 008A (Recharge groundwater) **Sampling point 4** – Extraction well 006A (Recharged groundwater)
Facility C CO	Treatment capacity: 50,000,000 gallons per day (MGD) Operating conditions: N/A	Reservoir and River	1) Riverbank filtration / infiltration via surface spreading (soil-aquifer treatment) 2) Water softening 3) Ultraviolet light-advanced oxidation process 4) Biofiltration Activated carbon adsorption	**Sampling point 1** – Secondary effluent **Sampling point 2** – Piped water after SAT (BWPF influent) **Sampling point 3** – Filtered water **Sampling point 4** - Purified water (after activated carbon)
Facility D VA	Treatment capacity: 150 MGD per day		1) Bar screen, grit chamber, sedimentation tank 2) Activated sludge, Aeration (final clarifier) 3) Chlorine/UV disinfection 4) Ozone 5) Biological activated carbon 6) Granular activated carbon 7) Ultraviolet 8) Disinfection Chlorine disinfection	**Sampling point 1 –** Raw wastewater **Sampling point 2** – Secondary effluent **Sampling point 3** – Settled water **Sampling point 4 –** Ozone effluent **Sampling point 5** – GAC effluent **Sampling point 7** – UV effluent

				Sampling point 8 – Monitoring well – Soil Aquifer Treatment
Facility E UF/RO skid AZ	<u>Treatment capacity</u>: 57,000 gallons a day <u>Operating conditions</u>: 12-tape-wrapped DOW FILMTEC TW30-4040 spiral wound polyamide thin-film. Pseudo two-stage	Tertiary treated wastewater	1) Screening and grit removal 2) Dissolved air flotation 3) Four modified 5-stage Bardenpho 4) Disk filtration-Chlorination Ultrafiltration/Reverse Osmosis	**Sampling Point 1** – Tertiary effluent **Sampling Point 2** – UF filtrate **Sampling Point 3** – RO permeate

Advanced treatment of reclaimed effluent is achieved by soil-aquifer treatment. There are 11 recharge basins completed and used at different times and 10 extraction wells on both the west and east sides of the Santa Cruz River. Altogether, the basins sum up to approximately 162,000 m^2 of land that can be filled up to 1 m high. At present, the recharge basins receive Class A tertiary effluent treated by a combination of dissolved air flotation (DAF) clarification for primary treatment followed by two parallel-modified 5-stage Bardenpho activated sludge secondary treatment with step-feed aeration. Secondary effluent is disc filtered followed by chloramine-based disinfection prior to discharge or recharge operations. The production wells extract groundwater from the site and distribute it for park irrigation throughout the region. Figure 2.2 provides an overview of the recharge facility and the sampling points.

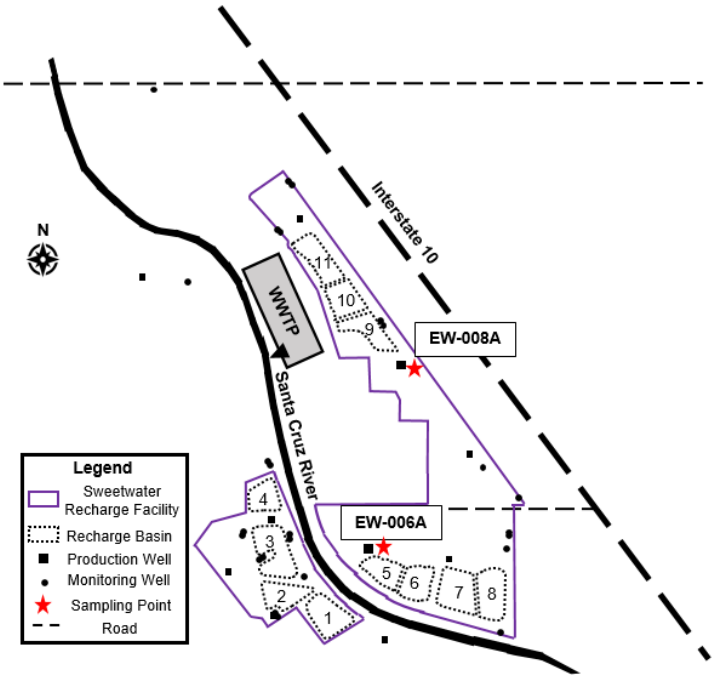


Figure 2.2. Map of the Recharge Facility with Sampling Points: Facility B.
Source: Reprinted from *Water Research*, 177; C.M. Morrison, W.Q. Betancourt, D.R. Quintanar, G.U. Lopez, I.L. Pepper, and C.P. Gerba; "Potential Indicators of Virus Transport and Removal during Soil Aquifer Treatment of Treated Wastewater Effluent"; Pages 115812, Copyright (2020), with permission from Elsevier.

Sampling sites included two extraction wells (EW-006A and EW-008A). EW-008A is a production well associated with the newest set of recharge basins (RB-9, RB-10, RB-11), which have only received Class A tertiary effluent. EW-006A is another production well associated with an older set of recharge basins (RB-5, RB-6, RB-7, RB-8) which initially received Class B secondary effluent from a city's decommissioned wastewater treatment plant. Consequently, individual recharge basins selected for this study differed in date of drilling and time of operation. EW-006A is down gradient of RB-5 while EW-008A sits directly adjacent to RB-9. The basin soils have been classified as sandy loam with a porosity of 0.39. The basins are underlain with a coarse sand and sandy gravel (Quanrud et al., 2003). The recharge facility is characterized by a moderate 37 m deep vadose zone. Characteristics of operational parameters for each extraction well are provided in Table 2.2.

Sampling was conducted between April 2021 and June 2022 to cover changing weather conditions in the Southwestern US (fall: Sep - Nov, winter: Dec – Feb, spring: Mar - May, dry early summer: June, monsoon: Jul - Aug, post-monsoon: Nov). Additionally, raw wastewater and tertiary effluent from the WWTP, representing the source water for recharge, was regularly collected from its distribution line to the basins at the recharge facility.

Table 2-2. Characteristics of Operational Parameters Associated with EW-006A and EW-008A.

Extraction Well [Drill Date]	Basin Area m^2	Basin Infiltration Rate	Basin Wet/Dry Ratio	Average Depth to Groundwater (m)	Quantity of Water Discharged into Basins
006A [01-01-1998]	11331	0.34 m/day 2021 0.49 m/day 2022	0.16 (2021) 0.20 (2022)	51-67	377.29 mg 541.68 mg
008A [05-21-2008]	12950	0.19 m/day 2021 0.22 m/day	0.12 (2021) 0.27 (2022)	83-90	241.59 mg 279.80 mg

Facility C is a state-of-the-art and carbon-based treatment train in Aurora, Colorado with two separate treatment processes that have a combined treatment capacity of 50 million gallons a day. The first treatment train utilizes a conventional treatment process and uses mountain water stored in a reservoir. The second treatment train is part of an innovative potable reuse system that delivers up to 10 million gallons per day of purified water. Riverbank filtration is the initial treatment in which secondary effluent travels for 10 days through 23 wells with hundreds of feet of sand and gravel. This process is followed by aquifer recharge and recovery in which water is pumped into basins where it percolates through more sand a gravel for additional travel time. After this natural cleansing step, the water is finally pumped to the water purification facility where it undergoes advanced treatment that involves precipitative softening, ultraviolet light coupled with advanced oxidation, biological activated carbon filtration and activated carbon adsorption. Although there were 10 sampling events planned for this facility, only three out of five sampling events were considered for analyses of viruses and physicochemical parameters. There were repeated delays of transportation of the samples that exceeded the recommended holding time (>72 hours) for initial sample processing. A schematic of Facility C with sampling points is provided in Figure 2.3 below.

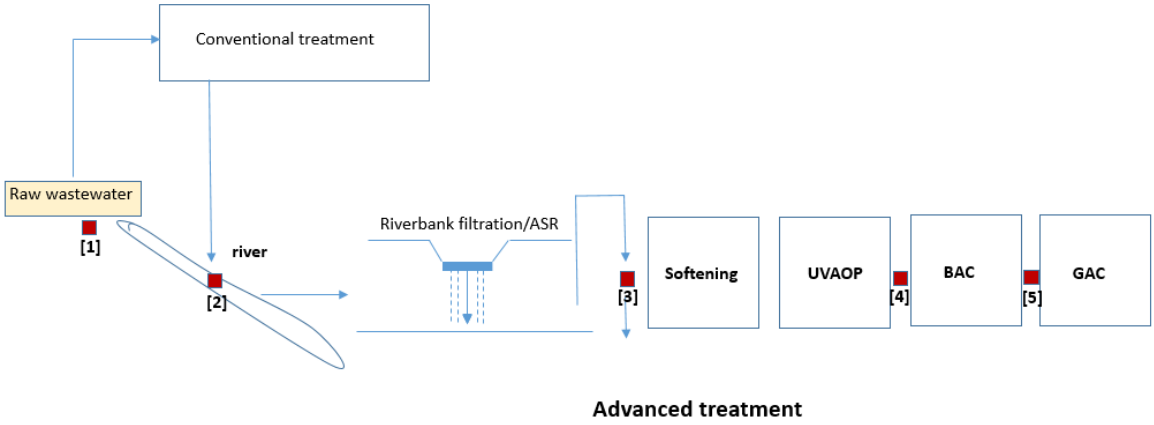


Figure 2-3. Schematic of the Treatment Trains Implemented in Facility C for Indirect Potable Reuse with Sampling Points (red squares)**.**

Facility D is part of an innovative water treatment initiative in eastern Virginia that consists of a carbon-based advanced treatment train that produces up to 1 million gallons a day of purified water of drinking water quality for groundwater replenishment. Highly treated wastewater generated through primary, secondary, and tertiary treatment is the feed water for the multi-step advanced treatment trains. Figure 2.4 below is a depiction of the advanced treatment trains and sampling points.

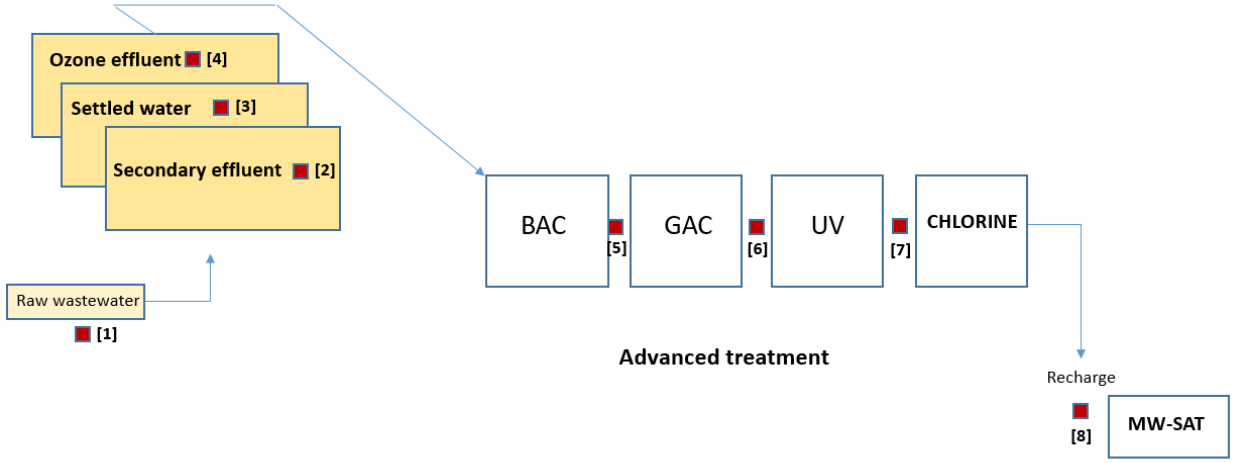


Figure 2-4. Schematic of Treatment Trains Implemented in Facility D for Indirect Potable Reuse with Illustrated Sampling Points (red squares)**.**

The integrated membrane system at The University of Arizona's WEST Center (**Facility E**) is an engineering-scale water reuse system that during this study was operated under two membrane configurations. The first configuration consisted of 12 tape-wrapped DOW FILMTEC TW30–4040 spiral wound polyamide thin-film composite RO membranes (7.2 m^2 active area each element) arranged in six pressure vessels containing two membranes per vessel. The

second configuration consisted of 12 tape-wrapped NF9 spiral wound nanofiltration membranes (7.2 m^2 active area each element) arranged in six pressure vessels containing two membranes per vessel.

The engineering-scale water reuse system is a 57 thousand liters (fifteen thousand gallons) per day UF-RO system (Applied Membranes, Inc., Vista, CA, USA) shown in Figure 2.5. The system is a pseudo two-stage configuration: the first stage contains two vessels in parallel and the second stage contains the remaining four vessels arranged in series, effectively mimicking a single vessel containing all the second stage elements. The engineering-scale integrated membrane system has been operating continuously since 2018. The RO apparent water permeability considerably decreases after 900 days of operation, although the RO skid still produces high-quality water with a constant apparent salt permeability and high observed salt rejection (Figure 2.6). The system is fully automated and constantly monitored for parameters operational performance.

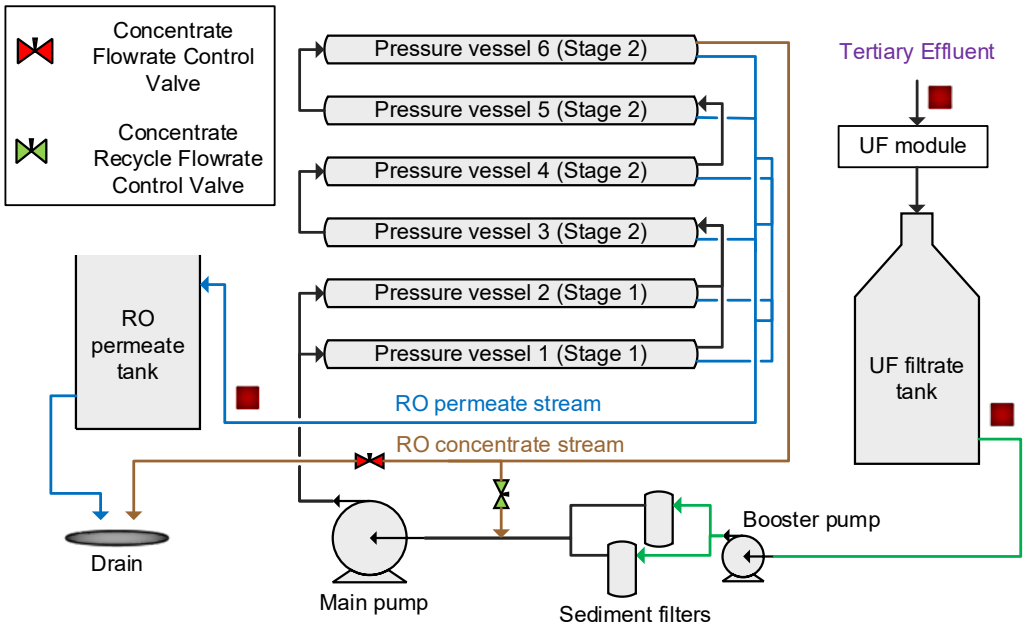


Figure 2-5. Schematic of the Integrated Membrane Engineering Skid with Illustrated Sampling Points (red squares)**.**

Source: Reprinted from *Journal of Membrane Science*, 642; B.M. Souza-Chaves, M.A. Alhussaini, V. Felix, L.K. Presson, W.Q. Betancourt, K.L. Hickenbottom, and A. Achilli; "Extending the Life of Water Reuse Reverse Osmosis Membranes Using Chlorination"; Pages 119897; Copyright (2022), with permission from Elsevier.

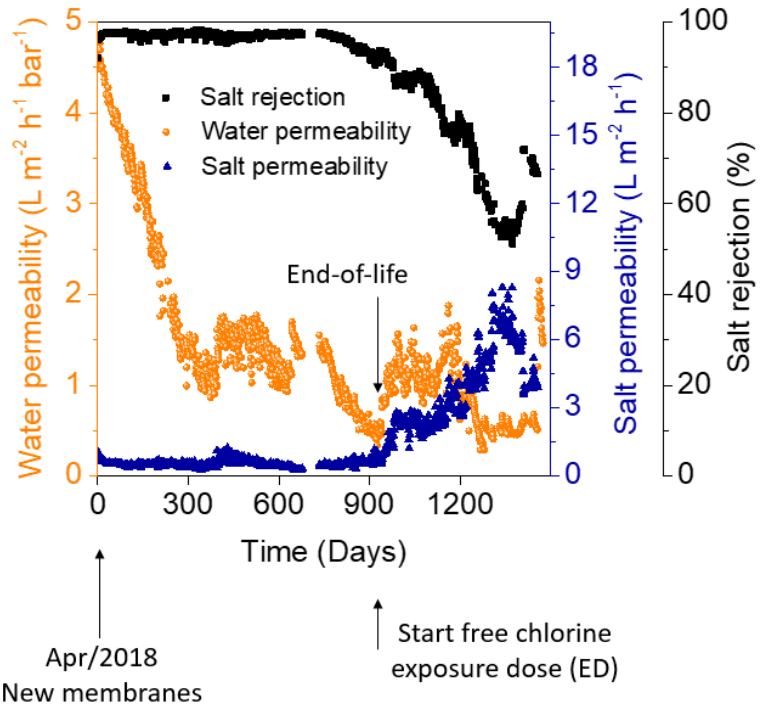


Figure 2-6. RO Apparent Water Permeability Coefficient (A), and Apparent Salt Permeability Coefficient (B) and Observed Salt Rejection Based on Conductivity.

Source: Reprinted from *Journal of Membrane Science*, 642; B.M. Souza-Chaves, M.A. Alhussaini, V. Felix, L.K. Presson, W.Q. Betancourt, K.L. Hickenbottom, and A. Achilli; "Extending the Life of Water Reuse Reverse Osmosis Membranes Using Chlorination"; Pages 119897; Copyright (2022), with permission from Elsevier.

2.2 Filtration and Concentration of Human Enteric Viruses and Virus Surrogates

Water samples of raw wastewater were collected in sterile 500-mL plastic bottles and processed by stepwise vacuum filtration through membrane filters of 0.8, 0.65, 0.45 and 0.2 µm pore sizes followed by centrifugal ultrafiltration (Centricon plus 70, 100 kDa, EMD Millipore, Billerica, MA) to recover and concentrate viruses from 70 to 140 mL (Betancourt et al., 2021).

Water samples from secondary effluent, tertiary effluent and all subsequent advanced treatment trains were filtered through five-inch sterilized electropositive NanoCeram VS2.5-5 (Argonide Corporation, Sanford, FL) cartridge filters at an average flow rate of 4.77 ± 1.40 L/min using pressurized taps from sample ports where available at each facility or a diaphragm pump to pass the water through the cartridge filter. Grab samples from the feed water intake in Facility A were collected in disposable plastic containers and transported to the laboratory for filtration.

Grab samples were collected simultaneously in sterile 1-L bottles from each sampling point listed in Table 2.1 to evaluate physicochemical parameters. Daily and hourly records of RO skid data on flow and conductivity were provided by plant operators and used (where applicable) to compare with the parameters measured at The University of Arizona WEST Center.

2.2.1 Virus Elution and Recovery

Viruses were eluted from the NanoCeram filters by passing 350 mL of 1.0% (wt/vol) sodium polyphosphate (NaPP; Sigma-Aldrich, St. Louis, MO) solution with 0.05 M glycine (pH 9.0) through the filter under positive pressure (N_2 gas) as previously described (Betancourt et al., 2018; Ikner et al., 2011). The total eluent volume was divided into two aliquots of 100 mL each for analysis of male specific (F+) and somatic coliphages using a modification of the single agar layer method to process large volume water samples (McMinn et al., 2018). Water volumes of 70 or 140 mL were reserved for secondary concentration of viruses using Centricon Plus-70 centrifugal ultrafilters (100 kDa cutoff; Millipore, Billerica, MA). Briefly, one or two aliquots of 70 mL volumes of the NanoCeram filter eluate were added to Centricon filters and concentrated via centrifugation (3,500 × *g* for 30 min). The viral concentrate (300 –700 µL) was collected via inversion of the filter followed by centrifugation for 2 min at 1,000 X *g*. This volume corresponded to the final concentrate sample volume (V_{FCSV}) that was used for viral nucleic acid extraction. At this point, water concentrates were immediately used for nuclease treatment and viral nucleic acid extraction.

2.3 Nuclease Treatment and Viral Nucleic Acid Extraction

2.3.1 Nuclease Treatment

Viral concentrates underwent pretreatment with nucleases prior to nucleic acid extraction to digest unprotected nucleic acids, thereby reducing the detection of viral DNA/RNA by RT-dPCR/dPCR from virions with degraded capsids. Briefly, free nucleic acids from the viral concentrates were removed by incubating aliquots of 165 µL at 37°C for 30 minutes with a nuclease cocktail consisting of 1X Turbo DNase Buffer (Ambion), 21U of Turbo DNase (Ambion), 4.5U of Baseline-ZERO DNase (Epicenter), 112.5U Benzonase (EMD Millipore), and 10 µg/mL RNase A (Sigma-Aldrich) (Gilling et al., 2014; Ng et al., 2012; Victoria et al., 2009).

2.3.2 Viral Nucleic Acid Extraction

Viral nucleic acids were extracted using the AllPrep PowerViral DNA/RNA kit (QIAGEN Inc, Valencia, CA) following the manufacturer's instructions. Nucleic acids were eluted in RNase-free water. For elution of viral nucleic acid from spin columns, 100 µL of RNase-Free water was used in order to maximize DNA/RNA yield. The volumes used for nucleic acid extraction varied depending on the concentrate volumes obtained from the centrifugal ultrafiltration step (secondary virus concentration). For example, water concentrate volumes >680 µL allowed up to four extractions per sample for a final DNA/RNA volume of 400 µL. The AllPrep PowerViral DNA/RNA Kit was selected for this study due to fast and easy purification of viral and microbial total nucleic acids from samples with high levels of PCR inhibitors, including wastewater. The kit uses Inhibitor Removal Technology® (IRT) to ensure complete removal of the inhibitory substances associated with environmental water matrices.

2.4 Quality Assurance/Quality Control

This section covers all the methods that were applied as quality control points during the conduction of the research for the concentration and recovery of viruses as well as for the detection and quantification of virus genomes by digital PCR using QIAcuity Software Suite 1.2. of the QIAcuity dPCR instrument for data acquisition. Key parameters to ensure the quality of

the data are also described for each method.

A flow diagram below (Figure 2.7) illustrates all the steps involved in sample processing from filtration to virus detection and quantification following standard procedures and methods validated in the UArizona WEST Center laboratory of Environmental Microbiology. For virus recovery and concentration, key parameters considered in this research included large-volume virus-spiking experiments for direct evaluation of the recovery yield, matrix spikes to determine virus recovery yields for different water matrices, and negative QC sample/equipment blanks. For absolute quantification of virus genomes, all preanalytical procedures were considered including evaluation of the viral RNA/DNA extraction efficiency, virus capsid integrity assay via digestion of unencapsidated RNA/DNA, and control reactions required in dPCR.

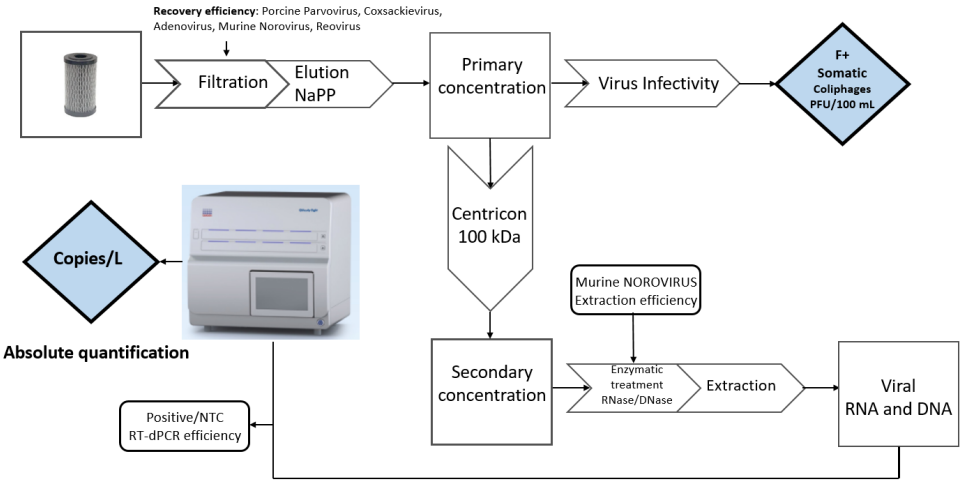


Figure 2-7. Flow Diagram Describing the Steps for Virus Detection and Quantification Including Steps for Quality Assurance and Quality Control.

2.4.1 Primary Virus Concentration Method Efficiency

The filtration method applied for virus recovery and concentration has been thoroughly validated and applied for the capture and recovery of multiple viruses in large volume water samples (>100 L) with variable virus recovery efficiencies based on the water matrices analyzed, including secondary and tertiary treated wastewater effluents and highly treated wastewater by integrated membrane systems (e.g., ultrafiltration or microfiltration and reverse osmosis) groundwater, and surface water (Betancourt et al., 2018; Betancourt et al., 2014; Ikner et al., 2011).

Through the execution of this study and through previous research conducted at The UArizona WEST Center Laboratory of Environmental Microbiology, the recovery efficiency of multiple enteric viruses was evaluated for tap water, secondary treated and tertiary-treated wastewater produced at two of the facilities included in this study. Virus recovery efficiencies in ultrafiltration permeate, reverse osmosis permeate, and recharged groundwater were further evaluated in this study.

One-thousandth-scale laboratory and onsite experiments were conducted to evaluate the efficiency of the primary virus concentration method (recovery yield). Large volume water

samples (1000 L) from RO permeate and recharged groundwater were collected into disinfected large capacity plastic containers and spiked with a suspension of Murine norovirus 1 (ATCC VR-1937), a laboratory strain of Coxsackievirus B5, Reovirus 3 (ATCC VR-232) a laboratory strain of Adenovirus 4, and Porcine parvovirus (ATCC VR-742™) to obtain a final copy number of approximately 10^6-10^7 virus targets per liter. Water samples were passed through the NanoCeram filters using a diaphragm pump and the viruses were eluted and recovered following procedures previously described. Viral nucleic acid was extracted from concentrated samples and subjected to RT-(dPCR) as described in subsequent sections. Recovery yields (Y) were calculated as follows:

$$Y = X/(Co \times V) \times 100$$

Y: Recovery yield of the primary concentration method
X: Recovery virus copy number (copies)
Co: Stock virus copy number added into test water (copy/µL)
V: Added stock virus volume (µL)

2.4.2 Disinfection Procedures

The equipment and supplies for sample collection (e.g., diaphragm pumps, filter housings, tubing, adapters, flow meters, carboys) were designated for each sampling location. In addition, negative QC samples consisting of reagent grade water passed through NanoCeram filters were used throughout the study in order to ensure the quality of the results. Filter apparatus modules were disinfected before and after use by recirculating or immersing the items in 0.525% sodium hypochlorite followed by dechlorination with sodium thiosulfate according to SOPs in place in the laboratory. Filter housings and tubing used for sample collection at Facilities located in Colorado and Virginia were shipped to the UArizona WEST Center for disinfection and returned to each facility for the next sampling event.

2.4.3 Analytical Sensitivity and Specificity of Virus Quantification by dPCR

The analytical sensitivity and specificity of the dPCR assays that were used for absolute quantification of viruses in this study were established prior to the conduction of these assays. Table 2.3 shows the assay limit of detection (ALoD) for the human enteric viruses that were monitored in this project and for surrogate viruses used for recovery efficiency assays and nucleic acid extraction efficiency assays. Ten-fold serial dilutions of gBlock gene fragments (IDT Technologies) or viral nucleic acid for three culturable viruses (Adenovirus, Enterovirus, Reovirus) and ATCC virus stocks were used to determine the ALoD. The ALoD for each synthetic virus template or viral nucleic acid templates are associated with 3 to 4 partitions with data acquisition obtained by the QIAcuity Software Suite 1.2 of the QIAcuity dPCR instrument. The QIAcuity systems are designed to determine absolute amounts of target DNA in a sample by using a digital PCR (dPCR) approach. In addition, the QIAcuity systems were used only in combination with QIAGEN kits including QIAcuity Nanoplates and QIAcuity PCR Reagents. The QIAcuity performs a fully automated processing of the QIAcuity Nanoplates, including all necessary steps of plate priming, sealing of partitions, thermocycling, and image analysis.

Based on the slightly different ALoD for the viruses monitored in this study as given in Table 2.3, a normalized ALoD for all viruses was performed using a set of guidelines for the determination of limits of detection and limits of quantification approved for clinical laboratory methods (NCCLS. Protocols for Determination of Limits of Detection and Quantitation; Approved Guideline. NCCLS document EP17-A [ISBN 1-56238-551-8]. NCCLS, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898 USA, 2004).

This guideline takes into account a series of results on blank samples and a series of results on very low-level samples. The guideline assumes that the dispersions of the results from blanks and low-level samples are due to random measurement error. An α value of 5% corresponds to using the 95th percentile of the distribution of blank values as the limit for declaring a measured value significantly higher than the blank. Given a Gaussian distribution of blank values, this limit corresponds to:

$LoB = \mu_B + 1.645\ \sigma_B$

where μ_B and σ_B are the mean and standard deviation of the blank measurements, respectively.

The ALoD is the lowest concentration that can be detected reliably. Considering that the low-level sample distribution is Gaussian and the 5th percentile of the distribution corresponds to the LoB, then:

$LoB = \mu_S - 1.645\ \sigma_S$

where μ_S and σ_S are the mean and standard deviation of the population of the low sample measurements.

Based on the above and considering that the distribution of blank values was Gaussian the $LoB = \mu_B + 1.645\ \sigma_B$, consequently the ALoD for the viruses monitored in this study was derived by the formula below:

$ALoD = \mu_B + 1.645\ \sigma_B + 1.645\ \sigma_S$.

Table 2-3. Assay Limit of Detection (ALoD) for Virus Indicators Monitored in this Project and for Surrogate Viruses Used for Recovery Efficiency Assays and Nucleic Acid Extraction Efficiency Assays.

Virus	ALoD	
	Template Type	
	Viral Nucleic Acid	Synthetic DNA (gBlock)
Adenovirus	0.653 copies/ µl	0.143 copies/µl
WCDV-1		0.143 copies/ µl
WCDV-2		0.191 copies/ µl
WCDV-3		0.143 copies/ µl
crAssphage		0.143 copies/ µl
Microviridae		0.143 copies/ µl
Lake Sarah-like		0.191 copies/ µl
Human Bocavirus		0.143 copies/ µl
Enterovirus	0.191 copies/ µl	0.191 copies/ µl
Reovirus Serotype 3 (ATCC® - VR-232)	0.22 copies/µl	0.385 copies/ µl
Pepper Mild Mottle Virus		0.191 copies/ µl

Genogroup I Norovirus		0.191 copies/ µl
Genogroup II Norovirus		0.169 copies/µl
Aichi virus		0.169 copies/µl
Murine Norovirus S7-PP3 strain	0.191 copies/µl	0.169 copies/µl
Porcine parvovirus		0.191 copies/µl

The ALoD was based on the lowest concentration of the gBlock or viral nucleic acid associated with 3 partitions.

2.4.4 Features of the dPCR System Used for Quantification of Virus Genomes

The thermal cycler of the QIAcuity is a plate thermocycler that features high speed and precision temperature control of the temperature cycling steps. Several Peltier elements are used for the temperature generation and control. For an optimal thermal contact between plate and thermocycler, the plate is being clamped on the heating surface during cycling. The QIAcuity Eight features two thermocyclers that are operated in parallel. The thermal cycler has the following specification:

- Process temperature: 40–99°C
- Ramp rate: approx. 3.0 C/s
- Accuracy: ±1°C
- Homogeneity: ±1°C

The optical system of the QIAcuity is a camera-based fluorescence microscopy system. The excitation source for the fluorescence dyes is a high-power white LED. This source in combination with a specific excitation filter is used to illuminate a whole well at a time. The fluorophores in the single partitions absorb that light and emit light that is being filtered by a detection filter, collected and imaged through an objective lens on a CMOS-camera chip. The configuration of the instrument used for this project was a 5plex with 5 selectable detection channels (Table 2.4). An additional channel was used for detecting the base fluorescence of the master mix, to determine the exact number of filled partitions and normalization of fluorescence data.

Table 2-4. Available Channels in QIAcuity.

Channel	Excitation (nm)	Emission (nm)	Fluorophores
Green	463-503	518-548	FAM™, EvaGreen®
Yellow	514-535	550-564	HEX™, VIC®, JOE™
Orange	543-565	580-606	TAMRA™
Red	570-596	611-653	ROX™, Texas Red®
Crimson	590-640	654-692	Cy5®

The fluorescence signal in the reference channel was measured to determine the number of valid partitions in a well. Differences in the signal intensities between partitions were normalized and the fluorescence signals in the target channels were corrected accordingly.

The QIAcuity Software Suite is designed to work with Windows® 10 operating system. The following analysis options are available in the software:

- Absolute Quantification
- Mutation Detection
- Genome Editing
- Copy Number Variation
- Gene Expression

The analysis option used to evaluate virus genomes during the course of this study was absolute quantification.

2.4.5 Primers and Probes for Quantification of Virus Genomes

The sequence of primers and probes that were used for absolute quantification of the virus genomes are included in Table 2.5. For real time PCR, these assays provided good reaction efficiencies (between 90% and 110%) and through assay optimization using different annealing temperatures as well as primer and probe concentrations, these assays were adapted for dPCR.

Table 2-5. Primers and Probes Used for Quantification of Virus Genomes By dPCR.

Virus	Primer and Probe	Sequence (5'→3')[a, b]	ALoD (RT-[qPCR]) Gc/rxn	Reference
Adenovirus	AQ2 AQ1 AP	GCCCCAGTGGTCTTACATGCACATC GCCACGGTGGGGTTTCTAAACTT FAM-TGCACCAGACCCGGGCTCAGGTACTCCGA-BHQ1	12.32	(Heim et al., 2003)
Aichi virus	AiV-AB-F AiV-AB-R AiV-AB-TP	GTCTCCACHGACACYAAYTGGAC GTTGTACATRGCAGCCCAGG FAM-TTYTCCTTYGTGCGTGC-MGB-NFQ	1.909	(Kitajima et al., 2013)
PMMoV	PMMV-F PMMV-R PMMV-P	GAGTGGTTTGACCTTAACGTTTGA TTGTCGGTTGCAATGCAAGT FAM-CCTACCGAAGCAAATG-BHQ1	14.06	(Haramoto et al., 2013)
Enterovirus	EV1F EV1R EV	CCCTGAATGCGGCTAAT TGTCACCATAAGCAGCCA FAM-ACGGACACCCAAAGTAGTCGGTTC-BHQ1	17.78	(Gregory et al., 2006)
Murine Norovirus	MNV-S MNV-AS MNV-TP	CCGCAGGAACGCTCAGCAG GGYTGAATGGGGACGGCCTG FAM-ATGAGTGATGGCGCA-MGB-NFQ	22	(Kitajima et al., 2010)
Reovirus	Reov-F Reov-R Reov-P	AGTTGCTGAACGCAAATTATTTTG TGCGAATCATCAGATTAACCTCTGT FAM-TATTGCGACTAAAAATACC-MGB-3	44.3	(Qiu et al., 2018)
crAssphage sp	crAssph-F crAssph-R crAssph-P	CAGAAGTACAAACTCCTAAAAAACGTAGAG GATGACCAATAAACAAGCCATTAGC [FAM] AATAACGATTTACGTGATGTAAC [MGB]	16.63	(Stachler et al., 2017)
Norovirus GI	COG1F COG1R RING1(a)-TP RING1(b)-TP	CGYTGGATGCGNTTYCATGA CTTAGACGCCATCATCATTYAC FAM-AGATYGCGATCYCCTGTCCA-BHQ1 FAM-AGATCGCGGTCTCCTGTCCA-BHQ1	2.22	(Kageyama et al., 2003)
Norovirus GII	COG2F COG2R RING2-TP	CARGARBCNATGTTYAGRTGGATGAG TCGACGCCATCTTCATTCACA FAM-TGGGAGGGCGATCGCAATCT-BHQ1	17.75	(Kageyama et al., 2003)

Virus	Primer and Probe	Sequence (5'→3')[a, b]	ALoD (RT-[qPCR]) Gc/rxn	Reference
Human Bocavirus	HuBoCV-F HuBoCV-R HBocV-P	CTGGTCTCTTGGTGGCAT TA ATT TCCTAGAGCAGGAGCCA FAM-CAA GTT TCT TTA AAC TTA AGC GCG CG-BHQ	16.58	(Iaconelli et al., 2016)
WCDV-1	WCDV-1F WCDV-1R WCDV-1p	GATAGTGTTTGCCGTGTTTGG TCAAGCAGTATGCATCGACTAC FAM-CCAGGTAGTGACTGTGTGCCGAATT-BHQ-1	7.65	This study
WCDV-2	WCDV-2F WCDV-2R WCDV-2p	GCACTGGTACAATCTTCCATCT CACAACCTTAGTCCCACGATACC FAM-AGCCGTGTCTATCGGCGTAATTGA-BHQ-1	8.23	This study
WCDV-3	WCDV-3F WCDV-3R WCDV-3p	CGTCCAGCATCTAAGTCTTCAA GTGATGCCAGACAGAGGAATAG FAM-AGAAGACTGCTCACTCCCAGACTGT-BHQ-1	7.27	This study
HUDISAVIRUS	Hud-Fa2 Hud-Reva1 Hud-Pb	CTTCAGTTCAGGCCGGTAAARR ACATCGACCCTCAGGTCTTDGCA TTGTAAGCTGCCATGAACCCAGGA	16.6	This study

a Mixed base in degenerate primer and probe is as follows: Y = C, T; D is A, G, or T; and R is an A or G
b The FAM (6-carboxyfluorescein) quencher is BHQ-1 (Black Hole Quencher). The FAM quencher is a minor groove binder nonfluorescent quencher (MGBNFQ).
PMMV - Pepper mild mottle virus
MNV - Murine norovirus
CGMMV – Cucumber green mosaic mottle virus
*Double quenched fluorescent hydrolysis probes

Primers and probes as well as gBlock gene fragments used as positive controls were purchased from Integrated DNA Technology (Integrated DNA Technology, Coralville, IA). All the gBlock gene fragments as well as the assays for WCDV1, WCDV2, WCDV3, and Hudisavirus were designed by IDT DNA Technology Synthetic Biology Specialists. A modification of the original primers developed by IDT DNA Technology for detection of Hudisavirus (shown in red in Table 2.5) was required in order to enable identification of all of the hudisaviruses and some closely related circular DNA viruses. These primers are listed in Table 2.5.

2.4.6 RT-dPCR and dPCR Reactions for Quantification of Virus Genomes

For absolute quantification of RNA viruses, the QIAcuity® One-Step Viral RT-PCR kit was used as this kit has been optimized for quantification of RNA viral targets with hydrolysis probes in a singleplex or multiplex reaction for the QIAGEN's QIAcuity instruments in digital PCR (dPCR) applications. According to the dPCR MIQE guidelines, in a one-step strategy, RNA is partitioned with both reverse transcription and PCR occurring sequentially in the same partition. Even if multiple cDNA copies are generated from each RNA molecule, results are not overestimated (Huggett, 2020). The QI Acuity® Probe PCR kit was used for absolute quantification of DNA viral targets as this kit has been optimized using hydrolysis probes in a singleplex or multiplex reaction using the QIAGEN's QIAcuity instruments for digital PCR (dPCR).

For each virus target, 40 µL reaction mixtures were used on the 26000 24-well Nanoplates. These are microfluidic dPCR plates designed for 24 samples and capable of generating up to 26000 partitions per well. Each partition is an independent PCR reaction with a partition

volume of 0.91 nL. The RT-dPCR reaction mix for RNA viruses consisted of 10 µL of master mix, specific concentrations of primers (400 to 500 nM) and probes (100 to 300 nM), depending on the virus, 0.4 µL of 100X Multiplex Reverse Transcription enzyme, 10 µL of 1X or 1:10 to 1:100 dilution of the template, and molecular grade water to complete 40 µL. The dPCR reaction mix for DNA viruses consisted of 10 µL master mix, specific concentrations of primers (400 to 500 nM) and probes (100 to 300 nM), depending on the virus target, 1X or 1:10 to 1:100 dilution of the template, and molecular grade water to complete 40 µL.

Although dPCR is more resilient to inhibition than RT-qPCR as demonstrated in numerous studies, matrix effects and inhibitors that may reduce the fluorescent intensity of the positive partitions were examined by using 1X and 1:10 to 1:100 dilutions of the template. For these purposes, duplicate wells of each dilution in most cases were assessed by dPCR or RT-dPCR. Our results demonstrated that there were samples in which dPCR/RT-dPCR was indeed influenced by PCR inhibitors associated with the different water matrices. Quantification accuracy for copy number measurements was dependent on both completeness of molecular count and accurate definition of the unit volume of sample and total reaction (i.e., number of partitions of accurately defined volume). dPCR has the capability of counting all intact (equal or larger than the amplicon) DNA molecules containing a specific target sequence (Huggett, 2020).

Negative control reactions that contained the reaction mix without nucleic acid template were used to identify cross contamination between samples, as well as carry-over contamination from previous amplified product. An equally important negative control that was included in dPCR experiments was the same DNA/RNA background, but without the target sequence. These controls were used to assess specificity as well as contamination. Positive controls that ideally reflect real samples in complexity, integrity, purity, and concentration were also important. Positive controls (both internal and external) of defined concentration provided quality assurance and were particularly useful for evaluating preanalytical steps. The number of positive partitions in low-level concentration analysis may be so low that the appropriate threshold setting is best determined from a more concentrated positive control (Huggett, 2020).

For the evaluation of the rejection efficiency of the organic compounds and ions, the following equation was used:

Rejection efficiency (%) = (1-Cf/Ci) x 100 (Basumatary et al., 2015)

Where, Cf and Ci represent the concentration of the constituent or virus in the final treated water and initial, respectively.

In addition, the rejection efficiency in terms of log reduction value was evaluated using the following equation:

Log reduction value (LRV) = -log (Cf/Ci).

2.5 Analytical Methods of Non-microbial Surrogates

2.5.1 Bulk Parameters

In-line conductivity measurements were acquired directly from the RO systems operating at full-scale and the engineering-scale system by online conductivity sensors (GF Signet 3-2850 conductivity/resistivity electrode). pH values were acquired with a pH-meter accumet AE150 (Fisher Scientific, Waltham, MA). Chloride was analyzed with a Dionex ion chromatography (ICS-5000 Dionex Ion Pac, 2 x 250 mm Microbore Analytical column) using 22 mM of potassium hydroxide as eluent, 0.35 mL/min flow rate, and 240 µL injection volume. UV absorbance at 254 nm wavelength was analyzed by UV-Visible spectrophotometer Varian Cary 50 Conc and turbidity was measured using a HACH portable turbidimeter model 2100Q. ATP was evaluated by a test kit (Quench-Gone, LuminUltra, New Brunswick, Canada) applying a conversion of cellular ATP (cATP) to microbial equivalents (ME; i.e. cell counts) that is based on the amount of ATP found in an *E. coli*-sized cell (0.001 picograms).

2.5.2 Ion Analysis

An inductively coupled plasma mass spectrometry (ICP-MS; Agilent 7800, Santa Clara, CA) was used to measure sodium, potassium, calcium, and magnesium ions. Trace metal grade nitric acid (67-70% HNO_3, Fisher Scientific, Fair Lawn, NJ) was used to acidify ICP samples for analysis, and deionized (DI) water was used for sample dilution. All samples were filtered through a glass fiber 0.45 um syringe filter (25 mm, Tisch Scientific, North Bend, OH) before analysis. Sulfate standard solution (1,000 mg/L as SO_4, Hach, Loveland, CO) and sulfate reagent pillows (SulfaVer® 4 sulfate, Hach, Loveland, CO) were used to analyze sulfates with the DR900 portable colorimeter.

2.5.3 Organic Analysis

DOC size fractions and concentrations were quantitatively measured by size-exclusion chromatography (SEC) using a high-performance liquid chromatographer (HPLC; Agilent Technologies, model 1260 Infinity II, Santa Clara, CA) coupled with an organic carbon detector (OCD; Sievers M9 Portable TOC Analyzer, Suez Water Technologies and Solutions, Trevose, PA). The instrument is equipped with a custom-made 250 x 20mm column with Toyopearl HW-50S packing material (Tosoh Bioscience, Tokyo, Japan). The injection volume was 500 µL, and the mobile phase condition was 1.0 mL/min phosphate buffer at pH 6.8 to suppress ionic interaction. A mixture of 25 mM sodium phosphate monobasic monohydrate (>98%, Santa Cruz Biotechnology, Dallas, TX) and 50 mM sodium sulfate (VWR International LLC, Radnor, PA) were used as size exclusion chromatography eluents. Polyethylene oxide standards (PSS-USA, Amherst, MA) at different molecular weights (11.4 kDa, 3.5 kDa, 1.02 kDa, 400 Da, and 194 Da) were used for the SEC column calibration. All samples were filtered in a 0.45-micron glass fiber filter.

The SEC-OCD chromatograms were classified into four major fractions (Cai et al., 2020; Huber et al., 2011), which include proteinaceous biopolymers (fraction A, apparent molecular weight (AMW) > 10 kDa), humic substances (fraction B, AMW = 10 – 1.5 kDa), building blocks of humic substances (fraction C, AMW = 1.5 – 0.5 kDa), and low molecular weight (LMW) acid and neutral substances (fraction D, AMW < 0.5 kDa).

DOC was qualitatively measured using excitation-emission matrix (EEM) fluorescence spectroscopy (Duetta fluorescence and absorbance spectrometer, Horiba, Kyoto, Japan). Fluorescence was scanned by excitation wavelengths from 250 to 450 nm and emission wavelengths from 250 to 550 nm in 5 nm step increments (0.1 integration time, 1 detector accumulation, and 10 excitation/emission total band pass). The light scattering (inner filter effect correction and Rayleigh masking) was eliminated through Duetta's EzSpec software. The Raman peak of distilled water was used to normalize the fluorescence spectra as described elsewhere (Chen et al., 2003b). The fluorescence spectra were examined for excitation wavelengths > 250 nm, where two regions were assigned: Region IV, associated with compounds with a limited number of aromatic rings and representing soluble microbial byproducts (SMP)-like substances (emission wavelength < 380 nm), and Region V, associated with polycyclic aromatic compounds and representing the humic acid-like substances (emission wavelength > 380 nm) (Park and Snyder, 2018).

2.6 Statistical Analyses

Microsoft Excel spreadsheets were used for entering, organizing, and storing the virus and physicochemical data followed by exploratory data and statistical analyses using the Statistical Package for the Social Sciences (IBM SPSS Statistics, Version 28.0), employing descriptive statistics, test for assumptions of normality, scatterplots, hypothesis testing, regression analyses (quantitative variables) chi-square tests (qualitative variables) for interpretation of the results. Additional statistical analysis and data interpretation was conducted in R (version.string R version 4.2.1 (2022-06-23 ucrt, nickname "Funny-Looking Kid").

Spearman's rho nonparametric correlation was employed to evaluate the strength and direction of monotonic association between two ranked variables corresponding to each of the viruses monitored in this study. Nonparametric correlations for the full-advanced treatment train were explored for both untransformed and log transformed values. Chi-square test of association (independence) were used to ascertain whether there was any statistically significant associations among the monitored viral indicators and the non-microbial surrogates evaluated at the different trains of full advanced treatment. Only correlations at the 0.01 level (2-tailed) with >7 detected values for viruses were considered for interpretation of the results. Contingency tables generated by SPSS allowed the initial analysis of the data and Chi-square provided considerable information about how each of the monitored viruses performed in this study, again in most cases only sewage and WRP had enough observations to reliably develop correlations among viruses and physicochemical data.

2.7 Log Reduction Values

Base 10 logarithms were taken, and log reductions calculated as the different in the log values of the influent and effluent for a given day. Hence, for Facility A there were three different types of log reduction values calculated:

1) Removal from sewage to tertiary effluent
2) Removal from tertiary effluent to reverse osmosis permeate
3) Removal from reverse osmosis permeate to UV treated effluent

As physicochemical parameters were not measured in sewage, only the latter two were calculated for the physicochemical parameters. Rather than calculating log reductions for temperature and pH, differences in upstream versus downstream levels were calculated. It was not feasible to stagger influent and effluent sampling by the residence time of the treatment processes. Hence, temporal variability in influent would introduce some random variation into the estimated reductions. This would tend to expand the range of the observed reduction estimates.

If either the influent or effluent was below detection, then the log reduction was not calculated for that pair of observations. From a practical perspective, very little information on reductions can be obtained in cases where one of the observations is below detection (even when using maximum likelihood approaches for censored observations). The computation of reductions based on half the detection limit being substituted for non-detected values was avoided because this method can easily lead to biased estimates. For example, if influent observations are near the detection limit and effluent concentrations are non-detectable, then substitution of half the detection limit would produce reduction estimates on the order of 50%, while in fact many processes achieve >99% removals.

A linear mixed effect analysis was conducted in R (version.string R version 4.2.1 (2022-06-23 ucrt, nickname “Funny-Looking Kid”) and lmer (Bates et al., 2015) to develop a model that predicts LRV with respect to treatment, virus, and nucleic acid content as fixed effects and dates as random effects for Facilities A and B where more observations were available for analysis. Date was considered a random effect in the mixed effect model based on the differences on virus reductions that may occur from variations in operational conditions at different dates. Maximum likelihood estimates of the parameters in the linear mixed-effects model were determined using the lmer function in the lme4 package for R. The LRV of Adenovirus that was statistically significantly different from 0, was set as a fixed intercept.

CHAPTER 3

Results and Discussion

3.1 Virus Indicators of Physical Treatment: Facility A

3.1.1 Virus Concentrations and Frequencies of Detection

Tables 3.1, 3.2, 3.3, 3.4, and 3.5 summarize the frequencies of detection and log concentrations per liter of human enteric viruses and virus surrogates in raw wastewater, tertiary effluent as source water for advance treatment, and advanced treatment trains in Facility A consisting of ultrafiltration-reverse osmosis membranes and ultraviolet-based photolysis disinfection. In raw wastewater, Human Bocavirus Adenovirus, and crAssphage (all three human-associated DNA viruses) exhibited the highest concentrations (6-$\log_{10}$-8-$\log_{10}$ GC/L) among all viruses while concentrations of 4-$\log_{10}$ to 5-$\log_{10}$ GC/L were more commonly observed for the rest of the viruses. High concentrations of virus genomes detected in raw wastewater in most cases were also associated with the detection of viruses across the advanced treatment trains.

The relative standard deviation (RSD) of the mean log concentration of each virus detected in raw wastewater and across the treatment trains was used for initial evaluation of virus indicator performance. Mean virus concentrations with an arbitrary RSD <30% were deemed as more precise estimates of viral loads than mean virus concentrations with a RSD >50%. The former was predominantly observed at early stages of treatment while the latter occurred at the advanced treatment trains where few viruses were infrequently detected at relatively low concentrations (1-$\log_{10}$-2-$\log_{10}$ GC/L). Mean log concentrations of male specific (F+) and somatic coliphages evaluated by the plaque assay consistently exhibited the lowest RSD among all viruses evaluated as indicators to confirm physical treatment, even at low levels of detection observed in advanced treated recycled water.

The Spearman's rank correlation test was performed to determine the association between the indicator viruses present in raw wastewater, source water (tertiary treated effluent) and advanced treated recycled water. As expected, higher correlations were revealed for those viruses that were consistently detected at the highest concentration in raw wastewater and source water for advanced treatment. Only raw wastewater and source water had enough observations to reliably developed correlations. Thus, non-detects or values that were <ALoD at the advanced treatment trains were substituted by half the limit of detection to evaluate potential correlations as the most sensitive approach. Although this approach revealed correlations among viruses, those correlations were based on non-detect virus data and therefore disregarded for further analyses.

Table 3-1. Frequencies of Detection of Human Enteric Viruses and Virus Surrogates at Different Levels of Water Treatment Trains for Groundwater Augmentation.

Virus	Sewage (%)	TEFF (%)	RO Permeate (%)	UV Permeate (%)
Aichi virus	71	71	7	0
crAssphage	79	86	14	29
WCDV1	50	36	0	0
WCDV2	43	79	7	0
WCDV3	57	57	0	0
Norovirus GI	71	21	14	14
Norovirus GII	71	36	0	0
H. Bocavirus	71	57	7	14
Hudisavirus	64	50	0	0
Adenovirus	64	57	7	7
Reovirus	36	50	7	21
Enterovirus	79	0	0	0
PMMoV	79	71	21	14
SOMATIC	100	100	43	36
Male specific	93	86	43	29

Table 3-2. Log Concentrations Per Liter (GC-PFU Log_{10}/L) of Human Enteric Viruses and Virus Surrogates in Raw Wastewater.

Virus	N	Minimum Log Concentration	Maximum Log Concentration	Mean Log Concentration	Std. Deviation	RSD
Aichi virus	10	3	5	4	.8	20
crAssphage	11	5	8	7	1	17
WCDV1	7	3	5	4	1	15
WCDV2	6	4	5	4	.5	11
WCDV3	8	3	5	4	1	20
Norovirus GI	10	3	5	4	1	21
Norovirus GII	10	3	5	4	1	20
H. Bocavirus	10	3	6	5	1	17
Hudisavirus	9	3	6	5	1	18
Adenovirus	9	3	6	4	1	24
Reovirus	5	3	5	4	1	17
Enterovirus	11	3	4	4	1	17
PMMoV	11	4	7	5	1	22
Somatic	14	6	7	7	0.4	5
Male specific	13	6	7	6	.4	7

RSD: Relative standard deviation

Table 3-3. Log Concentrations Per Liter (GC-PFU Log_{10}/L) of Human Enteric Viruses and Virus Surrogates in Tertiary Treated Recycled Water: Facility A.

Virus	N	Minimum Log Concentration	Maximum Log Concentration	Mean Log Concentration	Std. Deviation	RSD
Aichi virus	10	1	3	2	1	46
crAssphage	12	3	4	3	.4	14
WCDV1	5	2	3	2	.5	22
WCDV2	11	1	4	3	1	30
WCDV3	8	2	4	3	1	19
Norovirus GI	3	1	2	1	.3	24
Norovirus GII	5	.2	2	1	1	69
H. Bocavirus	9	1	3	2	1	52
Hudisavirus	7	1	3	2	.5	26
Adenovirus	8	.1	2	1	1	56
Reovirus	7	.2	3	2	2	73
Enterovirus	0					
PMMoV	10	2	5	3	1	32
Somatic	14	.5	1	.5	.4	89
Male specific	12	.1	2	1	1	100

Table 3-4. Log Concentrations Per Liter (GC-PFU Log_{10}/L) of Human Enteric Viruses and Virus Surrogates in RO Permeate: Facility A.

Virus	N	Minimum Log Concentration	Maximum Log Concentration	Mean Log Concentration	Std. Deviation	RSD
Aichi virus	1	0	-.5			
crAssphage	2	-1	1	-.3	1	119
WCDV1	0					
WCDV2	1	0	.03			
WCDV3	0					
Norovirus GI	2	-.8	.3	-.2	.8	84
Norovirus GII	0					
H. Bocavirus	1	0	-1			
Hudisavirus	0					
Adenovirus	1	0	-.9			
Reovirus	1	0	0	-.9		
Enterovirus	0					
PMMoV	3	.2	2	.7	.8	84
Somatic	6	-2	-1	-2	.4	23
Male specific	5	-2	-1.8	-2	.2	14

Table 3-5. Log Concentrations Per Liter (GC-PFU Log_{10}/L) of Human Enteric Viruses and Virus Surrogates in UV Disinfected RO Permeate: Facility A.

Virus	N	Minimum Log Concentration	Maximum Log Concentration	Mean Log Concentration	Std. Deviation	RSD
Aichi virus	0					
crAssphage	4	-.9	.7	.1	.7	645
WCDV1	0					
WCDV2	0					
WCDV3	0					
Norovirus GI	2	-1.5	-.6	-1.1	.6	54
Norovirus GII	0					
H. Bocavirus	2	0	0	.10	.117	117
Hudisavirus	0					
Adenovirus	1	0	-.9			
Reovirus	3	-.5	.3	-.1	.4	279
Enterovirus	0					
PMMoV	2	.7	2	1.5	1	68
Somatic	5	-2	-.8	-2	.5	32
Male specific	4	-2	-1.6	-1.8	.23	13

In tertiary treated recycled water, the frequencies of detection of most human enteric viruses and virus surrogates remained relatively constant, with the exception of enteroviruses that were not detected at this level. A 4-fold reduction of detection was observed for Norovirus GI and GII while the frequency of detection of Aichivirus, crAssphage, WCDV2, PMMoV, Hudisavirus, Adenovirus, Human Bocavirus, and coliphages remained at ≥50%. Reovirus and WCDV2 were relatively more common in tertiary effluent than in raw wastewater with a 1.4-fold and 1.8-fold increase in detection, respectively. WCDV1 decreased in concentration and frequency of detection (0.3-fold decrease) while the concentration and frequency of detection of WCDV3 remained constant. In general, the concentrations of viruses detected by dPCR/RT-dPCR in tertiary treated recycled water ranged from 3-log_{10} to 5-log_{10} GC/L and from 1-log_{10} to 2-log_{10} PFU/L for male specific and somatic coliphages detected by the plaque assay.

The efficiency of the method for the recovery of viruses spiked in tertiary treated recycled water and ultrafiltration permeate, source waters upstream of the advanced treatment by reverse osmosis and nanofiltration membranes, exhibited high variations with RSD exceeding 70%. Recycled water and ultrafiltration permeate were the water matrices characterized by the highest complexity in terms of particulate and dissolved organic compounds. Thus, variations and low levels of virus recoveries are expected as demonstrated in previous studies (Betancourt et al., 2018). Readings of TDS and EC in recycled water and ultrafiltration permeate consistently exceeded 1000 mg/L and 1000 µS/cm, respectively. Moreover, humic substances as indicated by size-exclusion chromatographs with DOC detection dominated DOC fraction concentrations in both water matrices. Further characterization of the type of organics by excitation emission matrix (EEM) fluorescence spectra revealed predominantly fluorescent intensities associated with Region V corresponding to polycyclic aromatic components, including humic acids, quinones, aromatic ketones, fluorescent whitening agents, and pharmaceutical compounds of colloidal organic matter (see section 3.1.4 Non-microbial surrogates of advanced treatment performance).

Studies have shown that in solid-water interfaces the presence of negatively charged dissolved organic matter competitively suppresses the adsorption of viruses to positively charged sorbent surfaces (Armanious et al., 2016c). Preliminary studies toward this research demonstrated a gradual decrease in the retention efficiency of bacteriophage MS2 spiked in recycled water using electropositive charged NanoCeram cartridge filtration. An increase in the volume of water filtered through the NanoCeram cartridges resulted in a decrease in the retention of MS2 coliphages to the filter matrix. Based on these preliminary assays, our virus recovery and concentration approach involved the filtration of <100 L of both recycled water and ultrafiltration permeate.

For this study, the mean recovery efficiency of Reovirus in recycled water was 23±22% (N=4, RSD 96%) and 25±18 (N=2, RSD 74%) in ultrafiltration permeate. The mean recovery efficiency of Porcine parvovirus, surrogate for Human Parvovirus, was 20±21% (N=4, RSD 100%) in tertiary effluent and 25±28 (N=2, RSD 112%) in ultrafiltration permeate. High variations in virus recoveries were also observed for Coxsackievirus B5 with mean recoveries of 22±15% (N=4, RSD 70 %) in recycled water and 20±17% (N=2, RSD 88) in ultrafiltration permeate. The mean recovery efficiency of Adenovirus in recycled water was 21±14 (N=4, RSD 71%) and 5±5% (N=2, RSD 100%) in ultrafiltration permeate. Large variations in recovery were also observed for MNV, surrogate for Norovirus GI and GII, with mean recoveries of 11±15% (N=2, RSD 140) in recycled water and 20±17 (N=2, RSD 88%) in ultrafiltration permeate. Due to the large variations in recoveries, the virus data obtained from this study were not adjusted to the percentage efficiencies described above.

3.1.2 Log Reduction Values

Log reduction values of human enteric viruses and virus surrogates from raw wastewater to full advanced treated water from Facility A were estimated as follows:

1) Removal from sewage to tertiary effluent
2) Removal from tertiary effluent to reverse osmosis permeate
3) Removal from reverse osmosis permeate to UV treated effluent

Virus reductions by the advanced treatment train consisting of reverse osmosis and UV-based photolysis was evaluated during fourteen sampling events that comprised changing weather conditions in the Southwestern US (fall: Sep - Nov, winter: Dec – Feb, spring: Mar - May, dry early summer: June, monsoon: Jul - Aug, post-monsoon: Nov).

Boxplots of LRV of viruses for each treatment train in Facility A as depicted in Figures 3.1, 3.2, and 3.3, were initially examined. It is important to note that the data in the boxplot are the LRVs derived from paired influent and effluent concentrations, excluding all non-detects. Non-detects were found in both influent and effluent data, not always concurrently, implying that actual variation of LRVs is higher than depicted by the boxplots. Obviously, more non-detects were found in the more downstream treatments steps as shown in the boxplots, thus a great part of the viruses was not detectable at all. It may imply that there are no significant differences of LRVs between viruses, based on detectable concentrations only. At the first treatment stage (raw wastewater to tertiary), the median reduction of somatic and male specific coliphages (PFU/L) was around 6-$\log_{10}$, while the median reduction of the human

enteric viruses was around 3-log_{10}-4-log_{10}. The median of LRV of virus surrogates was around 1-log_{10} and 2-log_{10} for CRESS viruses (WCDV1-WCDV2, WCDV3) and from 2-log_{10}-4-log_{10} for Hudisavirus, crAssphage and PMMoV. In all the subsequent treatment stages, LRVs of each virus were slightly similar, with some exceptions. Infrequent and highly variable detections of viruses were observed after full advanced treatment with evidence of virus breakthrough for human enteric viruses of public health significance. For instance, Reovirus and Norovirus GI were detected simultaneously in full advanced treated water in two sampling events, associated or not associated with the virus surrogates. Norovirus GI concentrations in product water expressed per 100 L ranged from 0.5-log_{10} to 1-log_{10} with overall reductions from raw wastewater to full advanced treated water (raw wastewater-UVP) between 5-log_{10}-7-log_{10}. Reovirus concentrations in product water expressed per 100 L were consistently 2-log_{10}. Two of these detections were deemed as virus breakthrough as previously mentioned since there was no upstream detection and therefore no LRVs could be attributed to the advanced treatment train for these two viruses. The overall LRV of Reovirus from raw wastewater to advanced treated water was 4-log_{10}. Human Bocavirus was also detected twice without evidence of other viruses present in the advanced treatment train. The detection outcome resulted in either no removal (virus breakthrough) or a LRV of 2-log_{10} from one time-paired influent (from raw wastewater) to UV-permeate observation. Among all the human viruses, Reovirus was the enteric virus most frequently detected (3 of 14) in product water.

Among the virus surrogates, crAssphage was detected in full advanced treated water at log concentrations expressed per 100 L ranging from 1-log_{10} to 3-log_{10} with and overall LRV of 8-log_{10}. Hudisavirus and CRESS viruses (WCDV1, WCDV2, and WCDV3) were not detected after full advanced treatment. Log concentrations of PMMoV and coliphages in full advanced treated water expressed per 100 L corresponded to 3-log_{10} and 0.1-log_{10}, respectively. Overall LRV of PMMoV and both coliphages after full advanced treatment averaged 5-log_{10} and 8-log_{10}, respectively. A graphical summary of LRV of viruses in Facility A is given in Figure 3.4. Based on the regression model, LRV of WCDV2 and WCDV3 in tertiary effluent were statistically significant lower than LRV of Adenovirus ($p=0$) while the LRV of WCDV1 was significantly lower ($p<0.05$) than the LRV of Adenovirus. However, the LRV of both coliphages were statistically significant higher than the LRV of Adenovirus ($p=0$). On the other hand, the LRV of crAssphage was just significantly higher ($p<0.1$) while for the rest of the viruses, including the human enteric viruses (Norovirus GI and GII, Human Bocavirus, Reovirus) the LRV were not significantly different. The same linear mixed effect analysis applied for LRV of virus in RO permeate and UV permeate revealed no statistically significant differences. In addition, the linear mixed effect analysis revealed no statistically significant difference among viruses.

One-thousandth-scale onsite experiments (N=2) were conducted in order to evaluate the recovery efficiencies of the five viruses in RO permeate using product water generated by the engineering-scale integrated membrane system treating recycled water for on-site non-potable water reuse. NanoCeram filtration provided less variation (RSD <30%) in recoveries for Porcine parvovirus and Murine Norovirus in RO product water with corresponding average recoveries of 31±4% (N=2, RSD 14%) and 20±4% (N=2, RSD 18%), respectively. Mean recoveries of Reovirus type 3, Coxsackievirus B5, and Adenovirus 4 by NanoCeram filtration indicated noticeable variations (RSD >50%) in performance efficiency. The mean recoveries of these viruses were

70±37% (N=2, RSD 53%) for Reovirus type 3, 14±9% (N=2, RSD 68%) for Coxsackievirus B5, and 30±21 (N=2, RSD 70%) for Adenovirus 4. Due to these variations in virus recoveries and the limited number of assays performed, the dPCR data was not adjusted to the recovery efficiencies described above.

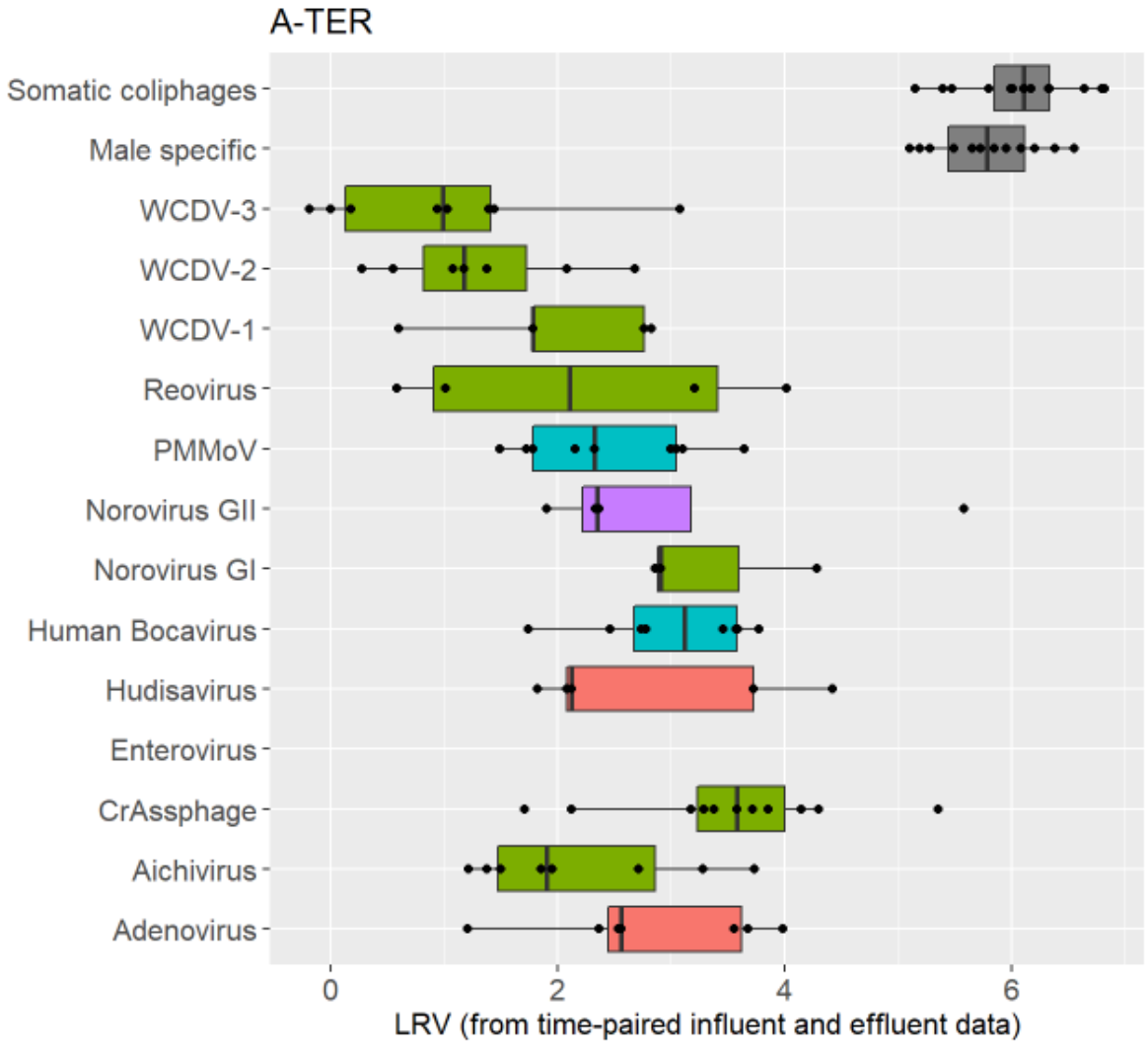


Figure 3-1. Log Reduction Values of Human Enteric Viruses and Virus Surrogates from Raw Wastewater to Tertiary Effluent: Facility A (dark dots: LRV, solid vertical line: median, box: quartiles, whiskers: minimum and maximum LRV)**.**

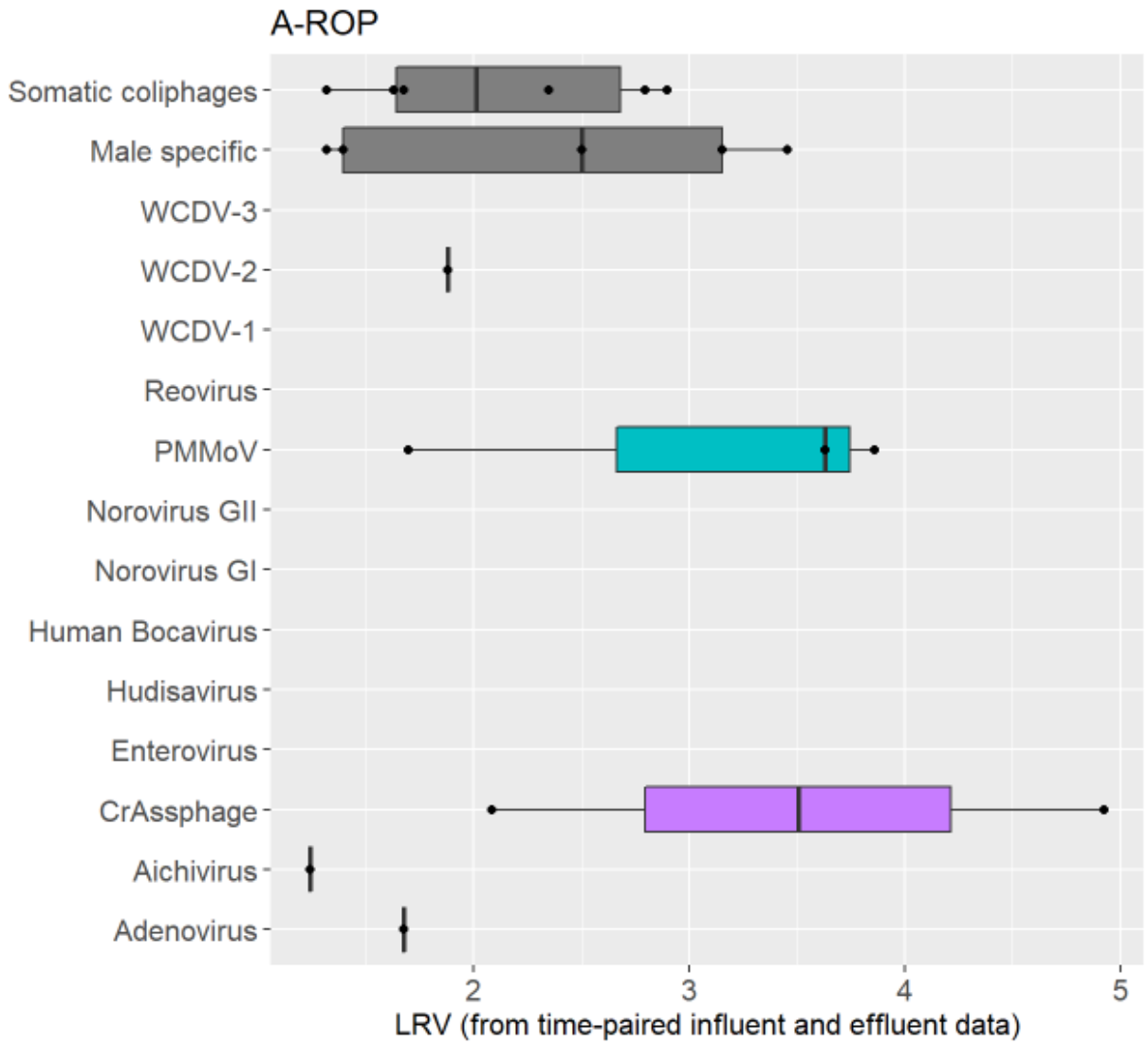


Figure 3-2. Log Reduction Values of Human Enteric Viruses and Virus Surrogates from Tertiary Effluent to Ultrafiltration-Reverse Osmosis: Facility A (dark dots: LRV, solid vertical line: median, box: quartiles, whiskers: minimum and maximum LRV)**.**

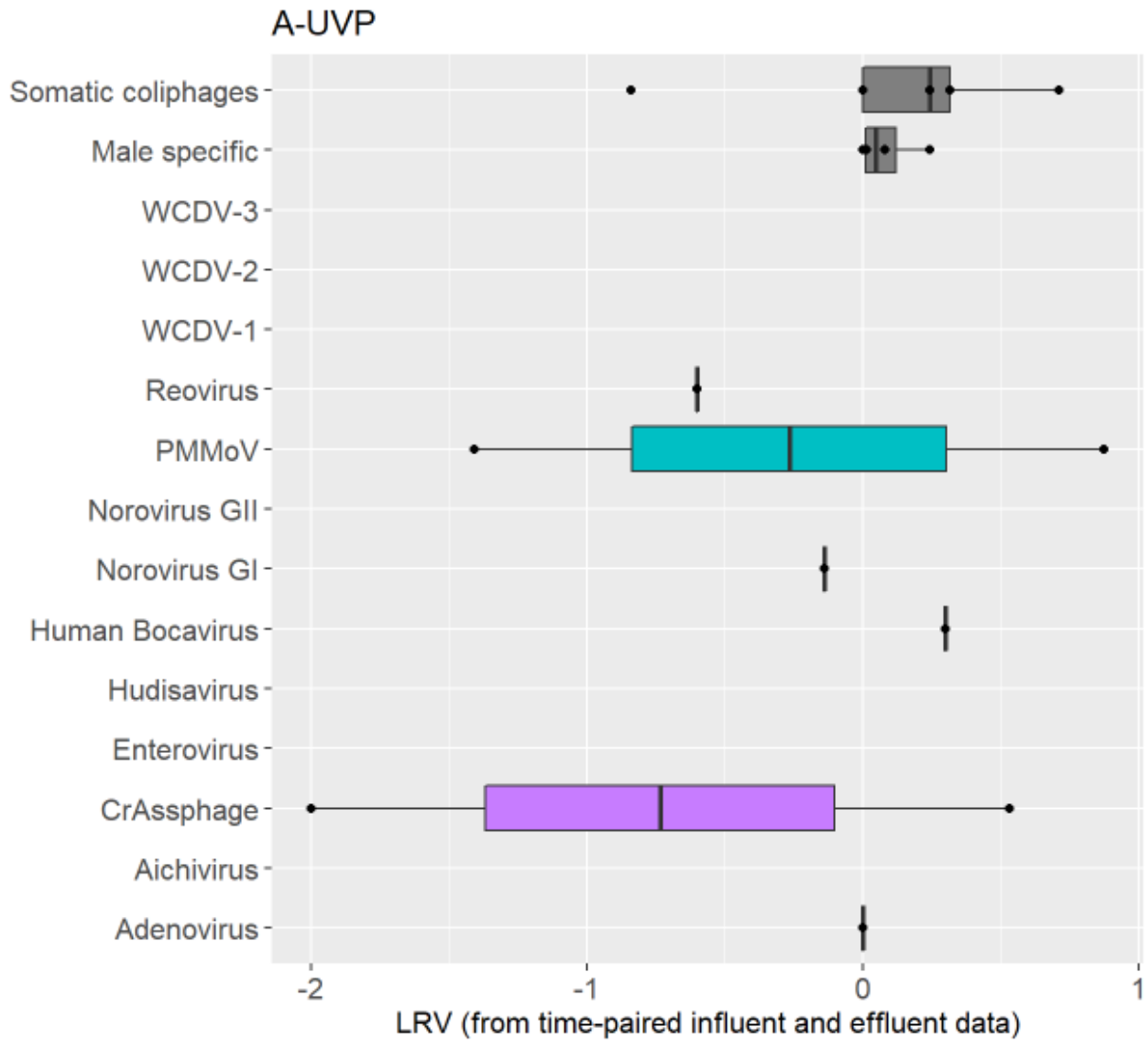


Figure 3-3. Log Reduction Values of Human Enteric Viruses and Virus Surrogates in UV Permeate: Facility A
(dark dots: LRV, solid vertical line: median, box: quartiles, whiskers: minimum and maximum LRV)**.**

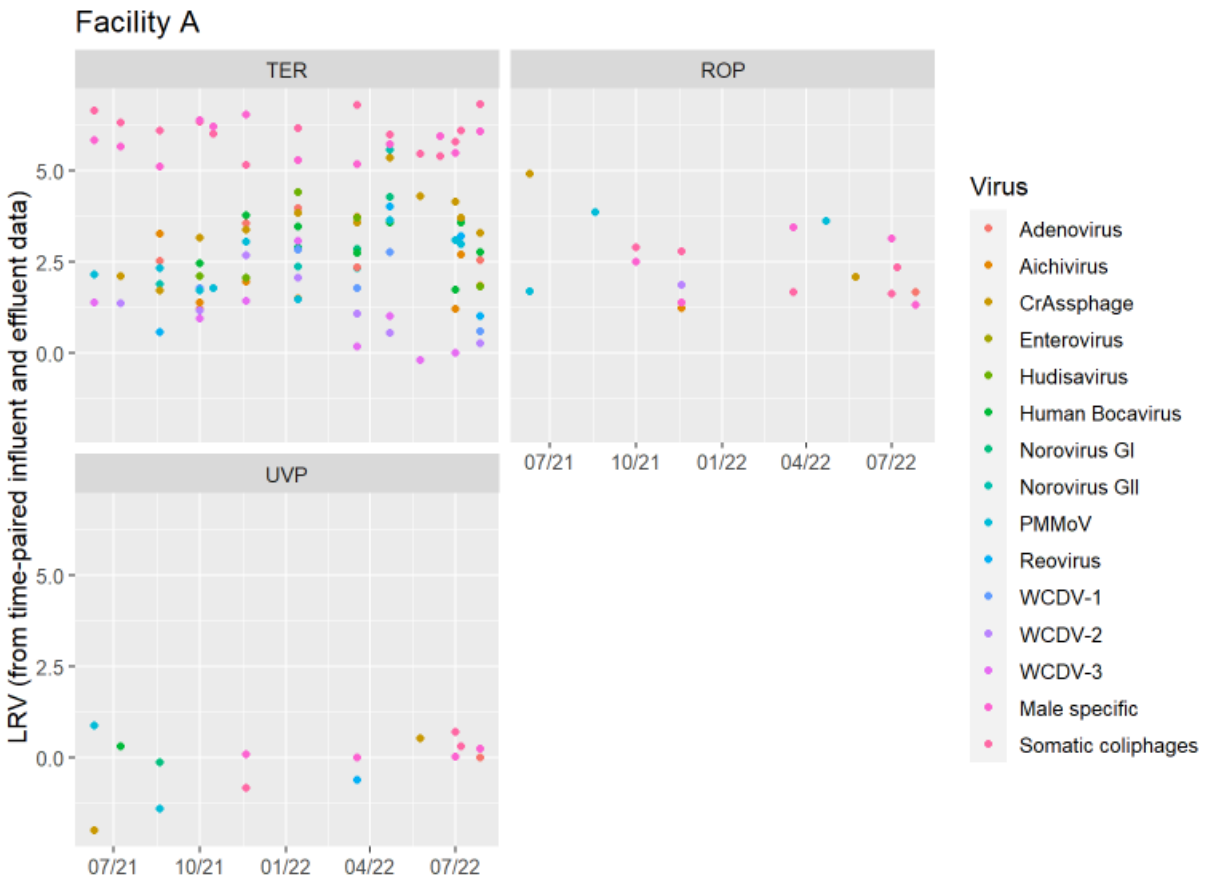


Figure 3-4. Graphical Summary of LRV of Viruses from Raw Wastewater to Full Advanced Treated Water: Facility A.

3.1.3 Performance Features of Indicator Viruses to Confirm Physical Treatment

From the results described above, virus abundance in raw wastewater, virus persistence upstream of the advanced treatment trains, virus documented resilience to disinfection technologies in water treatment, and virus structural complexity were useful features of virus indicator performance to confirm physical treatment. These features were observed for five human enteric viruses (Human Bocavirus, Norovirus GI, Adenovirus, Reovirus, Aichi virus) and six virus surrogates (crAssphage, WCDV2, Hudisavirus, PMMoV, male specific and somatic coliphages).

Among all viruses, somatic coliphages were the only endogenous wastewater viruses capable of demonstrating up to 9-$\log_{10}$ reduction credit, close but not to the level of the recommended 12-$\log_{10}$ reduction credit of viruses required for treated wastewater intended for indirect potable reuse, i.e., surface water augmentation or groundwater recharge (Title 22 and 17 California Code of Regulations State Board, 2015, (CCR, 2015). In addition, eight endogenous wastewater viruses, including three human enteric viruses (Norovirus GI, Adenovirus and Human Bocavirus) and four virus surrogates (crAssphage, WCDV2, PMMoV, somatic and male specific coliphages), were able to demonstrate the 2-$\log_{10}$ reduction credit for pathogen removal by RO membranes (from tertiary effluent to RO permeate), the regulatory credit for groundwater replenishment

projects in California. Physical removal after full advanced treatment (greater than LRV) was achieved for some viruses while virus breakthrough was revealed for at least four human enteric viruses (Adenovirus, Reovirus, Norovirus GI, and Human Bocavirus) and four virus surrogates (crAssphage, PMMoV, Somatic, and F+ coliphages).

Most of the enteric viruses that were found after reverse osmosis and UV advanced oxidation have isoelectric points (pI) between 4.5 and 7.4, except Reovirus, which possesses a pI of 3.9. The pH of product water at Facility A ranged from 4.46 to 6.99, meaning that all these viruses existed as negatively charged nanoparticles (<100 nm) that would have been rejected by the polyamide layer of the RO membrane due to dielectric exclusion. These viruses were not even efficiently rejected by the water channels on the non-porous polyamide layer of the RO membrane, also known as intramolecular and intermolecular spaces, with sizes of 2.1 – 2.4 A and 3.5 – 4.5 A, respectively (equivalent to 0.21 – 0.24 nm and 0.35 – 4.5 nm). The size of these spaces or water channels are substantially smaller than the size of the smallest viruses evaluated in this study (Table 1.3). Consequently, defects in the RO membrane that are inevitable to some extent during membrane manufacturing were more likely responsible for virus leak through the full-scale RO membrane. Defects larger than 20 nm in membrane, o-rings, or glue lines of membrane element have been reported (Yoon, 2019).

Further statistical analyses were conducted in order to evaluate correlations among the different human viruses and virus surrogates at different levels of treatment. The results of the Spearman's rho nonparametric correlation are shown in Table 3.6. Only the virus concentrations detected in sewage and tertiary treated water had enough observations to reliably develop correlations.

Table 3-6. Spearman's Correlation Coefficients Employed to Evaluate the Strength of Association between Human Enteric Viruses and Virus Surrogates Upstream of Advanced Treatment: Facility A.

Virus in Raw Wastewater	Number of Observations	Virus [Level of Treatment]	Spearman's Rho Correlation Coefficient	*P* Value
PMMoV	9	PMMoV [Tertiary]	0.833**	0.005
WCDV-1	7	crAssphage [raw wastewater]	0.964**	<0.001
Hudisavirus	9	crAssphage [raw wastewater]	0.850**	0.004
Hudisavirus	9	crAssphage [Tertiary]	0.850**	0.004
Adenovirus	8	Hudisavirus [raw wastewater]	1**	0
Enterovirus	10	Aichivirus [raw wastewater]	0.842**	0.002
GI Norovirus	10	crAssphage [raw wastewater]	0.754**	0.01
Human Bocavirus	9	Hudisavirus [raw wastewater]	0.993**	<0.001
Human Bocavirus	9	Adenovirus [raw wastewater]	0.993**	<0.001
Human Bocavirus	10	crAssphage [raw wastewater]	0.855**	0.002
Human Bocavirus	10	crAssphage [Tertiary]	0.794**	0.006
Somatic coliphage	10	Human Bocavirus [raw wastewater]	0.794**	0.006
crAssphage	9	Aichivirus [Tertiary]	0.817**	0.007

**Indicates highly significant as given by the software.

Statistically significant correlations were revealed among the different viruses in raw wastewater and tertiary treated wastewater. However, the most significant correlations were identified among DNA viruses in raw wastewater and to a lesser extent in tertiary treated wastewater. High statistical correlation was found between crAssphage and Human Bocavirus

and with most of the DNA viruses including somatic coliphages evaluated by culture methods. Only crAssphage was correlated with two RNA viruses (GI Norovirus and Aichi virus) in raw wastewater. Enterovirus and Aichi virus were the only two RNA viruses showing strong positive correlations while PMMoV was not correlated with any of the viruses monitored in this study.

The Mann-Whitney-U Test was used to compare between samples with detectable virus genomes by dPCR or plaque forming units (coliphages) and those without detectable levels of virus genomes or plaque forming units with the levels of non-microbial surrogates (see for a list of parameters considered) at the two advanced treatment trains (reverse osmosis and UV-based photolysis) (Table 3.7).

Table 3-7. Independent-Samples Mann-Whitney U Test Summary Comparing Mean Distributions between Viruses and Physicochemical Parameters Evaluated at Two Advanced Treatment Trains in Facility A.

Level of Treatment Versus Parameter	*P (*Exact Significance 2-Sided Test)	Mean Rank for Detects	Mean Rank for Non-Detects
ROPSECDOC-SOMATICROPDET	0.005	0.144387 (N=6)	1.08980 (N=8)
ROPSECDOC-F+ROPDET	0.039	0.193512 (N=6)	1.043961 (N=8)
ROPSECDOC-CRASSPROPDET	0.028	2.00758 (N=2)	0.45813 (N=12)
POSTUVREGIV-BOCAVIRUSPOSTUVDET	0.044	72420000.0 (N=2)	14460750.0 (N=12)
POSTUVREGIV-SOMATICPOSTUVDET	0.039	11983180.0 (N=5)	28717011.1 (N=9)
PostUVSECDOC-BocavirusPostUVDET	0.028	0.924072 (N=2)	0.236722 (N=12)
POSTUVSECDOC-SOMATICPOSTUVDET	0.028	0.09079 (N=5)	0.470540 (N=9)
POSTUVC+D-PMMoVPOSTUVDET	0.044	3832.63 (2)	1416.77 (N=12)
POSTUVTEMP-SOMATICPOSTUVDET	0.014	18.980 (N=5)	12.922 (N=9)

ROP: reverse osmosis permeate, SECDOC: size exclusion chromatography dissolve organic carbon, DET: detected, REGVI: region IV, C+D: DOC fractions C and D combined

The results of the Mann-Whitney U test revealed statistically significant differences for the mean DOC concentrations in the RO permeate with respect to plaque forming units per liter of somatic coliphages ($U=2.000$, $p<0.05$) male specific (F+) coliphages ($U=8$, $p<0.05$) and crAssphage ($U=24$, $p<0.05$). In the UV permeate, there were statistically significant differences for the mean fluorescence regional intensity integrated at fluorescent region IV (soluble microbial byproducts-like matter) with respect to the mean concentrations of Human Bocavirus ($U=23$, $p<0.05$) and Somatic coliphages ($U=7$, $p<0.05$). In addition, statistically significant differences were revealed in the UV permeate for the mean residual DOC concentration and the mean concentration of Human Bocavirus ($U=24$, $p<0.05$) as well as for plaque forming units of somatic coliphages ($U=6$, $p<0.05$). The Mann-Whitney U test results also revealed statically significant associations between fractions C+D of the SEC-OCD fingerprint and virus genome levels of PMMoV ($U=23.000$, $p<0.05$). Fraction C+D includes humic substances (fraction C, AMW = 1.5-0.5 kDa) low molecular weight (LMW) acids and neutral substances (fraction D, AMW <0.5 kDa). In addition, a statistically significant association was found between mean temperature registered at the UV permeate and plaque forming units of somatic coliphages ($U=41$, $p<0.05$) thereby indicating a temporal association between PFU detection of somatic coliphages and temperature variations that was not observed for F+ coliphages.

The Chi-Square Test indicated that at the advanced treatment level, there were only significant associations between somatic coliphages and F+ coliphages detected at ROP (Pearson Chi-Square X^2 (1) =7.024, p<0.05, N = 5). Additional significant associations were observed but only for virus occurrence at the tertiary effluent level (source water prior to advanced treatment) where crAssphage occurrence was associated with WCDV2 (X^2(1) =8.556, p<0.05, N = 11) and with Aichi virus (X2(1)=5.833, <0.05, N=10).

It is important to mention that these tests were conducted on an exploratory basis, and no effort was made to control alpha for multiple comparisons. Hence, these associations would need to be confirmed by subsequent research.

Spearman's rho nonparametric correlation was also used to evaluate statistically significant associations between log reductions of the different viruses at different stages of treatment and between log reductions of viruses with log reductions of the different physicochemical parameters. No statistically significant correlations were found between log reduction levels of each of the viruses monitored in this study at full advanced treatment (tertiary-ROP, ROP-UV). Upstream of the advanced treatment more associations were revealed for log reduction levels of viruses, specifically among DNA viruses. These associations were observed for observations equal or lower to 6. For instance, Adenovirus and Hudisavirus (Spearman's rho 0.943, p=0.005, N=6), Adenovirus and WCDV1 (Spearman's rho 1, p=0.000, N=4), WCDV2 and Human Bocavirus (Spearman's rho 1.000, p=0.00, N=6).

For the evaluation of correlations between log reduction values of viruses and rejection efficiency of non-microbial surrogates, only the viruses with relatively large number of observations (somatic and F+ or male-specific coliphages) with detections were considered. However, there were no statistically significant correlations among the variables evaluated in the analyses, more likely associated with lot of variability, which is inherently associated with ordinary measurements. Interestingly, the scatter plot generated for evaluation of somatic coliphage removal and TOC removal at full advanced treatment (ROP-UV) demonstrated a negative association between these two variables (Figure 3.5). Differences in rejection efficiencies of soluble contaminants and nanoparticles may be associated with this outcome. A possible explanation is that the virus absorption capacity of the polyamide membrane once saturated with organic components cannot efficiently reject other charged molecules or nanoparticles, including viruses (Warsinger et al., 2018). While this phenomenon was observed for only somatic coliphages, the infrequent occurrence of viruses after advanced treatment may be associated with a similar outcome. Somatic coliphages were the virus most frequently detected after full advanced treatment.

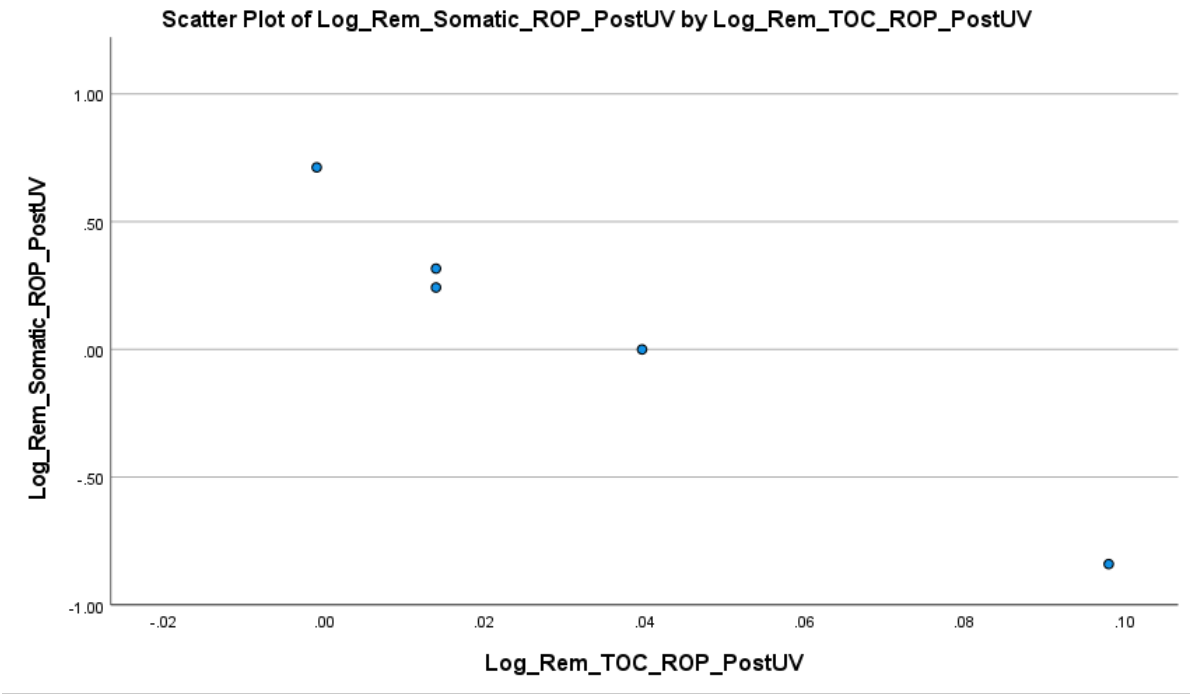


Figure 3-5. Scatter Plot Depicting the Distribution of LRV of Somatic Coliphages and LRV of TOC Concentrations in ROP Oxidized Permeate: Facility A.

3.1.4 Non-Microbial Surrogates of Advanced Treatment Performance

Advanced treatment trains for potable reuse applications should achieve robust removal of target constituents (pathogens, organic, and inorganic compounds) to ensure protection of public health. Previous studies have addressed the potentials and limitations of several non-microbial indicators and operational parameters to demonstrate the recommended pathogen reduction credits by individual or combined advanced treatment trains (Antony et al., 2014; Pype et al., 2016b; Yoon, 2019). This was also part of a previous WRF Report (Jacangelo, 2019) that evaluated scientifically proven methods for integrity monitoring of RO membranes for a 4-$\log_{10}$ or greater verification of microorganisms including viruses. There are limited studies at full-scale evaluating non-microbial surrogates for the purpose above. A recent WRF Report (Polanco et al., 2022) provided evidence for the use of strontium, sulfate, and/or free ATP as surrogates for LRV credit of reverse osmosis membranes, maintaining TOC and EC monitoring as back-ups and for other performance monitoring purposes. In this study, several non-microbial surrogates were investigated at full-scale using the level of reduction or rejection efficiency of each corresponding unit as a performance-based indicator to demonstrate treatment efficiency and reduction of virus indicator.

Total living biomass measured by the adenosine triphosphate (ATP) content of microorganisms in water as well as total organic carbon (TOC, DOC), UV absorbance and total fluorescence were used to determine overall biological load. In addition, size exclusion chromatography in combination with organic carbon detection (SEC-OCD) was evaluated in order to monitor rejection of high molecular weight compounds. The reduction of fluorescence values along with

TOC/DOC concentrations and UV_{254} absorbance detections were evaluated as potential bulk organic surrogates to determine correlations with virus removal.

Size exclusion chromatography and UV_{254} detection have been used to evaluate the change in both DOC size fractions and concentrations through advanced treatment processes at pilot and full scale (Henderson et al., 2010; Souza-Chaves et al., 2022). Our results indicated that humic substances (fraction B) contributed in greater proportion (49%) than building blocks (proteins, low molecular weight acid, and neutral substances, fractions C+ D) to source water upstream of the advanced treatment trains (Figures 3.6, 3.7, 3.8, 3.9). Figure 3.9 shows DOC concentrations calculated from SEC chromatograms. These fractions were subsequently removed by 95 ± 0.04 % after RO and UV photolysis with rejection efficiencies between 87% and 99% (or 0.9log to 2.0log) to produce water of less than 0.33 ± 0.31 mg/L. The concentration of fraction A in tertiary effluent was on average 0.36 ± 0.51 mg/L mostly associated with particle sizes greater than 10 kDa from proteinaceous biopolymers entering the RO system. These components were subsequently removed to negligible concentrations (0.008 ± 0.02 mg/L) by reverse osmosis as demonstrated in this and previous studies (Liu et al., 2020; Souza-Chaves et al., 2022). The concentration of fractions B and C+D combined in tertiary effluent corresponding to humic substances (fraction B, AMW =10-1.5 kDa) building blocks of humic substances and low molecular weight (LMW) acid and neutral substances (fraction C, AMW = 1.5 – 0.5 kDa; fraction D, AMW <0.5 kDa) were 3.91 ± 0.74 mg/L and 1.76 ± 1.27 mg/L, respectively. Relative to tertiary effluent, these fractions decreased to 0.12 ± 0.13 mg/L and 0.20 ± 0.31 mg/L, respectively in product water, with corresponding DOC rejection efficiencies of fractions B ranging from 88% to 99.98% (or 0.93-$\log_{10}$ to 3.9-$\log_{10}$) and fractions C+D ranging from 80% to 99.9% (or 0.7-$\log_{10}$ to 3.1-$\log_{10}$). Consequently, all DOC fractions were considerable reduced in product water with only organics of apparent molecular weights lower than 2000 Da (fractions C+D, 500 – 1500 Da) being detected in advanced treated water.

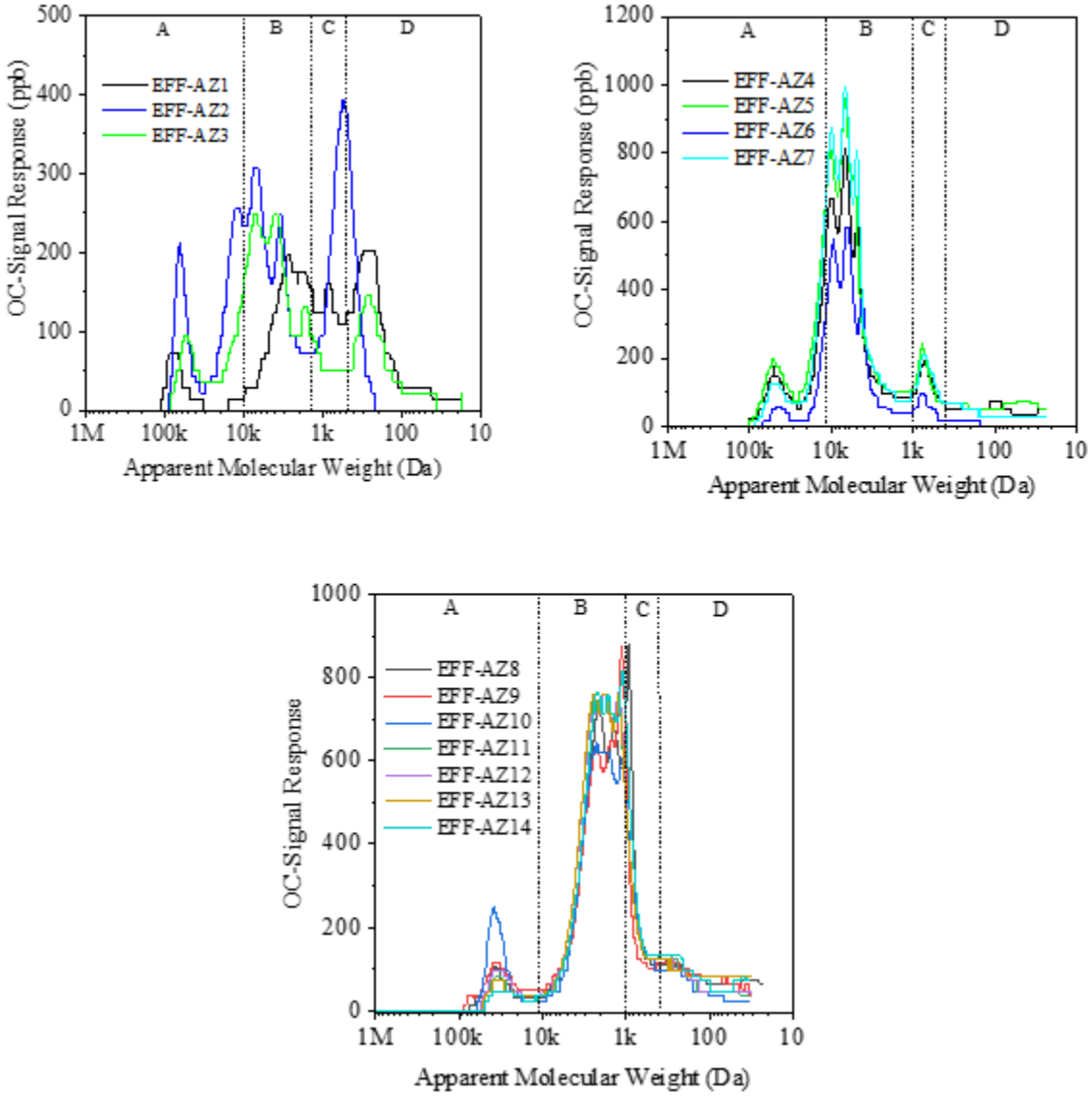


Figure 3-6. Size Exclusion Chromatograph – Organic Carbon Detector (SEC-OCD) Fingerprints of Tertiary Treated Effluent Upstream of Advanced Treatment (Fraction A: proteinaceous biopolymers; Fraction B: Humic substances; Fraction C: Building blocks of humic substances; Fraction D: Low molecular weight acids and neutrals).

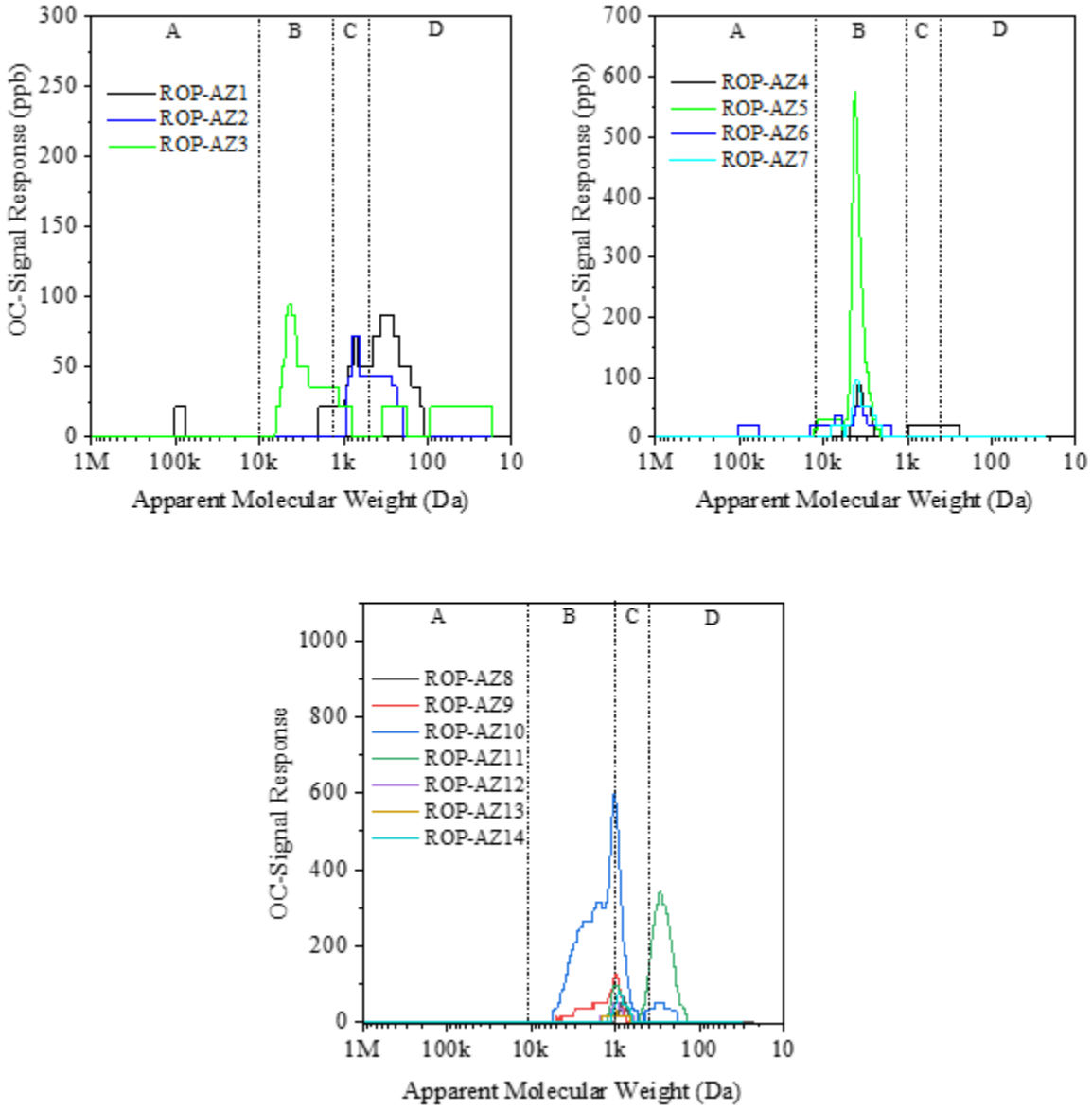


Figure 3-7. Size Exclusion Chromatograph – Organic Carbon Detector (SEC-OCD) Fingerprints of Product Water after Reverse Osmosis (Fraction A: proteinaceous biopolymers; Fraction B: Humic substances; Fraction C: Building blocks of humic substances; Fraction D: Low molecular weight acids and neutrals).

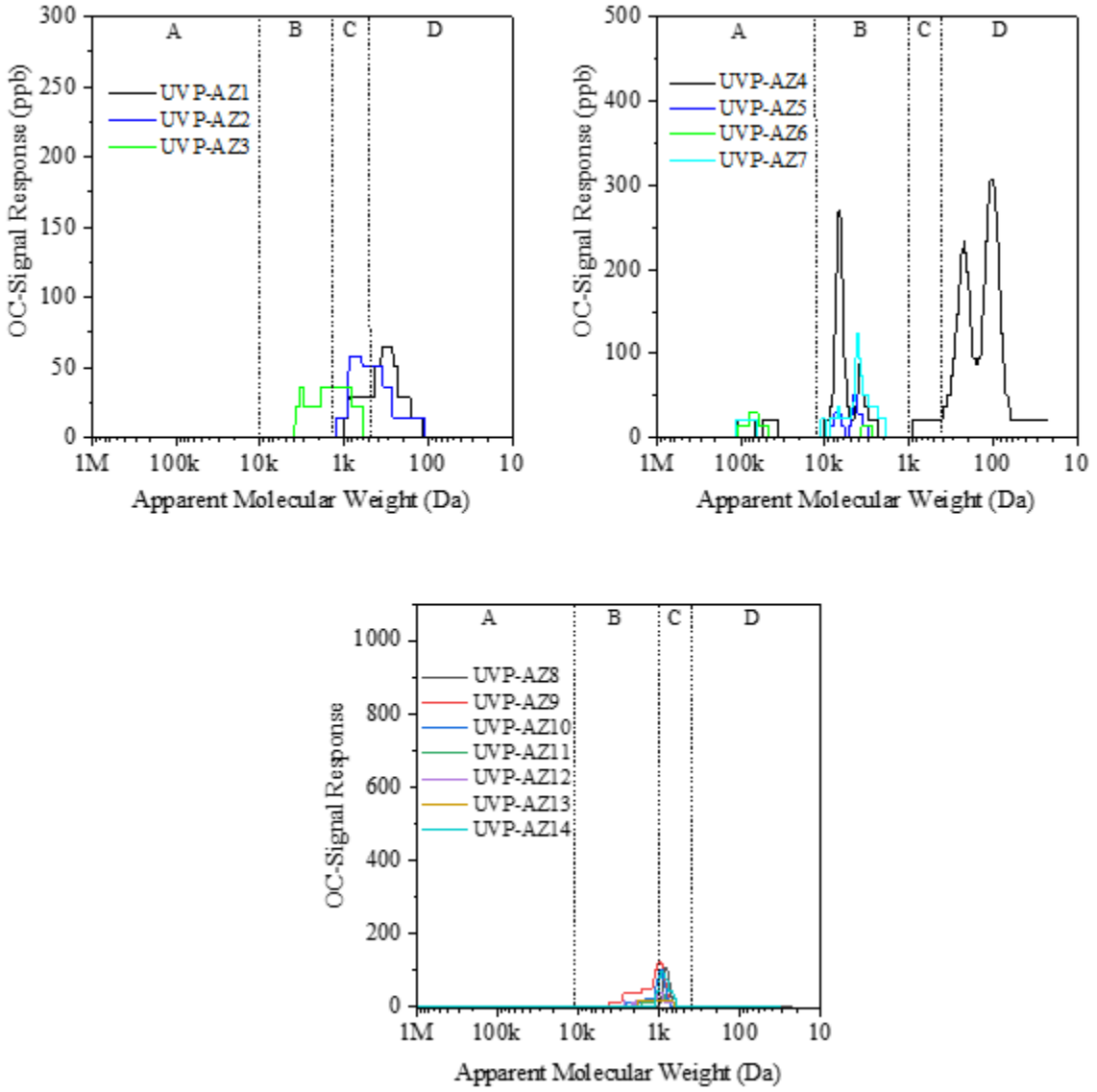


Figure 3-8. Size Exclusion Chromatograph – Organic Carbon Detector (SEC-OCD) Fingerprints of Product Water after UV-Based Photolysis (Fraction A: proteinaceous biopolymers; Fraction B: Humic substances; Fraction C: Building blocks of humic substances; Fraction D: Low molecular weight acids and neutrals).

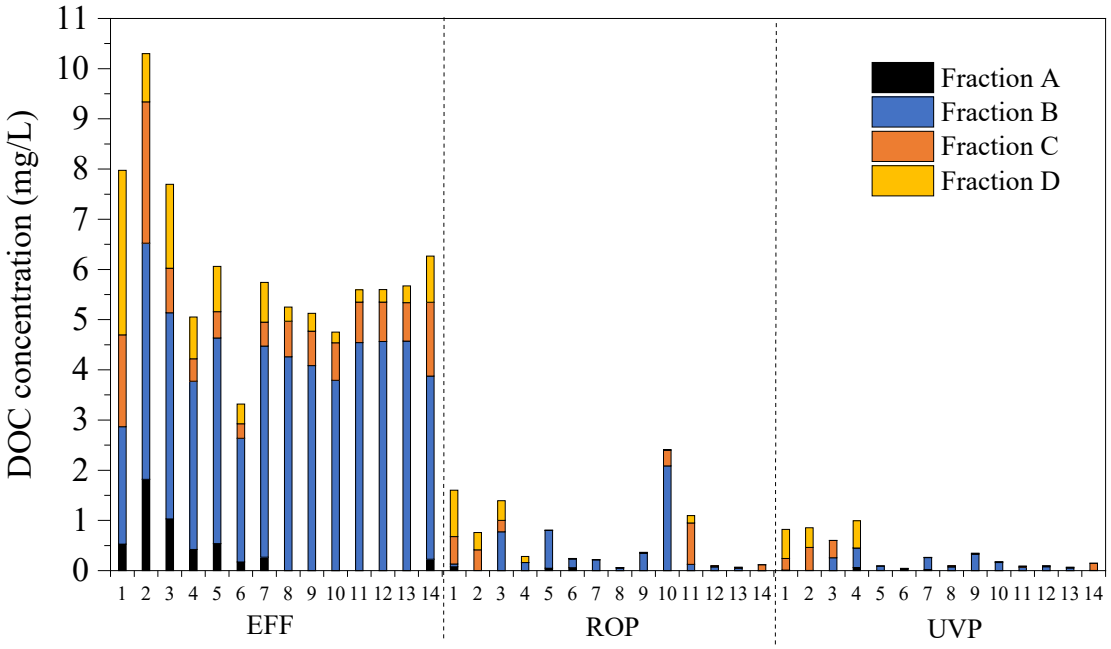


Figure 3-9. Dissolved Organic Carbon Concentrations Calculated from SEC Chromatograms from Tertiary Treated (Source Water, EFF), RO Permeate (ROP), and UV Disinfected RO Permeate (UVP).

Additional analyses were performed using excitation-emission matrix (EEM) fluorescence to further characterize the type of organics remaining after full advanced treatment. Figure 3.10 shows EEM fluorescence intensities and observed total fluorescence (TF) rejection following advanced treatment by reverse osmosis and UV-based photolysis. A normalization technique using a Raman peak of DI water was applied as previously described (Chen et al., 2003a) and fluorescence intensity unit is presented in Raman units square nanometers (R.U. nm^2). The regional integration and TF are displayed as two regions (Table 3.8): Region IV – soluble microbial metabolic by-products (Excitation [Ex] > 250 nm, Emission [Em] < 380 nm) and Region V – humic-like acids (Ex > 250 nm, Em > 380 nm) (Park and Snyder, 2018). Region IV is usually associated with fluorophores containing a limited number of aromatic rings, such as phenols, indoles, mono and polyaromatic hydrocarbons, DNA, aromatic amino acids, and lignin degradation products. Region V is associated with polycyclic aromatic components including humic acids, quinones, aromatic ketones, fluorescent whitening agents, and pharmaceutical compounds of colloidal organic matter (Carstea et al., 2016).

Table 3-8. Excitation-Emission Boundaries of Regions IV and V.

Excitation-emission type	Region IV (soluble microbial byproduct-like matter)	Region V (humic-acid like matter)
Excitation boundary (nm)	250-400	250-400
Emission boundary (nm)	280-380	380-550

Source: Reprinted from Chemosphere, 193, M. Park and S.A. Snyder, Sample Handling and Data Processing for Fluorescent Excitation-Emission Matrix (EEM) of Dissolved Organic Matter (DOM), Pages 530-537, Copyright (2018), with permission from Elsevier.

The significant loss of intensity in both regions of EEMs as depicted in Figure 3.10 reveals the high rejection efficiency of DOC by the full advanced treatment process (additional EEM

fingerprints can be found in Appendix). Rejection rates of total fluorescence ranged from 97% to 99.3% (or 1.5-log_{10} to 2.15-log_{10}). For Region IV the background fluorescence based rejection efficiencies ranged from 97% to 99% (or 1.5-log_{10} to 2.35-log_{10}) and from 97% to 99.9% for Region V (or 1.5-log_{10} to 3.1-log_{10}). The relative standard deviation of these rejection efficiencies was less than 1%, meaning that these rejection efficiencies were relatively constant. The SEC-OCD chromatograms support the results of EEM fluorescence. The size distribution of DOM changed towards lower molecular weight from tertiary effluent to advanced treated water due to increased removal of high molecular weight compounds. These results indicate that the contribution of microbial byproduct-like and humic acid-like fractions to the organic carbon content was minimal for product water. These results are consistent with the UV_{254} absorbance and DOC concentrations, although rejection efficiencies of DOC ranged from 80% to 99% (0.7-log_{10} to 2-log_{10}) while rejection efficiencies evaluated by UV_{254} absorbance ranged from 60% to 99% (0.4-log_{10} to 2-log_{10}).

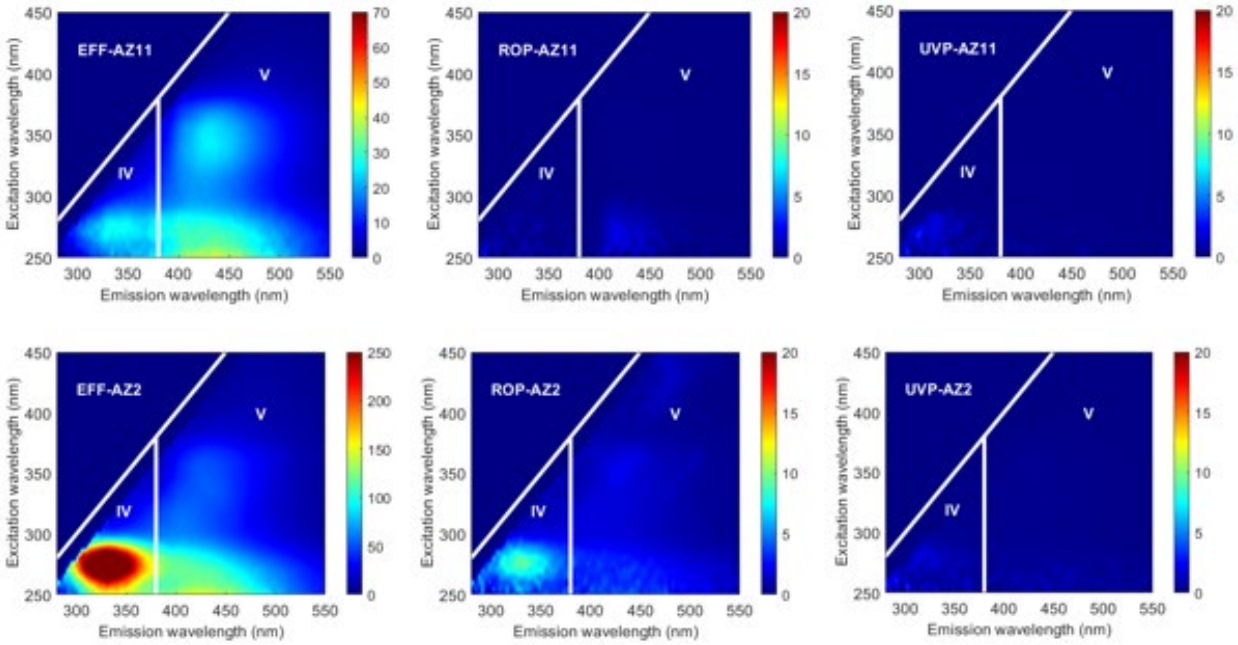


Figure 3-10. Fluorescence Regional Intensity Integrated at Each Fluorescent Region: Facility A (Regions IV and V and the respective total fluorescence (TF) intensity (summation of regional fluorescence intensities) with observed rejection from tertiary treated (source water) and RO permeate through UV disinfected RO permeate).

Ion permeability and rejection by the advanced treatment train was evaluated by measurements of monovalent and divalent ions. Among the studied divalent ions, calcium and magnesium have a higher hydrated radius (Table 3.9), which may occasionally lead to higher rejections (calcium 75% to 99.9% equivalent to 0.61-log_{10} to 3-log_{10}; magnesium 87% to 99% equivalent to 0.9-log_{10} to 2-log_{10}) than sulfate (89% to 0.98%, 0.98-log_{10} to 1.83-log_{10}). Lower to no rejections were observed for the monovalent ions (potassium, sodium, and chloride) likely due to their lower hydrated radius.

Table 3-9. Ion and Hydrated Radius of Selected Cations and Anions.

Ion	Hydrated Radius (nm)
Na^+	0.276 – 0.360
K^+	0.201 – 0.331
Mg^{2+}	0.300 – 0.470
Ca^{2+}	0.412 – 0.420
Cl^-	0.324 – 0.332
SO_4^{2-}	0.300 – 0.379

Source: Reprinted from *Journal of Membrane Science*, 642; B.M. Souza-Chaves, M.A. Alhussaini, V. Felix, L.K. Presson, W.Q. Betancourt, K.L. Hickenbottom, and A. Achilli; "Extending the Life of Water Reuse Reverse Osmosis Membranes Using Chlorination"; Pages 119897; Copyright (2022), with permission from Elsevier.

The rejection efficiencies of most of the parameters evaluated at FAT in Facility A were relatively similar; nevertheless, average rejection efficiencies of ATP, Region V of the regional integrated total fluorescence, and DOC fraction A were slightly higher. These measurements, occasionally reached up to 4-log_{10} rejection efficiency, particularly for ATP with an average of 2.38±0.76-log_{10}. The rejection efficiency for Region V corresponded to 2.26±0.43-log_{10} and 2.22±0.85-log_{10} for DOC fraction A. Changes in size distribution of organic molecules towards lower molecular weight fractions and decrease in fluorescence intensity from tertiary to advanced treatment was consistent with the rejection mechanisms of soluble contaminants in water purification processes associated with non-porous membranes.

ATP measurements of environmental water samples provide information as to the physiological activity state of microbial communities. Higher ATP levels indicate robust microbial metabolism, as well as high cell numbers and healthy communities. In contrast, low ATP levels point to lower population numbers or possibly communities under metabolic stress. Reductions of ATP and microbial equivalents by the advanced treatment process can thus be translated into treatment performance. These results indicate that non-viral surrogates that target reductions of soluble organic compounds and microbial activity were able to achieve a level of resolution close or similar to LRV of most but not all of the human enteric viruses and virus indicators. However, there were no statistically significant correlations among the reductions of viruses and physicochemical parameters (non-viral or non-microbial surrogates) evaluated at full advanced treatment.

Another important point is that at full-scale advanced treatment the rejection efficiencies evaluated of these parameters by the integrated membrane system were noticeable consistent as indicated by the corresponding RSD, thereby reflecting the overall stability of the advanced treatment process: fraction C+D, RSD: 7%; size exclusion chromatographs and DOC concentrations, RSD: 4%; Turbidity, RSD: 9%;, TDS, RSD: 6%; UV254, RSD: 11%. The rejection efficiency of divalent ions also showed low RSD for Sodium (RSD: 0.1%) Magnesium (RSD: 4%) Sulfates (RSD: 3%), Calcium (RSD: 7%), and Chloride (RSD: 12%).

3.2 Virus Indicators of Physical Treatment: Facility B (Soil-Aquifer Treatment)

3.2.1 Virus Concentrations and Frequencies of Detection

From April 2021 to June 2022, recharged groundwater from two extraction wells were collected from Facility B, the recharge basin located in Tucson, Arizona, on sampling events that accounted for any differences in seasonality and recharge patterns. Tertiary effluent and plant influent were also collected to evaluate virus indicator occurrence and removal by the advanced tertiary treatment process that provides Class A+ recycled water for infiltration, which has been evaluated in previous studies (Betancourt et al., 2014; Morrison et al., 2020; Schmitz et al., 2016). As previously reported (Morrison et al., 2020; Quanrud et al., 2003), specific information regarding travel time from basin discharge to well head could not be estimated and implemented into the sampling scheme due to the complexities of the pumping configurations as well as the presence of impermeable clay lenses located throughout the vadose zone. Moreover, the recharge facility contains several production and monitoring wells, with many production wells pumping concurrently during peak months, which can interfere with groundwater travel times and trajectories. Operational parameters and characteristics associated with the two extraction wells such as changes in DOC concentrations upon treatment, infiltration rates, wetting/drying cycles, average depth to groundwater, and quantity of water discharged into the basins were considered along with virus removal to determine whether statistically significant correlations could be elucidated.

Table 3.10, 3.11, 3.12, 3.13, and 3.14 lists the frequencies of detection and log concentrations per liter (log GC/L) of human enteric viruses and virus surrogates in samples of raw wastewater, tertiary treated effluent, and recharged groundwater from extraction wells 006A and 008A. Both human enteric viruses and virus surrogates were found in raw wastewater and tertiary treated recycled water at relatively similar frequencies and concentrations to those observed in Facility A, both located in Arizona.

The highest concentration of human enteric viruses found in raw wastewater (6-$\log_{10}$ GC/L) corresponded to Aichi virus, Human Bocavirus and Norovirus GI and GII plus five virus surrogates (crAssphage, WCDV1, PMMoV, somatic and male specific coliphages). Concentrations of 5-log GC/L were observed for the rest of the viruses. After tertiary treatment, LRV of 2-$\log_{10}$ to 3-$\log_{10}$ were demonstrated among all viruses with concentrations between 4-$\log_{10}$ and 6-$\log_{10}$ GC/L still present in tertiary treated recycled water. Although the concentrations of viruses found in this study were relatively similar to concentrations reported in a previous study, there was a marked difference in the frequency of detection and in the proportion of potentially viable viruses as determined by the nuclease treatment assay. Variations in absolute numbers of virus genome copies by dPCR were observed when comparing nuclease–treated versus untreated samples prior to viral nucleic acid extraction. For some viruses, there was a 10 to 100-fold decrease in levels of genome copies associated with nuclease-treated samples that was not observed among all the viruses present in the same sample. These results demonstrated that the application of nuclease treatment prior to viral nucleic acid extraction yielded variable concentrations of viruses across samples, thereby

indicating the proportion of viruses with intact or degraded capsids varied on a virus-to virus and on a sample-to-sample basis.

Table 3-10. Frequencies of Detection of Human Enteric Viruses and Virus Surrogates in Raw Wastewater (RW), Recycled Water, and Recharged Groundwater in Extraction Wells 006A and 008A.

Virus	RW	SW	EW-006A	EW-008A
Aichi virus	78	78	11	11
crAssphage	100	67	11	11
WCDV1	67	33	11	
WCDV2	11	11		11
WCDV3	44	44		
Norovirus GI	67	33		11
Norovirus GII	67	33	11	11
Human Bocavirus	78	56		
Hudisavirus	100	50		
Adenovirus	78	33		
Reovirus	56	22		22
Enterovirus	89	22	11	11
PMMoV	100	100	22	11
Somatic coliphages	100	86	14	29
Male-specific coliphages	100	100	37	25

Table 3-11. Log Concentrations Per Liter (GC-PFU Log_{10}/L) of Human Enteric Viruses and Virus Surrogates in Raw Wastewater: Facility B.

Virus	N	Minimum Log Concentration	Maximum Log Concentration	Mean Log Concentration	Std. Deviation	RSD
Aichivirus	7	3	6	5	0.7	16
crAssphage	9	5	7	6	0.5	9
WCDV1	6	4	6	5	0.9	19
WCDV2	1	6				
WCDV3	4	4	6	5	0.6	12
Norovirus GI	6	5	6	5	0.4	8
Norovirus GII	6	5	6	5	0.4	8
H. Bocavirus	7	3	6	5	0.9	18
Hudisavirus	6	4	5	5	0.4	9
Adenovirus	7	3	5	4	0.6	14
Reovirus	5	3	5	4	0.7	17
Enterovirus	8	4	5	4	0.4	8
PMMoV	9	5	6	5	0.7	13
Somatic coliphages	9	6	9	8	0.7	9
Male specific	9	6	7	6	0.5	7

RSD: Relative standard deviation

Table 3-12. Log Concentrations Per Liter (GC-PFU Log_{10}/L) of Human Enteric Viruses and Virus Surrogates in Tertiary Treated Recycled Water: Facility B.

Virus	N	Minimum Log Concentration	Maximum Log Concentration	Mean Log Concentration	Std. Deviation	RSD
Aichivirus	7	1	4	2	1	46
crAssphage	6	2	4	3	1	30
WCDV1	3	1	4	3	2	57
WCDV2	2	2	3	3	1	35
WCDV3	4	1	4	2	1	47
Norovirus GI	3	2	3	2	0.6	26
Norovirus GII	3	2	4	3	0.9	31
H. Bocavirus	5	2	4	2	0.8	47
Hudisavirus	2	2	3	3	1	25
Adenovirus	3	2	4	2	1	45
Reovirus	2	1	2	2	0.2	13
Enterovirus	2	2	2	2	0.3	18
PMMoV	9	1	5	3	1	35
Somatic	9	1	2	1	0.4	30
Male specific	9	1	2	1	0.2	20

Table 3-13. Log Concentrations Per Liter (GC-PFU Log_{10}/L) of Human Enteric Viruses and Virus Surrogates in Recharged Groundwater from Extraction Well 006A: Facility B.

Virus	N	Minimum Log Concentration	Maximum Log Concentration	Mean Log Concentration	Std. Deviation	RSD
Aichivirus	1	0	.1			
crAssphage	1	0	.2			
WCDV1	1	1				
WCDV2						
WCDV3						
Norovirus GI						
Norovirus GII	1		-.2			
H. Bocavirus						
Hudisavirus						
Adenovirus						
Reovirus						
Enterovirus	1	-.05				
PMMoV	2	.6	2	1	1	82
Somatic coliphages	1	.2				
Male specific	3	0.04	0.04	1	0.4	57

Table 3-14. Log Concentrations Per Liter (GC-PFU Log_{10}/L) of Human Enteric Viruses and Virus Surrogates in Recharged Groundwater from Extraction Well 008A: Facility B.

Virus	N	Minimum Log Concentration	Maximum Log Concentration	Mean Log Concentration	Std. Deviation	RSD
Aichivirus	1		1			
crAssphage	1		2			
WCDV1	1		1			
WCDV2	1		+[a]			
WCDV3						
Norovirus GI	1		+[a]	1		
Norovirus GII	1		+[a]			
H. Bocavirus						
Hudisavirus						
Adenovirus						
Reovirus	2	+[a]	-.3	-.7	.4	143
Enterovirus	1		1			
PMMoV	1		1			
Somatic	2	-.2	-.06	.2	.1	67
Male specific	3	-.1	.3	.06	.2	300

[a] Close to the ALoD

Upon soil-aquifer treatment, seven of the thirteen virus indicators were present in EW-006A while ten out of the thirteen virus indicators were present in EW-008A. Attempts were made to sample the same effluent as it traveled from the basins to the extraction wells, which corresponds to synoptic sampling. However, due to COVID-19 restrictions sampling was adjusted to one day for the three sampling locations within the recharge basin.

Despite the infrequent detection of human enteric viruses from extraction wells 006A and 008A, multiple detection of viruses was revealed in May 2021 from EW-008A associated with the newest set of recharge basins (RB-9, RB-10, and RB-11). Reovirus, Enterovirus, Norovirus GI and GII were detected in recharged groundwater from this site at concentrations ranging from 0.01-log_{10} to 1-log_{10} GC/L. Samples collected from EW-006A associated with the oldest set of recharge basins (RB-5, RB-006, RB-007, RB-008) were found positive for Enterovirus, Aichi virus, and Norovirus GI in at least one of the nine samples evaluated from this site. The highest virus concentrations from EW-006A ranged from 0.6-log_{10} to 1-log_{10} GC/L. None of the recharged groundwater samples showed evidence of Human Bocavirus or Adenovirus.

Among the virus surrogates, PMMoV was the only virus concomitantly detected with human enteric viruses in recharged groundwater from both extraction wells at concentrations ranging from 1-log_{10} to 2-log_{10} GC/L. Somatic and male specific coliphages were found simultaneously at slightly different concentrations in EW-006A and EW-008A with higher levels corresponding to male specific coliphages, however these concentrations were relatively low and expressed as per 100 L to calculate meaningful log concentrations. Log concentrations per 100 liter of somatic coliphages in EW-006A were in the order of 0.03-log_{10} PFU/100 L ($1.1x10^0$ PFU/100 L). Two of the nine samples from EW-006A were not analyzed for somatic coliphages due to the inability of the host to grow in the liquid media. Male specific coliphages were detected at log concentrations per 100 liter ranging from 0.04-log_{10} to 1-log_{10}. EW-008A also showed evidence of both somatic and male specific coliphages at relatively similar concentrations described for

EW-006A. A similar outcome was associated with crAssphage found only once in each well, however at a higher concentration close to $2\log_{10}$ GC/L. Hudisavirus, WCDV1, and WCDV3 were never present in recharged groundwater, while WCDV2 was detected once from EW-008A close to the ALoD.

Previous studies demonstrated the presence of Aichi virus, PMMoV, and crAssphage in EW-008A, which is associated with the set of recharge basins that have been in operation for less than ten years (Morrison et al., 2020). EW-008 has exclusively received Class A+ tertiary treated recycled water. EW-006A had not been the subject of investigations on virus occurrence and was selected for this study due to ease access and to its location down gradient of RB-5 in operation for over 25 years. Differences in the quantity of water discharged to each basin, dynamic of wet and dry cycles, infiltration rates, and size of each basin area were the operational parameters used for selection of these two extraction wells. According to Hydrologists who have developed groundwater-flow models across the State of Arizona, there are no good pair wells with nearby deep and shallow sampling areas at the recharge facility.

Contrary to our previous studies at this MAR site (Betancourt et al., 2014; Morrison et al., 2020) wetting/drying cycles and infiltration rates were not linked to virus removal during SAT of recycled water, more likely due to differences in operational parameters associated with these and previous sampling campaigns. Studies conducted at this facility have demonstrated that fate and transport processes are complicated by the vadose zone and dynamic recharge operations at managed aquifer recharge sites. Up to 57,000 m^3/day plus extra water not required during times of low demand is used to fill the recharge basins (Cáñez et al., 2021).

Basins at the recharge facility operate under different wet and dry cycles in order to maintain high infiltration rates. Infiltration rates at the recharge basins have averaged approximately 0.7 m/day under full-scale operation with infiltration rates of 0.19-0.2 m/day and 0.34-0.49 m/day for RB-9 (EW-008A) and RB-5 (EW-006A), respectively. These rates may vary due to rainfall impact. In this case, the differences in infiltration rates of these two individual basins do not explain the relatively more frequent detection of human enteric viruses from EW-008A (RB-9) than from EW-006A (RB-5). As demonstrated in previous studies, higher infiltration rates facilitate greater virus transport due to reduction in retention time (Betancourt et al., 2019). Moreover, during the sampling campaign of 2021, basins 5 through 8 (EW-006A) had a wet to dry ratio of 3 days wet followed by 27 dry days while basins 9 through 11 (EW-008A) were operating under a mixed wet and dry cycle plan due to failures of hydrological monitoring equipment. RB-9 and RB-10 operated under an extended dry cycle of no recharge for up to three months preceding the sampling event with multiple detection of viruses and two additional months after that. In January 2021, RB-9 received the largest volume of tertiary effluent (4.2 million liters) among all the basins. RB-11 was operating at a variable wet to dry ratio of 8 to 13 wet days followed by 17 to 22 dry days with most of the recharge operations occurring at this basin during March, April, May, June and July 2021. Infiltration rates at RB-11 were similar to infiltration rates that occurred at RB-5 and therefore virus transport within adjacent recharge basins is one among the different mechanisms that could explain the presence of multiple viruses in groundwater extracted from EW-008A (RB-9) under specific operating conditions. RB-11 (EW-008A) was receiving the largest volumes of effluent during

recharge operations in March, April, and May 2021 at infiltration rates equal or greater than those measured for RB-5 (EW-006A). Groundwater flow in the recharged basins is complex due to production wells pumping at any given time. EW-008A (RB-9 through 11) captures water at a deeper depth than EW-006A (RB-5 through 8). The basins consist of mostly gravel and sand, however, clay lenses are present below all of recharge sites but mostly deeper below the newest basins (RB-9, RB-10, RB-11) (Cáñez et al., 2021; Quanrud et al., 2003).

Previous studies demonstrated the role of travel and residence time in the reduction efficiency of viruses at full-scale MAR systems. Longer residence and travel times (>15 days) exceeded 2.8-$\log_{10}$ reduction of viruses with an order of one log in less than a day of travel time during SAT of recycled water (Betancourt et al., 2014). These two operational parameters did not differ between the two recharge basins surveyed for indicator viruses in this project. Studies have shown that the fate and transport of contaminants in the subsurface environment is affected by a combination of physical and chemical processes (Bradford et al., 2017), with infiltration contributing to the distribution of contaminants in groundwater under site-specific mechanisms capable of spreading the contamination (Schijven and Hassanizadeh, 2000). Virus removal in MAR systems, as previously discussed, results from the interaction of two major mechanisms, adsorption and inactivation, in which virus adsorption plays a key role (Bradford et al., 2017; Schijven and Hassanizadeh, 2000). Virus adsorption processes to solid-water interfaces are also governed by the physicochemical properties of the viruses which are highly heterogeneous and diverse (Armanious and Mezzenga, 2022) and therefore virus-type-dependent (John and Rose, 2005).

Figure 3.11 displays the LRV of human enteric viruses and virus surrogates from raw wastewater to tertiary effluent of Facility B. The LRV of the different viruses could be determined if the virus was detected in the recycled water used for recharge. Determination of the degree of virus occurrence and reduction at full-scale MAR systems requires the combination of multiple components: (i) the ability to determine the concentration of the viruses in water before and after recharge operations, (ii) the volume of concentrate assayed from both water matrices, (iii) the recovery efficiency of the methods for virus concentration, and (iv) the sensitivity of the method applied for virus detection. The highest LRV of viruses from raw wastewater to tertiary treated water were observed for somatic and male specific coliphages corresponding to 6-$\log_{10}$ to 9-$\log_{10}$ PFU/L. LRV of human enteric viruses and the rest of the virus surrogates were 3 to 8 orders of magnitude lower than the coliphages, ranging from 1-$\log_{10}$ to 4-$\log_{10}$ for human enteric viruses, 1-$\log_{10}$ to 3-$\log_{10}$ for WCDV viruses plus Hudisavirus, and 1-$\log_{10}$ to 5-$\log_{10}$ for crAssphage and PMMoV, respectively.

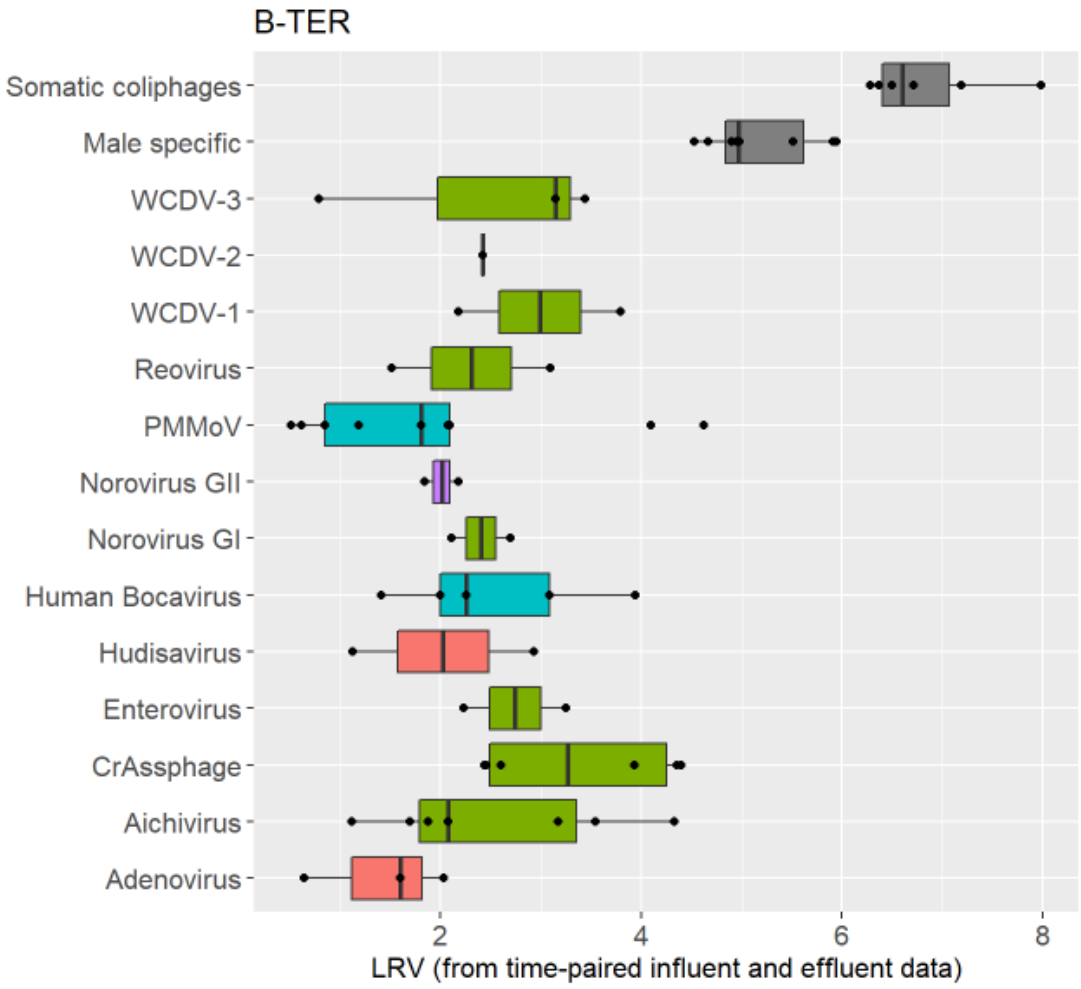


Figure 3-11. Log Reduction Values of Human Enteric Viruses and Virus Surrogates from Raw Wastewater to Tertiary Treated Effluent: Facility B (dark dots: LRV, solid vertical line: median, box: quartiles, whiskers: minimum and maximum LRV)**.**

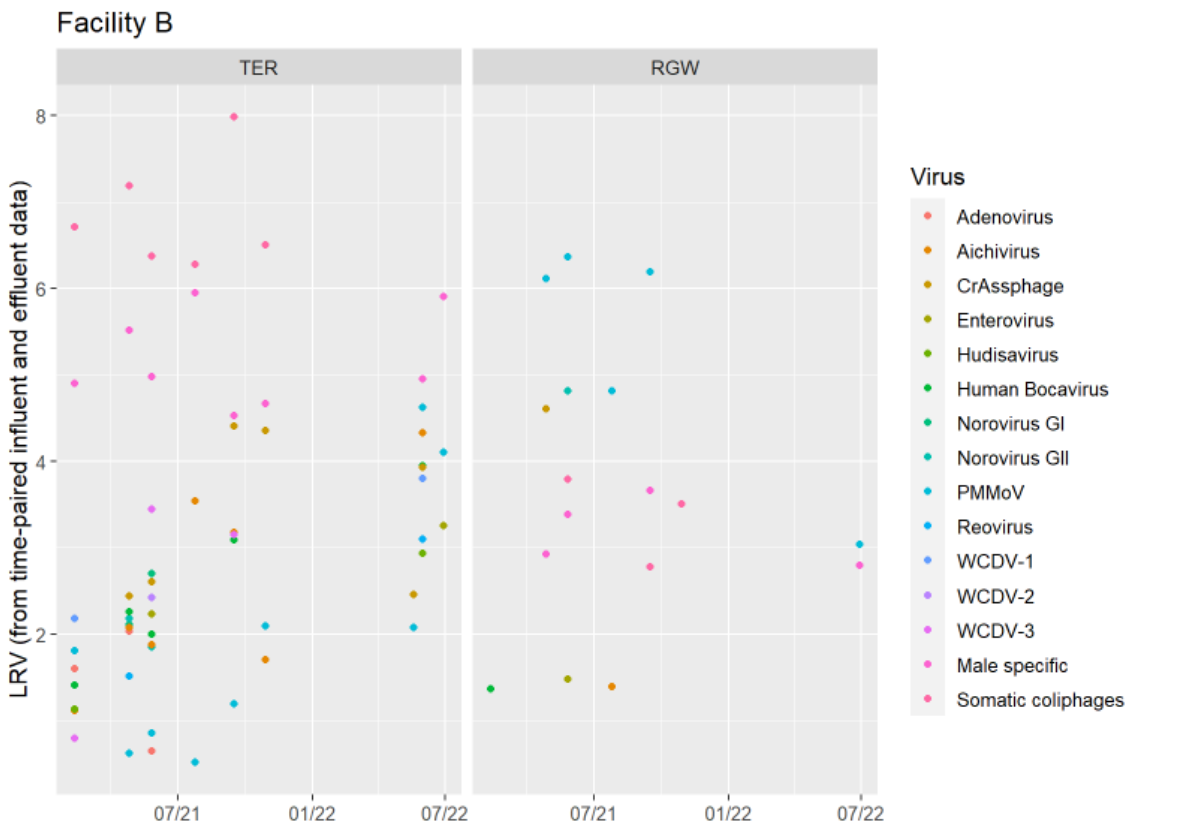


Figure 3-12. Log Reduction Values of Human Enteric Viruses and Virus Surrogates from Tertiary Treated Effluent to Recharged Groundwater from EW-006A and EW-008A: Facility B.

Four enteric viruses (Enterovirus, Reovirus, Norovirus GI and GII) were detected in recharged groundwater from EW-008A, which is associated with the newer set of recharge basins. Three human enteric viruses (Enterovirus, Norovirus GII, and Aichi virus) were detected in recharged groundwater from EW-006A, which is associated with an older set of recharge basins. LRVs of human enteric viruses from tertiary to recharged groundwater were one to two orders of magnitude different among the four viruses detected at these sites (Figure 3.12). For Enterovirus a LRV of 1-$\log_{10}$ was observed from tertiary to recharged groundwater while Aichi virus and Norovirus GII showed LRVs of 0.2-$\log_{10}$ and 3-$\log_{10}$, respectively. Norovirus GI and GII were both recovered from recharged groundwater with LRVs of 3-$\log_{10}$ and 4-$\log_{10}$ for Norovirus GI and GII, respectively. Reovirus was detected only in recharged water from EW-008A at a concentration of 2-$\log_{10}$ GC/L with a LRV of 0.1-$\log_{10}$ from tertiary effluent. WCDV1, WCDV2, and WCDV3, corresponding to the single-stranded circular DNA viruses (i.e., CRESS viruses), were infrequently detected in tertiary effluent and in recharged groundwater either concurrently or individually. One or two of these WCDV viruses were recovered and detected from all the Facilities included in this study although at very low frequencies. The persistence of these viruses through wastewater treatment and the associated implications for safe and sustainable water reuse are topics that warrant further investigations.

Among the virus surrogates, PMMoV exhibited LRVs of 3-$\log_{10}$ to 4-$\log_{10}$ and was still detected, although infrequently (2 of 9 in EW-008A and 1 of 9 in EW-008A), in recharged groundwater at

concentrations of 2-$\log_{10}$ to 3-$\log_{10}$ GC/L. CrAssphage was detected only once in recharged groundwater from both extraction wells at a concentration of 2-$\log_{10}$ GC/L. Both detections occurred during the same sampling event, however crAssphage was not detected in tertiary effluent for that particular event and therefore there was no LRV associated. On average and considering the concentration of crAssphage detected in tertiary effluent, the LRV would have been in the order of 1-$\log_{10}$ to 2-$\log_{10}$.

Based on the linear regression model analysis, the LRV of male specific and somatic coliphages from raw wastewater to tertiary treatment was statistically significantly higher that the LRV of Adenovirus ($p = 0$) while the LRV of crAssphage was significantly higher ($p<0.01$) than that of Adenovirus. For the rest of the viruses, there was no statistically significant difference among LRVs with respect to Adenovirus.

In this study, nuclease-protected genomes of five human enteric viruses were present in recharged groundwater with the highest concentrations (2-$\log_{10}$ GC/L) associated with Reovirus in EW-008A. Reovirus has been the most frequently detected culturable viruses in groundwater sources in the United States, particularly in aquifers considered as low-risk sites (Fout et al., 2003; Johnson et al., 2011). Additional studies have also demonstrated the presence of infectious enteroviruses in deep confined aquifers, thereby indicating effective transport and survival of human pathogenic viruses in subsurface environments (Borchardt et al., 2007) which fulfills the criteria of these human viruses of public health significance as self-indicators to confirm physical treatment by SAT and related wastewater impacts on groundwater sources.

Given that these viruses were detected along with other enteric viruses (Noroviruses) without evidence of virus surrogates, LRV for enteric viruses in MAR systems may be more safely estimated using the enteric viral pathogens themselves. Somatic and male specific coliphages used as surrogate indicators of virus infectivity were more consistently detected at these sites but never in association with human enteric viruses. These findings warrant further investigations.

The consistency of detection of viruses was also evaluated in terms of the RSD with a RSD <30% indicating less variation in virus concentrations at different stages of water treatment. This threshold remained for most viruses from raw wastewater to recycled water, except for WCDV1 and WCDV3. Moreover, decreases in frequencies of detection upstream of SAT were observed for multiple virus indicators that were not necessarily associated with efficient removal after treatment.

One-thousandth-scale and one-hundredth-scale onsite experiments (N=2) were conducted in order to evaluate the recovery of viruses in recharged groundwater. The recovery efficiency of all viruses were above 20% with relatively fewer variations in virus recoveries as determined by the RSD. The recovery efficiency of Reovirus was 67% in 100 L and 29% in 1000 L of recharge water for an average recovery of 48±27% (N=2, RSD 56%). Porcine parvovirus was more efficiently recovered from this matrix than the rest of the viruses, with recovery efficiencies of 100% in 100 L and 57% in 1000 L for an average recovery of 79±30% (N=2, RSD 39%). Murine norovirus showed recovery efficiencies of 34% in 100 L and 20% in 1000 L for an average recovery of 27±10% (N=2, RSD 37%). The recovery efficiency of Coxsackievirus B5 was 56% in

100 L and 27% in 1000 L with an average recovery of 42±21% (N=2, RSD 49%). Finally, Adenovirus showed recoveries of 66% in 100 L and 32% in 1000 L with an average recovery of 49±24% (N=2, RSD 49%).

Large-volume sampling combined with absolute quantification of nuclease-protected virus genomes by digital PCR proved to be more applicable for investigations of virus reductions at potentially low-level virus contamination scenarios such as at full-scale MAR systems, particularly human enteric viruses, than real time PCR applied in previous studies (Betancourt et al., 2014; Morrison et al., 2020). A rigorous side-by-side comparison between the two detection approaches was not feasible due to the allocation of sample volumes solely for dPCR assays. However, an increase in the detection of virus genomes was observed while transitioning from real time PCR into dPCR, which was subsequently adopted to confirm physical removal of viruses by advanced treatment trains. QA/QC procedures for real time PCR quantification analysis are more demanding and costly, particularly for relative quantification of viral targets and internal controls based on the quantification of cycle (Cq) value observed in the amplification curves. Periodical evaluations of the PCR amplification efficiency require multiple standard curves throughout monitoring evaluations and PCR efficiencies determined from individual amplification curves are more appropriate in order to report accurate and reproducible quantitative results (Ruijter et al., 2021).

The degree of sample inhibition by dPCR detection of virus genomes in recharged groundwater was negligible and the extraction efficiencies of viral nucleic acid were consistently high (>60%). These conditions may perform differently in virus surveys from MAR systems in geographically distinct regions of the United States. These results indicate that variations in virus recovery efficiencies are site-specific as previously discussed in tertiary treated recycled water, which along with virus abundance in source waters upstream of the advanced treatment trains, virus resilience to treatment linked to viral structure (size, rigidity, isoelectric point, stability against pH, temperature, UV light, and susceptibility to various chemical agents including chlorine and ozone, solvents and detergents), sensitivity of the detection assay, and treatment plants' operational conditions define the outcome of the monitoring framework to determine virus occurrence and reduction in potable water reuse schemes viewed as environmental scenarios of low-level virus contamination.

3.2.2 Non-Microbial Surrogates of Advanced Treatment Performance: SAT

As previously addressed for Facility A, total living biomass measured by the adenosine triphosphate (ATP) content of microorganisms in water as well as total organic carbon (TOC, DOC), UV absorbance and total fluorescence were used to determine overall biological load. In addition, size exclusion chromatography in combination with organic carbon detection (SEC-OCD) was evaluated in order to monitor rejection of high molecular weight compounds by SAT. The reduction of fluorescence values along with TOC/DOC concentrations and UV_{254} absorbance detections were evaluated as potential bulk organic surrogates to determine correlations with virus reductions. Among the non-microbial parameters evaluated in this study, the analysis and comparison of major cations (Mg^{2+}, Ca^{2+}, Na^{+}, K^{+}) and anions (Cl^{-}, NO_3^{-}, and SO_4^{2-}) have been used to determine if the samples collected beneath and downstream of the test basin resulted from the same slug of infiltrating reclaimed water (WRRF-10-05). During

our sampling campaigns, SAT of recycled water resulted in the attenuation of only K^+ while the rest of the ions either increased in concentration or remained constant with no evidence of attenuation during infiltration of recycled water. Rejection efficiencies around 85% (0.83log) were revealed for K^+ from recharged groundwater collected from EW-006A and EW-008A. The paired sample Wilcoxon signed rank test was conducted in order to compare non-microbial parameters between extraction wells EW-006A and EW-008A before and after SAT. The results revealed statistical differences in the reduction values of Nitrate concentrations ($p = 0.021$), UV_{254} ($p = 0.024$) and Magnesium ($p = 0.00004$). In most cases, the reduction efficiencies of these parameters were slightly higher in EW-008A associated with the newest set of recharge basins.

Changes in organic carbon concentrations and attenuation during SAT were evaluated by SEC-DOC and by excitation-emission matrix (EEM) fluorescence spectroscopy. From tertiary treated recycled water and recharged groundwater SEC-OCD chromatograms (Figure 3.13), the dissolved organic matter (DOM) size distribution changed towards lower molecular weight from tertiary effluent to recharged water due to increased removal of high molecular weight compounds.

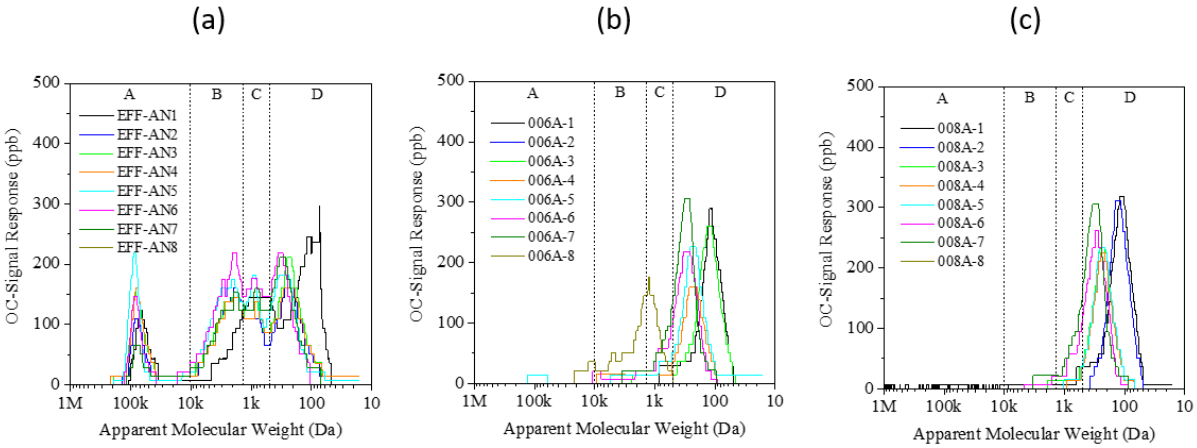


Figure 3-13. Size Exclusion Chromatography – Organic Carbon Detector (SEC-OCD) Fingerprints for Tertiary Effluent and Recharged Groundwater: Facility B.

In Figure 3.13, (a) tertiary effluent (EFF-AN1 through 8), (b) recharged groundwater from EW-006A, (c) recharged groundwater from EW-008A. Fraction A (AMW >10 kDa): proteinaceous biopolymers; Fraction B (10 kDa > AMW > 1.5 kDa): Humic substances; Fraction C (1.5 kDa > AMW > 0.5 kDa): Building blocks of humic substances; Fraction D (AMW < 0.5 kDa): Low molecular weight acids and neutrals. High molecular weight fractions (fraction A and B) are associated with tertiary effluent (EFF) and UF permeate (UFP), while low molecular weight acids and neutrals (fraction C+D) are predominant in recharged groundwater (extraction wells 006A and 008A). This trend was consistent over the course of the monitoring evaluation. The total DOC progressively decreases from tertiary treated wastewater (6 – 7 mg/L) to recharge groundwater (2 – 3 mg/L) in which concentrations were predominantly below 1.5 mg/L as determined by dissolved organic carbon concentrations calculated from SEC chromatograms (Figure 3.14). The latter DOC fraction was largely composed of low molecular weight dissolved

organic compounds (<1 kDa). The data displayed in Figure 3.14 also includes for comparison purposes DOC concentrations from SEC chromatograms from ultrafiltration permeate (UFP) and reverse osmosis permeate (ROP) derived from the same source water (tertiary treated effluent). Rejection efficiencies of total DOC concentrations ranged from 35% to 92% (equivalent to 0.2-log_{10} and 1.1-log_{10}, respectively). Rejection efficiencies of organic compounds associated with fraction A ranged from 90% to 99% (1-log_{10} to 2-log_{10}) while rejection efficiencies of components from fractions B and C+D ranged from 69% to 72% (0.5-log_{10} to 0.6-log_{10}) and from 50% to 87% (0.3-log_{10} to 0.9-log_{10}), respectively.

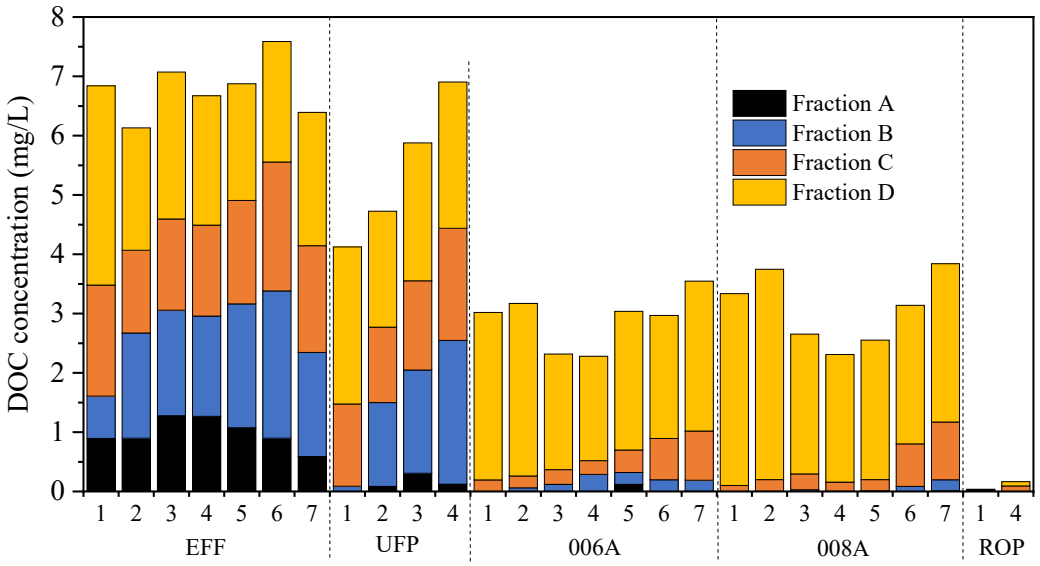


Figure 3-14. Dissolved Organic Carbon Concentrations Calculated from SEC Chromatograms from Tertiary Effluent (EFF), Ultrafiltration Permeate (UFP), Recharged Groundwater from Extraction Wells 006A and 008A, and Reverse Osmosis Permeate (ROP) from the UF/RO Engineering-Scale System Operating at the UArizona's WEST Center.

Figures 3.15 and 3.16 display the EEM fluorescence spectra (Fig. 3.15) and the regional/total fluorescence intensities (Fig. 3.15) from tertiary effluent to recharged groundwater. The contribution of microbial byproduct-like and humic acid-like fractions to the organic carbon content is high for tertiary treated effluent (EFF) in all samples, but it is minimal for recharged groundwater, ultrafiltration permeate (UFP) and reverse osmosis permeate (ROP) included in this Figure for comparison purposes since these waters all derived from the same source used for recharge. The results indicate a 1.1-fold decrease of total fluorescence from final effluent (EFF) to UFP, followed by a 7.1-fold decrease for 006A, 10.9-fold decrease for 008A, and a 22-fold decrease for ROP. The SEC-OCD chromatograms support the results of EEM fluorescence, which are consistent with the UVA_{254} and DOC results, where the UVA_{254} and DOC results for final effluent are in average, 9.5-fold and 6-fold higher, respectively, when compared to recharged groundwater.

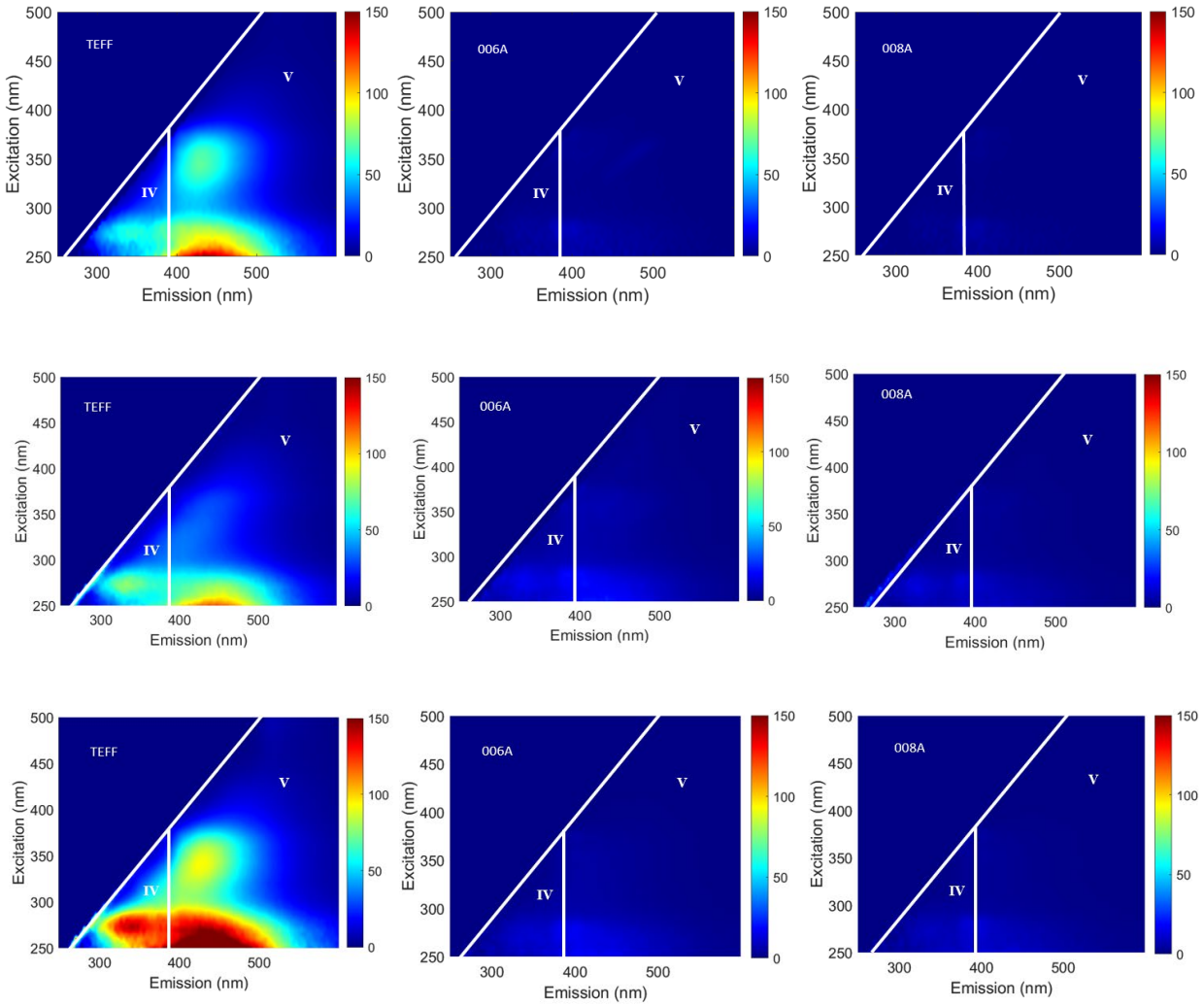


Figure 3-15. Excitation-Emission Matrix (EEM) Fluorescence Spectra Examples of Tertiary Effluent Samples (TEFF) and Recharged Groundwater from Extraction Wells 006A and 008A. (Region IV: Soluble microbial byproduct-like matter; Region V: Humic acid-like matter).

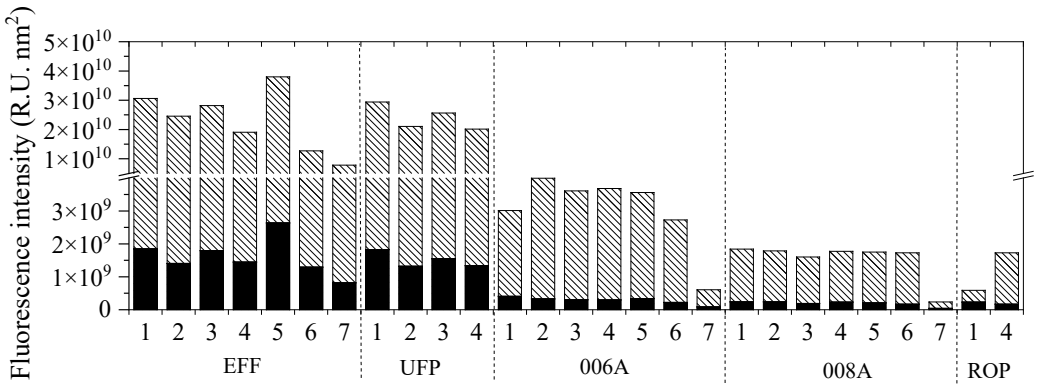


Figure 3-16. Regional and Total Fluorescence Intensities of Tertiary Effluent (EFF), Ultrafiltration Permeate (UFP), Reverse Osmosis Permeate (ROP), and Recharged Groundwater from Extraction Wells 006A and 008A. (Black bar: Region IV (soluble microbial byproduct-like matter) and Dashed bar: Region V (Humic acid-like matter). R.U. = Raman units).

Electrical conductivity and TDS concentrations did not show any substantial changes as recycled water infiltrated through the soil (Table 3.15) while the attenuation of water turbidity was variable and relatively low with rejection efficiencies between 3% and 72% (equivalent to 0.01-log_{10} and 0.56-log_{10}). ATP measurements from recycled water through recharged groundwater demonstrated rejection efficiencies between 79% and 89% (equivalent to 0.7-log_{10} to 0.9-log_{10}) from EW-006A and between 63% and 80% (equivalent to 0.4-log_{10} and 0.7-log_{10}) from EW-008A.

Correlation matrices were generated to analyze the relationships, both presence and reductions, among viruses and non-microbial surrogates and among non-microbial surrogates themselves at the advanced treatment level. The strongest correlations were observed for parameters expected to be correlated as they are derived from the same measurements, for instance reductions of Region IV-V and total fluorescence or reductions of ions. No significant correlations were revealed among viruses and non-microbial parameters at the advanced treatment trains, more likely associated with the infrequent detection of viruses at this level.

Table 3-15. Water Quality Parameters for Samples Collected from Tertiary Treated Effluent Water (TEFF) and Recharged Groundwater from Extraction Wells 006A and 008A.

Sampling location	Date of sample collection	pH	Temperature Celsius	EC µS/cm	TDS mg/L	Turbidity NTU
TEFF	4/23/2021	6.96	7.0	1273	n.a.	1.06
EW-006A	4/26/2021	6.98	9.3	1194	n.a.	0.80
EW-008A	4/26/2021	7.05	9.3	1274	n.a.	0.18
TEFF	5/28/2021	8.12	9.5	1284	677	0.96
EW-006A	5/26/2021	7.10	11.5	1250	676	0.28
EW-008A	5/26/2021	7.10	11.4	1258	669	0.22
TEFF	7/28/2021	7.21	11.0	1417	705	1.36
EW-006A	7/27/2021	7.15	10.7	1444	720	0.30
EW-008A	7/27/2021	7.10	10.5	1473	741	0.43
TEFF	9/16/2021	7.29	10.5	1210	437	0.79
EW-006A	9/16/2021	7.37	9.0	654	429	0.65
EW-008A	9/16/2021	7.40	9.4	712	457	0.45
TEFF	10/29/2021	7.31	6.4	599	622	0.60
EW-006A	10/29/2021	7.23	7.3	695	704	0.24
EW-008A	10/29/2021	7.23	9.6	781	753	0.26
TEFF	2/11/2022	6.93	22.5	757	379	1.44
EW-006A	2/11/2022	6.73	21.3	727	363	0.16
EW-008A	2/11/2022	6.85	21.2	736	368	1.39
TEFF	6/29/2022	7.88	21.1	1290	839	1.06
EW-006A	6/29/2022	7.38	21.1	1212	788	0.21
EW-008A	6/29/2022	7.79	21.1	1274	828	0.24
TEFF	6/1/2022	7.50	14.4	1454	947	0.82
EW-006A	6/1/2022	7.21	14.6	1186	772	0.18
EW-008A	6/1/2022	7.33	14.8	1309	853	0.28

n.a.: not available

3.3 Virus Indicators of Physical Treatment and Non-microbial Surrogates: Facility C (Riverbank Filtration-Aquifer Recharge and Recovery-BAC)

Facility C combines two natural treatment processes and multiple engineered advanced treatment trains to generate product water for potable reuse. As discussed previously, there were ten sampling events planned for this facility, however only three out of five sampling events were considered for analyses due to repeated delays of transportation of the samples that exceeded the recommended holding time (>72 hours) for initial processing. In addition, the small number of sampling events from this facility precluded the application of a meaningful statistical analysis for making statistical inferences.

Figure 3.17 displays the log concentrations of viruses present from raw wastewater to advanced treatment in Facility C. Only one sample of raw wastewater was shipped for analysis of viruses. Detections of viruses from two or more samples of advanced treated water were averaged (geometric mean) for estimations of LRVs. Aichi virus, Reovirus, and Human Bocavirus were recovered and detected from the advanced treatment trains. For most human enteric viruses, two or more detection events were associated with similar log concentrations. Coliphages and PMMoV were the only virus surrogates present in advanced treatment trains, particularly coliphages that were present twice in BAC product water along with Human Bocavirus.

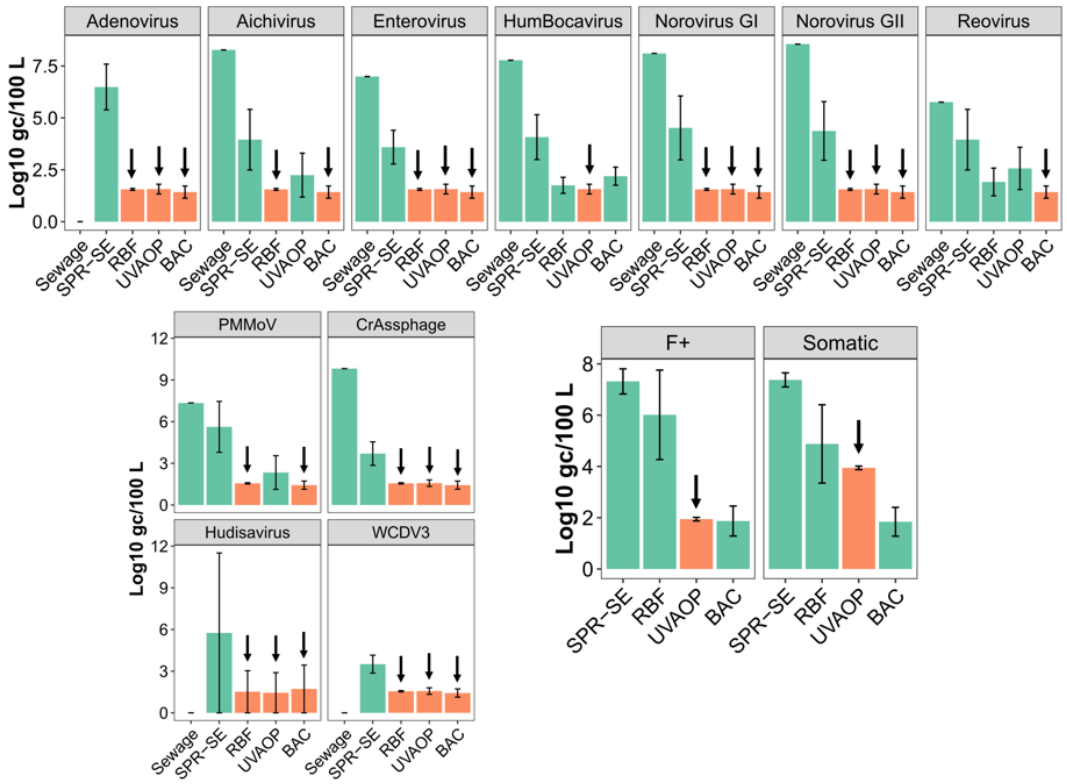
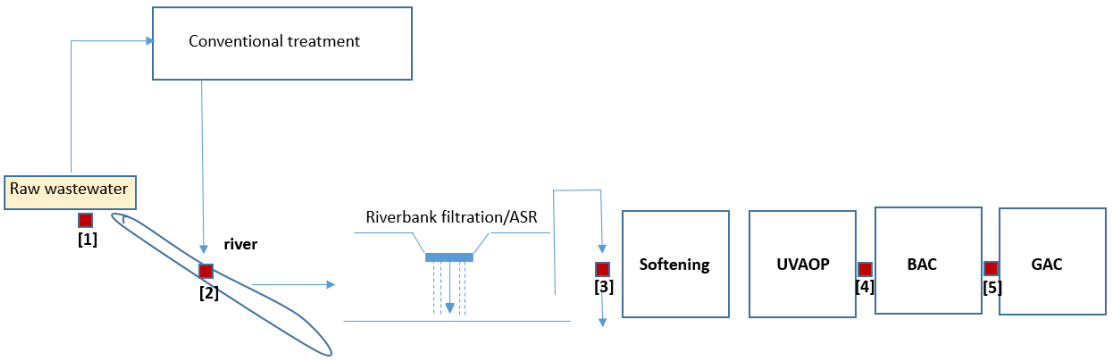


Figure 3-17. Average Concentrations (Log_{10} Genome Copies Per 100 L) of Human Enteric Viruses and Virus Surrogates in Treatment Trains from Facility C.

High concentrations of human enteric viruses and virus surrogates in raw wastewater or efficiencies in reduction upstream of FAT were not necessarily associated with the detection of viruses across the advanced treatment trains. Log reduction values of human enteric viruses and virus surrogates are summarized in Figures 3.18 and 3.19. More extensive sampling would more likely have revealed the presence of other human enteric viruses from carbon-based advanced treatment trains (BAC/BAF and GAC) where either some removal or no removal of viruses is anticipated. However, virus surveys in FAT based on the detection of multiple viruses, although a matter of significant relevance, are limited by the specific requirements for each virus type. For instance, enhanced detection of Reovirus viral RNA was achieved in this study by the application of a heat shock treatment (110 °C for 5 min) prior to RT-dPCR as demonstrated for other dsRNA viruses (Gendron et al., 2010). This step required the allocation of at least 20 µl of viral RNA for dsRNA denaturation, which increased the volume of water concentrates (consequently the cost) for analyses of higher equivalent volumes for each virus. In addition, carbon-based treatment trains as evidenced in this study, do not demonstrate high rejection efficiencies of organic compounds that may interfere with the methods for primary concentration of viruses.

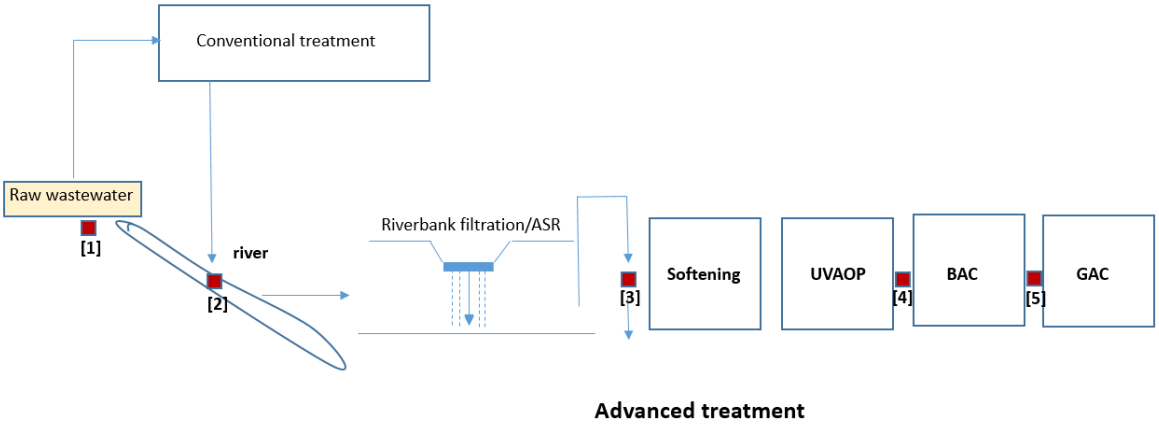
On site experiments for virus recovery efficiencies were not conducted at this Facility. Two of the sampling trips proposed for virus spikes were postponed and finally canceled due to travel restrictions from COVID-19. Notwithstanding, samples from all the advanced treatment trains required from 1:10 to 1:100 dilutions for better resolution of partitions by dPCR. The extraction efficiencies of viral nucleic acids for these samples varied from 0.7% to 60%, which indicated the potential losses of nucleic acids and interferences during extractions more likely due to the presence of high loads of particulate and dissolved organic carbon as demonstrated by EEM and SEC-DOC fingerprints, which can also affect the UV disinfection process for virus inactivation.



Virus	Log_{10} GC/L	Log_{10} GC/L	Log_{10} GC/L	Log GC/L	LRV
Aichi virus					
6.3 (1)	4.1 (1)	<ALoD	1.4 (1) NR	<ALoD	>5
Adenovirus					
5.1 (1)	4.5 (4)	<ALoD	<ALoD	<ALoD	>4
Norovirus GI					
6.1 (1)	3.8 (2)	<ALoD	<ALoD	<ALoD	>4
Norovirus GII					
6.5 (1)	3.5 (2)	<ALoD	<ALoD	<ALoD	>3
Enterovirus					
4.9 (1)	2.8 (1)	<ALoD	<ALoD	<ALoD	>2
Reovirus					
3.7 (1)	4.1 (1)	0.7 (1)	1.1 (2) NR	<ALoD	>3
Human Bocavirus					
5.7 (1)	2.4 (3)	0.2 (1)	<ALoD	0.4 (1) NR	5

<ALoD: less than the assay limit of detection; NR: no removal

Figure 3-18. Schematic of Human Enteric Virus Concentrations (GC/L) and Overall Log Reduction Values for Advanced Treatment Trains at Facility C (number of detections are shown in parenthesis).



Virus	Log_{10} GC/L	Log_{10} GC/L	Log_{10} GC/L	Log GC/L	LRV
CrAssphage					
7.8 (1)	2.2(1)	<ALoD	<ALoD	<ALoD	>6
WCDV3					
3.3 (1)	2.5 (1)	<ALoD	<ALoD	<ALoD	>1
Hudisavirus					
4.6 (1)	3.7 (1)	<ALoD	<ALoD	<ALoD	>1
PMMoV					
5.3 (1)	4.4 (2)	<ALoD	1.7 (1)	<ALoD	>3.6
Somatic* coliphages					
NAs	5.4 (4)	4.6 (1)	<ALoD	0.1 (1)	5
Male specific*					
NAs	5.3 (4)	5.0 (2)	<ALoD	0.2(2)	5

*There was no sample volume available for the assessment of coliphages. <ALoD: less than the assay limit of detection; NA: not applicable; NAs: not assayed; WCDV2 and WCDV3 were not assayed.

Figure 3-19. Schematic of Virus Surrogate Concentrations (GC/L) and Overall LRV of Viruses for Advanced Treatment Trains at Facility C (number of detections are shown in parenthesis).

In a previous study, Reovirus was the only virus detected from the combined riverbank filtered water (the mixture of water from production wells) that is conveyed to surface spreading basins for infiltration as part of the aquifer storage recharge and recovery treatment process (Betancourt et al., 2014). The ASR location was the last treatment train evaluated in our previous study. Our current study demonstrated that Reovirus traveled further down the advanced treatment trains where other viruses of public health significance such as Aichi virus and Human Bocavirus were also found.

With respect to the non-microbial surrogates, SEC analysis from samples collected at Facility C in Colorado demonstrated that UVAOP and BAC treatments did not remove organic contents efficiently. Dissolved organic concentration of the river water ranged from 3.5 to 5.7 mg/L and the DOC of BAC treated water was on average 3.0 mg/L (SD±0.6), which indicated a low DOC removal that varied from 37% to 58% (equivalent to 0.2-log_{10} to 0.4-log_{10} mg/L). The SEC chromatograms also revealed that the high molecular weight fraction (fraction A) detected in river water was removed along the advanced treatment train, based on a decrease in fractions

B and C. Rejection efficiencies of fraction A ranged from 62% to 99% (equivalent to 0.4-$\log_{10}$ and 2.5-$\log_{10}$) while rejection efficiencies of fractions B and C+D ranged between 46% and 54% (B: equivalent to 0.3-$\log_{10}$) and between 10% and 46% (C+D: 0.04-$\log_{10}$ and 0.3-$\log_{10}$). However, an increase in fraction D occurred after RBF, UVAOP and BAC treatment, which may be related to the addition of small organic particles during the RBF process, indicating that these contaminants are not removed by UVAOP and BAC processes (Figure 3.20 and 3.21).

Figure 3.22 displays the EEM fluorescence spectra for water samples collected from sampling locations at Facility C. Changes in the regional/total fluorescence intensities from river water to BAC product water were observed. River water and water after riverbank filtration displayed the highest fluorescence intensity, and a decrease in both regions was observed during the water purification process. These results indicate that a proportion of both microbial byproducts-like (Region IV) and humic-acid-like (Region V) moieties are reduced but not efficiently by the treated stages. The rejection efficiencies of TF ranged from 53% to 73% (equivalent to 0.3-$\log_{10}$ to 0.6-$\log_{10}$). Region IV displayed a much higher rejection efficiency corresponding to 63% and 81% (equivalent to 0.4-$\log_{10}$ and 0.7-$\log_{10}$) while Region V showed rejection efficiencies between 52% and 71% (equivalent to 0.3-$\log_{10}$ and 0.5-$\log_{10}$).

The reduction efficiencies of ATP ranged from 73% to 96% (equivalent to 0.6-$\log_{10}$ and 1.2-$\log_{10}$) from river water to BAC product water while the reduction of water turbidity ranged from 56% to 96% (equivalent to 0.4-$\log_{10}$ and 1.4-$\log_{10}$). The levels of UV_{254} absorbance were highly variable with negligible reductions to reductions of 70% (0.5-$\log_{10}$). The reduction of ions by the advanced treatment trains was also negligible.

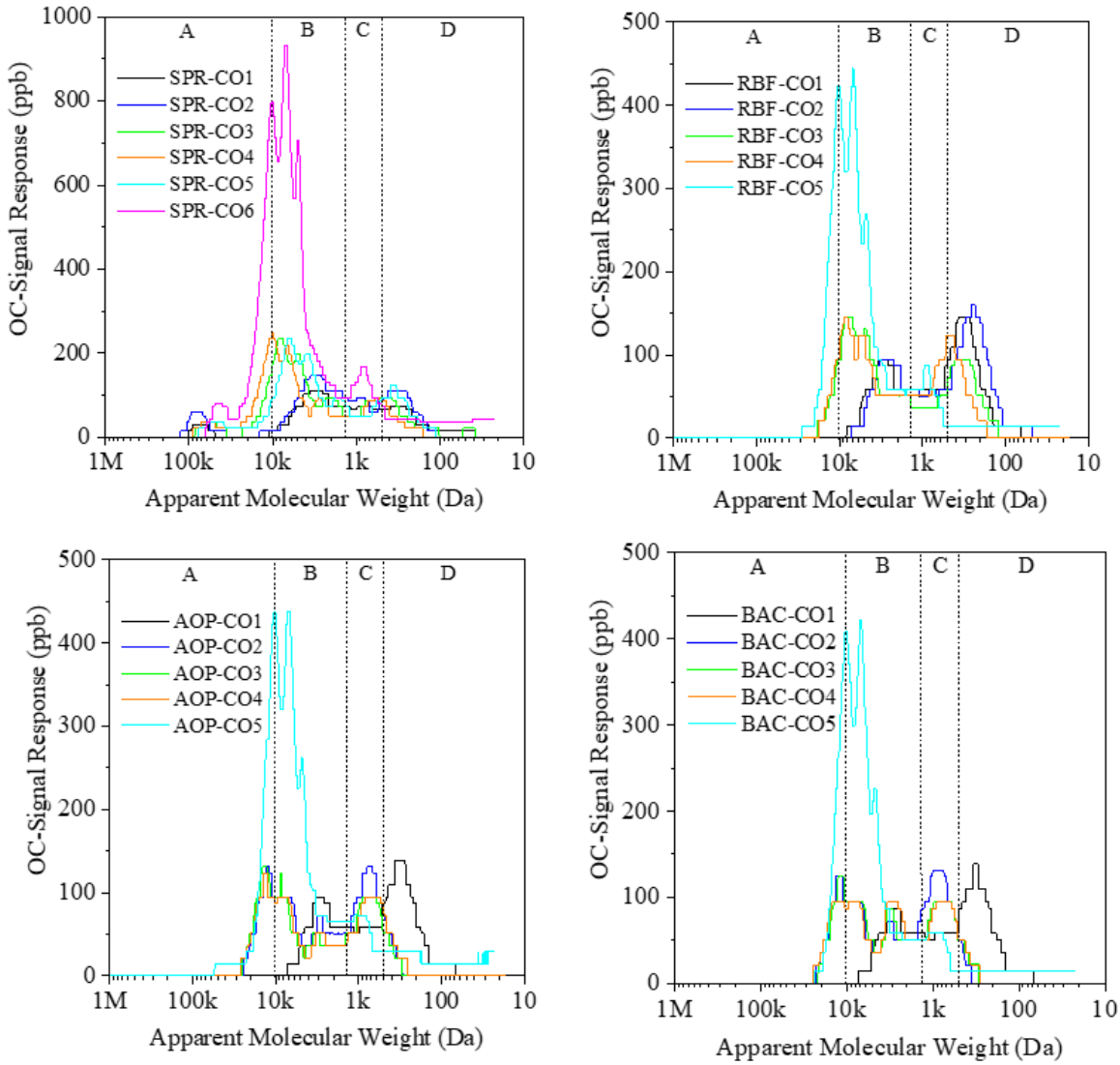


Figure 3-20. Size Exclusion Chromatograph – Organic Carbon Detector (SEC-OCD) Fingerprints of River Water, Product Water after Riverbank Filtration, Ultraviolet Light with Advanced Oxidation Product Water, and Product Water from Biological Activated Carbon Filtration.

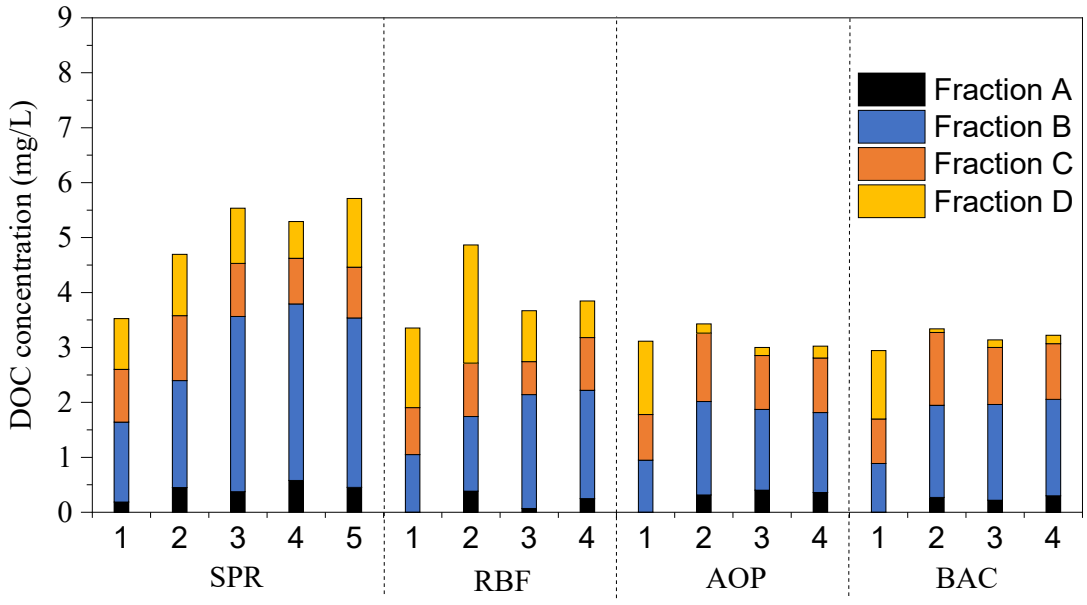


Figure 3-21. Dissolved Organic Carbon Concentrations Calculated from SEC Chromatograms from River Water (SPR), Product Water after Riverbank Filtration (RBF), Ultraviolet Light with Advanced Oxidation Product Water (AOP), and Water from Biological Activated Carbon Filtration (BAC).

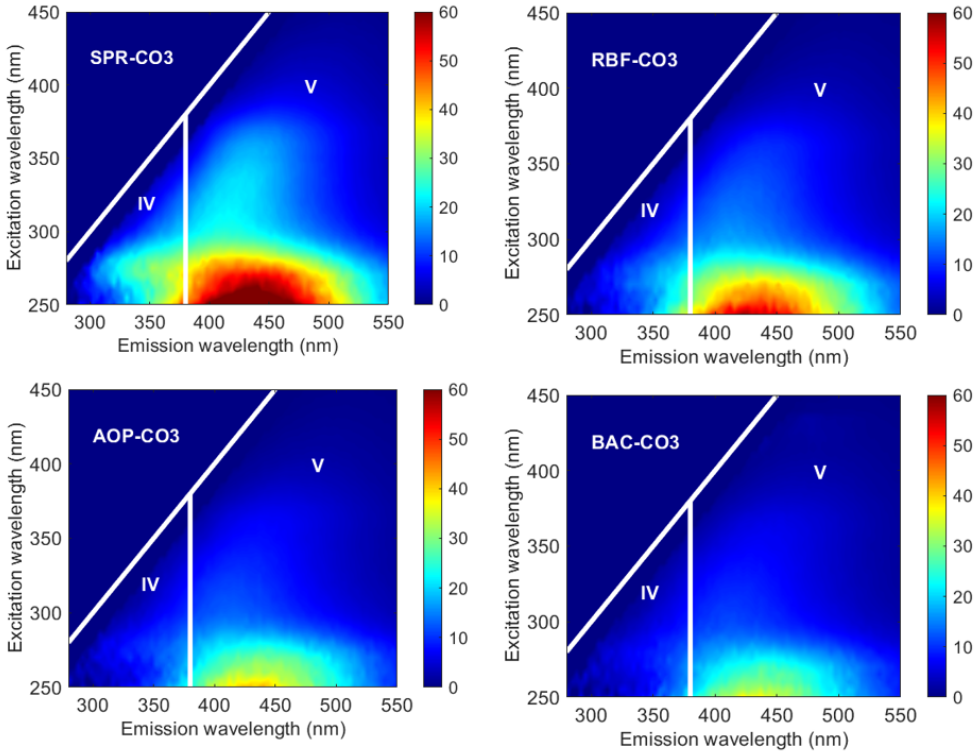


Figure 3-22. Excitation-Emission Matrix (EEM) Fluorescence Spectra Examples of River Water, Product Water after Riverbank Filtration, Ultraviolet Light with Advanced Oxidation Product Water, and Water from Biological Activated Carbon Filtration (Region IV: Soluble microbial byproduct-like matter; Region V: Humic acid-like matter).

3.4 Virus Indicators of Physical Treatment and Non-microbial Surrogates: Engineering-scale Ultrafiltration-Reverse Osmosis and Ultrafiltration-Nanofiltration

The virus indicators were evaluated in the engineering-scale system operating under two integrated membrane configurations that involved ultrafiltration (UF) and reverse osmosis (RO) elements versus ultrafiltration and nanofiltration elements.

Samples of tertiary effluent, ultrafiltration permeate and reverse osmosis permeate or nanofiltration permeate were collected and analyzed for virus genomes and culturable bacteriophages using methods previously described in this report. One major goal of these analyses was to track virus breakthrough under the two integrated membrane configurations specified above.

Figure 3.23 and Figure 3.24 display the concentrations of human enteric viruses and virus surrogates detected at different levels of treatment from the engineering scale system under two configurations. Multiple detections of viruses were observed from the ultrafiltration permeate with up to twelve of the fifteen virus indicators still present at this treatment train. These findings showed that the viruses were not rejected by microporous membranes. Log reduction credits for viruses by microporous membranes have not been allowed under the California's regulatory framework for Groundwater Replenishment Projects. The concentrations of human enteric viruses varied from 1-$\log_{10}$ to 4-$\log_{10}$ GC/L with LRVs showing evidence of either no removal (Human Bocavirus not found in feed water during these sampling campaigns) or greater than LRV as well as averages of 1-$\log_{10}$, 2-$\log_{10}$, and 3-$\log_{10}$ GC/L for Aichi virus, Adenovirus, and Reovirus, respectively. The concentrations of virus surrogates were either close to the ALoD or between 1-$\log_{10}$ and 3-$\log_{10}$ GC/L with corresponding LRVs ranging from 2-$\log_{10}$ to 4-$\log_{10}$. Reovirus, Adenovirus, and Human Bocavirus were simultaneously detected at least once in the UF permeate along with the majority of the virus surrogates, except coliphages that were only present along with Human Bocavirus.

The trend of virus detection changed from UF permeate to RO permeate where none of the human enteric viruses and coliphages were detected after RO membrane treatment. Hudisavirus was not evaluated under the UF-RO configuration since the primers and probe for this virus had not been developed during those sampling campaigns. PMMoV, WCDV2, WCDV3, and crAssphage were all detected from RO permeate at concentrations that varied from either close to the ALoD to 1-$\log_{10}$-2-$\log_{10}$ GC/L, with corresponding LRVs showing evidence of either no removal (WCDV2, present more frequently in RO permeate) or average LRVs between 0.3-$\log_{10}$-0.4-$\log_{10}$ (WCDV3, crAssphage) and 1-$\log_{10}$-2-$\log_{10}$ (PMMoV, crAssphage).

Similarly, the trend of virus detection from the engineering-scale system changed from UF-RO to UF-NF, the latter configuration leading to more often occurring virus genome and culturable bacteriophage detections as summarized in Table 3.16.

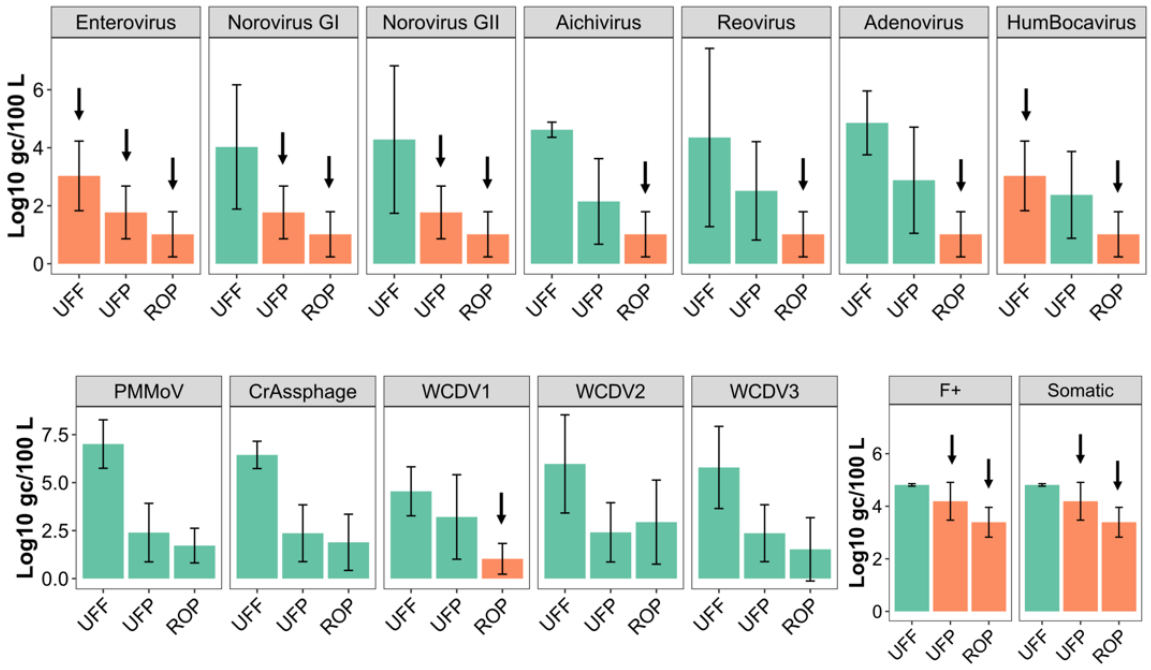


Figure 3-23. Average Concentrations (Log Concentrations in Genome Copies Per 100 Liters) of Human Enteric Viruses and Virus Surrogates in Water Samples Collected from the Engineering-Scale System: Ultrafiltration-Reverse Osmosis Configuration.

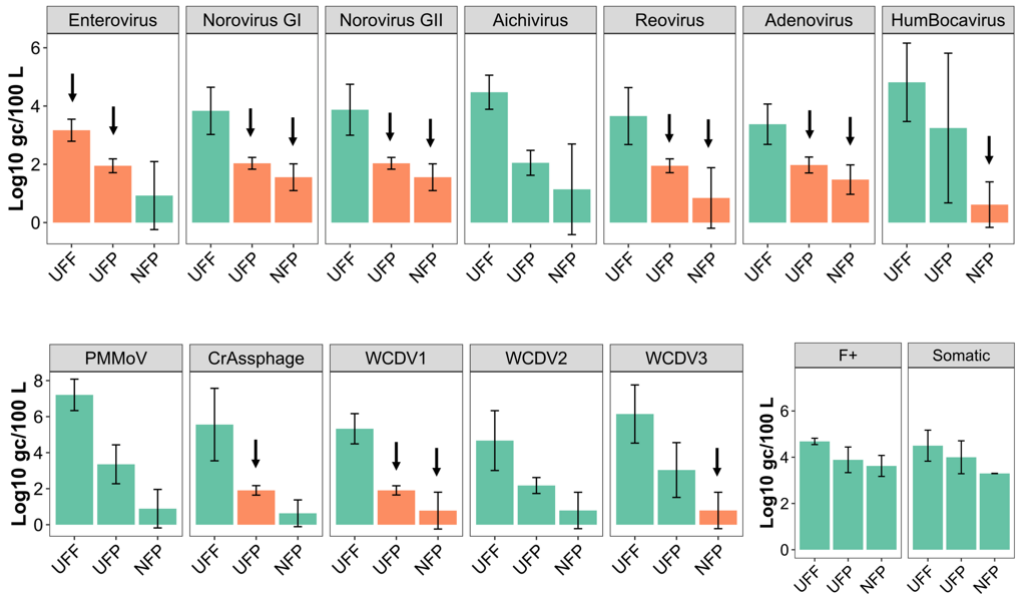


Figure 3-24. Average Concentrations (Log Concentrations in Genome Copies Per 100 Liters) of Human Enteric Viruses and Virus Surrogates in Water Samples Collected from the Engineering-Scale Integrated Membrane System: Ultrafiltration-Nanofiltration.

Table 3-16. Changes in Virus Detection from the Engineering-Scale Integrated Membrane System Treating Tertiary Effluent under Two Membrane Configurations: Ultrafiltration-Reverse Osmosis Versus Ultrafiltration-Nanofiltration.

Virus	Advanced Treatment Train		
	UFP	ROP	NFP
Human enteric virus			
Reovirus	1+	<ALoD	<ALoD
Aichi virus	1+1	<ALoD	1+
Enterovirus	-	<ALoD	1+
Human Bocavirus	1+1	<ALoD	<ALoD
Adenovirus	1+1	<ALoD	<ALoD
Norovirus GI	<ALoD	<ALoD	<ALoD
Norovirus GII	<ALoD	<ALoD	<ALoD
Virus surrogate			
CrAssphage	1+	1+1	1+
WCDV1	1+1	<ALoD	<ALoD
WCDV2	1+1+1	1+1	<ALoD
WCDV3	1+1+1+1	1+1	<ALoD
Hudisavirus	1+1		1+1
Male specific	1+	<ALoD	1+
Somatic	1+	<ALoD	1+
PMMoV	1+1+1+1	1+	1+1

UFP: ultrafiltration permeate, ROP: reverse osmosis permeate, NFP: nanofiltration permeate (1+, denotes the number of positive detection events for the specific virus; <ALoD, less than the assay limit of detection

Two human-associated RNA viruses, Aichi virus and Enterovirus, were detected simultaneously from NF permeate at least once (1 out 4) in association with virus genomes of two surrogates (PMMoV and Hudisavirus) and plaque forming units of culturable coliphages. These results differ from those corresponding to the full-scale integrated membrane system where virus breakthrough occurred more frequently and for multiple viruses (Table 3.17).

Table 3-17. Trends in Virus Detection from High-Pressure Integrated Membrane System at Engineering-Scale and Full-Scale.

Virus	Advanced Treatment Train		
	Engineering-scale		Full-scale
	ROP	NFP	ROP
Human enteric virus			
Reovirus	<ALoD	<ALoD	1+
Aichi virus	<ALoD	1+	1+
Enterovirus	<ALoD	1+	<ALoD
Human Bocavirus	<ALoD	<ALoD	1+
Adenovirus	<ALoD	<ALoD	1+
Norovirus GI	<ALoD	<ALoD	1+1
Norovirus GII	<ALoD	<ALoD	<ALoD
Virus surrogate			
CrAssphage	1+1	1+	1+1
WCDV1	<ALoD	<ALoD	<ALoD
WCDV2	1+1	<ALoD	1+
WCDV3	1+1	<ALoD	<ALoD
Hudisavirus		1+1	<ALoD
Male specific	<ALoD	1+	1+1+1+1+1+1
Somatic coliphage	<ALoD	1+	1+1+1+1+1+1
PMMoV	1+	1+1	1+1+1

Source water for advanced treatment at these two facilities consists of Class A+ recycled water and the concentrations of viruses upstream of advanced treatment trains were relatively similar. Therefore, the differences in detection are more likely a reflection of the number of samples collected and analyzed for viruses from the full-scale and engineering-scale integrated membrane systems. LRV for human enteric viruses and virus surrogates from the engineering-scale integrated membrane system are given in Table 3.18.

Table 3-18. Log Reduction Values of Human Enteric Viruses and Virus Surrogates (Log_{10}) by Two Membrane-Based Treatment Processes (Ultrafiltration-Reverse Osmosis Versus Ultrafiltration-Nanofiltration) at Engineering-Scale.

Virus	Membrane Process			
	Reverse Osmosis		Nanofiltration	
	UFF TO UFP	UFP TO ROP	UFF TO NFF	NFFP TO NFP
Reovirus	3.5	>3.5	2.7	>2.7
Aichi virus	1.1	>1.1	2.4	NR
Enterovirus				breakthrough
Human Bocavirus	breakthrough		>3.4-NR	
Adenovirus	1.5 - 1.6	>1.5 - >1.6	>2.1	
Norovirus GI	>2.6 - >3.8		>2.8	
Norovirus GII	>2.5 - >4.7		>2.9	
CrAssphage	2.1	1.8	>1.4	breakthrough
WCDV1	1.7	>1.7	>3.8	
WCDV2	1.7	NR	2.8 - 3.4	>2.8 - >3.4
		1.3 - 4.6		
WCDV3	2.1 - 4.2	0.31	2.4 - 3.2	>2.4 - >3.2
Hudisavirus	NA	NA	2.8	NR
				2.5
Male specific	>4.8		2.3	0.3
Somatic	>4.8		2.5	1.2
PMMoV	1.7	1.3	3.2 - 3.7	2.3

UFF: ultrafiltration feed; UFP: ultrafiltration permeate; ROP: reverse osmosis permeate; NFF: nanofiltration feed; NFP: nanofiltration permeate. NA denotes not applicable since assay for detection was not available, NR: no reduction.

The mechanisms of separation or removal by nonporous membranes is a function of diffusivity and solubility in the membrane material, independent of molecular size (Warsinger et al., 2018). The size of intramolecular spaces of polyamide layer on non-porous RO membranes ranges at 3.5-4.5 A (Yoon, 2019). A theoretical pore size of <0.001 µm has been assigned to these types of membranes. The size of the viruses surveyed in this study ranges from 15 nm to 100 nm in diameter (corresponding to 1.5 to 595 MDa, Table 1.3), which is higher than intramolecular spaces and theoretical pore size of these nonporous membranes. Considering the multiple detection of viruses, these results indicate that virus leak may be a common outcome associated with membrane-based treatment processes. Convective transport through membrane defects is considered the dominant mechanism of virus leak that occur when there are defects larger than 20nm in membrane, o-rings, or glue lines of membrane elements.

Our results demonstrated more detections of viruses under the UF-NF operation of the engineered-scale system. Both NF and RO membranes are designed to remove dissolved chemical constituents although many of the same constituents are removed by NF to a lesser extent than by RO membranes. NF membranes are considered as an alternative “loose” RO membrane with higher water permeability, therefore differences in rejection efficiencies of viruses and chemical constituents are expected (Warsinger et al., 2018; Yoon, 2019).

With respect to the non-microbial surrogates as previously discussed in this report, total living biomass measured by the adenosine triphosphate (ATP) content of microorganisms in water as well as total organic carbon (TOC, DOC), UV absorbance and total fluorescence were used to determine overall biological load. In addition, size exclusion chromatography in combination with organic carbon detection (SEC-OCD) was evaluated in order to monitor rejection of high molecular weight compounds. The reduction of fluorescence values along with TOC/DOC concentrations and UV_{254} absorbance detections were evaluated as potential bulk organic surrogates to determine correlations with virus removal.

SEC-OCD fingerprints of ultrafiltration feed (UFF), nanofiltration feed (NFF), and nanofiltration permeate (NFP) are given in Figure 3.25. Only fingerprints associated with nanofiltration membranes are displayed in this section. Fingerprints from all samples analyzed for this study are included in Appendix A. The relative absence of fraction A in the NFF indicates that dissolved organic carbon (DOC) entering the RO or NF systems are lower than 10 kDa. As previously discussed for the full-scale membrane-based treatment train, all DOC fractions are considerably reduced in the RO or NF permeate.

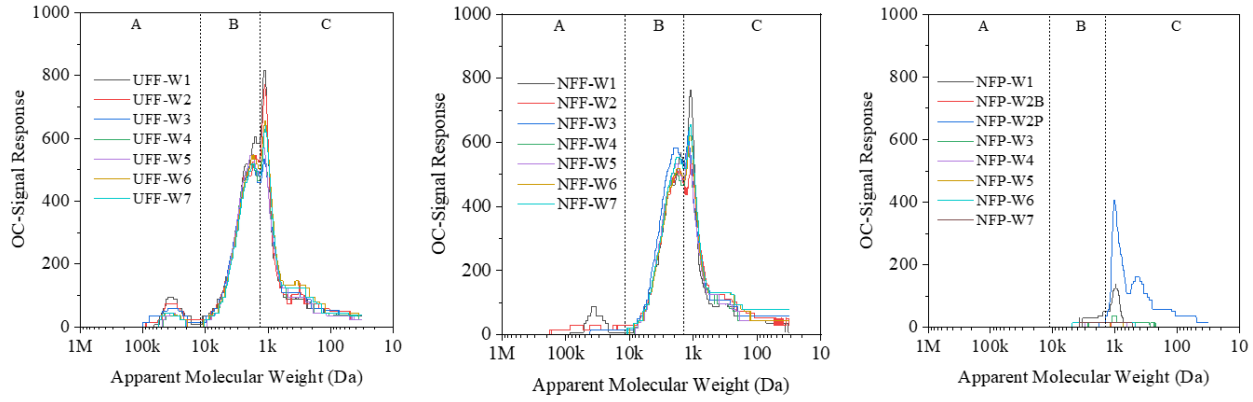


Figure 3-25. Size Exclusion Chromatography – Organic Carbon Detector (SEC-OCD) Fingerprints for Ultrafiltration Feed (UFF; Corresponding to Tertiary Effluent), Nanofiltration Feed (NFF), and Nanofiltration Permeate (NFP).

The size distribution of DOM changed towards lower molecular weight from tertiary effluent to NF and RO permeate due to increased removal of high molecular weight compounds. These results also indicate that the contribution of microbial byproduct-like and humic acid-like fractions to the organic carbon content was minimal for product water. These results are in agreement with the UV_{254} absorbance, DOC concentrations, and EEM fingerprints, the latter shown in Figure 3.26.

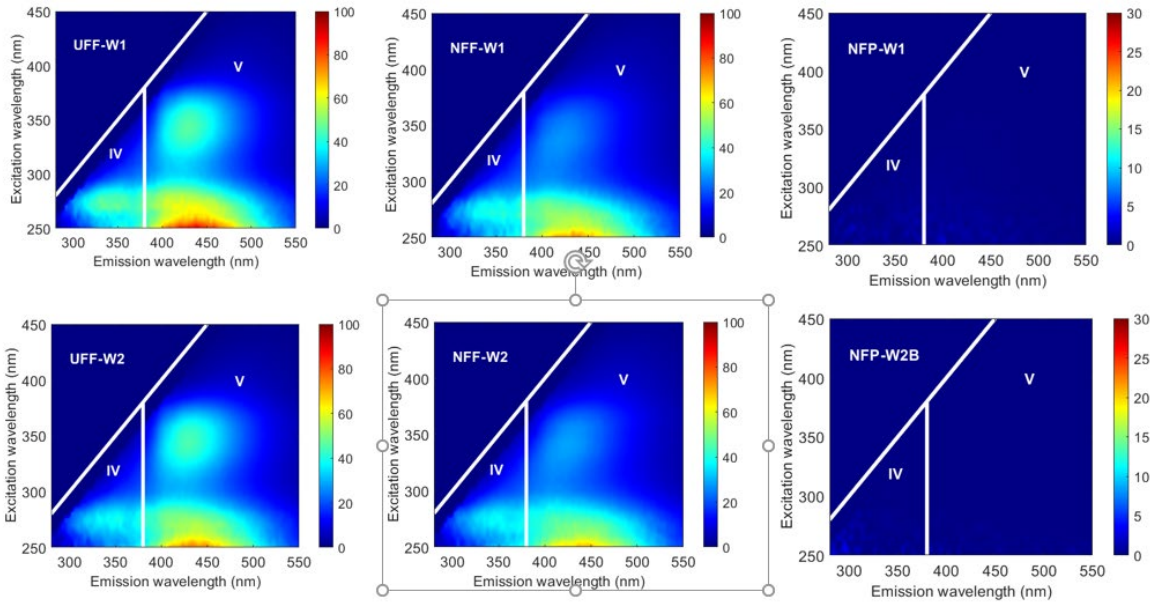


Figure 3-26. Excitation-Emission Matrix Fluorescence Contours of (A) UF Feed, (B) NF Feed, (C) NF Permeate.

Changes in size distribution of organic molecules towards lower molecular weight fractions and decrease in fluorescence intensity from tertiary to advanced treatment was consistent with the rejection mechanisms of soluble contaminants in water purification processes associated with non-porous membranes that have been previously covered in this report.

Similar to the full-scale membrane system, ion rejection increased from UF feed to NF and RO permeates, with lower rejections observed for monovalent ions (potassium, sodium, chloride) likely due to their lower hydrated radius as previously discussed. For the engineering-scale system there were three samples under the UF-RO configuration and three more under the UF-NF configuration, which precluded the application of a meaningful statistical analysis for making statistical inferences. Therefore, the evaluation of the level of resolution of non-microbial parameters to achieve a level close or similar to LRV of viruses was less informative in this case. For instance, out of the three ATP measurements reductions of 78% and 97% (0.6-$\log_{10}$ to 1.6-$\log_{10}$) were observed. At full scale, ATP reductions of up to 99.99% (4-$\log_{10}$) were more evident.

3.5 Virus Indicators of Physical Treatment and Non-microbial Surrogates: Carbon-based Advanced Treatment Trains (Facility D)

There were three sampling campaigns from Facility D located in the Southeast of the U.S. Water samples were collected from the initial stages of wastewater treatment to the advanced treatment trains that generate product water for recharge operations. Figure 3.27 shows the concentrations of viruses that were detected at the different sampling points.

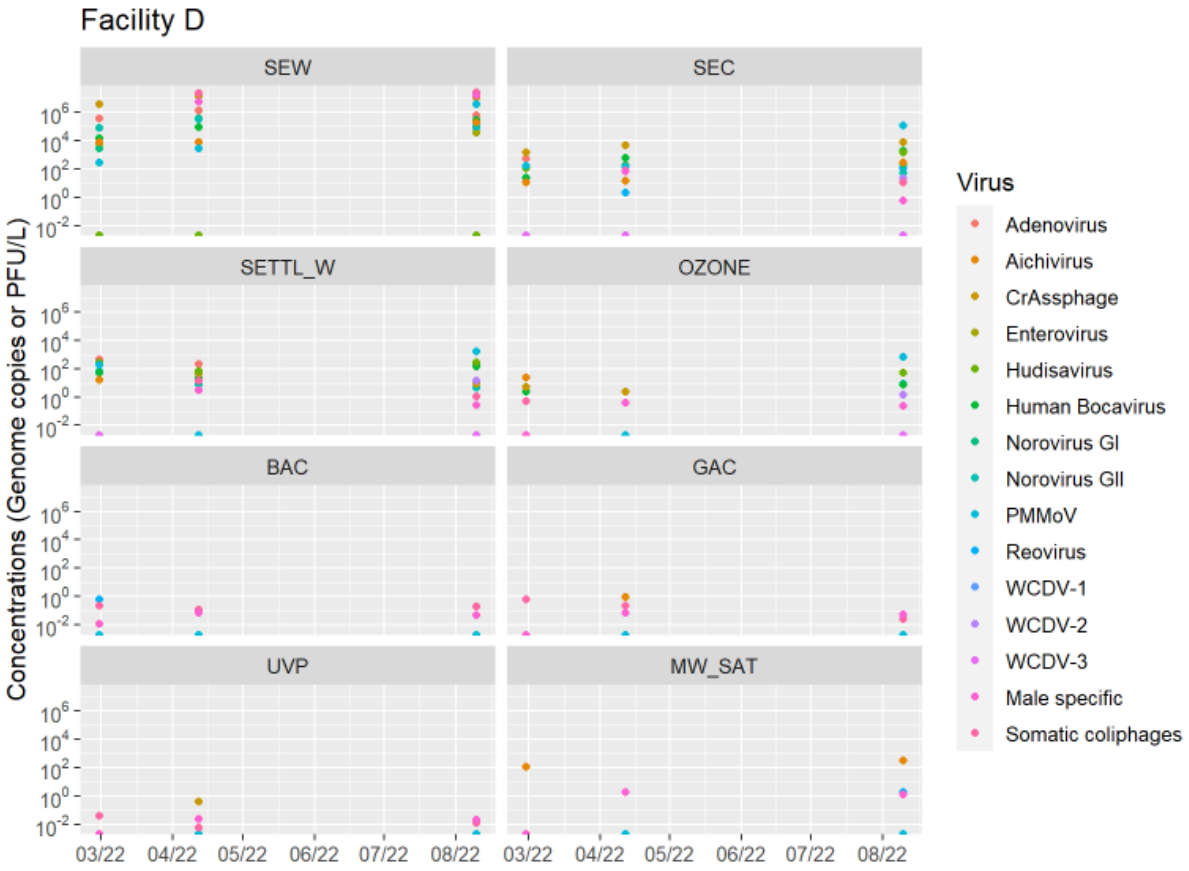


Figure 3-27. Concentrations (Log_{10} Genome Copies or PFU Per Liter) of Human Enteric Viruses and Virus Surrogates in Water Samples Collected from Facility D.

In raw wastewater, all the human enteric viruses were found at similar concentrations to those observed for the rest of the facilities included in this study, thereby indicating a homogenous distribution of these viruses in three different regions of the continental United States. Enterovirus was not detected upon secondary treatment, a similar pattern of detection observed for this virus from the rest of the facilities. This outcome may be the result of low recovery efficiencies of enterovirus in wastewater matrices using the adsorption-elution approach applied in this study. Mean Enterovirus recoveries of 15±22% (70% RSD) were obtained from tertiary effluent and ultrafiltration permeate matrix spikes from Facility B in Arizona. Enterovirus was detected from recharged groundwater and from RO permeate in this study. Enterovirus has also been detected in aquifers deemed “low risk” based on prior monitoring of fecal indicators and factors such as presence of thick layers of overlying sediments (Johnson et al., 2011), which indicates the environmental persistence of these viruses and its resilience to conventional and advanced physical treatment. Improvements in Reovirus detection were noted after using of a thermal heat shock treatment of viral RNA prior to RT-dPCR based on previous studies conducted with bacteriophage Phi 6 (dsRNA). Consequently, improvements in viral detection approaches are required to truly understand the presence and fate of Enterovirus and other enteric viruses in the water reuse infrastructure.

Figure 3.28 displays the LRVs of human enteric viruses and virus surrogates for the treatment trains monitored from Facility D.

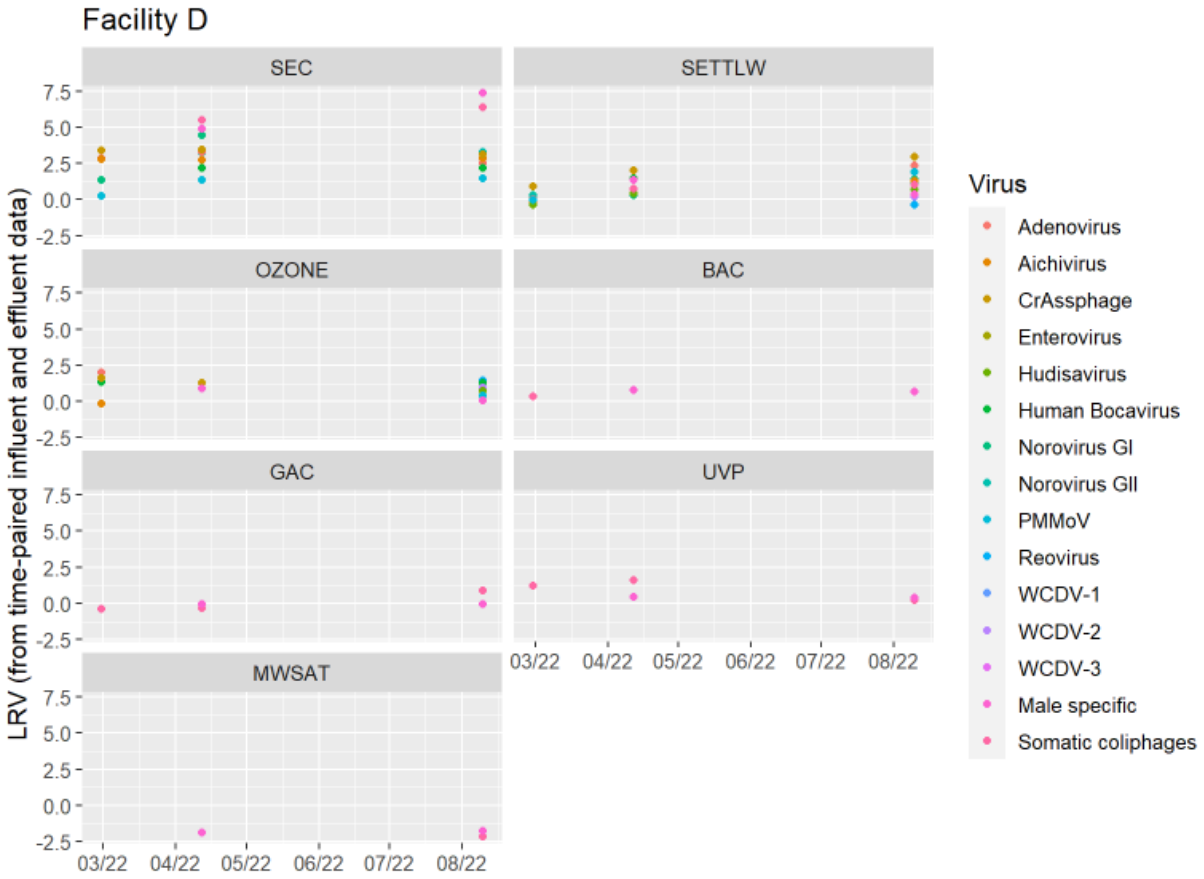


Figure 3-28. Boxplot of LRV of Viruses in Facility D (Carbon-Based Advanced Treatment Train).

As it occurred in previous Facilities, the LRV of somatic and male-specific coliphages in Facility D during the first treatment train varied from 6-$\log_{10}$ to 7-$\log_{10}$, whereas the reduction of the rest of the viruses (reduction of genome copies) was on average 3-$\log_{10}$. Prior to advanced treatment, the ozone-treated secondary effluent showed evidence of multiple human enteric viruses, including Reovirus, Adenovirus, Human Bocavirus, and Aichi virus. Noroviruses were not detected after flocculation-sedimentation and therefore these viruses were reduced to a level <ALoD. During the subsequent advanced treatment trains, including BAC, GAC, UV photolysis, Reovirus and Aichi virus were the only human enteric viruses detected, although at very low concentrations that were expressed as GC/100 L yielding log concentration of 2-$\log_{10}$ GC/100 L. Virus breakthrough occurred for Aichi virus, which was the only virus detected at the GAC treatment train and in the recharged groundwater after SAT.

Log reduction credits of viruses for carbon-based advanced treatment trains have been developed based on pilot-scale and full-scale installations, as reported in WRFF-11-02 (Trussell, 2012). The Texas Commission on Environmental Quality uses these log reduction values as a basis for granting credits to advanced treatment technologies. Under these criteria, a 5-$\log_{10}$ reduction is granted to the Ozone-Biological Activated Carbon (BAC) treatment train. Human

Bocavirus, Adenovirus, Reovirus, and Aichi virus were detected in the ozone-treated effluent along with multiple virus surrogates including coliphages, Hudisavirus, crAssphage and PMMoV. The Ozone-BAC treatment process demonstrated >LRV for most viruses except for Reovirus, Adenovirus, and male (F+) coliphages which exhibited LRV of up to 3-log_{10}. On the other hand, reductions of somatic coliphages were not observed after Ozone-BAC treatment and both coliphages were subsequently detected in GAC and post-UV product water. Crassphage was detected once from GAC product water and subsequently detected along with coliphages, Aichi virus, and Hudisavirus in recharged groundwater samples collected from the monitoring well. These results differ from those observed for the membrane-based treatment process at full-scale where Reovirus, Adenovirus, Human Bocavirus, Norovirus GI and Aichi virus were detected after FAT. However, only three samples from each treatment train were collected from Facility D (carbon-based scheme) while fourteen samples were collected from Facility A (membrane-based scheme). Therefore, additional sampling from Facility D may reveal a similar or a different outcome.

With respect to the non-microbial surrogates, the SEC-OCD fingerprints revealed a similar pattern of DOM change that resulted in rejection of high molecular weight compounds along the advanced treatment train from secondary effluent to post-UV product water (Figure 3.29). Fraction A (high molecular weight compounds) revealed rejection efficiencies of 99.9% (3-log_{10}) while fractions B and C+D exhibited rejection efficiencies of 50% and 65% (0.3-log_{10}, 0.4-log_{10}), respectively. These results were consistent with the rejection efficiencies of organic compounds evaluated by UV_{254} absorbance (rejection efficiency: 84%-90%, equivalent to 0.8-log_{10}-0.99-log_{10}) DOC concentrations (rejection efficiencies: 55%-58% equivalent to 0.35-log_{10}-0.38-log_{10}), TOC concentrations (59%, 0.39-log_{10}) and EEM fingerprints, the latter shown in Figure 3.30.

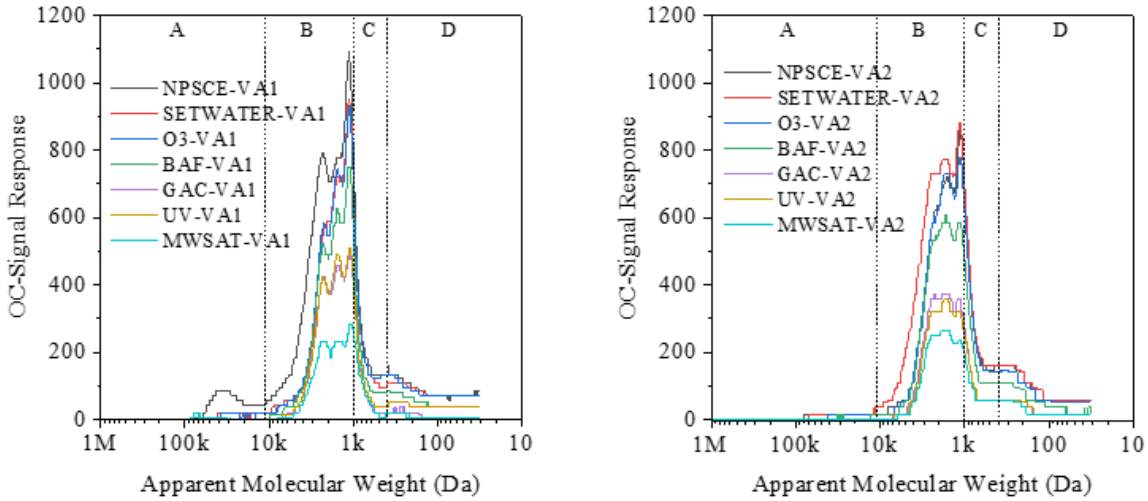


Figure 3-29. Size Exclusion Chromatography – Organic Carbon Detector (SEC-OCD) Fingerprints of Secondary Treated Water (NPSCE), Settled Water (SETWATER), Ozone Product Water (O3), BAC Effluent (BAF), GAC Product Water, UV Product Water, and Recharged Groundwater. (Fraction A (AMW >10 kDa): proteinaceous biopolymers; Fraction B (10 kDa > AMW > 1.5 kDa): Humic substances; Fraction C (1.5 kDa > AMW > 0.5 kDa): Building blocks of humic substances; Fraction D (AMW < 0.5 kDa): Low molecular weight acids and neutrals).

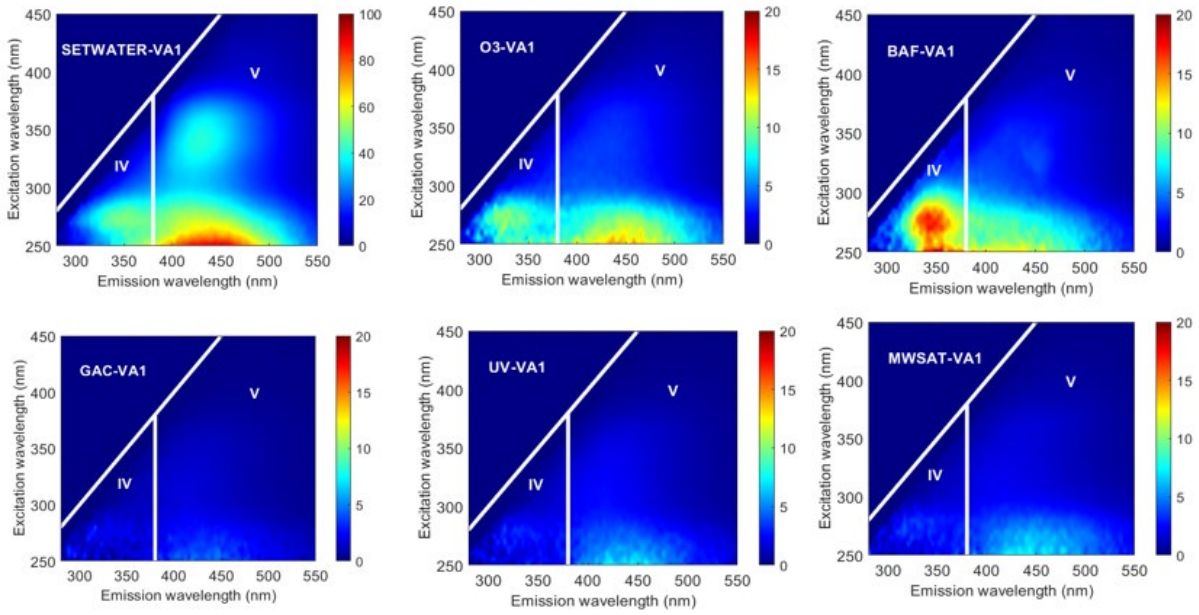


Figure 3-30. Excitation-Emission Matrix Fluorescence Contours of Secondary Treated Water (NPSCE), Settled Water (SETWATER), Ozone Product Water (O_3), BAC Effluent (BAF), GAC Product Water, UV Product Water, and Recharged Groundwater.

Changes in the regional/total fluorescence intensities from secondary effluent to GAC-UV treated product water were observed. The highest fluorescence intensity was exhibited upstream of the advanced treatment with a decrease in TF and the excitation-emission boundaries corresponding to Regions IV and V during the water purification process. As previously discussed, these results indicate that a proportion of both microbial byproducts-like (Region IV) and humic-acid-like (Region V) moieties were reduced by the advanced treated trains but to a lesser extent than the membrane-based treatment train that exhibited rejection efficiencies of up to 99.9% (3-$\log_{10}$). The rejection efficiencies of TF, Region IV, and Region V were on average 92% (1.12-$\log_{10}$) from secondary effluent to post-UV disinfected GAC effluent. This was the highest rejection of organic contents throughout the treatment trains.

Ion rejection by the advanced treatment train was evaluated by measurements of monovalent and divalent ions. Both monovalent and divalent ion exhibited limited rejection efficiencies that ranged from no rejection for NO^-_3, SO_4^{2} and Cl^- to negligible rejection efficiencies for K^+, Mg^{2+}, Ca^{2+}, and Na^+ between 8% and 23% equivalent to 0.03-$\log_{10}$ to 0.1-$\log_{10}$, respectively. The rejection efficiency of total microbial activity along the advanced treatment train measured by ATP exhibited values of 94%-96.8% (1.2-$\log_{10}$-1.5-$\log_{10}$) from secondary effluent to Ozone-BAC product water and 95%-99.8% (equivalent to 1.31-$\log_{10}$-2.8-$\log_{10}$) from secondary effluent to GAC-UV product water. The small sample size from this Facility precluded any statistical analysis.

CHAPTER 4

Conclusions

The results of this study reveal several features of virus indicators performance to confirm advanced physical treatment. Firstly, a guidance framework is provided for the selection of appropriate virus indicators to confirm advanced physical treatment that considers virus abundance in raw wastewater, virus persistence upstream of the advanced treatment trains, virus documented resilience to disinfection technologies in water treatment, and virus structural complexity. The efficiency of virus recovery and concentration as well as the detection approach applied for the estimation of virus genomes or virus infectious units at each advanced treatment train are also critical components of this guidance framework for appropriate assessment of log reduction values of viruses.

The boxplot shown in Figure 4.1 depicts the LRVs of all viruses evaluated in this study at different treatment trains from Facilities A, B, and D. It is important to note that the data in the boxplot are the LRVs derived from paired influent and effluent concentrations, excluding all non-detects. Non-detects were found in both influent and effluent data, not always concurrently, implying that actual variation of LRVs may be higher than the LRVs depicted by the boxplot. The median reductions of somatic and male specific coliphages (reduction of PFU) from raw wastewater to either secondary or tertiary effluent was around 6-$\log_{10}$, whereas the median reductions of the rest of the viruses (reduction of genome copies) were around 2-$\log_{10}$ to 3-$\log_{10}$. There did not seem to be an effect on virus reduction based on the type of viral nucleic acid content.

The reduction of dPCR counts points out to complete virus particle removal. Prior to virus detection and quantification by RT-dPCR/dPCR, water concentrates were treated with nucleases in order to remove free nucleic acid and viral nucleic acid of viruses with compromised capsids. Therefore, the reduction of genome copies reflects physical removal of intact virus particles. The reduction of somatic and male-specific coliphages, based on the reduction of plaque-forming units, can be attributed to physical removal of virus particles as well as to damaged virus particles that were unable to replicate in vitro.

Endogenous wastewater viruses, including human enteric viruses such as noroviruses, human bocavirus, adenoviruses, and reoviruses were recovered and detected after full advanced treatment from the integrated membrane-based process train. Simultaneous detection of reoviruses and noroviruses occurred during two sampling events while adenoviruses and human Bocavirus were detected during one and two separate sampling events, respectively. Reoviruses were detected more frequently (3 of 14) than noroviruses and human bocavirus (2 of 14). Aichi virus was predominantly found in highly treated water and recharged groundwater from the carbon-based treatment trains. Interestingly, the detection of human enteric viruses was associated with the detection of infectious coliphages used as virus surrogates of infectivity. Despite the differences between the equivalent volumes examined for the detection of human enteric viruses by RT-dPCR/dPCR and coliphages by the plaque assay, these results

highlight the importance of monitoring multiple virus indicators under different detection approaches in order to confirm physical treatment. In particular, monitoring for viruses of public health significance such as human enteric viruses that based on the results of this study are proposed as self-indicators to confirm advanced physical treatment. Enteric viruses are proposed as self-indicators because they were found, although intermittently, in fully advanced treated water either simultaneously or in separate sampling events in association or not with virus surrogates such as coliphages. Enteric viruses are human pathogens (at least the ones found in this study) while coliphages are virus surrogates that infect enteric bacteria. Therefore, looking at virus pathogens is more protective of public health for potable reuse purposes than looking at virus surrogates.

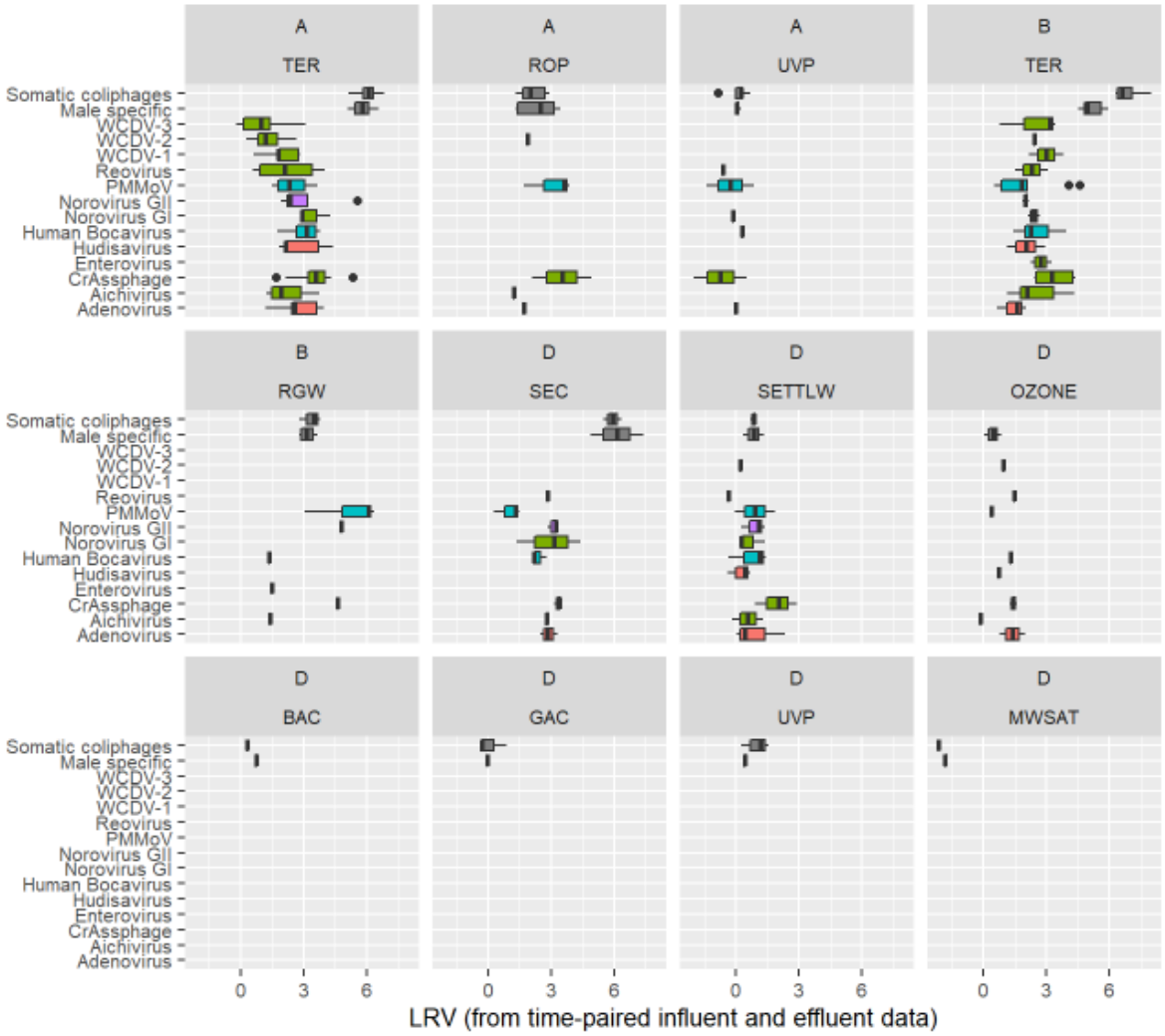


Figure 4-1. Boxplot of LRV of Viruses for Facilities A, B, and D.

Based on the outcome of our previous results (Souza-Chaves et al., 2022), our current study demonstrates the capabilities of the molecular detection approaches to reveal concentrations of viruses in expected low-level environmental treatment trains. Large-volume sampling combined with absolute quantification of nuclease-protected virus genomes by digital PCR proved to be applicable for investigations of virus reductions in these environments. Digital PCR

enabled the detection of nuclease-protected virus genomes to a level of resolution capable of demonstrating LRV (>7) for viruses of public health significance. However, our capability to demonstrate high levels of reduction for all viruses are still limited by the physical properties of the viruses themselves. This goes along with the inefficiencies of the methods for recovery and concentration of viruses that once again demonstrated to be both virus- and site specific. The limitations associated with the allocation of log reduction credits required for safe potable reuse applications at full-scale may be better addressed using long-term monitoring campaigns for multiple viruses. This study provides a framework for monitoring virus indicators at full-scale and engineering-scale installations to confirm physical treatment. However, the evaluation of the log-reduction performance of advanced treatment trains requires long-term monitoring at full-scale in order to capture the complexity of operational parameters and site-specific conditions that have an important impact on the virus-monitoring framework. In this regard, the implementation of efficient and sensitive methods of virus recovery and detection are fundamental to more clearly understand whether the infrequent detection of low levels of enteric viruses in highly treated recycled waters is a true reflection of low levels of virus occurrence or method deficiencies associated with water matrix effects and/or virus complexity. Large-volume sampling (>1000 L) is considered a suitable approach for recovery and concentration of viruses from full-advanced treated water in which viruses are expected to be present at very low concentrations, however virus recovery efficiencies may vary depending on the overall quality of the water. Low-molecular weight organic compounds present in full-advanced treated water may affect virus recoveries by competing with adsorption sites that may be saturated and therefore unavailable for virus adsorption to the filter matrix. All of these factors above must be thoroughly evaluated and thus seen as important components of an expanding research agenda to ensure current and future sustainable, reliable and safe water reuse.

Among the virus surrogates, crAssphage was found at the highest concentration in source waters prior to advanced treatment and therefore was also found throughout the advanced treatment trains, however the detection of this surrogate occurred independently of the detection of viruses of public health significance. None of the CRESS viruses (WCDV1, WCDV2, and WCDV3) were detected in the advanced treatment trains at full scale, except a single detection in recharged groundwater and in reverse osmosis permeate from the engineering-scale system. Both treatment processes rely on tertiary treated recycled water from the same facility where these viruses were discovered by viral metagenomics sequencing. The development of an assay for simultaneous detection of all three viruses may provide better insights about the distribution of these viruses within the water reuse infrastructure. The taxonomic classification and host prediction of these viruses were derived by bioinformatics analyses, which classified all three viruses within the *Circoviridae* family with an unknown eukaryotic host present in sewage. Those were major reasons to select these viruses for monitoring as indicators to confirm physical treatment. Members of the *Circoviridae* family are small (15 – 25 nm), highly resistant eukaryotic CRESS DNA viruses with widespread detection, which suggests that this viral type thrives in many environments (Rosario et al., 2012a). Members of the families of ssDNA viruses associated with human diseases have been recently discovered by metagenomics DNA sequencing and investigated in wastewater (Abbas et al., 2019; Kerr et al., 2018; Martin et al., 2023; Pérot et al., 2023). Eukaryotic CRESS DNA viruses

only recently gained recognition commensurate with their ubiquity, diversity, and impact (Zhao et al., 2019). The persistence of these viruses through wastewater treatment and the associated implications for safe and sustainable water reuse are topics that warrant further investigations.

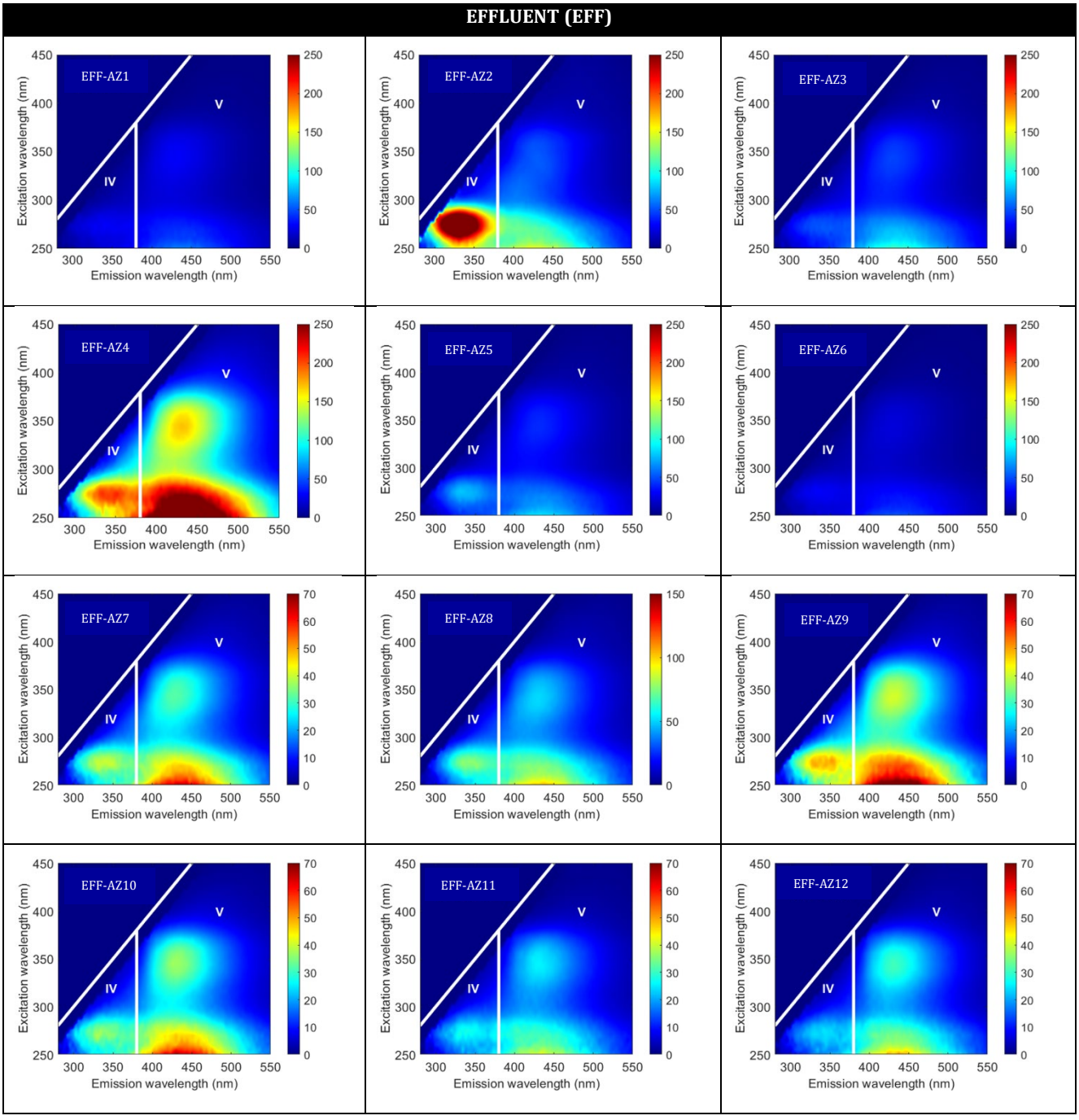
A key objective of this study was to collect full-scale data in order to determine source concentrations and log reduction values of selected viruses investigated in combination with potential online surrogates that may correlate with full-scale virus data. There were no statistically significant correlations among the variables evaluated in the analyses, more likely associated with lot of variability, which is inherently associated with ordinary measurements. As mentioned elsewhere in this report, previous studies have addressed the potentials and limitations of several non-microbial surrogates and operational parameters to demonstrate the recommended pathogen reduction credits by individual or combined advanced treatment trains (Antony et al., 2014; Pype et al., 2016b; Yoon, 2019). The results of this study indicate that non-viral surrogates that target reductions of soluble organic compounds and microbial activity were able to achieve a level of resolution close or similar to LRV of most but not all of the human enteric viruses and virus indicators. However, there were no statistically significant correlations among the reductions of viruses and non-microbial surrogates evaluated at full advanced treatment. These aspects are covered in section 3.1.4.

To fully understand virus presence and fate in the water reuse infrastructure, advancements in viral detection methods are essential.

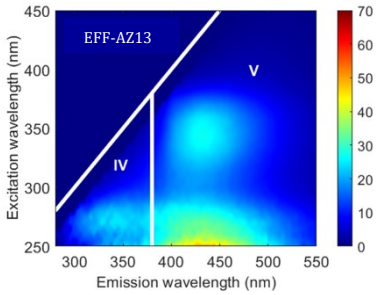
The generated LRV data presented in this report as well as the virus concentrations in raw wastewater provide an extensive base for Quantitative Microbial Risk Assessment (QMRA) of exposure to viruses in potable reuse scenarios.

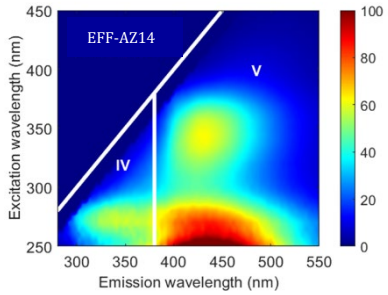
APPENDIX A

Facility A

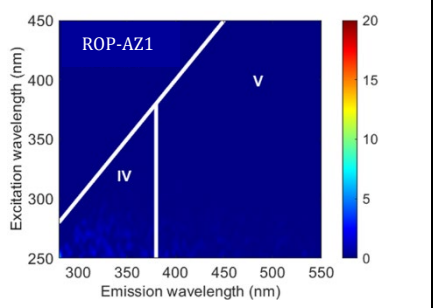
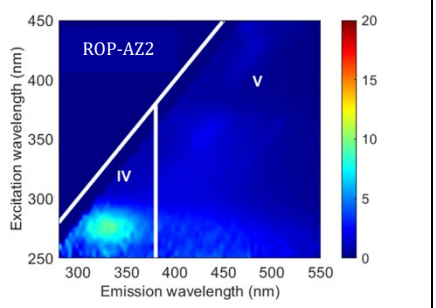
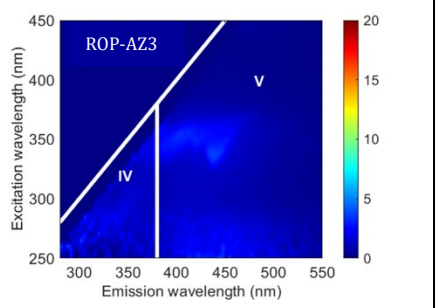
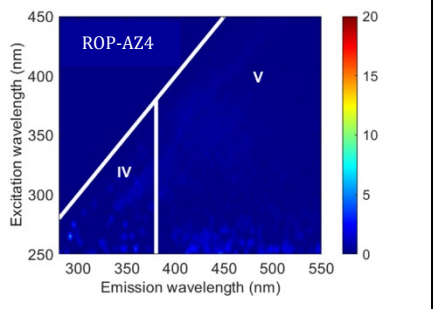
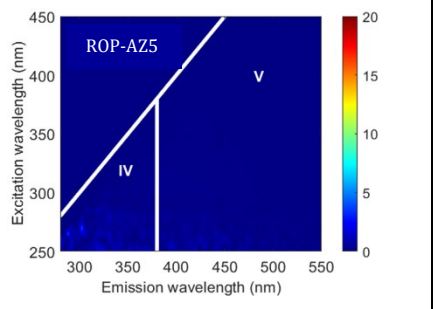
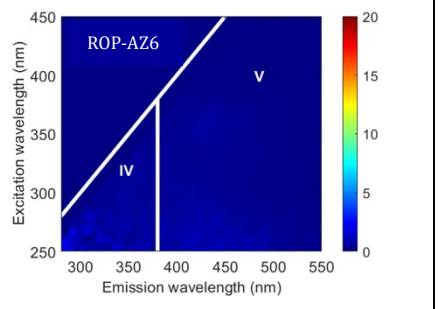
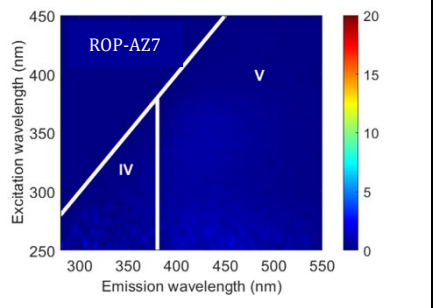
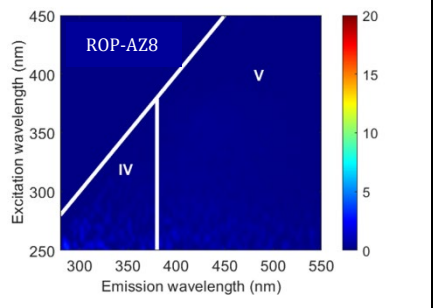
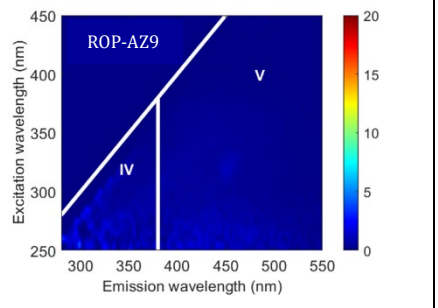
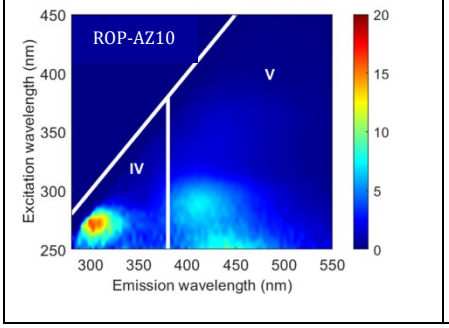
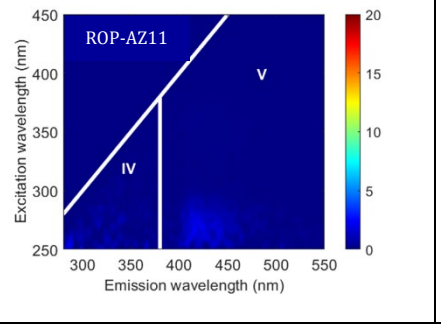
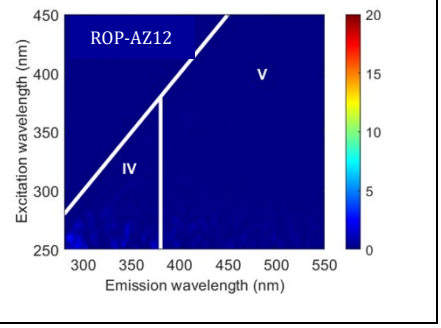


Facility A (continued)

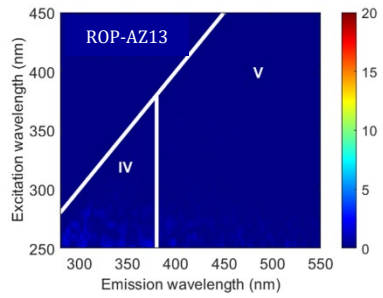


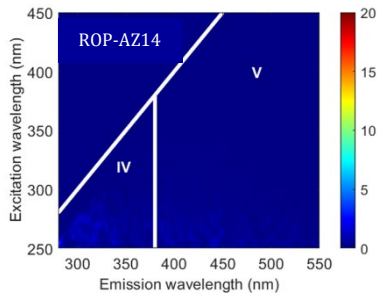


Facility A (continued)

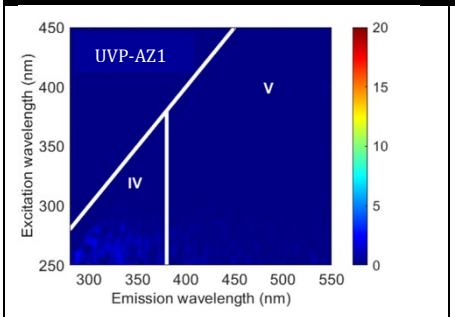
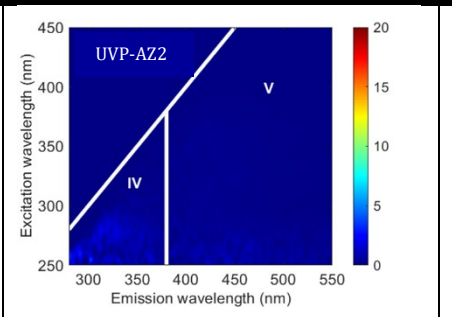
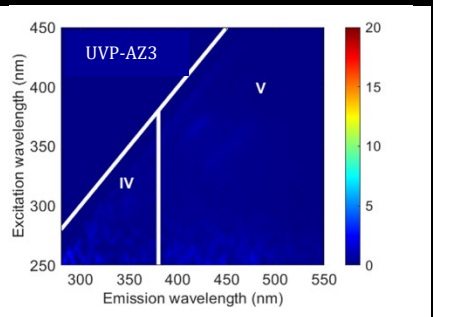
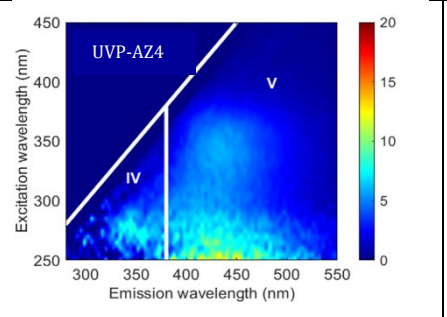
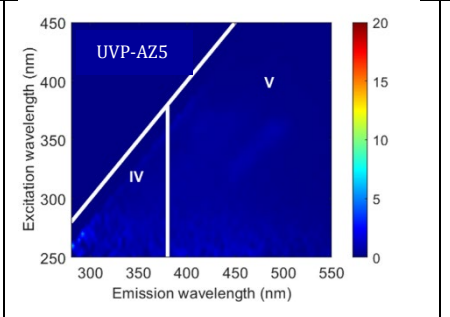
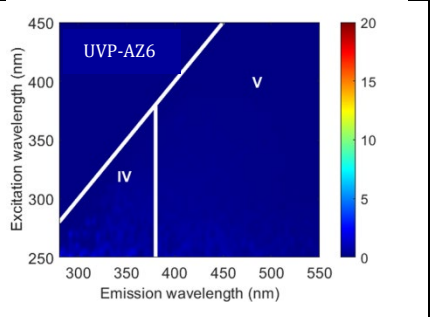
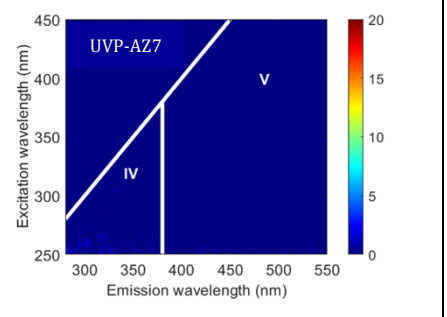
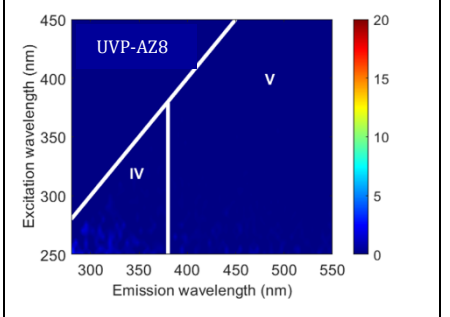
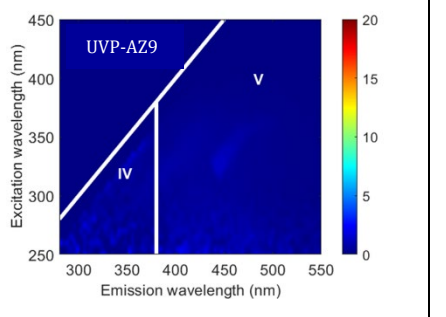
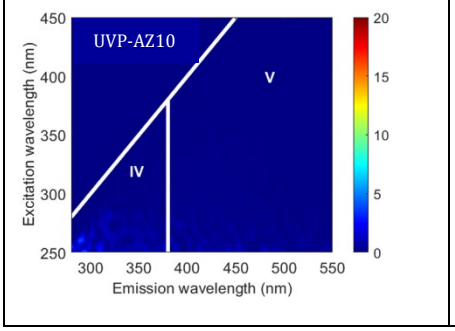
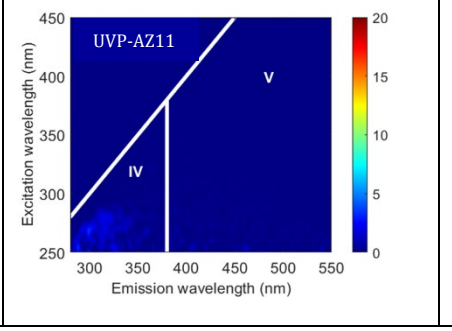
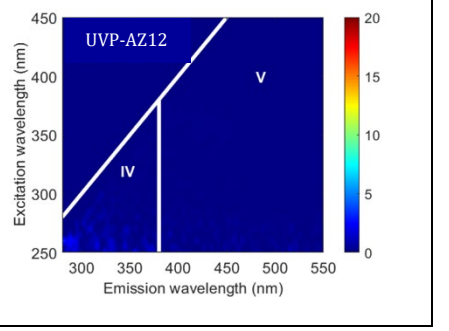
REVERSE OSMOSIS PERMEATE (ROP)		
		
		
		
		

Facility A (continued)

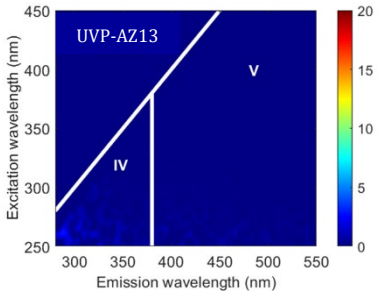


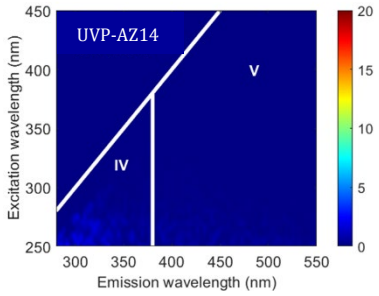


Facility A (continued)

UV PERMEATE (UVP)		
		
		
		
		

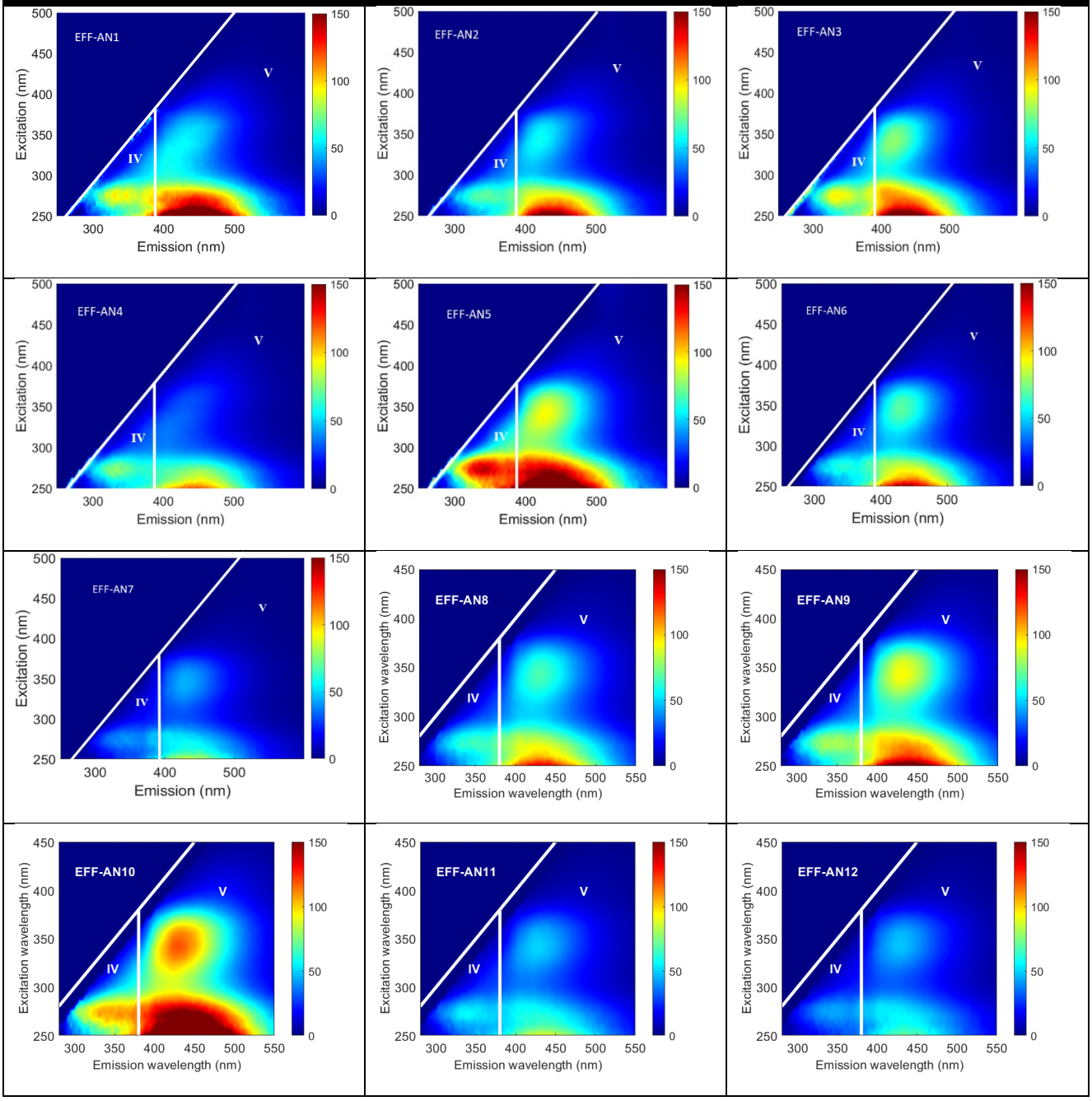
Facility A (continued)



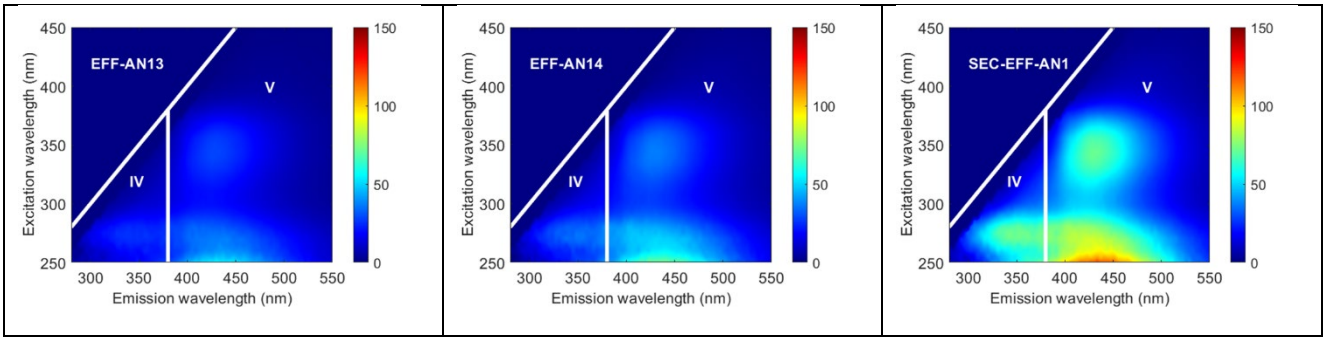


Facility B

SECONDARY EFLUENT (SEC-EFF) / TERTIARY EFFLUENT (EFF)

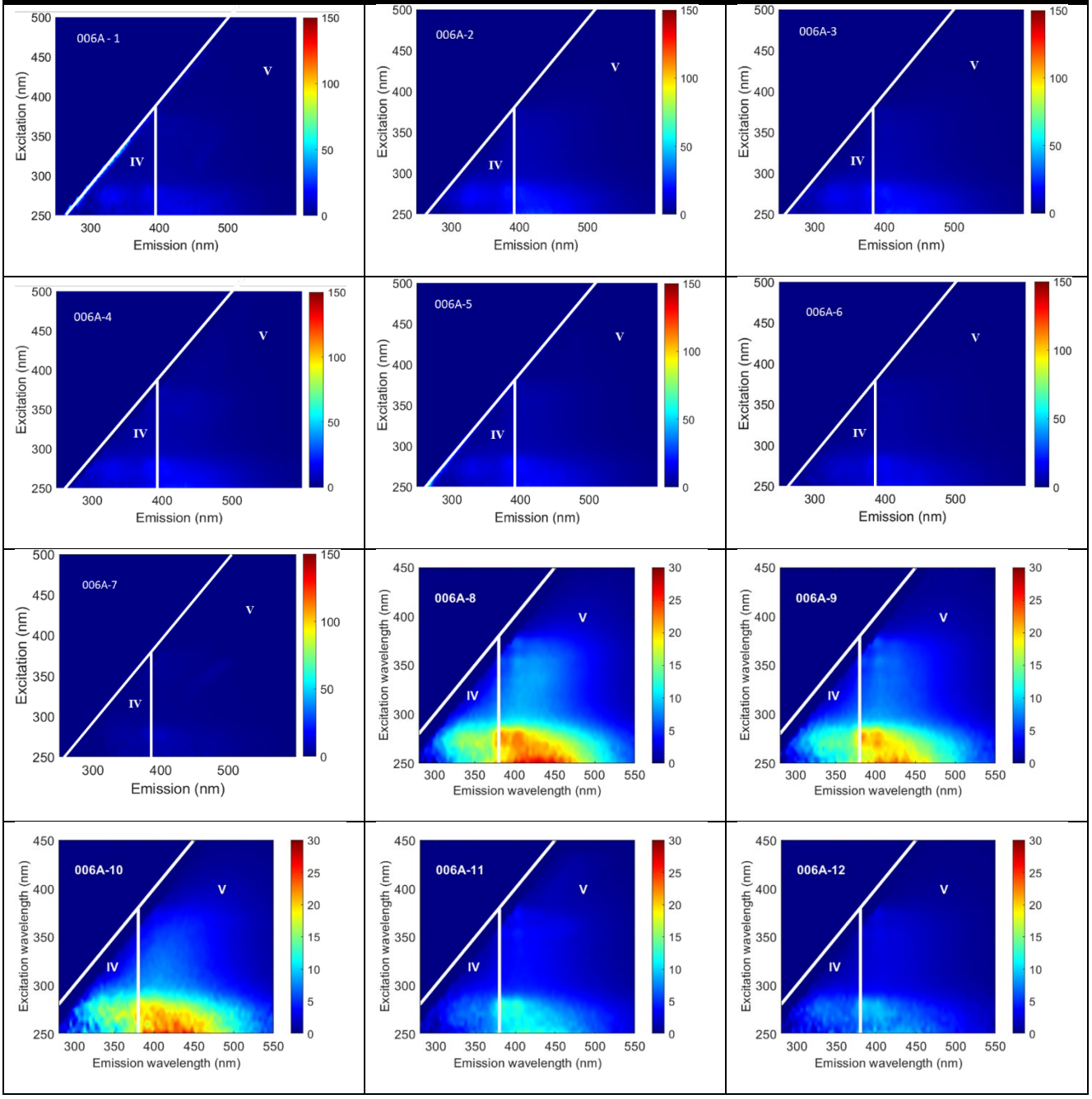


Facility B (continued)

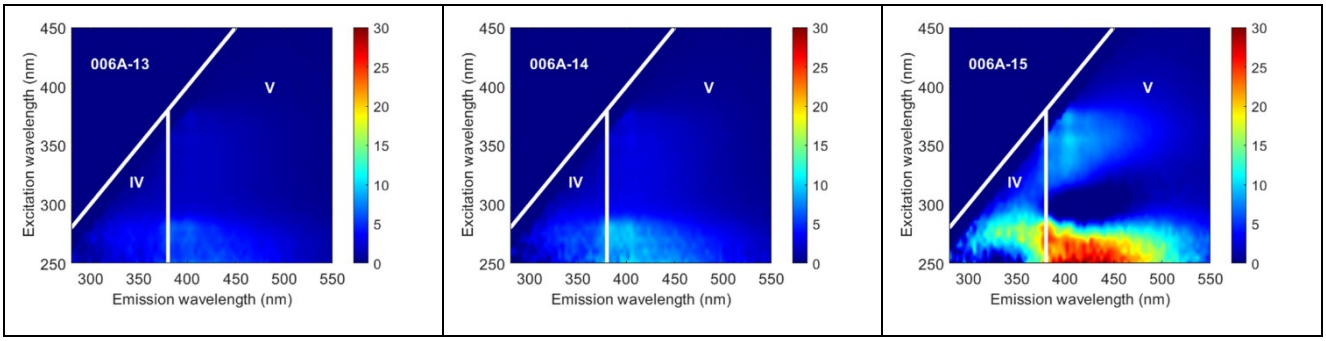


Facility B (continued)

RECHARGED GROUNDWATER FROM EXTRACTION WELL 006A

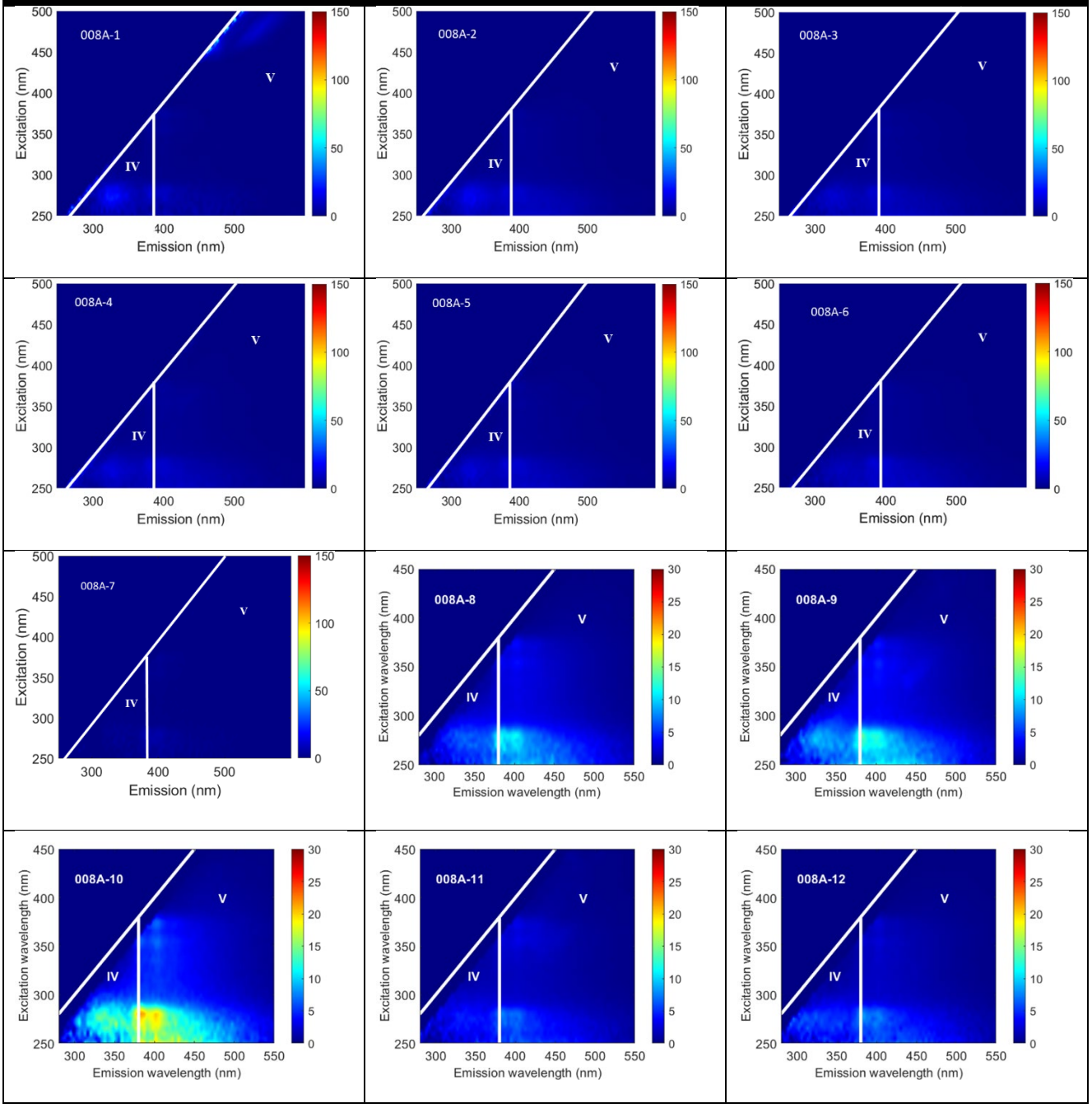


Facility B (continued)

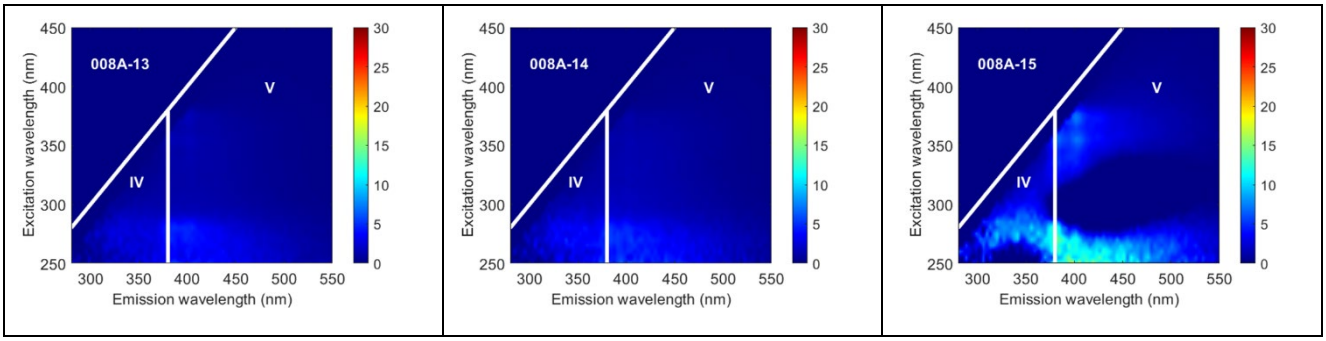


Facility B (continued)

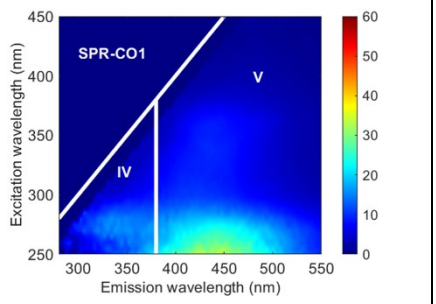
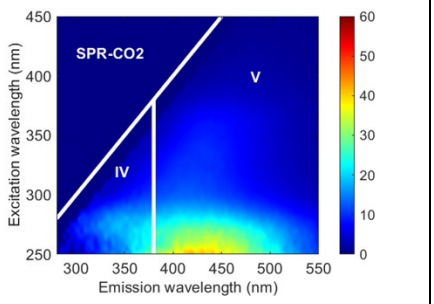
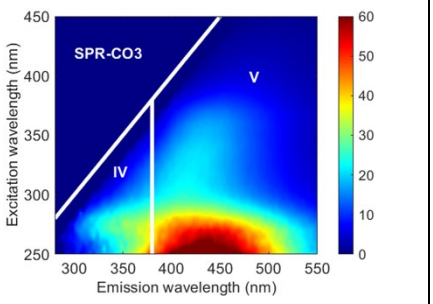
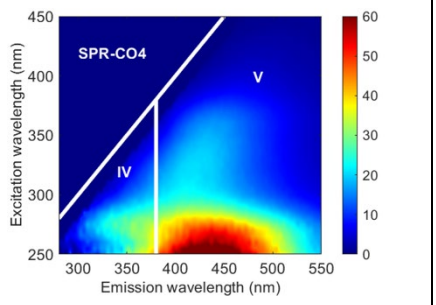
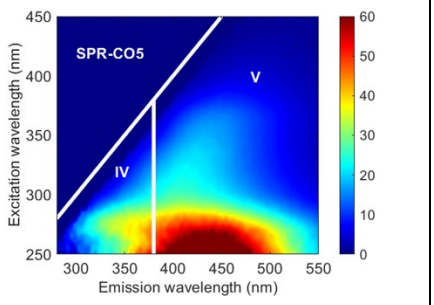
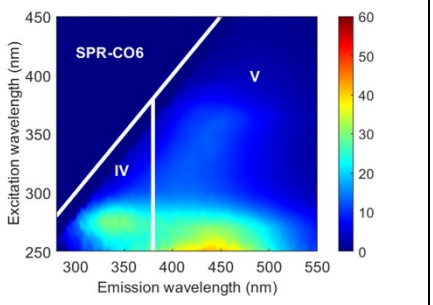
RECHARGED GROUNDWATER FROM EXTRACTION WELL 008A

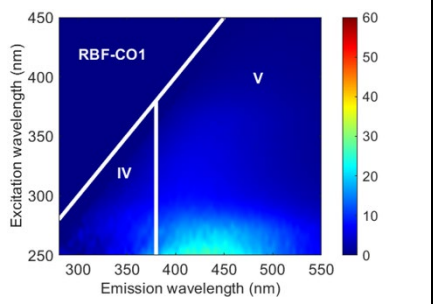
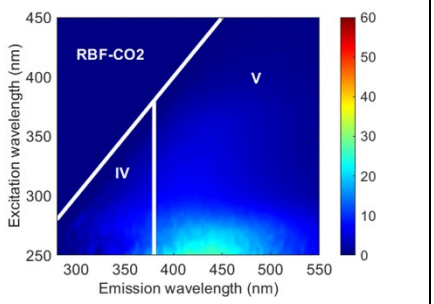
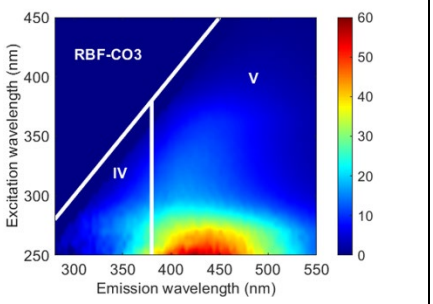
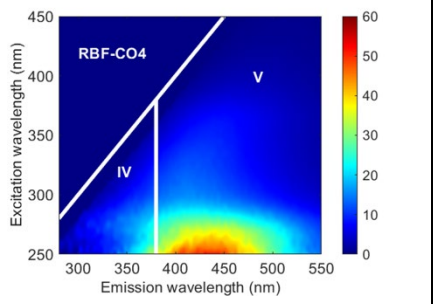
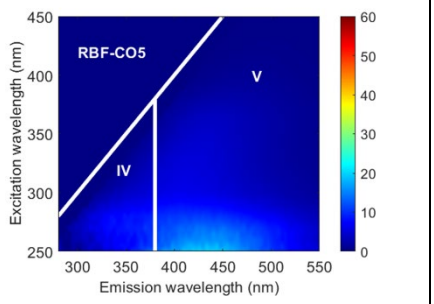


Facility B (continued)

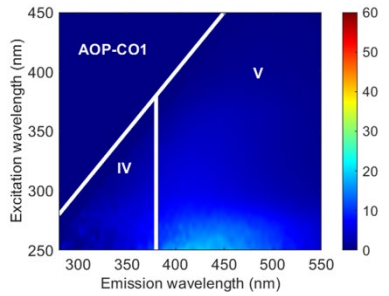
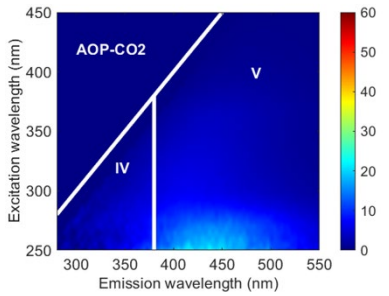
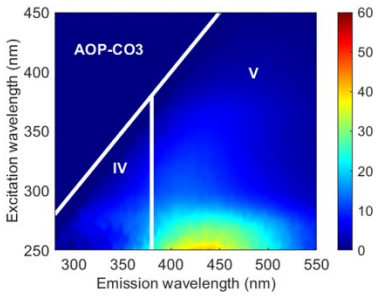
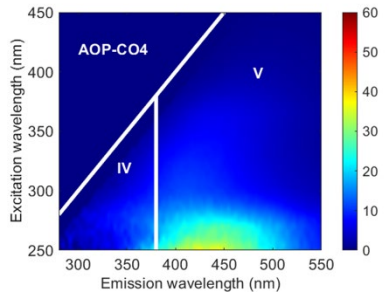
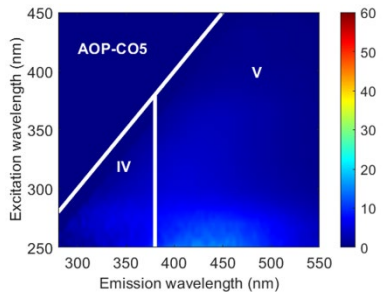


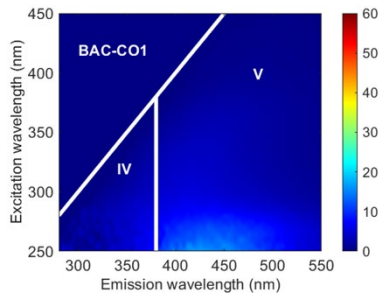
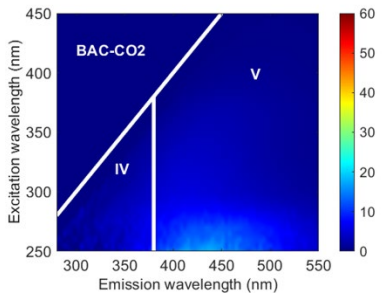
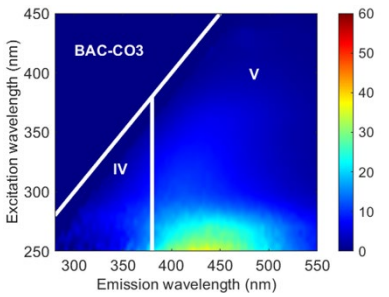
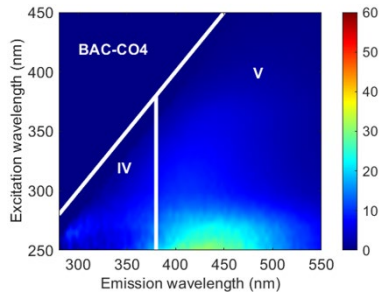
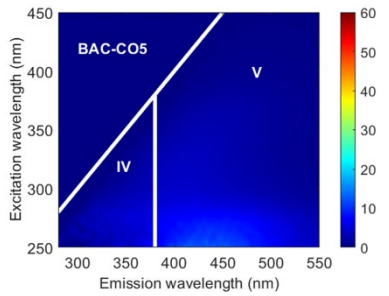
Facility C

SOUTH PLATTE RIVER (SPR)		
		
		

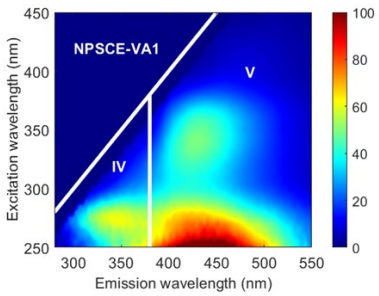
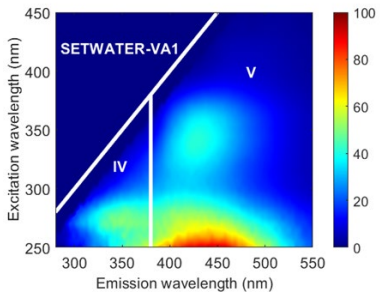
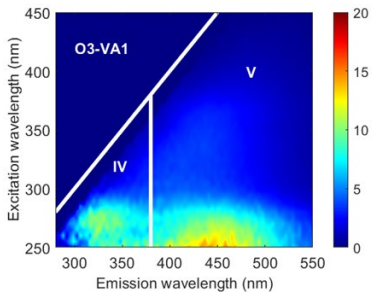
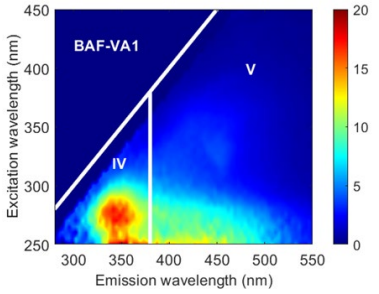
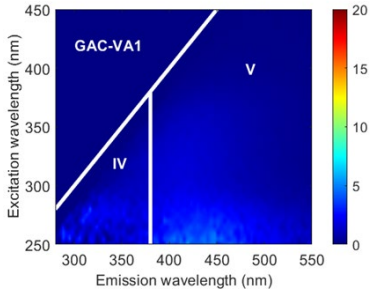
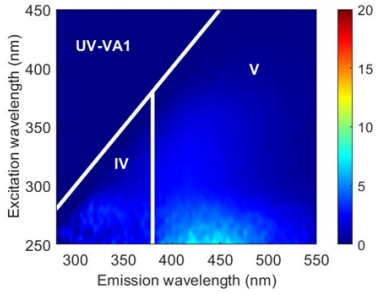
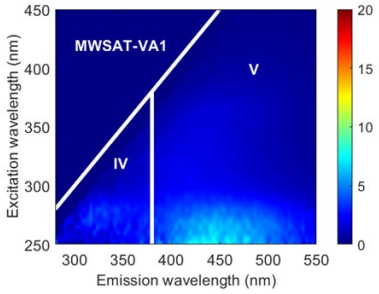
RIVERBANK FILTRATION (RBF)		
		
		

Facility C (continued)

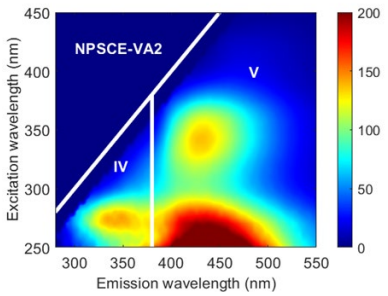
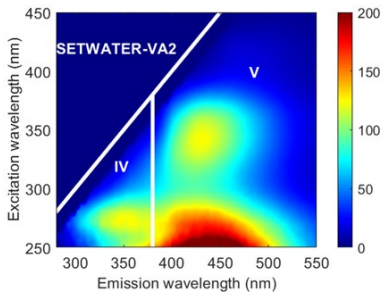
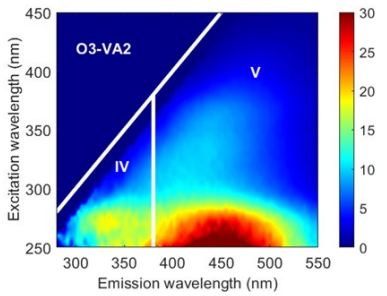
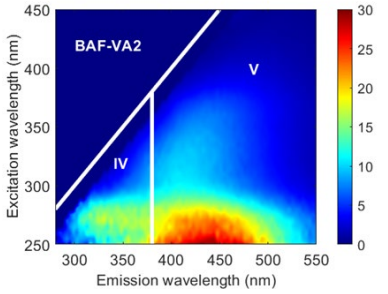
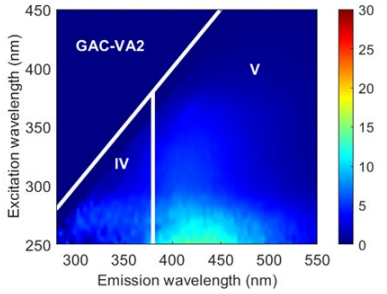
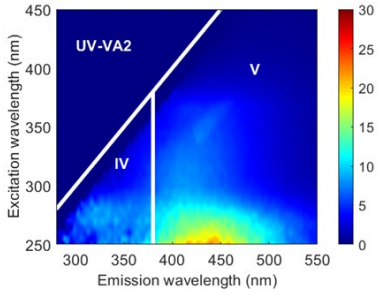
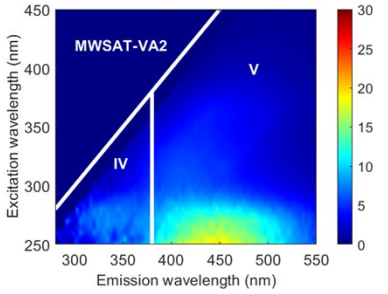
UV DISINFECTION – ADVANCED OXIDATION PROCESS (AOP)		
		
		

BIOLOGICAL ACTIVATED CARBON (BAC)		
		
		

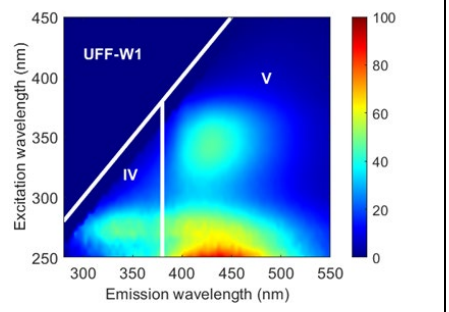
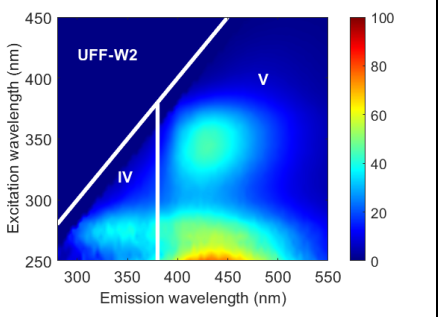
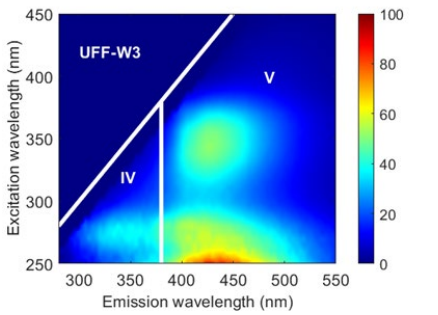
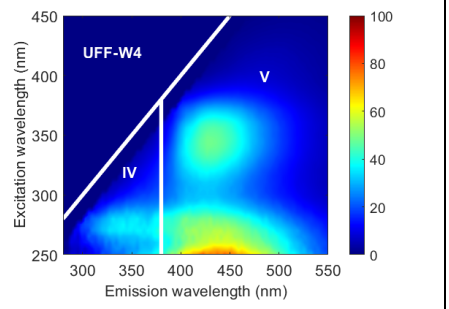
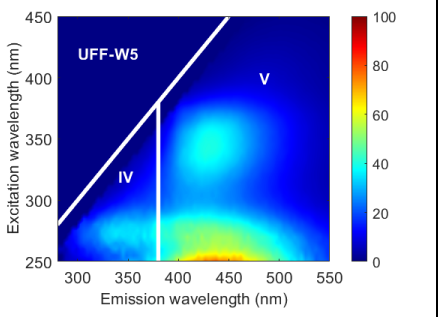
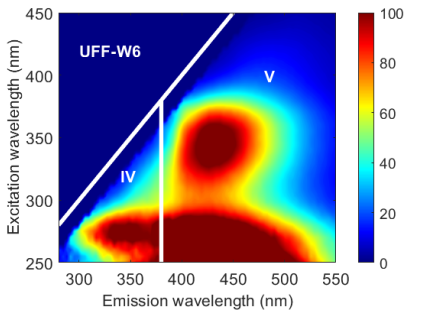
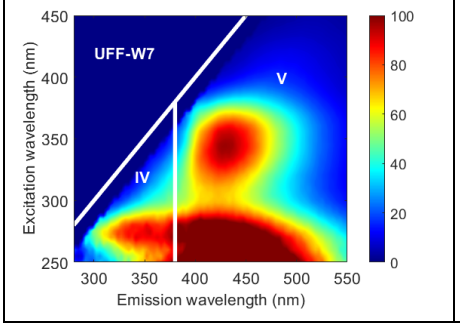
Facility D

SEQUENTIAL TREATMENT – SAMPLE 1		
		
		
		

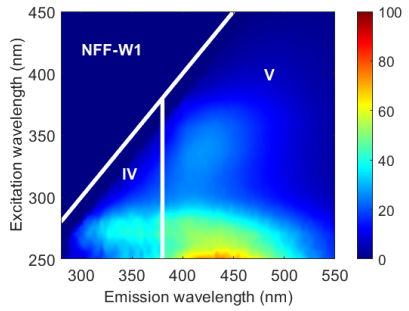
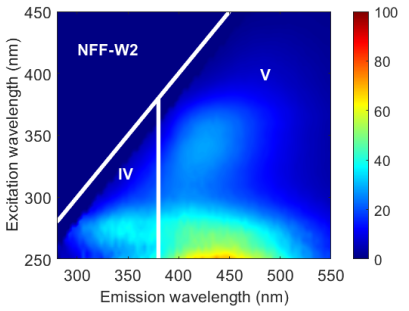
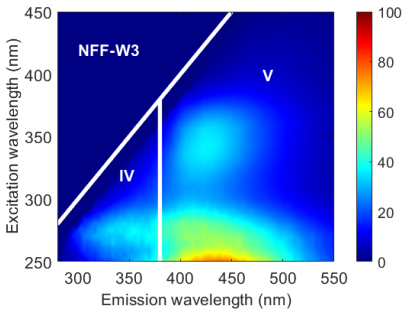
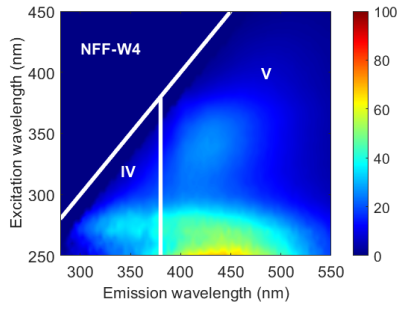
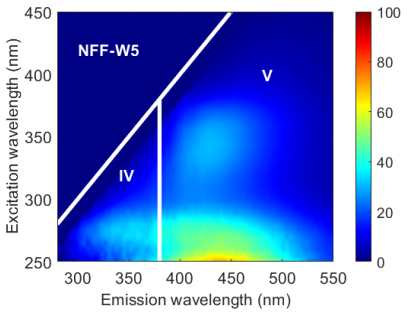
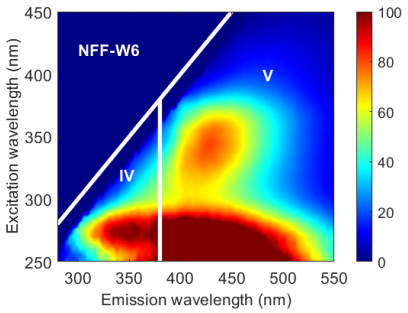
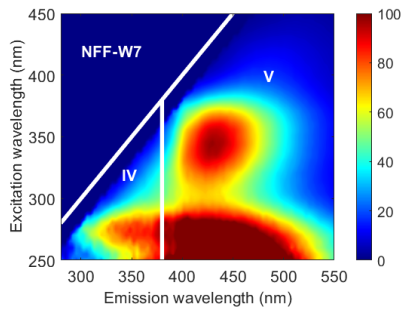
Facility D (continued)

SEQUENTIAL TREATMENT – SAMPLE 2		
		
		
		

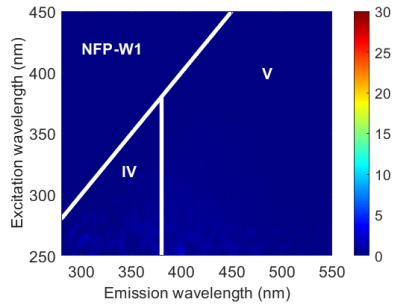
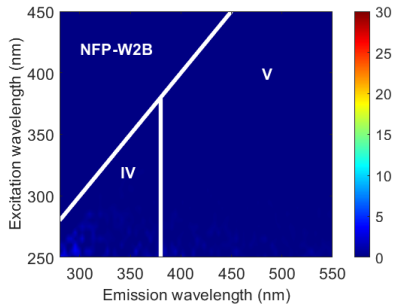
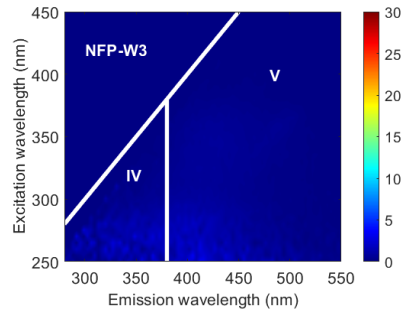
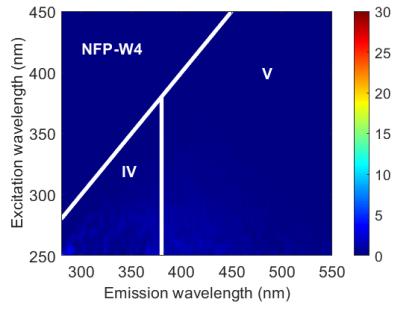
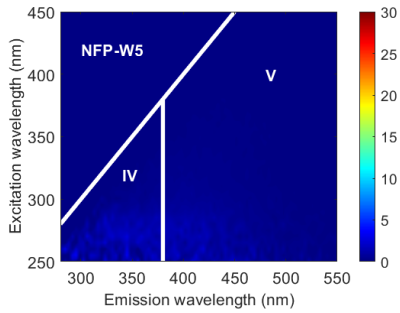
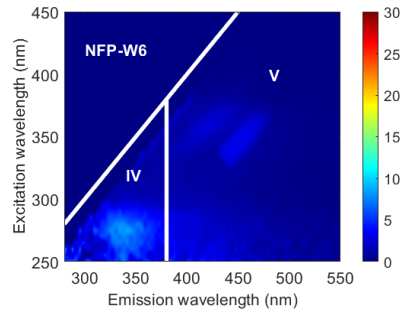
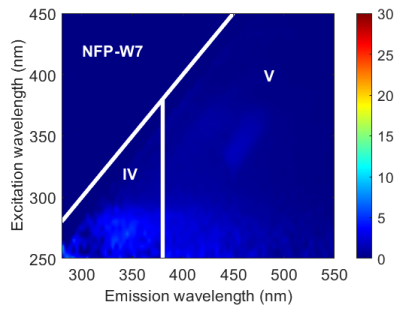
Engineering-Scale Integrated Membrane System

TERTIARY EFFLUENT – ULTRAFILTRATION FEED (UFF)		
		
		
		

Engineering-Scale Integrated Membrane System (continued)

NANOFILTRATION FEED (NFF)		
		
		
		

Engineering-Scale Integrated Membrane System (continued)

NANOFILTRATION PERMEATE (NFP)		
		
		
		

References

Abbas, A. A., L. J. Taylor, M. I. Dothard, J. S. Leiby, A. S. Fitzgerald, L. A. Khatib, R. G. Collman, and F. D. Bushman. 2019. "*Redondoviridae*, a Family of Small, Circular DNA Viruses of the Human Oro-Respiratory Tract Associated with Periodontitis and Critical Illness." *Cell Host & Microbe* 25 (5): 719-729.e4. https://doi.org/10.1016/j.chom.2019.04.001.

Adams, D. J., D. N. Ridinger, R. S. Spendlove, and B. B. Barnett. 1982. "Protamine Precipitation of Two Reovirus Particle Types from Polluted Waters." *Applied and Environmental Microbiology* 44 (3): 589-596. https://doi.org/10.1128/aem.44.3.589-596.1982.

Adriaenssens, E. M., K. Farkas, J. E. McDonald, D. L. Jones, H. E. Allison, and A. J. McCarthy. 2021. "Tracing the Fate Of Wastewater Viruses Reveals Catchment-Scale Virome Diversity and Connectivity." *Water Research* 203: 117568. https://doi.org/10.1016/j.watres.2021.117568.

Al-Abri, M., B. Al-Ghafri, T. Bora, S. Dobretsov, J. Dutta, S. Castelletto, L. Rosa, and A. Boretti. 2019. "Chlorination Disadvantages and Alternative Routes for Biofouling Control in Reverse Osmosis Desalination." *npj Clean Water* 2: 2. https://doi.org/10.1038/s41545-018-0024-8.

Allan, I. J., B. Vrana, J. de Weert, A. Kringstad, A. Ruus, G. Christensen, P. Terentjev, and N. W. Green. 2021. "Passive Sampling and Benchmarking to Rank HOC Levels in the Aquatic Environment." *Scientific Reports* 11: 11231. https://doi.org/10.1038/s41598-021-90457-3.

Amarasiri, M., M. Kitajima, T. H. Nguyen, S. Okabe, and D. Sano. 2017. "Bacteriophage Removal Efficiency as a Validation and Operational Monitoring Tool for Virus Reduction in Wastewater Reclamation: Review." *Water Research* 121: 258-269. https://doi.org/10.1016/j.watres.2017.05.035.

Amy, G., and J. Drewes. 2007. "Soil Aquifer Treatment (SAT) as a Natural and Sustainable Wastewater Reclamation/Reuse Technology: Fate of Wastewater Effluent Organic Matter (Efom) and Trace Organic Compounds." *Environmental Monitoring and Assessment* 129: 19-26. https://doi.org/10.1007/s10661-006-9421-4.

Antony, A., J. Blackbeard, M. Angles, and G. Leslie. 2014. "Non-Microbial Indicators for Monitoring Virus Removal by Ultrafiltration Membranes." *Journal of Membrane Science* 454: 193-199. https://doi.org/10.1016/j.memsci.2013.11.052.

Antony, A., A. Branch, G. Leslie, and P. Le-Clech. 2016. "Impact of Membrane Ageing on Reverse Osmosis Performance – Implications on Validation Protocol." *Journal of Membrane Science* 520: 37-44. https://doi.org/10.1016/j.memsci.2016.07.036.

Armanious, A., M. Aeppli, R. Jacak, D. Refardt, T. Sigstam, T. Kohn, and M. Sander, M. 2016a. "Viruses at Solid–Water Interfaces: A Systematic Assessment of Interactions Driving Adsorption." *Environmental Science & Technology* 50 (2): 732-743. https://doi.org/10.1021/acs.est.5b04644.

Armanious, A., and R. Mezzenga. 2022. "A Roadmap for Building Waterborne Virus Traps." *JACS Au* 2 (10): 2205-2221. https://doi.org/10.1021/jacsau.2c00377.

Armanious, A., M. Münch, T. Kohn, and M. Sander. 2016b. “Competitive Coadsorption Dynamics of Viruses and Dissolved Organic Matter to Positively Charged Sorbent Surfaces.” *Environmental Science & Technology* 50 (7): 3597-3606. https://doi.org/10.1021/acs.est.5b05726.

Asano, T., and J. A. Cotruvo. 2004. “Groundwater Recharge with Reclaimed Municipal Wastewater: Health and Regulatory Considerations.” *Water Research* 38 (8): 1941-1951. https://doi.org/10.1016/j.watres.2004.01.023.

Aw, T. G., A. Howe, and J. B. Rose. 2014. “Metagenomic Approaches for Direct and Cell Culture Evaluation of the Virological Quality Of Wastewater.” *Journal of Virological Methods* 210: 15-21. https://doi.org/10.1016/j.jviromet.2014.09.017.

AWWA (American Water Works Association). 2018. M62, Membrane Applications for Water Reuse, First Edition. Denver, CO: American Water Works Association.

Basumatary, A. K., R. V. Kumar, A. K. Ghoshal, and G. Pugazhenthi. 2015. “Synthesis and Characterization of MCM-41-Ceramic Composite Membrane for the Separation of Chromic Acid from Aqueous Solution.” *Journal of Membrane Science* 475: 521-532. https://doi.org/10.1016/j.memsci.2014.10.055.

Bates, D., M. Mächler, B. Bolker, and S. Walker. 2015. “Fitting Linear Mixed-Effects Models Using lme4.” *Journal of Statistical Software* 67 (1): 1-48. https://doi.org/10.18637/jss.v067.i01.

Bekele, E., S. Toze, B. Patterson, and S. Higginson. 2011. “Managed Aquifer Recharge of Treated Wastewater: Water Quality Changes Resulting from Infiltration Through the Vadose Zone.” *Water Research* 45 (17): 5764-5772. https://doi.org/10.1016/j.watres.2011.08.058.

Betancourt, W. Q. 2020. “Waterborne Plant Viruses of Importance in Agriculture.” In: *Encyclopedia of Sustainability Science and Technology*, edited by R. A. Meyers, 1-15. New York: Springer New York.

Betancourt, W. Q., S. Abd-Elmaksoud, and C. P. Gerba. 2018. “Efficiency of Reovirus Concentration from Water with Positively Charged Filters.” *Food and Environmental Virology* 10 (2): 209-211. https://doi.org/10.1007/s12560-017-9332-2.

Betancourt, W. Q., M. Kitajima, A. D. Wing, J. Regnery, J. E. Drewes, I. L. Pepper, and C. P. Gerba. 2014. “Assessment of Virus Removal By Managed Aquifer Recharge at Three Full-Scale Operations.” *Journal of Environmental Science and Health A*, *Toxic/Hazardous Substances & Environmental Engineering* 49 (14): 1685-1692. https://doi.org/10.1080/10934529.2014.951233.

Betancourt, W. Q., J. Schijven, J. Regnery, A. Wing, C. M. Morrison, J. E. Drewes, and C. P. Gerba. 2019. “Variable Non-Linear Removal of Viruses During Transport Through a Saturated Soil Column.” *Journal of Contaminant Hydrology* 223: 103479. https://doi.org/10.1016/j.jconhyd.2019.04.002.

Betancourt, W. Q., B. W. Schmitz, G. K. Innes, S. M. Prasek, K. M. Pogreba Brown, E. R. Stark, A. R. Foster, R. S. Sprissler, D. T. Harris, S. P. Sherchan, C. P. Gerba, and I. L. Pepper. 2021. “COVID-19 Containment on a College Campus via Wastewater-Based Epidemiology, Targeted Clinical Testing and an Intervention.” *Science of The Total Environment* 779: 146408. https://doi.org/10.1016/j.scitotenv.2021.146408.

Biagini, P. 2004. “Human Circoviruses.” *Veterinary Microbiology* 98 (2): 95-101. https://doi.org/10.1016/j.vetmic.2003.10.004.

Bibby, K., K. Crank, J. Greaves, X. Li, Z. Wu, I. A. Hamza, and E. Stachler. 2019. “Metagenomics and the Development of Viral Water Quality Tools.” *npj Clean Water* 2, 9. https://doi.org/10.1038/s41545-019-0032-3.

Bibby, K., and J. Peccia. 2013. “Identification of Viral Pathogen Diversity in Sewage Sludge by Metagenome Analysis.” *Environmental Science & Technology* 47 (4): 1945-1951. https://doi.org/10.1021/es305181x.

Blatchley, E.R. 3rd, W. L. Gong, J. E. Alleman, J. B. Rose, D. E. Huffman, M. Otaki, and J. T. Lisle. 2007. “Effects of Wastewater Disinfection on Waterborne Bacteria and Viruses.” *Water Environment Research* 79: 81-92. https://doi.org/10.2175/106143006X102024.

Boehm, A. B., A. I. Silverman, A. Schriewer, and K. Goodwin. 2019. “Systematic Review and Meta-Analysis of Decay Rates of Waterborne Mammalian Viruses and Coliphages in Surface Waters.” *Water Research* 164: 114898. https://doi.org/10.1016/j.watres.2019.114898.

Borchardt, M. A., K. R. Bradbury, M. B. Gotkowitz, J. A. Cherry, and B. L. Parker. 2007. “Human Enteric Viruses in Groundwater From a Confined Bedrock Aquifer.” *Environmental Science & Technology* 41 (18): 6606-6612. https://doi.org/10.1021/es071110+.

Boudaud, N., C. Machinal, F. David, A. Fréval-Le Bourdonnec, J. Jossent, F. Bakanga, C. Arnal, M. P. Jaffrezic, S. Oberti, and C. Gantzer. 2012. “Removal of MS2, Qβ and GA bacteriophages During Drinking Water Treatment at Pilot Scale.” *Water Research* 46 (8): 2651-2664. https://doi.org/10.1016/j.watres.2012.02.020.

Bradford, S. A., F. J. Leij, J. Schijven, and S. Torkzaban. 2017. “Critical Role of Preferential Flow in Field-Scale Pathogen Transport and Retention.” *Vadose Zone Journal* 16: 1-13. https://doi.org/10.2136/vzj2016.12.0127.

Brown, M., H. L. Wilson-Friesen, and F. Doane. 1992. “A Block in Release Of Progeny Virus and a High Particle-to-Infectious Unit Ratio Contribute to Poor Growth of Enteric Adenovirus Types 40 and 41 in Cell Culture.” *Journal of Virology* 66 (5): 3198-3205. https://doi.org/10.1128/jvi.66.5.3198-3205.1992.

Burrell, C. J., C. R. Howard, and F. A. Murphy. 2017. “Virion Structure and Composition.” In *Fenner and White’s Medical Virology*, Fifth Edition. Edited by C. J. Burrell, C. R. Howard, and F. A. Murphy. Academic Press.

Cai, M. H., Y.P. Wu, W. X. Ji, Y. Z. Han, Y. Li, J. C. Wu, C. D. Shuang, G. V. Korshin, A. M. Li, and W. T. Li. 2020. “Characterizing Property and Treatability of Dissolved Effluent Organic Matter Using Size Exclusion Chromatography with an Array of Absorbance, Fluorescence, Organic Nitrogen and Organic Carbon Detectors.” *Chemosphere* 243: 125321-125321. https://doi.org/10.1016/j.chemosphere.2019.125321.

Cáñez, T. T., B. Guo, J. C. McIntosh, and M. L. Brusseau. 2021. “Perfluoroalkyl and Polyfluoroalkyl Substances (PFAS) in Groundwater at a Reclaimed Water Recharge Facility.” *Science of The Total Environment* 791: 147906. https://doi.org/10.1016/j.scitotenv.2021.147906.

Canh, V. D., M. Liu, J. Sangsanont, and H. Katayama. 2022. “Capsid Integrity Detection of Pathogenic Viruses in Waters: Recent Progress Aand Potential Future Applications.” *The Science of the Total Environment* 827: 154258. https://doi.org/10.1016/j.scitotenv.2022.154258.

Carstea, E. M., J. Bridgeman, A. Baker, and D. M. Reynolds. 2016. “Fluorescence Spectroscopy for Wastewater Monitoring: A Review.” *Water Research* 95: 205-219. https://doi.org/10.1016/j.watres.2016.03.021.

Cashdollar, J. L., N. E. Brinkman, S. M. Griffin, B. R. McMinn, E. R. Rhodes, E. A. Varughese, A. C. Grimm, S. U. Parshionikar, L. Wymer, and G. S. Fout. 2013. “Development and Evaluation of EPA Method 1615 for Detection of Enterovirus and Norovirus in Water.” *Applied and Environmental Microbiology* 79 (1): 215-223. https://doi.org/10.1128/AEM.02270-12.

Cashdollar, J. L., and L. Wymer. 2013. “Methods for Primary Concentration of Viruses from Water Samples: A Review and Meta-Analysis of Recent Studies.” *Journal of Applied Microbiology* 115 (1): 1-11. https://doi.org/10.1111/jam.12143.

CCR (California Code of Regulations). 2015. Water Recycling Criteria, Title 17, Division 1, State Department of Health Services. State Water Resources Control Board Regulations Related to Recycled Water. Sacramento, CA. https://www.waterboards.ca.gov/drinking_water/certlic/drinkingwater/documents/lawbook/RWregulations_20150716.pdf.

Chaitanya, K. V. 2019. “Structure and Organization of Virus Genomes.” In *Genome and Genomics: From Archaea to Eukaryotes*. Edited by K. V. Chaitanya. Singapore: Springer. pp. 1-30.

Chaudhry, R. M., K. A. Hamilton, C. N. Haas, and K. L. Nelson. 2017. “Drivers of Microbial Risk for Direct Potable Reuse and de Facto Reuse Treatment Schemes: The Impacts of Source Water Quality and Blending.” *International Journal of Environmental Research and Public Health* 14 (6): 635. https://doi.org/10.3390/ijerph14060635.

Chen, W., P. Westerhoff, J. A. Leenheer, and K. Booksh. 2003. “Fluorescence Excitation-Emission Matrix Regional Integration to Quantify Spectra for Dissolved Organic Matter.” *Environmental Science and Technology* 37 (24): 5701-5710. https://doi.org/10.1021/es034354c.

Chowdhari, S., S. Rana, S. Rana, C. M. Morrison, S. E. Abney, R. Singh, P. L. Gurian, A. Kumar, A. Kumar, W. Q. Betancourt, and P. Vivekanandan. “Quantitative Assessment of Microbial Pathogens and Indicators of Wastewater Treatment Performance for Safe and Sustainable Water Reuse in India.” *Microbiology Spectrum* 10 (6): e01720-01722. https://doi.org/10.1128/spectrum.01720-22.

Corpuz, M. V. A., A. Buonerba, G. Vigliotta, T. Zarra, F. Ballesteros Jr., P. Campiglia, V. Belgiorno, G. Korshin, and V. Naddeo. 2020. “Viruses in Wastewater: Occurrence, Abundance and Detection Methods.” *The Science of the Total Environment* 745: 140910. https://doi.org/10.1016/j.scitotenv.2020.140910.

Costantini, V., E. K. Morantz, H. Browne, K. Ettayebi, X. L. Zeng, R. L. Atmar, M. K. Estes, and J. Vinje. 2018. “Human Norovirus Replication in Human Intestinal Enteroids as Model to Evaluate Virus Inactivation.” *Emerging Infectious Diseases* 24 (8): 1453-1464. https://doi.org/10.3201/eid2408.180126.

Cotmore, S. F., M. Agbandje-McKenna, M. Canuti, J. A. Chiorini, A.-M. Eis-Hubinger, J. Hughes, M. Mietzsch, S. Modha, M. Ogliastro, J. J. Pénzes, D. J. Pintel, J. Qiu, M. Soderlund-Venermo, P. Tattersall, P. Tijssen, and I. R. Consortium. 2019. "ICTV Virus Taxonomy Profile: Parvoviridae." *Journal of General Virology* 100 (3): 367-368. https://doi.org/10.1099/jgv.0.001212.

Cromeans, T., G. W. Park, V. Costantini, D. Lee, Q. Wang, T. Farkas, A. Lee, and J. Vinje. 2014. "Comprehensive Comparison of Cultivable Norovirus Surrogates in Response to Different Inactivation and Disinfection Treatments." *Applied and Environmental Microbiology* 80 (18): 5743-5751. https://doi.org/10.1128/AEM.01532-14.

Deininger, R., and J. Lee. 2001. "Rapid Determination of Bacteria in Drinking Water Using an ATP Assay." *Field Analytical Chemistry & Technology* 5 (4): 185-189. https://doi.org/10.1002/fact.1020.

Delanka-Pedige, H. M. K., X. Cheng, S. P. Munasinghe-Arachchige, I. S. A. Abeysiriwardana-Arachchige, J. Xu, N. Nirmalakhandan, and Y. Zhang. 2020. "Metagenomic Insights into Virus Removal Performance of an Algal-Based Wastewater Treatment System Utilizing *Galdieria Sulphuraria*." *Algal Research* 47: 101865. https://doi.org/10.1016/j.algal.2020.101865.

Derx, J., A. P. Blaschke, A. H. Farnleitner, L. Pang, G. Blöschl, and J. F. Schijven. 2013. "Effects of Fluctuations in River Water Level on Virus Removal by Bank Filtration And Aquifer Passage — A Scenario Analysis." *Journal of Contaminant Hydrology* 147: 34-44. https://doi.org/10.1016/j.jconhyd.2013.01.001.

Dias, E., J. Ebdon, and H. Taylor. 2019. "Estimating the Concentration of Viral Pathogens and Indicator Organisms in the Final Effluent of Wastewater Treatment Processes Using Stochastic Modelling." *Microbial Risk Analysis* 11: 47-56. https://doi.org/10.1016/j.mran.2018.08.003.

Dika, C., J. F. L. Duval, G. Francius, A. Perrin, and C. Gantzer. 2015. "Isoelectric Point is an Inadequate Descriptor of MS2, Phi X 174 and PRD1 Phages Adhesion on Abiotic Surfaces." *Journal of Colloid and Interface Science* 446: 327-334. https://doi.org/10.1016/j.jcis.2014.08.055.

Eftim, S. E., T. Hong, J. Soller, A. Boehm, I. Warren, A. Ichida, and S. P. Nappier. 2017. "Occurrence of Norovirus in Raw Sewage - A Systematic Literature Review and Meta-Analysis." *Water Research* 111: 366-374. https://doi.org/10.1016/j.watres.2017.01.017.

ElHadidy, A. M., S. Peldszus, and M. I. Van Dyke. 2013. "An Evaluation of Virus Removal Mechanisms by Ultrafiltration Membranes Using MS2 and ϕX174 Bacteriophage." *Separation and Purification Technology* 120: 215-223. https://doi.org/10.1016/j.seppur.2013.09.026.

ElHadidy, A. M., S. Peldszus, and M. I. Van Dyke. 2014. "Effect of Hydraulically Reversible and Hydraulically Irreversible Fouling on the Removal of MS2 and ϕX174 Bacteriophage by an Ultrafiltration Membrane." *Water Research* 61: 297-307. https://doi.org/10.1016/j.watres.2014.05.003.

Elkayam, R., M. Michail, O. Mienis, T. Kraitzer, N. Tal, and O. Lev. 2015. "Soil Aquifer Treatment as Disinfection Unit." *Journal of Environmental Engineering* 141 (12): 05015001. https://doi.org/10.1061/(ASCE)EE.1943-7870.0000992.

EPA (U.S. Environmental Protection Agency). 2012. Guidelines for Water Reuse, EPA/600/R-12/618. Washington, D.C.: National Risk Management Research Laboratory, Office of Research and Development.

EPA (U.S. Environmental Protection Agency). 2005. Membrane Filtration Guidance Manual, EPA 815-R-06-009. Washington, D.C.: Office of Groundwater and Drinking Water.

Erickson, H. P. 2009. "Size and Shape of Protein Molecules at the Nanometer Level Determined by Sedimentation, Gel Filtration, and Electron Microscopy." *Biological Procedures Online* 11: 32-51. https://doi.org/10.1007/s12575-009-9008-x.

Ettayebi, K., S. E. Crawford, K. Murakami, J. R. Broughman, U. Karandikar, V. R. Tenge, F. H. Neill, S. E. Blutt, X.-L. Zeng, L. Qu, B. Kou, A. R. Opekun, D. Burrin, D. Y. Graham, S. Ramani, R. L. Atmar, and M. K. Estes. 2016. "Replication of Human Noroviruses in Stem Cell-Derived Human Enteroids." *Science* 353: 1387-1393. https://www.science.org/doi/10.1126/science.aaf5211.

Fermin, G. 2018. "Chapter 2 – Virion Structure, Genome Organization, and Taxonomy of Viruses." In *Viruses*. Edited by P. Tennant, G. Fermin, and J. E. Foster. Academic Press. pp. 17-54. https://doi.org/10.1016/B978-0-12-811257-1.00002-4.

Ferrer, O., R. Casas, C. Galvañ, F. Lucena, A. Vega, O. Gibert, J. Jofre, and X. Bernat. 2013. "Challenge Tests with Virus Surrogates: An Accurate Membrane Integrity Evaluation System?" *Desalination and Water Treatment* 51: 4947-4957. https://doi.org/10.1080/19443994.2013.795339.

Ferrer, O., S. Casas, C. Galvañ, F. Lucena, A. Bosch, B. Galofré, J. Mesa, J. Jofre, and X. Bernat. 2015. "Direct Ultrafiltration Performance and Membrane Integrity Monitoring by Microbiological Analysis." *Water Research* 83: 121-131. https://doi.org/10.1016/j.watres.2015.06.039.

Fierer, N., H. Holland-Moritz, A. Alexiev, H. Batther, N. B. Dragone, L. Friar, M. J. Gebert, S. Gering, J. B. Henley, S. Jech, E. M. Kibby, T. Melie, W. B. Patterson, E. Peterson, K. Schutz, E. Stallard-Olivera, J. Sterrett, C. Walsh, and C. Mansfeldt. 2022. "A Metagenomic Investigation of Spatial and Temporal Changes in Sewage Microbiomes across a University Campus." *mSystems* 7 (5): e00651-00622. https://doi.org/10.1128/msystems.00651-22.

Filter, J., V. Zhiteneva, C. Vick, A. S. Ruhl, M. Jekel, U. Hübner, and J. E. Drewes. 2021. "Varying Attenuation of Trace Organic Chemicals in Natural Treatment Systems – A Review Of Key Influential Factors." *Chemosphere* 274: 129774. https://doi.org/10.1016/j.chemosphere.2021.129774.

Flannery, J., S. Keaveney, P. Rajko-Nenow, V. O'Flaherty, and W. Dore. 2012. "Concentration of Norovirus During Wastewater Treatment and its Impact on Oyster Contamination." *Applied and Environmental Microbiology* 78 (9): 3400-3406. https://doi.org/10.1128/AEM.07569-11.

Folkins, M. A., R. Dey, and N. J. Ashbolt. 2020. "Interactions between Human Reovirus and Free-Living Amoebae: Implications for Enteric Virus Disinfection and Aquatic Persistence." *Environmental Science & Technology* 54 (16): 10201-10206. https://doi.org/10.1021/acs.est.0c02896.

Fout, G. S., B. C. Martinson, M. W. Moyer, and D. R. Dahling. 2003. “A Multiplex Reverse Transcription-PCR Method for Detection of Human Enteric Viruses in Groundwater.” *Applied and Environmental Microbiology* 69 (6): 3158-3164. https://doi.org/10.1128/AEM.69.6.3158-3164.2003.

Fox, P., and R. Makam. 2009. “Surface Area and Travel Time Relationships in Aquifer Treatment Systems.” *Water Environment Research* 81 (11): 2337-2343. https://www.jstor.org/stable/40575508.

Francy, D. S., E. A. Stelzer, R. N. Bushon, A. M. Brady, A. G. Williston, K. R. Riddell, M. A. Borchardt, S. K. Spencer, and T. M. Gellner. 2012. “Comparative effectiveness of Membrane Bioreactors, Conventional Secondary Treatment, and Chlorine and UV Disinfection to Remove Microorganisms from Municipal Wastewaters.” *Water Research* 46 (13): 4164-4178. https://doi.org/10.1016/j.watres.2012.04.044.

Frohnert, A., S. Apelt, S. Klitzke, I. Chorus, R. Szewzyk, and H. C. Selinka. 2014. “Transport and Removal of Viruses in Saturated Sand Columns Under Oxic and Anoxic Conditions - Potential Implications for Groundwater Protection.” *International Journal of Hygiene and Environmental Health* 217 (8): 861-870. https://doi.org/10.1016/j.ijheh.2014.06.004.

Fundneider, T., V. Acevedo Alonso, A. Wick, D. Albrecht, and S. Lackner. 2021. “Implications of Biological Activated Carbon Filters for Micropollutant Removal in Wastewater Treatment.” *Water Research* 189: 116588. https://doi.org/10.1016/j.watres.2020.116588.

Gall, A. M., B. J. Marinas, Y. Lu, and J. L. Shisler. 2015. “Waterborne Viruses: A Barrier to Safe Drinking Water.” *PLoS Pathogens* 11 (6): e1004867. https://doi.org/10.1371/journal.ppat.1004867.

Gendron, L., D. Verreault, M. Veillette, S. Moineau, and C. Duchaine. 2010. “Evaluation of Filters for the Sampling and Quantification of RNA Phage Aerosols.” *Aerosol Science and Technology* 44 (10): 893-901. https://doi.org/10.1080/02786826.2010.501351.

Gentile, G. J., M. C. Cruz, V. B. Rajal, and M. M. Fidalgo de Cortalezzi. 2018. “Electrostatic Interactions in Virus Removal By Ultrafiltration Membranes.” *Journal of Environmental Chemical Engineering* 6 (1): 1314-1321. https://doi.org/10.1016/j.jece.2017.11.041.

Gerba, C., D. Powelson, M. Yahya, L. G. Wilson, and G. Amy. 1991. “Fate of Viruses in Treated Sewage Effluent During Soil Aquifer Treatment Designed for Wastewater Reclamation and Reuse.” *Water Science & Technology* 24 (9): 95-102. https://doi.org/10.2166/wst.1991.0239.

Gerba, C. P., and W. Q. Betancourt. 2017. “Viral Aggregation: Impact on Virus Behavior in the Environment.” *Environmental Science & Technology* 51 (13): 7318-7325. https://doi.org/10.1021/acs.est.6b05835.

Gerba, C. P., and W. Q. Betancourt. 2019. “Assessing the Occurrence of Waterborne Viruses in Reuse Systems: Analytical Limits and Needs.” *Pathogens* 8 (3): 107. https://doi.org/10.3390/pathogens8030107.

Gerba, C. P., W. Q. Betancourt, and M. Kitajima. 2017. “How Much Reduction of Virus is Needed for Recycled Water: A Continuous Changing Need for Assessment?” *Water Research* 108: 25-31. https://doi.org/10.1016/j.watres.2016.11.020.

Gerba, C. P., W. Q. Betancourt, M. Kitajima, and C. M. Rock. 2018. “Reducing Uncertainty in Estimating Virus Reduction By Advanced Water Treatment Processes.” *Water Research* 133: 282-288. https://doi.org/10.1016/j.watres.2018.01.044.

Gibson, K. E., and M. A. Borchardt. 2016. “Methods for Virus Recovery in Water.” In *Viruses in Foods. Food Microbiology and Food Safety*. Edited by S. Goyal and J. Cannon. Springer. https://doi.org/10.1007/978-3-319-30723-7_10.

Gilling, D. H., M. Kitajima, J. R. Torrey, and K. R. Bright. 2014. “Mechanisms of Antiviral Action of Plant Antimicrobials Against Murine Norovirus.” *Applied and Environmental Microbiology* 80 (16): 4898-4910. https://doi.org/10.1128/AEM.00402-14.

Girones, R., M. A. Ferrus, J. L. Alonso, J. Rodriguez-Manzano, B. Calgua, A. Correa Ade, A. Hundesa, A. Carratala, and S. Bofill-Mas. 2010. “Molecular Detection of Pathogens in Water--The Pros and Cons of Molecular Techniques.” *Water Research* 44 (15): 4325-4339. https://doi.org/10.1016/j.watres.2010.06.030.

Gonzales-Gustavson, E., M. Rusiñol, G. Medema, M. Calvo, and R. Girones. 2019. “Quantitative Risk Assessment of Norovirus and Adenovirus for the Use of Reclaimed Water to Irrigate Lettuce in Catalonia.” *Water Research* 153: 91-99. https://doi.org/10.1016/j.watres.2018.12.070.

Goswami, K. P., and G. Pugazhenthi. 2020. “Credibility of Polymeric and Ceramic Membrane Filtration in the Removal of Bacteria and Virus from Water: A Review.” *Journal of Environmental Management* 268: 110583. https://doi.org/10.1016/j.jenvman.2020.110583.

Gregory, J. B., R. W. Litaker, and R. T. Noble. 2006. “Rapid One-Step Quantitative Reverse Transcriptase PCR Assay with Competitive Internal Positive Control for detection of enteroviruses in Environmental Samples.” *Applied and Environmental Microbiology* 72 (6): 3960-3967. https://doi.org/10.1128/AEM.02291-05.

Grondahl-Rosado, R. C., E. Yarovitsyna, E. Trettenes, M. Myrmel, and L. J. Robertson. 2014. “A One Year Study on the Concentrations of Norovirus and Enteric Adenoviruses in Wastewater and A Surface Drinking Water Source in Norway.” *Food and Environmental Virology* 6: 232-245. https://doi.org/10.1007/s12560-014-9161-5.

Haak, L., B. Delic, L. Li, T. Guarin, L. Mazurowski, N. G. Dastjerdi, A. Dewan, and K. Pagilla. 2022. “Spatial and Temporal Variability and Data Bias in Wastewater Surveillance of SARS-CoV-2 in a Sewer System.” *Science of the Total Environment* 805: 150390. https://doi.org/10.1016/j.scitotenv.2021.150390.

Hammes, F., F. Goldschmidt, M. Vital, Y. Wang, and T. Egli. 2010. “Measurement and Interpretation of Microbial Adenosine Tri-Phosphate (ATP) in Aquatic Environments.” *Water Research* 44 (13): 3915-3923. https://doi.org/10.1016/j.watres.2010.04.015.

Haramoto, E., M. Kitajima, A. Hata, J. R. Torrey, Y. Masago, D. Sano, and H. Katayama. 2018. “A Review on Recent Progress in the Detection Methods And Prevalence of Human Enteric Viruses in Water.” *Water Research* 135: 168-186. https://doi.org/10.1016/j.watres.2018.02.004.

Haramoto, E., M. Kitajima, N. Kishida, Y. Konno, H. Katayama, M. Asami, and M. Akiba. 2013. "Occurrence of Pepper Mild Mottle Virus in Drinking Water Sources in Japan." *Applied and Environmental Microbiology* 79 (23): 7413-7418. https://doi.org/10.1128/AEM.02354-13.

Haramoto, E., and M. Otagiri. 2014. "Occurrence of Human Cosavirus in Wastewater and River Water in Japan." *Food and Environmental Virology* 6: 62-66. https://doi.org/10.1007/s12560-013-9120-6.

Harwood, V. J., A. D. Levine, T. M. Scott, V. Chivukula, J. Lukasik, S. R. Farrah, and J. B. Rose. 2005. "Validity of the Indicator Organism Paradigm for Pathogen Reduction in Reclaimed Water and Public Health Protection." *Applied and Environmental Microbiology* 71 (6): 3163-3170. https://doi.org/10.1128/AEM.71.6.3163-3170.2005.

Hata, A., M. Kitajima, and H. Katayama. 2013. "Occurrence and Reduction Of Human Viruses, F-specific RNA Coliphage Genogroups and Microbial Indicators at a Full-Scale Wastewater Treatment Plant in Japan." *Journal of Applied Microbiology* 114 (2): 545-554. https://doi.org/10.1111/jam.12051.

Hata, A., K. Matsumori, M. Kitajima, and H. Katayama. 2015. "Concentration of Enteric Viruses in Large Volumes of Water Using a Cartridge-Type Mixed Cellulose Ester Membrane." *Food and Environmental Virology* 7: 7-13. https://doi.org/10.1007/s12560-014-9169-x.

Hayes, E. K., A. K. Stoddart, and G. A. Gagnon. 2022. "Adsorption of SARS-CoV-2 onto Granular Activated Carbon (GAC) in wastewater: Implications for Improvements in Passive Sampling." *Science of the Total Environment* 847: 157548. https://doi.org/10.1016/j.scitotenv.2022.157548.

He, J. W., and S. Jiang. 2005. "Quantification of Enterococci and Human Adenoviruses in Environmental Samples by Real-Time PCR." *Applied and Environmental Microbiology* 71 (5): 2250-2255. https://doi.org/10.1128/AEM.71.5.2250-2255.2005.

Heffron, J., and B. K. Mayer. 2016. "Emerging Investigators Series: Virus Mitigation by Coagulation: Recent Discoveries and Future Directions." *Environmental Science: Water Research & Technology* 2: 443-459. https://doi.org/10.1039/C6EW00060F.

Heffron, J., and B. K. Mayer. 2020. "Improved Virus Isoelectric Point Estimation by Exclusion of Known and Predicted Genome-Binding Regions." *Applied and Environmental Microbiology* 86 (23): e01674-01620. https://doi.org/10.1128/AEM.01674-20.

Heffron, J., and B. K. Mayer. 2021. "Virus Isoelectric Point Estimation: Theories and Methods." *Applied and Environmental Microbiology* 87 (3): e02319-02320. https://doi.org/10.1128/AEM.02319-20.

Heim, A., C. Ebnet, G. Harste, and P. Pring-Akerblom. 2003. "Rapid and Quantitative Detection of Human Adenovirus DNA by Real-Time PCR." *Journal of Medical Virology* 70 (2): 228-239. https://doi.org/10.1002/jmv.10382.

Hellmér, M., N. Paxéus, L. Magnius, L. Enache, B. Arnholm, A. Johansson, T. Bergström, and H. Norder. 2014. "Detection of Pathogenic Viruses in Sewage Provided Early Warnings of Hepatitis A Virus and Norovirus Outbreaks." *Applied and Environmental Microbiology* 80 (21): 6771-6781. https://doi.org/10.1128/AEM.01981-14.

Henderson, R. K., R. M. Stuetz, and S. J. Khan. 2010. “Demonstrating Ultra-Filtration and Reverse Osmosis Performance using Size Exclusion Chromatography.” *Water Science and Technology : A Journal of the International Association on Water Pollution Research* 62 (12): 2747-2753. https://doi.org/10.2166/wst.2010.423.

Hewitt, J., M. Leonard, G. E. Greening, and G. D. Lewis. 2011. “Influence of Wastewater Treatment Process and the Population Size on Human Virus Profiles in Wastewater.” *Water Research* 45 (18): 6267-6276. https://doi.org/10.1016/j.watres.2011.09.029.

Hijnen, W. A., G. M. Suylen, J. A. Bahlman, A. Brouwer-Hanzens, and G. J. Medema. 2010. “GAC Adsorption Filters as Barriers for Viruses, Bacteria and Protozoan (Oo)Cysts in Water Treatment.” *Water Research* 44 (4): 1224-1234. https://doi.org/10.1016/j.watres.2009.10.011.

Hornstra, L. M., T. Rodrigues da Silva, B. Blankert, L. Heijnen, E. Beerendonk, E. R. Cornelissen, and G. Medema. 2019. “Monitoring the Integrity of Reverse Osmosis Membranes Using Novel Indigenous Freshwater Viruses and Bacteriophages.” *Environmental Science: Water Research & Technology* 5: 1535-1544. https://doi.org/10.1039/C9EW00318E.

Hornstra, L. M., J. F. Schijven, A. Waade, G. S. Prat, F. J. C. Smits, G. Cirkel, P. J. Stuyfzand, and G. J. Medema. 2018. “Transport of Bacteriophage MS2 and PRD1 in Saturated Dune Sand Under Suboxic Conditions.” *Water Research* 139: 158-167. https://doi.org/10.1016/j.watres.2018.03.054.

Hu, J. Y., S. L. Ong, L. F. Song, Y. Y. Feng, W. T. Liu, T. W. Tan, L. Y. Lee, and W. J. Ng. 2003. “Removal of MS2 Bacteriophage Using Membrane Technologies.” *Water Science and Technology : A Journal of the International Association on Water Pollution Research* 47 (12): 163-168. https://doi.org/10.2166/wst.2003.0642.

Huber, S. A., A. Balz, M. Abert, and W. Pronk. 2011. “Characterisation of aquatic humic and Non-Humic Matter with Size-Exclusion Chromatography - Organic Carbon Detection - Organic Nitrogen Detection (LC-OCD-OND).” *Water Res*. 45 (2): 879-885. https://doi.org/10.1016/j.watres.2010.09.023.

Huggett, J. F. 2020. “The Digital MIQE Guidelines Update: Minimum Information for Publication of Quantitative Digital PCR Experiments for 2020.” *Clinical Chemistry* 66 (8): 1012-1029. https://doi.org/10.1093/clinchem/hvaa125.

Iaconelli, M., M. Divizia, S. Della Libera, P. Di Bonito, and G. La Rosa. 2016. “Frequent Detection and Genetic Diversity of Human Bocavirus in Urban Sewage Samples.” *Food and Environmental Virology* 8: 289-295. https://doi.org/10.1007/s12560-016-9251-7.

Ikner, L. A., Gerba, C. P., and K. R. Bright. 2012. “Concentration and Recovery of Viruses from Water: A Comprehensive Review.” *Food and Environmental Virology* 4: 41-67. https://doi.org/10.1007/s12560-012-9080-2.

Ikner, L. A., M. Soto-Beltran, and K. R. Bright. 2011. “New method using a Positively Charged Microporous Filter and Ultrafiltration for Concentration of Viruses from Tap Water.” *Applied and Environmental Microbiology* 77 (10): 3500-3506. https://doi.org/10.1128/AEM.02705-10.

Ito, T., T. Kato, M. Hasegawa, H. Katayama, S. Ishii, S. Okabe, and D. Sano. 2016. “Evaluation of Virus Reduction Efficiency in Wastewater Treatment Unit Processes as a credit value in the Multiple-Barrier System for Wastewater Reclamation and Reuse.” *Journal of Water and Health* 14 (6): 879-889. https://doi.org/10.2166/wh.2016.096.

Jacangelo, J. G., A. Subramani, S. Gray, and M. Cran 2019. *Methods for Integrity Testing of NF and RO Membranes*. Project 4757. Denver, CO: The Water Research Foundation.

Jacangelo, J. G., J.-M. Laîné, K. E. Carns, E. W. Cummings, and J. Mallevialle., 1991. “Low-Pressure Membrane Filtration for Removing Giardia and Microbial Indicators.” *Journal AWWA* 83 (9): 97-106. https://doi.org/10.1002/j.1551-8833.1991.tb07219.x.

Jacangelo, J. G., A. Madec, K. J. Schwab, D. Huffman, and C. S. Mysore. 2005. “Advances in the Use of Low-Pressure, Hollow Fiber Membranes for the Disinfection of Water.” *Water Science and Technology: Water Supply* 5 (5): 109-115. https://doi.org/10.2166/ws.2005.0045.

Jiang, S. C., H. N. Bischel, R. Goel, D. Rosso, S. P. Sherchan, K. L. Whiteson, T. Yan, and H. M. Solo-Gabriele. 2022. “Integrating Virus Monitoring Strategies for Safe Non-Potable Water Reuse.” *Water* 14 (8): 1187. https://doi.org/10.3390/w14081187.

Jofre, J., F. Lucena, A. Blanch, and M. Muniesa. 2016. “Coliphages as Model Organisms in the Characterization and Management of Water Resources.” *Water* 8 (5): 199. https://doi.org/10.3390/w8050199.

John, D. E., and J. B. Rose. 2005. “Review of Factors Affecting Microbial Survival in Groundwater.” *Environmental Science & Technology* 39 (19): 7345-7356. https://doi.org/10.1021/es047995w.

Johnson, T. B., L. D. McKay, A. C. Layton, S. W. Jones, G. C. Johnson, J. L. Cashdollar, D. R. Dahling, L. F. Villegas, G. S. Fout, D. E. Williams, and G. Sayler. 2011. “Viruses and Bacteria in Karst and Fractured Rock Aquifers in East Tennessee, USA.” *Groundwater* 49 (1): 98-110. https://doi.org/10.1111/j.1745-6584.2010.00698.x.

Kageyama, T., S. Kojima, M. Shinohara, K. Uchida, S. Fukushi, F. B. Hoshino, N. Takeda, and K. Katayama. 2003. “Broadly Reactive and Highly Sensitive Assay for Norwalk-like Viruses Based on Real-time Quantitative Reverse Transcription-PCR.” *Journal of Clinical Microbiology* 41 (4): 1548-1557. https://doi.org/10.1128/jcm.41.4.1548-1557.2003.

Karthikeyan, S., N. Ronquillo, P. Belda-Ferre, D. Alvarado, T. Javidi, C. A. Longhurst, and R. Knight. 2021. “High-Throughput Wastewater SARS-CoV-2 Detection Enables Forecasting of Community Infection Dynamics in San Diego County.” *mSystems* 6 (2): e00045-00021. https://doi.org/10.1128/msystems.00045-21.

Kato, R., T. Asami, T., E. Utagawa, H. Furumai, and H. Katayama. 2018. “Pepper Mild Mottle Virus as a Process Indicator at Drinking Water Treatment Plants Employing Coagulation-Sedimentation, Rapid Sand Filtration, Ozonation, and Biological Activated Carbon Treatments in Japan.” *Water Research* 132: 61-70. https://doi.org/10.1016/j.watres.2017.12.068.

Kauppinen, A., and I. T. Miettinen. 2017. “Persistence of Norovirus GII Genome in Drinking Water and Wastewater at Different Temperatures.” *Pathogens* 6 (4): 48. https://doi.org/10.3390/pathogens6040048.

Kerr, M., K. Rosario, C. C. M. Baker, and M. Breitbart. 2018. “Discovery of Four Novel Circular Single-Stranded DNA Viruses in Fungus-Farming Termites.” *Genome Announcements* 6 (17). https://doi.org/10.1128/genomea.00318-18.

Kim, J., J. Lim, and C. Lee. 2013. “Quantitative Real-time PCR approaches for Microbial Community Studies in Wastewater Treatment Systems: Applications and Considerations.” *Biotechnology Advances* 31 (8): 1358-1373. https://doi.org/10.1016/j.biotechadv.2013.05.010.

Kim, M. B., S. Hwangbo, S. Jang, and Y. K. Jo. 2022. “Bioengineered Co-culture of Organoids to Recapitulate Host-Microbe Interactions.” *Materials Today Bio* 16: 100345. https://doi.org/10.1016/j.mtbio.2022.100345.

Kirby, A. E., P. F. Teunis, and C. L. Moe. 2014. “Two Human Challenge Studies Confirm High Infectivity of Norwalk Virus.” *The Journal of Infectious Diseases* 211 (1): 166-167. https://doi.org/10.1093/infdis/jiu385.

Kitajima, M., A. Hata, T. Yamashita, E. Haramoto, H. Minagawa, and H. Katayama. 2013. “Development of a Reverse Transcription-quantitative PCR System for Detection and Genotyping of Aichi Viruses in Clinical and Environmental Samples.” *Applied and Environmental Microbiology* 79 (13): 3952-3958. https://doi.org/10.1128/AEM.00820-13.

Kitajima, M., B. C. Iker, I. L. Pepper, and C. P. Gerba. 2014. “Relative Abundance and Treatment Reduction of Viruses During Wastewater Treatment Processes--Identification of Potential Viral Indicators.” *The Science of the Total Environment* 488-489: 290-296. https://doi.org/10.1016/j.scitotenv.2014.04.087.

Kitajima, M., T. Oka, H. Takagi, Y. Tohya, H. Katayama, N. Takeda, and K. Katayama. 2010. “Development and Application of a Broadly Reactive Real-Time Reverse Transcription-PCR Assay for Detection of Murine Noroviruses.” *J Virol Methods* 169 (2): 269-273. https://doi.org/10.1016/j.jviromet.2010.07.018.

Kitajima, M., A. T. Rachmadi, B. C. Iker, E. Haramoto, and C. P. Gerba. 2018. “Temporal Variations in Genotype Distribution of Human Sapoviruses and Aichi Virus 1 in Wastewater in Southern Arizona, United States.” *Journal of Applied Microbiology* 124 (5): 1324-1332. https://doi.org/10.1111/jam.13712.

Kitis, M., J. C. Lozier, J.-H. Kim, B. Mi, and B. J. Mariñas. 2003. “Microbial Removal and Integrity Monitoring of RO and NF Membranes.” *Journal AWWA* 95 (12): 105-119. https://doi.org/10.1002/j.1551-8833.2003.tb10515.x.

Kiulia, N. M., R. Gonzalez, H. Thompson, T. G. Aw, and J. B. Rose. 2021. “Quantification and Trends of Rotavirus and Enterovirus in Untreated Sewage Using Reverse Transcription Droplet Digital PCR.” *Food and Environmental Virology* 13: 154-169. https://doi.org/10.1007/s12560-020-09455-9.
Klasse, P. J. 2015. “Molecular Determinants of the Ratio of Inert to Infectious Virus Particles.” *Progress in Molecular Biology and Translational Science* 129: 285-326. https://doi.org/10.1016/bs.pmbts.2014.10.012.

Kobayashi, N., M. Oshiki, T. Ito, T. Segawa, M. Hatamoto, T. Kato, T. Yamaguchi, K. Kubota, M. Takahashi, A. Iguchi, T. Tagawa, T. Okubo, S. Uemura, H. Harada, T. Motoyama, N. Araki, and D. Sano. 2017. "Removal of Human Pathogenic Viruses in a Down-Flow Hanging Sponge (DHS) Reactor Treating Municipal Wastewater and Health Risks Associated with Utilization of the Effluent for Agricultural Irrigation." *Water Research* 110: 389-398. https://doi.org/10.1016/j.watres.2016.10.054.

Kreissel, K., M. Bösl, P. Lipp, M. Franzreb, and B. Hambsch. 2012. "Study on the Removal Efficiency of UF Membranes Using Bacteriophages in Bench-Scale and Semi-Technical Scale." *Water Science and Technology* 66 (6): 1195-1202. https://doi.org/10.2166/wst.2012.299.

Kruithof, J., P. C. Kamp, H. C. Folmer, M. M. Nedrelof, and S. C. J. M. Hoof. 2001. "Development of a Membrane Integrity Monitoring Strategy for the UF/RO Heemskerk Drinking Water Treatment Plant." *Water Supply* 1 (5-6): 261-271. https://doi.org/10.2166/ws.2001.0122.

Kuo, D. H., F. J. Simmons, S. Blair, E. Hart, J. B. Rose, and I. Xagoraraki. 2010. "Assessment of Human Adenovirus Removal in a Full-Scale Membrane Bioreactor Treating Municipal Wastewater." *Water Research* 44 (5): 1520-1530. https://doi.org/10.1016/j.watres.2009.10.039.

La Bella, T., S. Imbeaud, C. Peneau, I. Mami, S. Datta, Q. Bayard, S. Caruso, T. Z. Hirsch, J. Calderaro, G. Morcrette, C. Guettier, V. Paradis, G. Amaddeo, A. Laurent, L. Possenti, L. Chiche, P. Bioulac-Sage, J.-F. Blanc, E. Letouze, J.-C. Nault, and J. Zucman-Rossi. 2020. "Adeno-Associated Virus in the Liver: Natural History and Consequences in Tumour Development." *Gut* 69: 737-747. http://dx.doi.org/10.1136/gutjnl-2019-318281.

Lakretz, A., H. Mamane, H. Cikurel, D. Avisar, E. Gelman, and I. Zucker. 2017. "The Role of Soil Aquifer Treatment (SAT) for Effective Removal of Organic Matter, Trace Organic Compounds and Microorganisms from Secondary Effluents Pre-treated by Ozone The Role of Soil Aquifer Treatment (SAT) for Effective Removal of Organic Matter, Trace Organic Compounds and Microorganisms from Secondary Effluents Pre-treated by Ozone." *Ozone: Science & Engineering* 39 (5): 385-394. https://doi.org/10.1080/01919512.2017.1346465.

Langlet, J., F. Gaboriaud, J. F. L. Duval, and C. Gantzer. 2008. "Aggregation and Surface Properties of F-Specific RNA Phages: Implication for Membrane Filtration Processes." *Water Research* 42 (10-11): 2769-2777. https://doi.org/10.1016/j.watres.2008.02.007.

Lasareishvili, B., H. Shi, X. Wang, K. D. Hillstead, M. Tediashvili, E. Jaiani, and V. V. Tarabara. 2021. "Virus Recovery by Tangential Flow Filtration: A Model to Guide the Design of a Sample Concentration Process." *Biotechnology Progress* 37 (1): e3080. https://doi.org/10.1002/btpr.3080.

Lee, S., N. Yamashita, and H. Tanaka. 2019. "How Fiber Breakage Reduces Microorganism Removal in Ultrafiltration for Wastewater Reclamation." *Food and Environmental Virology* 11 (2): 167-177. https://doi.org/10.1007/s12560-019-09372-6.

Leifels, M., I. A. Hamza, M. Krieger, M. Wilhelm, M. Mackowiak, and L. Jurzik. 2016. "From Lab to Lake - Evaluation of Current Molecular Methods for the Detection of Infectious Enteric Viruses in Complex Water Matrices in an Urban Area." *PLoS One* 11 (11): e0167105. https://doi.org/10.1371/journal.pone.0167105.

Leifels, M., D. Shoults, A. Wiedemeyer, N. Ashbolt, E. Sozzi, A. Hagemeier, and L. Jurzik. 2019. "Capsid Integrity qPCR-An Azo-Dye Based and Culture-Independent Approach to Estimate Adenovirus Infectivity after Disinfection and in the Aquatic Environment." *Water Research X* 11: 100080. https://doi.org/10.1016/j.wroa.2020.100080.

Liu, J., M. Zhao, C. Duan, P. Yue, and T. Li. 2020. "Removal Characteristics of Dissolved Organic Matter and Membrane Fouling in Ultrafiltration and Reverse Osmosis Membrane Combined Processes Treating the Secondary Effluent of Wastewater Treatment Plant." *Water Science and Technology* 83 (3): 689-700. https://doi.org/10.2166/wst.2020.589.

Liu, P., V. R. Hill, D. Hahn, T. B. Johnson, Y. Pan, N. Jothikumar, and C. L. Moe. 2012. "Hollow-Fiber Ultrafiltration for Simultaneous Recovery of Viruses, Bacteria and Parasites from Reclaimed Water." *Journal of Microbiological Methods* 88 (1): 155-161. https://doi.org/10.1016/j.mimet.2011.11.007.

Liu, X., Y. Li, X. Wang, Y. Song, L. Wu, B. Yu, X. Ma, P. Ma, M. Liu, X. Huang, and X. Wang. 2022. "Rapid and Specific Detection of Active SARS-CoV-2 With CRISPR/Cas12a." *Frontiers in Microbiology* 12. https://doi.org/10.3389/fmicb.2021.820698.

Luo, M., M. Mietzsch, P. Chipman, K. Song, C. Xu, J. Spear, D. Sousa, R. McKenna, M. Söderlund-Venermo, and M. Agbandje-McKenna. 2021. "pH-Induced Conformational Changes of Human Bocavirus Capsids." *Journal of Virology* 95 (8): e02329-20. https://doi.org/10.1128/jvi.02329-20.

Madireddi, K., R. W. Babcock, B. Levine, T. L. Huo, E. Khan, Q. F. Ye, J. B. Neethling, I. H. Suffet, and M. K. Stenstrom. 1997. "Wastewater Reclamation at Lake Arrowhead, California: An Overview." *Water Environment Research* 69 (3): 350-362. https://doi.org/10.2175/106143097X125551.

Malathi, V. G., and P. Renuka Devi. 2019. "ssDNA Viruses: Key Players in Global Virome." *VirusDisease* 30: 3-12. https://doi.org/10.1007/s13337-019-00519-4.

Martin, N., G. Gonzalez, L. Reynolds, C. Bennett, C. Campbell, T. Nolan, A. Byrne, S. Fennema, N. Holohan, S. R. Kuntamukkula, N. Sarwar, L. Sala-Comorera, J. Dean, J. M. Urtasun-Elizari, D. Hare, E. Liddy, E. Joyce, J. O'Sullivan, J. Cuddihy, A. McIntyre, E. Robinson, D. Dahly, N. Fletcher, S. Cotter, E. Fitzpatrick, M. Carr, C. De Gascun, and W. Meijer. 2023. "Adeno-Associated Virus 2 and Human Adenovirus F41 in Wastewater during Outbreak of Severe Acute Hepatitis in Children, Ireland." *Emerging Infectious Disease journal* 29 (4): 751-760. https://doi.org/10.3201/eid2904.221878.

Mateu, M. G. 2013. "Introduction: The Structural Basis of Virus Function." In *Structure and Physics of Viruses. Sub-Cellular Biochemistry* 68: 3-51. https://doi.org/10.1007/978-94-007-6552-8_1.

Matsushita, T., N. Shirasaki, Y. Tatsuki, and Y. Matsui. 2013. "Investigating Norovirus Removal by Microfiltration, Ultrafiltration, and Precoagulation–Microfiltration Processes using Recombinant Norovirus Virus-Like Particles and Real-Time Immuno-PCR." *Water Research* 47 (15): 5819-5827. https://doi.org/10.1016/j.watres.2013.07.004.

Mattle, M. J., B. Crouzy, M. Brennecke, K. R. Wigginton, P. Perona, and T. Kohn. 2011. "Impact of Virus Aggregation on Inactivation by Peracetic Acid and Implications for Other Disinfectants." *Environmental Science & Technology* 45 (18): 7710-7717. https://doi.org/10.1021/es201633s.

Mattle, M. J., and T. Kohn. 2012. “Inactivation and Tailing during UV254 Disinfection of Viruses: Contributions of Viral Aggregation, Light Shielding within Viral Aggregates, and Recombination.” *Environmental Science & Technology* 46 (18): 10022-10030. https://doi.org/10.1021/es302058v.

Mayer, B. K., Y. Yang, D. W. Gerrity, and M. Abbaszadegan. 2015. “The Impact of Capsid Proteins on Virus Removal and Inactivation during Water Treatment Processes.” *Microbiology Insights* 8 (Supplement 2): 15-28. https://doi.org/10.4137/MBI.S31441.

McMinn, B. R., N. J. Ashbolt, and A. Korajkic. 2017. “Bacteriophages as Indicators of Faecal Pollution and Enteric Virus Removal.” *Letters in Applied Microbiology* 65 (1): 11-26. https://doi.org/10.1111/lam.12736.

McMinn, B. R., E. R. Rhodes, E. M. Huff, P. Wanjugi, M. M. Ware, S. P. Nappier, M. Cyterski, O. C. Shanks, K. Oshima, and A. Korajkic. 2018. “Comparison of Somatic and F+ Coliphage Enumeration Methods with Large Volume Surface Water Samples.” *J Virol Methods* 261: 63-66. https://doi.org/10.1016/j.jviromet.2018.08.007.

Mi, B., C. L. Eaton, J.-H. Kim, C. K. Colvin, J. C. Lozier, and B. J. Mariñas. 2004. “Removal of Biological and Non-Biological Viral Surrogates by Spiral-Wound Reverse Osmosis Membrane Elements with Intact and Compromised Integrity.” *Water Research* 38 (18): 3821-3832. https://doi.org/10.1016/j.watres.2004.07.004.

Mi, X., E. K. Bromley, P. U. Joshi, F. Long, and C. L. Heldt. 2020. “Virus Isoelectric Point Determination Using Single-Particle Chemical Force Microscopy.” *Langmuir* 36 (1): 370-378. https://doi.org/10.1021/acs.langmuir.9b03070.

Michen, B., and T. Graule. 2010. “Isoelectric Points of Viruses.” *Journal of Applied Microbiology* 109 (2): 388-397. https://doi.org/10.1111/j.1365-2672.2010.04663.x.

Miller, S., R. Rodriguez, and K. Nelson. 2020. “Removal and Growth of Microorganisms Across Treatment and Simulated Distribution at a Pilot-Scale Direct Potable Reuse Facility.” *Environmental Science: Water Research & Technology* 6: 1370-1387. https://doi.org/10.1039/C9EW01087D.

Montazeri, N., D. Goettert, E. C. Achberger, C. N. Johnson, W. Prinyawiwatkul, and M. E. Janes. 2015. “Pathogenic Enteric Viruses and Microbial Indicators During Secondary Treatment of Municipal Wastewater.” *Applied and Environmental Microbiology* 81 (18): 6436-6445. https://doi.org/10.1128/AEM.01218-15.

Morrison, C. M., W. Q. Betancourt, D. R. Quintanar, G. U. Lopez, I. L. Pepper, and C. P. Gerba. 2020. “Potential Indicators of Virus Transport and removal during Soil Aquifer Treatment of Treated Wastewater Effluent.” *Water Research* 177: 115812. https://doi.org/10.1016/j.watres.2020.115812.

Mushegian, A. R. 2020. “Are There 10^{31} Virus Particles on Earth, or More, or Fewer?” *Journal of Bacteriology* 202 (9). https://doi.org/10.1128/jb.00052-20.

Newcombe, G. 2006. Chapter 8 – "Removal of Natural Organic Material and Algal Metabolites Using Activated Carbon." In: *Interface Science and Technology* 10: 133-153. Edited by G. Newcombe and D. Dixon. https://doi.org/10.1016/S1573-4285(06)80077-3.

Ng, T. F., R. Marine, C. Wang, P. Simmonds, B. Kapusinszky, L. Bodhidatta, B. S. Oderinde, K. E. Wommack, and E. Delwart. 2012. "High Variety of Known and New RNA and DNA Viruses of Diverse Origins in Untreated Sewage." *Journal of Virology* 86 (22): 12161-12175. https://doi.org/10.1128/jvi.00869-12.

North Carolina Environmental Quality. 2011. "North Carolina Adm. Code 15A NCAC 2U Reclaimed Water." North Carolina Department of Environment and Natural Resources: Regulatory Review of North Carolina.

NRC (National Research Council). 2012. *Water Reuse: Potential for Expanding the Nation's Water Supply Through Reuse of Municipal Wastewater.* Washington, DC: The National Academic Press.

Nwachuku, N., C. P. Gerba, A. Oswald, and F. D. Mashadi. 2005. "Comparative Inactivation of Adenovirus Serotypes by UV Light Disinfection." *Applied and Environmental Microbiology* 71 (9): 5633-5636. https://doi.org/10.1128/AEM.71.9.5633-5636.2005.

Oakley, S., and M. von Sperling. 2017. "Media Filters: Trickling Filters and Anaerobic Filters." In: *Water and Sanitation for the 21st Century: Health and Microbiological Aspects of Excreta and Wastewater Management* (Global Water Pathogen Project). (J.R. Mihelcic and M.E. Verbyla (eds), Part 4: Management Of Risk from Excreta and Wastewater - Section: Sanitation System Technologies, Pathogen Reduction in Sewered System Technologies), Michigan State University, E. Lansing, MI, UNESCO. https://doi.org/10.14321/waterpathogens.64.

Otaki, M., K. Yano, and S. Ohgaki. 1998. "Virus Removal in a Membrane Separation Process." *Water Science and Technology* 37 (10): 107-116. https://doi.org/10.1016/S0273-1223(98)00300-X.

Ottoson, J., A. Hansen, T. Westrell, K. Johansen, H. Norder, and T. A. Stenstrom. 2006. "Removal of Noro- and Enteroviruses, Giardia Cysts, Cryptosporidium Oocysts, and Fecal Indicators at Four Secondary Wastewater Treatment Plants in Sweden." *Water Environment Research* 78 (8): 828-834. https://doi.org/10.2175/106143006X101719.

Palermo, C. N., R. R. Fulthorpe, R. Saati, and S. M. Short. 2019. "Metagenomic Analysis of Virus Diversity and Relative Abundance in a Eutrophic Freshwater Harbour." *Viruses* 11 (9): 792. https://doi.org/10.3390/v11090792.

Park, M., and S. A. Snyder. 2018. "Sample Handling and Data Processing for Fluorescent Excitation-Emission Matrix (EEM) of Dissolved Organic Matter (DOM)." *Chemosphere* 193: 530-537. https://doi.org/10.1016/j.chemosphere.2017.11.069.

Patterson, J. W., P. L. Brezonik, and H. D. Putnam. 1970. "Measurement and Significance of Adenosine Triphosphate in Activated Sludge." *Environmental Science & Technology* 4 (7): 569-575. https://doi.org/10.1021/es60042a003.

Pearson, V. M., S. B. Caudle, and D. R. Rokyta. 2016. “Viral Recombination Blurs Taxonomic Lines: Examination of Single-Stranded DNA Viruses in a Wastewater Treatment Plant.” *PeerJ 4*: e2585. https://doi.org/10.7717/peerj.2585.

Pecson, B. M., E. Darby, R. Danielson, Y. Dearborn, G. D. Giovanni, W. Jakubowski, M. Leddy, G. Lukasik, B. Mull, K. L. Nelson, A. Olivieri, C. Rock, and T. Slifko. 2022. “Distributions of Waterborne Pathogens in Raw Wastewater Based on a 14-Month, Multi-Site Monitoring Campaign.” *Water Research* 213: 118170. https://doi.org/10.1016/j.watres.2022.118170.

Pérot, P., J. Fourgeaud, C. Rouzaud, B. Regnault, N. Da Rocha, H. Fontaine, J. Le Pavec, S. Dolidon, M. Garzaro, D. Chrétien, G. Morcrette, T. J. Molina, A. Ferroni, M. Leruez-Ville, O. Lortholary, A. Jamet, and M. Eloit. 2023. “Circovirus Hepatitis Infection in Heart-Lung Transplant Patient, France.” *Emerging Infectious Diseases* 29 (2): 286-293. https://doi.org/10.3201/eid2902.221468.

Pierre, G., A. Furiga, M. Berge, C. Roques, P. Aimar, and C. Causserand. 2011. “Protocol for the Assessment of Viral Retention Capability of Membranes.” *Journal of Membrane Science* 381 (1-2): 41-49. https://doi.org/10.1016/j.memsci.2011.07.017.

Polanco, J., J. Safarik, and M. H. Plumlee. 2022. *Demonstrating Virus Log Removal Credit for Wastewater Treatment and Reverse Osmosis for Potable Reuse at OCWD*. Project 5401. Denver, CO: The Water Research Foundation.

Prado, T., A. de Castro Bruni, M. R. F. Barbosa, S. C. Garcia, A. M. de Jesus Melo, and M. I. Z. Sato. 2019. “Performance of Wastewater Reclamation Systems in Enteric Virus Removal.” *Science of The Total Environment* 678: 33-42. https://doi.org/10.1016/j.scitotenv.2019.04.435.

Pype, M.-L., B. Donose, L. Martí, D. Patureau, N. Wery, and W. Gernjak. 2016a. “Virus Removal and Integrity in Aged RO Membranes.” *Water Research* 90: 167-175. https://doi.org/10.1016/j.watres.2015.12.023.

Pype, M.-L., M. G. Lawrence, J. Keller, and W. Gernjak. 2016b. “Reverse Osmosis Integrity Monitoring in Water Reuse: The Challenge to Verify Virus Removal – A Review.” *Water Research* 98: 384-395. https://doi.org/10.1016/j.watres.2016.04.040.

Qiu, Y., B. E. Lee, N. Neumann, N. Ashbolt, S. Craik, R. Maal-Bared, and X. L. Pang. 2015. “Assessment of Human Virus Removal During Municipal Wastewater Treatment in Edmonton, Canada.” *Journal of Applied Microbiology* 119 (6): 1729-1739. https://doi.org/10.1111/jam.12971.

Qiu, Y., Q. Li, B. E. Lee, N. J. Ruecker, N. F. Neumann, N. J. Ashbolt, and X. Pang. 2018. “UV Inactivation of Human Infectious Viruses at Two Full-Scale Wastewater Treatment Plants in Canada.” *Water Research* 147: 73-81. https://doi.org/10.1016/j.watres.2018.09.057.

Quanrud, D. M., J. Hafer, M. M. Karpiscak, J. Zhang, K. E. Lansey, and R. G. Arnold. 2003. “Fate of Organics During Soil-Aquifer Treatment: Sustainability of Removals in the Field.” *Water Research* 37 (14): 3401-3411. https://doi.org/10.1016/S0043-1354(02)00489-X.

Queensland Government. 2005. Queensland Water Recycling Guidelines. Queensland Environmental Protection Agency. Brisbane. Australia. Accessed September 24, 2023.

https://www.hort360.com.au/wordpress/wp-content/uploads/2015/03/Queensland-Water-Recycling-Guidelines-Dec-2005.pdf.

Rachmadi, A. T., M. Kitajima, T. Kato, H. Kato, S. Okabe, and D. Sano. 2020. "Required Chlorination Doses to Fulfill the Credit Value for Disinfection of Enteric Viruses in Water: A Critical Review." *Environmental Science & Technology* 54 (4): 2068-2077. https://doi.org/10.1021/acs.est.9b01685.

Rames, E., A. Roiko, H. Stratton, and J. Macdonald. 2016. "Technical Aspects of Using Human Adenovirus as a Viral Water Quality Indicator." *Water Research* 96: 308-326. https://doi.org/10.1016/j.watres.2016.03.042.

Ramírez-Flores, C. J., and L. J. Knoll. 2021. "Breakthroughs in Microbiology Made Possible with Organoids." *PLoS Pathogens* 17: e1010080. https://doi.org/10.1371/journal.ppat.1010080.

Randazzo, W., A. Vasquez-Garcia, R. Aznar, and G. Sanchez. 2018. "Viability RT-qPCR to Distinguish Between HEV and HAV With Intact and Altered Capsids." *Frontiers in Microbiology* 9: 1973. https://doi.org/10.3389/fmicb.2018.01973.

Rauch-Williams, T., J. Mosher, J. E. Drewes, V. Zhiteneva, and C. Gerba. 2022. *State-of-the-Science Review: Evidence for Pathogen Removal in Managed Aquifer Recharge Systems*. Project 4957 Denver, CO: The Water Research Foundation.

Rayfield, W. J., D. J. Roush, R. A. Chmielowski, N. Tugcu, S. Barakat, and J. K. Cheung. 2015. "Prediction of Viral Filtration Performance of Monoclonal Antibodies Based on Biophysical Properties of Feed." *Biotechnology Progress* 31 (3): 765-774. https://doi.org/10.1002/btpr.2094.

Rexroad, J., T. T. Martin, D. McNeilly, S. Godwin, and C. R. Middaugh. 2006. "Thermal Stability of Adenovirus Type 2 as a Function of pH." *J Pharm Sci* 95 (7): 1469-1479. https://doi.org/10.1002/jps.20617.

Rivadulla, E., and J. L. Romalde. 2020. "A Comprehensive Review on Human Aichi Virus." *Virologica Sinica* 35: 501-516. https://doi.org/10.1007/s12250-020-00222-5.

Rosario, K., and M. Breitbart. 2011. "Exploring the Viral World Through Metagenomics." *Current Opinion in Virology* 1 (4): 289-297. https://doi.org/10.1016/j.coviro.2011.06.004.

Rosario, K., A. Dayaram, M. Marinov, J. Ware, S. Kraberger, D. Stainton, M. Breitbart, and A. Varsani. 2012a. "Diverse Circular ssDNA Viruses Discovered in Dragonflies (Odonata: Epiprocta)." *Journal of General Virology* 93 (12): 2668-2681. https://doi.org/10.1099/vir.0.045948-0.

Rosario, K., S. Duffy, and M. Breitbart. 2012b. "A Field Guide to Eukaryotic Circular Single-Stranded DNA Viruses: Insights Gained From Metagenomics." *Archives of Virology* 157: 1851-1871. https://doi.org/10.1007/s00705-012-1391-y.

Rosario, K., C. Nilsson, Y. W. Lim, Y. Ruan, and M. Breitbart. 2009. "Metagenomic Analysis of Viruses in Reclaimed Water." *Environmental Microbiology* 11 (11): 2806-2820. https://doi.org/10.1111/j.1462-2920.2009.01964.x.

Rose, J. B., S. R. Farrah, V. J. Harwood, A. D. Levine, J. Lukasik, P. Menendez, and T. M. Scott. 2004. *Reduction of of Pathogens, Indicator Bacteria, and Alternative Indicators by Wastewater Treatment.* Project 1031. Alexandria, VA: Water Environment Research Foundation.

Roux, S., N. Solonenko, V. Dang, B. Poulos, S. Schwenck, D. Goldsmith, M. Coleman, M. Breitbart, and M. Sullivan. 2016. "Towards Quantitative Viromics for both Double-Stranded and Single-Stranded DNA Viruses." *PeerJ* 4: e2777.

Ruijter, J. M., R. J. Barnewall, I. B. Marsh, A. N. Szentirmay, J. C. Quinn, R. van Houdt, Q. D. Gunst, and M. J. B. van den Hoff. 2021. "Efficiency Correction Is Required for Accurate Quantitative PCR Analysis and Reporting." *Clinical Chemistry* 67 (6): 829-842. https://doi.org/10.1093/clinchem/hvab052.

Ryu, H., Y. Addor, N. E. Brinkman, M. W. Ware, L. Boczek, J. Hoelle, J. H. Mistry, S. P. Keely, and E. N. Villegas. 2021. "Understanding Microbial Loads in Wastewater Treatment Works as Source Water for Water Reuse." *Water* 13 (11): 1452. https://doi.org/10.3390/w13111452.

San Martín, C. 2013. "Structure and Assembly of Complex Viruses." In: *Structure and Physics of Viruses: An Integrated Textbook*. Edited by M. G. Mateu. Dordrecht: Springer Netherlands. pp. 329-360.

SBDDW (State Board, Division of Drinking Water). 2018. Recycled Water Regulations. California Code of Regulations 60320.208 2014. Titles 22 and 17 California Code of Regulations. Sacramento, CA: https://www.waterboards.ca.gov/drinking_water/certlic/drinkingwater/documents/lawbook/RWregulations_20181001.pdf

Scheller, C., F. Krebs, R. Minkner, I. Astner, M. Gil-Moles, and H. Wätzig. 2020. "Physicochemical Properties of SARS-CoV-2 for Drug Targeting, Virus Inactivation and Attenuation, Vaccine Formulation and Quality Control." *Electrophoresis* 41 (13-14): 1137-1151. https://doi.org/10.1002/elps.202000121.

Schijven, J., and S. Hassanizadeh. 2002a. Virus Removal by Soil Passage at Field Scale and Groundwater Protection of Sandy Aquifers. *Water Sci Technol* 46 (3): 123-9.

Schijven, J.F., and S. M. Hassanizadeh. 2000. "Removal of Viruses by Soil Passage: Overview of Modeling, Processes, and Parameters." *Critical Reviews in Environmental Science and Technology* 30 (1): 49-127. https://doi.org/10.1080/10643380091184174.

Schijven, J. F., W. Hoogenboezem, M. Hassanizadeh, and J. H. Peters. 1999. "Modeling Removal of Bacteriophages MS2 and PRD1 by Dune Recharge at Castricum, Netherlands." *Water Resources Research* 35 (4): 1101-1111. https://doi.org/10.1029/1998WR900108.

Schmitz, B. W., M. Kitajima, M. E. Campillo, C. P. Gerba, and I. L. Pepper. 2016. "Virus Reduction During Advanced Bardenpho and Conventional Wastewater Treatment Processes." *Environmental Science & Technology* 50 (17): 9524-9532. https://doi.org/10.1021/acs.est.6b01384.

Scott, T., R. M. McLaughlin, V. Harwood, V. Chivukula, A. Levine, A. Gennaccaro, J. Lukasik, S. Farrah, and J. Rose. 2003. "Reduction of Pathogens, Indicator Bacteria, and Alternative Indicators by Wastewater Treatment and Reclamation Processes." *Wat Suppl* 3 (4): 247-252. https://doi.org/10.2166/ws.2003.0069.

Sedmak, G., D. Bina, J. Macdonald, and L. Couillard. 2005. “Nine-year study of the Occurrence of Culturable Viruses In Source Water for Two Drinking Water Treatment Plants and the Influent and Effluent of a Wastewater Treatment Plant in Milwaukee, Wisconsin (August 1994 through July 2003).” *Applied and Environmental Microbiology* 71 (2): 1042-1050. https://doi.org/10.1128/AEM.71.2.1042-1050.2005.

Sellwood, J., and J. V. Dadswell. 1981. “Viruses Isolated from Sewage 1974-77.” In: *Viruses and Wastewater Treatment*. Edited by M. Goddard and M. Butler. Pergamon, pp. 27-30. https://doi.org/10.1016/B978-0-08-026401-1.50008-7.

Shirasaki, N., T. Matsushita, Y. Matsui, and K. Murai. 2017. “Assessment of the Efficacy of Membrane Filtration Processes to Remove Human Enteric Viruses and the Suitability of Bacteriophages and a Plant Virus as Surrogates for those Viruses.” *Water Research* 115: 29-39. https://doi.org/10.1016/j.watres.2017.02.054.

Shulman, L. M., and I. Davidson. 2017. “Viruses with Circular Single-Stranded DNA Genomes are Everywhere!” *Annual Review of Virology* 4: 159-180. https://doi.org/10.1146/annurev-virology-101416-041953.

Simmons, F. J., D. H. Kuo, and I. Xagoraraki. 2011. “Removal of Human Enteric Viruses by a Full-Scale Membrane Bioreactor During Municipal Wastewater Processing.” *Water Research* 45 (9): 2739-2750. https://doi.org/10.1016/j.watres.2011.02.001.

Simmons, F. J., and I. Xagoraraki. 2011. “Release of Infectious Human Enteric Viruses By Full-Scale Wastewater Utilities.” *Water Research* 45 (12): 3590-3598. https://doi.org/10.1016/j.watres.2011.04.001.

Singh, R. 2015. “Chapter 1 - Introduction to Membrane Technology.” In: *Membrane Technology and Engineering for Water Purification* (Second Edition). Edited by R. Singh. Oxford: Butterworth-Heinemann. pp. 1-80.

Soller, J. A., S. E. Eftim, I. Warren, and S. P. Nappier. 2017. “Evaluation of Microbiological Risks Associated With Direct Potable Reuse.” *Microbial Risk Analysis* 5: 3-14. https://doi.org/10.1016/j.mran.2016.08.003.

Soller, J. A., A. W. Olivieri, J. Crook, R. C. Cooper, G. Tchobanoglous, R. T. Parkin, R. C. Spear, and J. N. S. Eisenberg. 2003. “Risk-Based Approach to Evaluate the Public Health Benefit of Additional Wastewater Treatment.” *Environmental Science & Technology* 37 (9): 1882-1891. https://doi.org/10.1021/es025774p.

Souza-Chaves, B. M., M. A. Alhussaini, V. Felix, L. K. Presson, W. Q. Betancourt, K. L. Hickenbottom, and A. Achilli. 2022. “Extending the Life of Water Reuse Reverse Osmosis Membranes Using Chlorination.” *Journal of Membrane Science* 642: 119897. https://doi.org/10.1016/j.memsci.2021.119897.

Stachler, E., C. Kelty, M. Sivaganesan, X. Li, K. Bibby, and O. C. Shanks. 2017. “Quantitative CrAssphage PCR Assays for Human Fecal Pollution Measurement.” *Environmental Science & Technology* 51 (16): 9146-9154. https://doi.org/10.1021/acs.est.7b02703.

Strauss, D., J. Goldstein, T. Hongo-Hirasaki, Y. Yokoyama, N. Hirotomi, T. Miyabayashi, and D. Vacante. 2017. “Characterizing the Impact of Pressure on Virus Filtration Processes and Establishing Design Spaces to Ensure Effective Parvovirus Removal.” *Biotechnology Progress* 33: 1294-1302. https://doi.org/10.1002/btpr.2506.

Stubbs, G., and A. Kendall. 2012. “Helical Viruses.” In *Viral Molecular Machines*. Edited by M. G. Rossmann and V. B. Rao. Boston, MA: Springer US. pp. 631-658.

Sun, C., L. Xie, X. Li, L. Sun, and H. Dai. 2015. “Study on Different Ultrafiltration-Based Hybrid Pretreatment Systems for Reverse Osmosis Desalination.” *Desalination* 371: 18-25. https://doi.org/10.1016/j.desal.2015.05.020.

SWRCB DWW (California State Water Resources Control Board Division of Drinking Water). 2021. “Proposed Framework of Regulating Direct Potable Reuse in California Addendum.” Prepared by State Water Board’s Division of Drinking Water.

Symonds, E. M., D. W. Griffin, and M. Breitbart. 2009. “Eukaryotic Viruses In Wastewater Samples from the United States.” *Applied and Environmental Microbiology* 75 (5): 1402-1409. https://doi.org/10.1128/AEM.01899-08.

TCEQ (Texas Commission of Environmental Quality). 2022. Water Supply Division. RG-634. Direct Potable Reuse for Public Water Systems. Austin, TX.

Templeton, M. R., R. C. Andrews, and R. Hofmann. 2008. “Particle-Associated Viruses in Water: Impacts on Disinfection Processes.” *Critical Reviews in Environmental Science and Technology* 38 (3): 137-164. https://doi.org/10.1080/10643380601174764.

Tesson, V., A. De Rougemont, L. Capowiez, and P. Renault. 2018. “Modelling the Removal and Reversible Immobilization of Murine Noroviruses in a Phaeozem Under Various Contamination and Rinsing Conditions.” *European Journal of Soil Science* 69 (6): 1068-1077. https://doi.org/10.1111/ejss.12719.

Torii, S., M.-H. Corre, F. Miura, M. Itamochi, K. Haga, K. Katayama, H. Katayama, and T. Kohn. 2022. “Genotype-Dependent Kinetics of Enterovirus Inactivation by Free Chlorine and Ultraviolet (UV) Irradiation.” *Water Research* 220: 118712. https://doi.org/10.1016/j.watres.2022.118712.

Torrey, J., U. von Gunten, and T. Kohn. 2019. “Differences in Viral Disinfection Mechanisms as Revealed by Quantitative Transfection of Echovirus 11 Genomes.” *Applied and Environmental Microbiology* 85 (14). https://doi.org/10.1128/AEM.00961-19.

Travis, B., and D. Tracey. 2016. “Biological Monitoring Improves Membrane Performance.” *Opflow* 42 (12): 26-29. https://doi.org/10.5991/OPF.2016.42.0074.

Trussell, R. 2012. *Equivalency of Advanced Treatment Trains for Potable Reuse.* Project 1695. Alexndria, VA: Water Reuse Research Foundation.

Turkeltaub, T., A. Furman, R. Mannheim, and N. Weisbrod. 2022. “Continuous Monitoring Of A Soil Aquifer Treatment System's Physico-Chemical Conditions To Optimize Operational Performance.” *Hydrol. Earth Syst. Sci.* 26 (6): 1565-1578. https://doi.org/10.5194/hess-26-1565-2022.

Van Der Bruggen, B., C. Vandecasteele, T. Van Gestel, W. Doyen, and R. Leysen. 2003. "A Review Of Pressure-Driven Membrane Processes In Wastewater Treatment And Drinking Water Production." *Environmental Progress* 22 (1): 46-56. https://doi.org/10.1002/ep.670220116.

Vickers, J. C., M. Dummer, T. Le, and J. B. Zoba. 2019. "Removal of MS-2 Coliphage In Full-Scale Reverse Osmosis Systems." *AWWA Water Science* 1 (6): e1158. https://doi.org/10.1002/aws2.1158.

Victoria, J. G., A. Kapoor, L. Li, O. Blinkova, B. Slikas, C. Wang, A. Naeem, S. Zaidi, and E. Delwart. 2009. "Metagenomic Analyses Of Viruses In Stool Samples From Children With Acute Flaccid Paralysis." *Journal of Virology* 83 (9): 4642-4651. https://doi.org/10.1128/jvi.02301-08.

Vinjé, J., M. K. Estes, P. Esteves, K. Y. Green, K. Katayama, N. J. Knowles, Y. L'Homme, V. Martella, H. Vennema, P. A. White, and I. R. Consortium. 2019. "ICTV Virus Taxonomy Profile: Caliciviridae." *Journal of General Virology* 100 (11): 1469-1470. https://doi.org/10.1099/jgv.0.001332.

Waldman, P., A. Meseguer, F. Lucas, L. Moulin, and W. Wurtzer. 2017. "Interaction of Human Enteric Viruses with Microbial Compounds: Implication for Virus Persistence and Disinfection Treatments." *Environmental Science & Technology* 51 (23): 13633-13640. https://doi.org/10.1021/acs.est.7b03875.

Walshe, G. E., L. Pang, M. Flury, M. E. Close, and M. Flintoft. 2010. "Effects of pH, ionic Strength, Dissolved Organic Matter, And Flow Rate On The Co-Transport Of MS2 Bacteriophages With Kaolinite In Gravel Aquifer Media." *Water Research* 44(4) : 1255-1269. https://doi.org/10.1016/j.watres.2009.11.034.

Warsinger, D. M., S. Chakraborty, E. W. Tow, M. H. Plumlee, C. Bellona, S. Loutatidou, L. Karimi, A. M. Mikelonis, A. Achilli, A. Ghassemi, L. P. Padhye, S. A. Snyder, S. Curcio, C. D. Vecitis, H. A. Arafat, and J. H. Lienhard. 2018. "A Review Of Polymeric Membranes And Processes For Potable Water Reuse." *Progress In Polymer Science* 81: 209-237. https://doi.org/10.1016/j.progpolymsci.2018.01.004.

Wigginton, K. R., and T. Kohn. 2012. "Virus Disinfection Mechanisms: The Role Of Virus Composition, Structure, And Function." *Current Opinion In Virology* 2 (1): 84-89. https://doi.org/10.1016/j.coviro.2011.11.003.

Wigginton, K. R., B. M. Pecson, T. Sigstam, F. Bosshard, and T. Kohn. 2012. "Virus Inactivation Mechanisms: Impact Of Disinfectants On Virus Function And Structural Integrity." *Environmental Science & Technology* 46 (21): 12069-12078. https://doi.org/10.1021/es3029473.

Wong, K., T. C. Voice, and I. Xagoraraki. 2013. "Effect of Organic Carbon On Sorption Of Human Adenovirus To Soil Particles And Laboratory Containers." *Water Research* 47 (10): 3339-3346. https://doi.org/10.1016/j.watres.2013.03.029.

Wylie, K. M., G. M. Weinstock, and G. A. Storch. 2012. "Emerging View Of The Human Virome." *Translational Research : The Journal Of Laboratory And Clinical Medicine* 160 (4): 283-290. https://doi.org/10.1016/j.trsl.2012.03.006.

Xagoraraki, I., Z. Yin, and Z. Svambayev. 2014. "Fate of Viruses in Water Systems." *Journal of Environmental Engineering* 140 (7): 04014020. https://doi.org/10.1061/(ASCE)EE.1943-7870.0000827.

Yang, J., M. Monnot, L. Ercolei, and P. Moulin. 2020. “Membrane-Based Processes Used in Municipal Wastewater Treatment for Water Reuse: State-Of-The-Art and Performance Analysis.” *Membranes* 10 (6): 131. https://doi.org/10.3390/membranes10060131.

Yasui, M., H. Iso, S. Torii, Y. Matsui, and H. Katayama. 2021. “Applicability of Pepper Mild Mottle Virus And Cucumber Green Mottle Mosaic Virus As Process Indicators Of Enteric Virus Removal By Membrane Processes At A Potable Reuse Facility.” *Water Research* 206: 117735. https://doi.org/10.1016/j.watres.2021.117735.

Yates, M. V., and S. R. Yates. 1988. “Virus Survival and Transport in Ground Water.” *Water Science and Technology* 20 (11-12): 301-307. https://doi.org/10.2166/wst.1988.0299.

Yates, M. V., S. R. Yates, and C. P. Gerba. 1988. “Modeling Microbial Fate In The Subsurface Environment.” *Critical Reviews in Environmental Control* 17 (4): 307-344. https://doi.org/10.1080/10643388809388339.

Ye, Y., L. Zhao, M. J. Imperiale, and K. R. Wigginton. 2019. “Integrated Cell Culture-Mass Spectrometry Method for Infectious Human Virus Monitoring.” *Environmental Science & Technology Letters* 6 (7): 407-412. https://doi.org/10.1021/acs.estlett.9b00226.

Yoon, S.-H. 2019. “Potential and Limitation Of Fluorescence-Based Membrane Integrity Monitoring (FMIM) For Reverse Osmosis Membranes.” *Water Research* 154: 287-297. https://doi.org/10.1016/j.watres.2019.02.001.

Yuan, B., M. Pham, and T. H. Nguyen. 2008. “Deposition Kinetics of Bacteriophage MS2 on a Silica Surface Coated with Natural Organic Matter in a Radial Stagnation Point Flow Cell.” *Environmental Science & Technology* 42 (20): 7628-7633. https://doi.org/10.1021/es801003s.

Yutin, N., S. Benler, S. A. Shmakov, Y. I. Wolf, I. Tolstoy, M. Rayko, D. Antipov, P. A. Pevzner, and E. V. Koonin. 2021. “Analysis of Metagenome-Assembled Viral Genomes From The Human Gut Reveals Diverse Putative Crass-Like Phages With Unique Genomic Features.” *Nature Communications* 12: 1044. https://doi.org/10.1038/s41467-021-21350-w.

Zell, R., E. Delwart, A. E. Gorbalenya, T. Hovi, A. M. Q. King, N. J. Knowles, A. M. Lindberg, M. A. Pallansch, A. C. Palmenberg, G. Reuter, P. Simmonds, T. Skern, G. Stanway, T. Yamashita, and I. R. Consortium. 2017. “ICTV Virus Taxonomy Profile: Picornaviridae.” *Journal of General Virology* 98 (10): 2421-2422. https://doi.org/10.1099/jgv.0.000911.

Zhang, M., N. Altan-Bonnet, Y. Shen, and D. Shuai. 2022. “Waterborne Human Pathogenic Viruses in Complex Microbial Communities: Environmental Implication on Virus Infectivity, Persistence, and Disinfection.” *Environmental Science & Technology* 56 (9): 5381-5389. https://doi.org/10.1021/acs.est.2c00233.

Zhao, L., K. Rosario, M. Breitbart, and S. Duffy. 2019. “Chapter Three - Eukaryotic Circular Rep-Encoding Single-Stranded DNA (CRESS DNA) Viruses: Ubiquitous Viruses With Small Genomes and a Diverse Host Range.” In: Advances in Virus Research. Edited by M. Kielian, T. C. Mettenleiter, and M. J. Roossinck. Academic Press. pp. 71-133.