Virus Monitoring Strategies for Wastewater Reuse

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Wastewater reclamation and reuse have the potential to supplement water supplies, offering resiliency in times of drought and helping to meet increased water demands associated with population growth. Non-potable water reuse represents the largest potential reuse market. Yet, economic constraints for new water reuse infrastructure and safety concerns due to microbial water quality, especially viral pathogen exposure, limit the widespread implementation of water reuse. Cost-effective, real-time methods to measure or indicate the viral quality of recycled water would do much to instill greater confidence in the practice. One of the greatest challenges of water-quality monitoring is that pathogens (including viruses as well as bacteria and protozoa) are often present at concentrations high enough to present disease risks but too low for direct detection. As a result, a variety of surrogate microorganisms are used as indicators of microbial water quality.

Keywords: viruses ; wastewater ; reuse

1. Current Technologies for Monitoring Viruses

1.1. Sample Concentration Methods

The quantities and types of human enteric viruses in wastewater vary widely and depend on several factors such as geographic location, season, and source of wastewater. High concentrations of human viruses can be detected easily from small amounts of wastewater or sludge samples, while greater volumes are generally required for detection for treated water due to lower viral concentrations. To improve detection, it is necessary to concentrate viruses in water samples.

Several different types of concentration methods are available. A single method is rarely capable of effectively concentrating all viruses in a water sample. As a result, using the right concentration approach can enhance virus detection [1]. Several studies summarized and compared concentration methods including virus adsorption and elution (VIRADEL), electronegative filtration, electropositive filtration, size-exclusion, and coagulation/flocculation [1][2][3][4]. Viral concentration methods that are useful for monitoring viruses in water reuse are highlighted below.

Electronegative membranes are commonly applied for virus concentration. Several studies demonstrated virus concentration using flat filter membranes with electronegative surface charge in electronegative filtration [5][6][7][8][9]. Haramoto et al. [2] successfully concentrated viruses and protozoa from wastewater, river water, and groundwater samples using electronegative mixed cellulose ester membranes (pore size, 0.45 µm). More recently, electronegative membranes are extensively used for concentrating Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2) from wastewater in efforts to document COVID-19 disease transmission [10][11]. The VIRADEL method has been used to concentrate viruses from a variety of water samples, including seawater, tap water, surface water, and wastewater [3]. Electropositive media and filters have also been applied in a variety of configurations for virus concentration. Examples include 1MDS filters (3M, Maplewood, MN USA) [1] and NanoCeram filters (Argonide, Sanford, FL, USA). The NanoCeram filter media is applied to concentrate viruses in drinking water [4], seawater [12] and wastewater [1][13] and are suggested as a less expensive alternative to the 1MDS filter [12].

In addition to surface-charged filters, size-exclusion filtration methods (e.g., ultrafiltration) allow for the simultaneous recovery of viruses and bacteria [1][4][14][15]. Another common ultrafiltration technique uses specialized cartridges designed for separation through membrane filters during centrifugation [7][16].

Among coagulation/flocculation methods, skimmed milk flocculation was shown to be a low-cost, one-step virus concentration approach. This procedure entails flocculating viruses with skimmed milk proteins in pre-acidified water samples (pH 3.5), stirring for 8 h, and gravity sedimentation of the floc for another 8 h. The sedimented floc is centrifuged to obtain a pellet, which is resuspended in a smaller volume of phosphate buffer after supernatant removal. Virus recoveries using this method are established at roughly 50% from 5 and 10 L samples of saltwater and river water [1][4].

The method is likely highly applicable to the treated wastewater for reuse. Another common coagulation/flocculation method utilizes polyethylene glycol precipitation (PEG) [17][18][19]. This method is similar to that of skimmed milk flocculation except that PEG and sodium chloride are added, and the centrifugation and sedimentation steps are slightly different [20].

As these studies show, no single strategy for concentrating human enteric viruses in wastewater appears to be completely efficient [21]. Given the attention to SARS-CoV-2 surveillance in wastewater, a recent inter-laboratory method comparison study in the recovery of SARS-CoV-2 from wastewater was conducted [11]. Three viral concentration methods (ultrafiltration, electronegative filtration, and PEG precipitation) did not present significant variability in the final outcomes [22]. The recent SARS-CoV-2 research also indicated that the virus was concentrated naturally by settled solids in wastewater treatment plants because of the affinity of viral lipophilic outer envelope [23]. Therefore, testing settled solids and primary sludge can provide highly sensitive detection of SARS-CoV-2 [24][25]. These methods are expected to be less applicable to the detection of viruses in finished water produced for reuse (low solids). Applications of automated virus concentration techniques, including magnetic bead-based virus capture [26][27], demonstrate the potential for high-throughput virus concentration.

Given the emergence of various new target viruses of interest (e.g., crAssphage), recovery efficiencies of different concentration approaches may need to be reevaluated [3]. The influence of viral shape, surface charge, hydrophobicity and other characteristics on recovery efficiencies of existing concentration methods should be examined. Given the wide range of viral recoveries from various water matrices, as well as the discoveries of new viruses, incorporating efficient viral concentration methods will be beneficial for future research and applications in practice.

1.2. Culture versus Molecular Detection

Cell culture methods are the gold standard for detecting infectious viruses, but next-generation molecular tools are now widely utilized for detecting enteric viruses in water samples [28]. Polymerase chain reaction (PCR)-based methods enable faster detection timeframes (within hours), higher sensitivity and specificity, and the capacity to detect unculturable viruses.

Multiplex quantitative PCR (qPCR) assays that use distinct fluorophores for various targets can detect several different viruses in a sample at the same time [29]. High-throughput qPCR using microfluidic technology is demonstrated as a direct multi-pathogen detection approach for environmental water samples. This technology makes use of microfluidic chips, which allow for high-throughput measurement of large sample quantities for a variety of enteric viruses and other pathogens [30][31].

A downside of PCR-based approaches is that they are susceptible to inhibitory compounds that are frequently co-concentrated with viruses, such as humic acids commonly found in environmental water samples. Various strategies are applied to reduce the effects of inhibitory substances. For instance, magnetic bead-based extraction methods may remove qPCR inhibitors more efficiently than spin column-based approaches [21].

Droplet digital PCR (ddPCR) is also shown to have improved performance in the presence of inhibitory compounds as compared to qPCR [31][32]. ddPCR performs better because it is an end-point positive/negative detection combined with Poisson statistics for quantification, so it has higher accuracy and precision against PCR inhibition. Furthermore, ddPCR directly quantifies viral gene copies in a sample without the need for calibration by known-concentration standards [33][34]. Since 2020, the adoption of ddPCR has accelerated due to increasing application for wastewater surveillance of SARS-CoV-2 during the COVID-19 pandemic [35].

2. Viruses and Viral Surrogates in Wastewater for Reuse

Risk-based assessments of wastewater treatment performance and water reuse applications should include both quantitative assessments of waterborne pathogenic human viruses known to be in circulation as well as non-pathogenic virus surrogates for human viral pathogens. The presence and loads of human viruses within treated wastewater will depend upon the health characteristics of the communities contributing to the wastewater and the efficacy of the treatment operations to remove the viruses. Hence, the number and type of human pathogenic viruses in untreated and treated wastewater will vary regionally and over time. Given the high level of variability of human viruses in wastewater, viral surrogates are often used to assess viral risks. The EPA defines viral surrogates as “Nonpathogenic (e.g., coliphage, pepper mild mottle virus [PMMoV], etc.) or pathogenic viruses (e.g., adenovirus, norovirus, etc.) and/or other types of indicators (e.g., enterococcus qPCR (EPA Method 1609, [36]), the human marker HF183, etc.) demonstrated to predict the presence of and/or risk of illness from human pathogenic viruses via co-occurrence studies and quantitative microbial risk

assessments.” Given this EPA definition, viral surrogates are surrogates of risk of illnesses from viruses as a whole, and thus pathogenic viruses themselves can serve as surrogates of risk.

2.1. Human Viruses

Human enterovirus, norovirus, and adenovirus are frequently used in risk-based water quality assessments because of their high abundance in wastewater, their importance in waterborne outbreaks, and the historical data on their prevalence in wastewater around the world. Enteroviruses including coxsackievirus, enterovirus 71, coxsackie A virus, DHV-1a, and DHV-3 are considered the most prevalent viruses in the world [37]. They cause a number of infectious illnesses, which are usually mild. Children, particularly those younger than 10 years old, are most likely to be infected. Human noroviruses are the leading cause of epidemic gastroenteritis in all age groups. They are the leading cause of acute gastroenteritis in the United States and are responsible for at least 50% of acute gastroenteritis outbreaks occurring worldwide each year [38]. Adenoviruses in water are extensively investigated and reviewed [39]. The high abundance (typically 10^8–10^{10} gc/L in raw wastewater) and relative ease of detection made adenovirus a popular target for monitoring viral quality in water. With a double-stranded DNA genome, adenovirus is more resistant to UV disinfection than other viral pathogens during wastewater reclamation [40]. Diverse serotypes of human adenoviruses are responsible for both enteric illnesses and respiratory and eye infections. Unlike the three viruses discussed above, Aichivirus was identified more recently in wastewater. High concentrations of Aichivirus were found in over 90% of wastewater tested in the Netherlands, Japan, and North America [41][42][43][44][45], suggesting that further investigation of Aichivirus to assess treatment performance is warranted. Most human viruses that are identified in high concentrations in wastewater are transmitted through fecal–oral pathways with the exception of human adenovirus. Amongst various serotypes of adenoviruses, serotypes 40 and 41 are enteric viruses and are transmitted through the fecal–oral route, while adenovirus serotype 5 causes respiratory infection and is transmitted by aerosols but also shed in human feces in high concentrations [39]. Understanding the viral transmission pathways has important implications on health risk assessment.

Enteric viruses in wastewater show clear seasonality in concentrations and are unlikely to be detected in wastewater at all times of year [44][46]. Human virus selected for risk-based monitoring of recycled water should thus attempt to capture known seasonality of regionally significant waterborne viruses. For instance, enteroviruses peak in the summer while noroviruses peak during winter in temperate climates. In contrast, human adenovirus and Aichivirus are frequently found in wastewater without any distinct seasonality. Data on the presence and removal of a suite of human viruses alongside other water treatment operations and water quality may thus provide a broad picture of viral pathogens and their removal during wastewater reclamation throughout a given year.

2.2. Viral Surrogates for Human Viruses

Various viral surrogates for human viruses are proposed to indicate the removal of infectious viruses during wastewater treatment. Among them, somatic and F-specific coliphage are top candidates. In fact, a large body of work evaluated the suitability of coliphages as indicators of human viral contamination in recreational water [47]. In comparison with human virus infectivity assays, coliphage assays are significantly faster, cheaper, and easier. Advancements in genome-based methods also identified new potential surrogates for human viruses in wastewater, with pepper mild mottle virus (PMMoV) and crAssphage rising as particularly promising candidates. In 2021, tomato brown rugose fruit virus (ToBRFV) was found to be the most abundant RNA virus in Southern California wastewater, in much greater abundance than PMMoV [48]. These potential human viral surrogates, although morphologically and physiologically distinct from human enteric viruses, are found in high concentrations in municipal wastewater. Furthermore, recent studies evaluating viral indicators [49][50] suggest gut-associated bacteriophages beyond crAssphage as additional potential viral surrogates, with the advantage of adding human specificity over the more abundant plant viruses.

2.2.1. Coliphages

Coliphages are bacterial viruses that infect *E. coli* and are found in human fecal waste. Coliphages are relatively easy and inexpensive to measure through culture-based techniques, which are based upon counts of plaque-forming units (PFU) on agar containing the host bacteria [51]. This technique provides an approximation of the presence and number of infective coliphage viruses. These analyses help overcome the limitations of PCR, which measures genetic material regardless of infectivity. Coliphages are considered better indicators for viral pathogens than traditional FIB (fecal indicator bacteria) due to their more similar physical structure and morphology [52][53][54]. Coliphages are generally expected to exhibit persistence in environmental waters and response to treatment that is similar to human enteric viruses, but extensive reviews of environmental data reveal varying patterns [55]. The detection of infectious coliphage in reuse water implies a potential presence of infectious human viruses in the same wastewater or the failure of treatment processes to inactive viruses.

Coliphages are separated into two classes: somatic and male-specific (otherwise known as F+ or F-specific) coliphages. Somatic coliphages are DNA viruses that infect host bacteria via the outer membrane. They consist of a broad range of coliphage types and have been included in many environmental studies. Male-specific coliphages (F+) were originally believed to contain a single-stranded RNA genome [56] but are now known to include viruses with DNA- or RNA-based genomes [57]. The male-specific coliphages (F+) infect host bacteria through an appendage, the F-pilus of male strains of *E. coli*, used for bacterial conjugation. Various studies suggest that somatic coliphages are more abundant than F-specific coliphages in untreated wastewater, primary and raw sludge. With few exceptions, similar relative proportions of somatic coliphages, F-specific bacteriophages, and RNA F-specific bacteriophages are measured in secondary effluents from wastewater treatment plants when counted using standardized methods in the same samples [53][58][59]. F-specific bacteriophages are inactivated by high temperature or high pH and have low persistence in warmer climates. F-specific bacteriophages thus perform more accurately as viral indicators in samples where they predominate, such as groundwater, clay sediments, and reclaimed waters [60]. MS2 is a strain of F+ RNA (group I) coliphage. Because of the resemblances of physical size and shape of MS2 and its genomic content to many human enteric viruses (i.e., enterovirus), MS2 is proposed as a viral surrogate by EPA for recreational water quality. Somatic coliphages are greatly affected both by UV radiation as well as chlorination. Chlorination may not significantly change the relative proportion of somatic and F- specific coliphages [59], but somatic coliphages are found to be lower in number than F-specific coliphages following UV treatment. F-specific coliphages may therefore be better indicators in effluents from facilities using UV treatment [52][61].

2.2.2. CrAssphage

CrAssphage is a group of dsDNA bacteriophages infecting Bacteroides spp. [62] and potentially other bacterial hosts. CrAssphage is highly abundant in wastewater (excreted by 50–70% of people). This group was named based on its metagenome-assembled genome and is thought to belong to the normal human gut virome [63]. Importantly, crAssphage can be specifically associated with humans and is a specific indicator of human waste, distinguishable from other animal waste. There is still much to be learned about crAssphage in wastewater, although some groups are already using it as a specific indicator of human fecal contamination [44][64][65][66][67][68][69][70]. In addition, qPCR comparisons of crAssphage abundance with PMMoV and Aichivirus show that crAssphage abundance correlates with human viral pathogens and is found in high abundance relative to other tested viruses [45].

2.2.3. Pepper Mild Mottle Virus

Pepper mild mottle viruses (PMMoV) are non-enveloped, rod-shaped plant pathogens that contain a single-stranded RNA (ssRNA) genome [71][72]. Several characteristics make PMMoV a valuable indicator of human fecal load in a water sample from diverse geographic regions. PMMoV is ubiquitous and present at high concentrations in human feces worldwide [73]. PMMoV virions are also stable over a range of environmentally relevant temperatures [73]. Since the presence of PMMoV is dietary in origin, PMMoV may be a more consistent indicator of fecal load than viruses that cause human disease [73]. Finally, PMMoV is rarely found in animal feces, limiting the potential for animal fecal contributions to bias PMMoV-based estimates of human fecal load [72]. PMMoV is used extensively as a measure of fecal strength in wastewater in analyses of SARS-CoV-2.

PMMoV does have several limitations as a water-quality indicator. PMMoV's morphology and surface charge are markedly different from enteric viruses. This could lead to differences between PMMoV and viruses of interest with respect to environmental behavior and removal/reduction rates under different treatment processes. The co-occurrence of PMMoV with human viruses is poorly understood, if not inconsistent, and requires further investigation. There are also concerns about underestimating viral removal efficiency due to the high stability of PMMoV genome fragments. On the other hand, PMMoV detection may offer a conservative estimation of viral risk in water reuse.

2.3. Metagenomics Approaches

Metagenomics can provide unique insights for selecting targeted viral surrogates for the non-potable reuse of wastewater. As sequencing and bioinformatics pipelines continue to rapidly evolve, they may offer more comprehensive input data for risk assessments. Already known to be the most abundant biological entity in the earth's biosphere [74], virus diversity is expected to be significantly larger than currently known. The current 10th report by the International Committee for the Taxonomy of Viruses identified 189 viral families and 9110 viral species [75], while one study estimated more than 320,000 viral species infecting mammals alone [76]. As municipal wastewater contains both fecal and other human bodily wastes, it is expected to contain viruses of diverse origins, including human viral pathogens, plant and animal viruses from dietary ingestion, and bacteriophages that infect the human microbiome. Metagenomics based on the emerging next-generation

sequencing (NGS) technologies requires no a priori knowledge of the targets and hence has the unique capability of providing more comprehensive mapping of the viral diversity in wastewater and identifying new potential viral surrogates.

Metagenomic characterization of viruses in wastewater reported a highly diverse wastewater virome with specific host affiliation profiles. Many studies reported that a significant portion of wastewater viral metagenomic sequences have no known matches in reference databases [77][78][79][80], indicating tremendous virus diversity in wastewater. Sequences assigned to human viral pathogens (either enteric or respiratory) are usually present but at very low abundance levels (e.g., often less than 1% of the total reads or contigs) [76][78][81][82]. For example, in a 2021 study of Southern California wastewater, norovirus was detected in the majority of unenriched or enriched wastewater samples, while PMMoV was detected in all samples regardless of enrichment [48]. Although the direct metagenomic detection of human pathogenic viruses may be the most unbiased approach for microbial risk assessment in water reuse, the low abundance and associated requirements for pre-processing of wastewater samples and post-sequencing bioinformatic analysis could present significant technical challenges. A resurgence of interest in wastewater monitoring of SARS-CoV-2 led to additional approaches for analyzing imperfect sequence data to assess the abundance and distribution of variants of concern, all of which may expand the utility of wastewater sequencing [83][84][85].

The metagenomic characterization of the wastewater viromes led to the identification of potential alternative viral surrogates. The analysis of human fecal metagenomes led to the discovery of the most abundant phage in human feces. The previously unknown *Bacteroides* phage, crAssphage [86], was also shown to be the most abundant phage in wastewater virome [87]. Given the high abundance of fecal bacteria in wastewater, not surprisingly, many viral sequences in wastewater virome were identified to belong to bacteriophages, including crAssphage [77][81][88][89]. The metagenomic sequencing of wastewater viromes also detected plant viruses as the largest group of eukaryotic viruses in wastewater viromes which is attributable to undigested plant matter in human fecal matter [90]. Among many different plant viral families, the PMMoV was previously detected by metagenomic sequencing as the dominant RNA virus in human feces [91], which has also been suggested as a viral surrogate in fecal pollution [72], and may also be potentially suitable for water quality monitoring in water reuse.

3. Non-Viral Indicators of Viral Quality

3.1. Physicochemical Water Quality Parameters

Physicochemical water quality parameters measured at wastewater treatment plants have the potential to support viral health risk assessments by informing expectations about treatment performance and by indicating virus removal efficiency (e.g., by the breakthrough of small molecules in a reverse osmosis system). Total organic carbon (TOC) and electrical conductivity (EC) are easily measurable water quality parameters that can serve as conservative surrogates for continuous monitoring of microbe removal for water reuse [92][93]. Other physicochemical parameters, such as pH, NH_4^+, turbidity, and adenosine triphosphate (ATP), also offer rapid and low-cost measures of water quality. New modeling approaches, such as Artificial Neural Network models, could potentially integrate diverse data inputs to determine which provide a meaningful indication of virus infectivity and removal.

3.2. Bacterial Surrogates

Bacterial surrogates for human viral pathogens are likely to provide an incomplete understanding of viruses in water reuse, but information from bacterial monitoring programs may ultimately provide utility in viral health risk assessments. Common bacterial surrogates include coliform bacteria (especially *Escherichia coli*), fecal streptococci, enterococci, and bacteria belonging to the genus *Bacteroides* [94][95]. Fecal indicator bacteria (FIB) have had a long history trying to establish their utility for microbial water quality monitoring. FIB are not pathogenic in themselves but are used to “indicate” the possible presence of pathogens. The coliform group of bacteria was the original FIB group, dating back to 1914 [96], used to regulate drinking water. This group is still used today to regulate drinking water supplies, except that regulations also require measurements of specific subcategories of total coliform, fecal coliform (which selects for coliforms of fecal origin by using a higher incubation temperature), and *E. coli* (based on the action of β-glucuronidase).

As for viruses, differences in source, size, morphology, persistence, stability, genome structure, and other characteristics of bacterial surrogates can (1) lead to differences in the ways that surrogates and viruses respond to different treatment processes and (2) can create inconsistent relationships between surrogates and viruses in different settings. Using multiple surrogates or surrogate approaches is often recommended to obtain a comprehensive and reliable water-quality assessment. For bacterial monitoring, this may mean combining the monitoring of one or more individual surrogate species with approaches that examine the broader bacterial community in a water sample. Examples of the latter include heterotrophic plate count (HPC) [97], the 16s rRNA gene assay [95], and flow cytometry (FCM) [98]. Such approaches are

especially useful for monitoring bacterial regrowth in drinking-water infrastructure [99] and generally for assessing water quality in highly treated waters where the concentration of any individual surrogate is expected to be low [100].

The use of coliforms for regulating recreational water is questioned as it was found that environmental sources other than feces can contribute to the presence of the coliform group of microbes. Alternative sources were observed in both tropical and subtropical climates [101][102][103][104], and most recently, within temperate regions [105][106]. Alternative bacteria were identified as *Clostridium perfringens* [107] and enterococci (previously known as fecal streptococci). Enterococci include a group of 26 species of *Enterococcus* [108]. These alternative indicators of fecal contamination can potentially be used to supplement viral surrogates in water reuse.

3.3. Virus-like Particles as Viral Removal and Viral Safety Indicator

An important remaining challenge associated with enumeration strategies for human viruses and viral surrogates is the lengthy time for analysis (from hours for PCR to days for bacteriophage culture, to more than a week for human virus culture). Flow cytometry (FCM), has the potential to quickly determine concentrations of biological particles in water samples. FCM refers to the analysis of particles (including cells, cell fragments, inorganic debris, and viruses) based on how they scatter light in the forward and side directions and/or fluoresce when passing through a laser beam. Switzerland's Federal Office of Public Health officially endorsed FCM as an acceptable method for obtaining total cell counts for freshwater samples [109], and many utilities and regulatory bodies around the world are considering the same. The successful application of FCM to enumerate bacteria in drinking water demonstrates that FCM can characterize microbial water quality in a rapid, reliable, and reproducible manner. The recent development of better instrumentation and new fluorescent dyes expanded the applications of FCM from bacteria to viruses. The total number of viruses in wastewater is estimated to be in the range of 10^{11}/L based on direct counting under the microscope and by FCM [110]. Ma et al. [111] and Huang et al. [110] both used FCM combined with sensitive nucleic-acid dyes to quantify the abundance of virus-like particles (VLPs) at various stages of wastewater treatment. A review by Safford and Bischel [98] of nearly 300 studies published in the past two decades concluded that "substantial progress" was made in the application of FCM to water treatment, distribution, and reuse. Nevertheless, research showed that FCM is only capable of detecting viral particles of relatively large physical and/or genome size [112]. Despite progress on the use of FCM to detect viruses, demonstration studies of FCM in wastewater treatment are needed to evaluate correlations between total virus removal as detected by flow cytometry and removal of human viruses. Such studies would provide much value to understand the potential role of FCM in supporting measurements of viral quality and risk in municipal reuse applications.

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