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Review

Integrating Virus Monitoring Strategies for Safe Non-Potable Water Reuse

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Abstract: 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. This manuscript discusses advancements in monitoring and modeling viral health risks in the context of water reuse. First, we describe current wastewater reclamation processes and treatment technologies with an emphasis on virus removal. Second, we review technologies for the measurement of viruses, both culture- and molecular-based, along with their advantages and disadvantages. We outline promising viral surrogates and specific pathogenic viruses that can serve as indicators of viral risk for water reuse. We suggest metagenomic analyses for viral screening and flow cytometry for quantification of virus-like particles as new approaches to complement more traditional methods. Third, we describe modeling to assess health risks through quantitative microbial risk assessments (QMRAs), the most common strategy to couple data on virus concentrations with human exposure scenarios. We then explore the potential of artificial neural networks (ANNs) to incorporate suites of data from wastewater treatment processes, water quality parameters, and viral surrogates. We recommend ANNs as a means to utilize existing water quality data, alongside new complementary measures of viral quality, to achieve cost-effective strategies to assess risks associated with infectious human viruses in recycled water. Given the review, we conclude that technologies will be ready to identify and implement viral surrogates for health risk reduction in the next decade. Incorporating modeling with monitoring data would likely result in a more robust assessment of water reuse risk.

Keywords: viruses; wastewater; reuse; surrogates; modeling

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1. Introduction

Municipal wastewater reclamation and reuse represent an important opportunity to meet human civilization's ever-increasing water demands. Compared with wastewater reuse efforts in other water-stressed regions around the world, water reuse in the United States has significant room to grow in both quantity and diversity of applications. Currently, roughly 7–8% of municipal wastewater in the U.S. is reclaimed for reuse [1], which is significantly less than the percentages in Israel and Singapore, where 85% and 35% of wastewater is treated for various reuse purposes [2]. Recognizing that traditional water supplies are no longer a certainty for many municipal water utilities across the U.S., a wave of investment was initiated in water reuse. So far, 17 U.S. states have planned reuse projects in the pipeline, exceeding $18 billion in total investment [3]. California and Florida continue to lead reuse development, while planned water reuse projects in Hawaii, Georgia, Wyoming, North Dakota, Pennsylvania, and Tennessee signal even more widespread adoption, according to Bluefield Research (Figure 1). Bluefield's nationwide database of reuse projects ballooned to 763 projects in 2017, in comparison to 135 projects just a few years prior [3]. The rapid development of reuse projects around the U.S. shows that water reuse is no longer just a drought mitigation strategy but instead a viable option for utilities to boost water supplies.

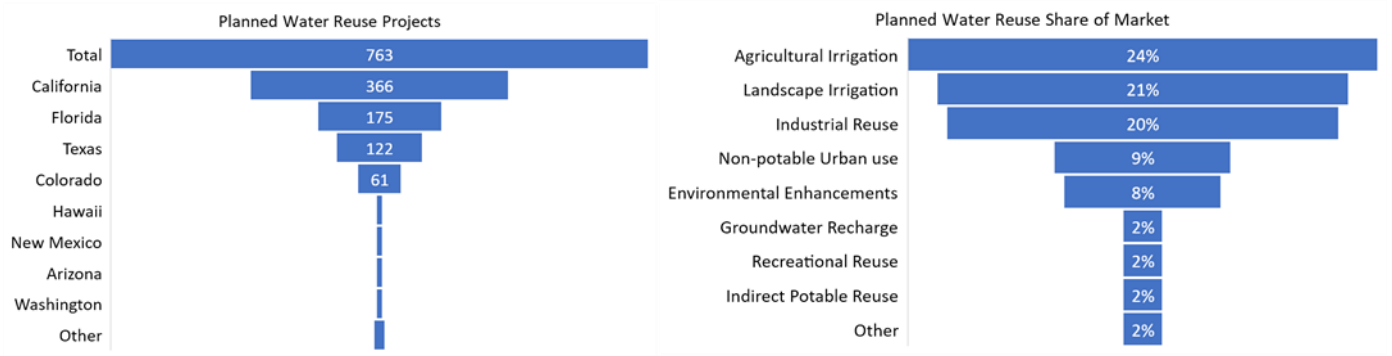

Figure 1. Planned water reuse projects by state and planned water reuse share of the market based on data collected by Blue Field Research [3].

Non-potable reuse is and will continue to be the dominant market share of reclaimed wastewater while drinking water production from wastewater is a very small fraction of the planned water reuse share of the market [4]. Non-potable reuse applications vary by region; the main wastewater reuse applications include agricultural irrigation, landscape irrigation, industrial use, and non-potable urban uses, including indoor plumbing (Figure 1). Indirect potable reuse represents less than 2% of the market share of the global planned water reuse market [5]. This trend is likely to continue due to the high cost of infrastructure investments, technology costs, and the low public acceptance to use recycled wastewater as a source of drinking water [6]. Standardized viral monitoring methods to assess treatment performance and risks of water reuse are critically needed for use in anticipated diverse non-potable reuse scenarios.

The treatment of wastewater for non-potable reuse varies significantly from region to region. There are no uniform engineering treatment processes or water quality standards at the national or international level. U.S. EPA guidelines for water reuse recommend secondary wastewater treatment followed by filtration and disinfection as technology processes for reclaiming municipal wastewater for urban uses, the irrigation of food crops eaten raw, and recreational impoundments. The filtration process is no longer mandatory when the water is intended for use with restricted human access [1], such as aesthetic impoundments, construction uses, processed food crops, industrial cooling, and other environmental uses where direct contact with humans is considered minimal.

Microbiological water quality guidelines for reuse water are based upon fecal coliform counts, with standards including no detectable fecal coliform/100 mL and less than 200 fecal coliforms/100 mL based on a 7-day median value for unrestricted and restricted reuse types, respectively [1]. However, a complicating factor is that wastewater is known to include pathogenic viruses, and viruses may be impacted differently than fecal bacteria when treated by traditional wastewater treatment processes. So, there is a need to investigate viral pathogens specifically to determine guidelines and regulatory criteria suitable to protect the public health of those who may come in contact with reused water [7].

Viruses are ubiquitous and persistent in raw and treated wastewater as well as in receiving water bodies [8]. Human feces from infected persons are the main source of human viruses [9,10]. A recent review captured the high abundance and diverse human viruses in human wastewater [11]. Due to their small size (20~220 nm), the low dose required for infection, and high resistance to wastewater treatment, including disinfection processes, viruses generally pose the highest health risk for water reuse [12,13].

To evaluate what is known and what is needed for wastewater reuse to gain acceptance from a microbiological safety perspective, this paper presents a review of the viral quality of reuse water. This review of viruses in reuse water differs from others in that it focuses on measurements of viral quality and describes conventional and new approaches for estimating risk. Other reviews focus on viruses in wastewater, including their occurrence, methods of detection, the potential to cause waterborne diseases [14,15], technologies available to remove viruses from wastewater [16], and the identification of viral surrogates [17]. Reviews on wastewater treatment for reuse focus on computing viral removal efficiencies based upon published datasets [18] and the reductions in viral loads to assure safety in the consumption of edible crops and drinking water [9]. This review differs in that it provides a review of water reclamation processes, human viruses, and viral surrogates in wastewater, followed by traditional and innovative technologies for viral measurement and methods for assessing risk. The intended audience for this review is both practitioners (wastewater treatment plant operators) and researchers. As such, the discussion at the end of this manuscript describes the advantages and disadvantages of the available technologies from a practical implementation viewpoint.

2. Water Reclamation Processes

As wastewater treatment is undergoing the transition to resource recovery, the previously known sewage or wastewater treatment facilities are now referred to as water resource recovery facilities (WRRFs). One of the main drivers to retrofit or upgrade facilities to WRRFs is water reclamation and reuse, which necessitates nutrient removal and filtration. In Figure 2, we illustrate the main unit operations responsible for water reclamation in most facilities in the United States. The treatment steps from left to right mirror the chronology of technology deployment; since a century ago, most facilities were mere screening plants that later upgraded to settling and ultimately added biological treatment following the infrastructural wave of the 1970s [19]. The treatment of wastewater can be accomplished with the goal of discharge to a water body (river, lake, ocean) by performing in series: screening and grit removal (in the head works); solids settling (in the primary clarifiers); biological oxidation of dissolved matter and non-settled solids (in the secondary process).

There exists a variety of secondary process options, with activated sludge having gained the majority of the treatment market worldwide since its invention by Ardern and Lockett [20]. The main benefits of this process are simplicity of design and operation and the ability to reach advanced levels of nutrient removal. Other processes exist, each with their benefits and peculiarity. Of those illustrated in Figure 2, membrane bioreactors represent the most recent technological development, having been on the market for less than three decades. This process is particularly suitable for water reclamation, despite its elevated energy intensity, because it combines the two unit operations of biological oxidation and filtration into one process.

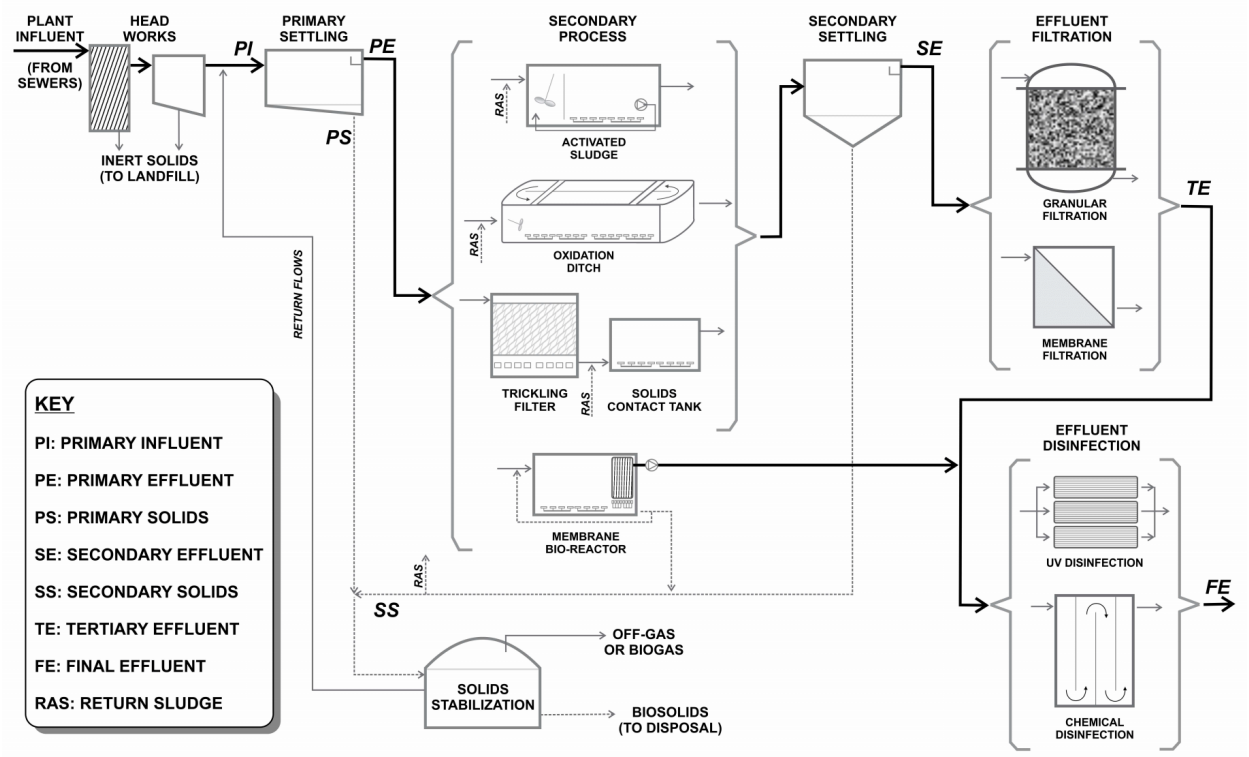

Figure 2. Treatment flow diagrams for water reclamation. Unit operations within parentheses perform similar treatment functions. Dashed lines are for sludge, while solid lines are for water flows. For suitable treatment trains, choose one among the unit operations within parentheses.

A typical water reclamation standard used as a reference worldwide is California's Title 22, which specifies filtration in the tertiary step of treatment [21]. One must remember that filtration is performed with microfiltration membranes (with pore diameters of the order of ~10^{-4} mm), and thus the barrier separation targets bacteria and protozoa but not viruses [19]. Disinfection is always required downstream of the filtration step (Figure 2). When reclamation is pushed forward to the step of potable water reuse, further barrier separation is used (e.g., reverse osmosis). The energy associated with the last step can be substantial [22], yet much lower than the option of long-distance water importation [23]. However, the quality of the effluent water fits the criteria for many additional uses, including potable reuse.

The reduction of pathogen counts is one of the primary criteria used to assess reuse options and are quantified as log-reduction credits for each specific treatment process [23,24]. Log-reduction credits are evaluated through the removal of reference human pathogens or surrogates but often underestimate the removal efficiency of microorganisms [25]. Norovirus and *Cryptosporidium* spp. were identified as important reference pathogens when comparing treatment process layouts due to the challenge for some treatment processes to abate them adequately [26]. Establishing a suite of viral surrogates for ongoing monitoring of water reuse will provide value in establishing appropriate credits for water reuse treatment processing.

3. Current Technologies for Monitoring Viruses

3.1. 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 (Table 1). 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 [11]. Several previous reviews summarized and compared concentration methods including virus adsorption and elution (VIRADEL), electronegative filtration, electropositive filtration, size-exclusion, and coagulation/flocculation [11,15,27,28]. Viral concentration methods that are useful for monitoring viruses in water reuse are highlighted below.

Table 1. Methods for Concentrating and Quantifying Viruses in Wastewater Samples.

Viral Concentration Methods		
	Advantages	**Disadvantages**
Virus adsorption and elution	Fast (hours).	Viruses must be subject to capture and elution, which may not be 100% efficient. Inhibition depends upon eluate chemistry.
Electronegative filtration	Fast (hours). Inexpensive. Supplies are easy to procure.	Viruses must be positively charged and captured, which may not be 100% efficient.
Size exclusion	Captured by size.	Cartridges are subject to availability and can be expensive. May be subject to extensive plugging limiting volume processed. Requires a specialized centrifuge in some cases.
Coagulation/flocculation	Fast (hours). Inexpensive. Supplies are easy to procure.	Viruses must be captured by process, which may not be 100% efficient. Inhibition depends upon eluate chemistry.
Magnetic Bead Based Capture	Fast (hours). Process is automated by commercial vendors. Less inhibition	Beads are subject to availability and can be expensive.
Viral Quantification Methods		
	Advantages	**Disadvantages**
Cell Culture	Measures potentially infectious viruses.	Slow to obtain results (weeks).
PCR	Fast (hours). Higher sensitivity and specificityDetects non-culturable viruses.	Does not measure infectiousViruses. Subject to inhibition.

Electronegative membranes are commonly applied for virus concentration. Several studies demonstrated viral filtration using flat filter membranes with electronegative surface charge in electronegative filtration [29–33]. Haramoto et al. [15] 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 [34,35]. The VIRADEL method has been used to concentrate viruses from a variety of water samples, including seawater, tap water, surface water, and wastewater [27]. Electropositive media and filters have also been applied in a variety

of configurations for virus concentration. Examples include 1MDS filters (3M, Maplewood, MN USA) [11] and NanoCeram filters (Argonide, Sanford, FL, USA). The NanoCeram filter media is applied to concentrate viruses in drinking water [28] and wastewater [11,36] and are suggested as a less expensive alternative to the 1MDS filter [37].

In addition to surface-charged filters, size-exclusion filtration methods allow for the simultaneous recovery of viruses and bacteria [11,28,38,39]. Another common ultrafiltration technique uses specialized cartridges designed for separation through membrane filters during centrifugation [31,40].

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 [11,28]. The method is likely highly applicable to the treated wastewater for reuse. Another common coagulation/flocculation method utilizes polyethylene glycol precipitation (PEG) [41–43]. 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 [44].

As these studies show, no single strategy for concentrating human enteric viruses in wastewater appears to be completely efficient [9]. Given the attention to SARS-CoV-2, the virus that causes COVID-19, in wastewater, a recent inter-laboratory method comparison study in the recovery of SARS-CoV-2 from wastewater was conducted [35]. Three viral concentration methods (ultrafiltration, electronegative filtration, and PEG precipitation) did not present significant variability in the final outcomes [45]. 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 [46]. Therefore, testing settled solids and primary sludge can provide highly sensitive detection of SARS-CoV-2 [47,48]. 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 [49,50], demonstrate the potential for high-throughput virus concentration.

Given the emergence of various new target viruses of interest (e.g., crAssphage, tomato mosaic virus), recovery efficiencies of different concentration approaches may need to be reevaluated [27]. 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.

3.2. 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 [51]. Polymerase chain reaction (PCR)-based methods enable faster detection timeframes (within hours), higher sensitivity and specificity, and the capacity to detect unculturable viruses (Table 1).

Multiplex quantitative PCR (qPCR) assays that use distinct fluorophores for various targets can detect several targets in a sample at the same time [52]. 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 [53,54].

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 [9].

Droplet digital PCR (ddPCR) is also shown to have improved performance in the presence of inhibitory compounds as compared to qPCR [54,55]. 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 [56,57]. Since 2020, the adoption of ddPCR has accelerated due to increasing application for wastewater surveillance of SARS-CoV-2 during the COVID-19 pandemic [58].

4. 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 "Non-pathogenic (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, [59]), 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.

Much research characterizes the occurrence and abundance of pathogenic human viruses and viral surrogates in wastewater. Table 2 shows virus panels that represent structurally diverse surrogates, including viruses with single-stranded (ss) or double-stranded (ds) DNA and RNA genomes as well as a range of sizes and morphologies. This list is not meant to be all-inclusive but is intended to capture a range of physicochemical properties that influence the inactivation and removal efficiency of viruses undergoing diverse treatment processes. Moreover, we present a combination of human viruses that we expect to have wide geographic relevance and comprise a range of disease etiologies with varying seasonal prevalence patterns. This section further describes types of viral surrogates in wastewater along with molecular- and culture-based assays for their detection. We also discuss the use and importance of metagenomics for virus discovery. Non-viral surrogates are described in Section 5.

4.1. 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 [61]. 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 [63]. Adenoviruses in water are extensively investigated and reviewed [64]. 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 [65]. Diverse serotypes of human adenoviruses are responsible for both enteric illnesses and respiratory and eye infections. Unlike the three viruses discussed above, Aichivirus (Table 2) 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 [66–70], 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 [64]. Understanding the viral transmission pathways has important implications on health risk assessment.

Table 2. Potential Human Viral Pathogens and Surrogates to Indicate Human Health Risks During Non-Potable Water Reuse.

Candidates	Host	Genome Type	Morphology	Transmission Pathway to Human	Criteria for an Ideal Surrogate					
					Presence in the Presence of Enteric Viruses	Similar Survival Rate to Hardiest Enteric Virus	Levels Observed in Raw Sewage [a,b,c,d] Copies/L or pfu/L	Ease of Infectivity Assay	Fast and Sensitive Detection Method	Globally Distributed and TEMPO-RALLY Stable
Enterovirus	Human	RNA+	small-icosahedral	Fecal–oral	Yes	No	10^5–10^6	No	No	No
Norovirus	Human	RNA+	small-icosahedral	Fecal–oral	Yes	No	10^4–10^9	No	No	No
Adenovirus	Human	dsDNA	medium-icosahedral	Fecal–oral and aerosol	Yes	Yes	10^4–10^9	No	No	Yes
Aichi virus	Human	RNA+	small-icosahedral	Fecal–oral	Not well studied	Not well studied	Not well studied	No	No	Yes
Somatic coliphage	*E. coli*	dsDNA	vary	Not Applicable [e]	Yes	Yes	10^4–10^6	Yes	No	Yes
F-specific coliphage	*E. coli*	ssRNA or ssDNA	small-icosahedral or filamentous	Not Applicable [e]	Yes	Yes	10^3–10^7	Yes	No	Yes
CrAssphage	*Bacteroides intestinalis*	Circular dsDNA	icosahedral head with a short tail	Not Applicable [e]	Yes	Not well studied	10^7–10^9	No	No	Not well studied
PMMoV	Pepper	RNA+	rod	Not Applicable [f]	Yes	Yes	10^6–10^9	No	No	Yes
ToBRFV	Tomato	RNA+	rod	Not Applicable [f]	Not well studied	Not well studied	Not well studied	No	No	Not well studied
virus-like particles	Mostly bacteria	Vary	vary	Vary	Yes	Yes	Not well studied	No	Yes	Yes

[a] Corpuz et al., 2020 [11], [b] Rusinol and Girones, 2017 [60], [c] Betancourt and Shulman, 2017 [61], [d] Ahmed et al., 2020 [62], [e] Found in the intestines of most humans, [f] Dietary source.

Enteric viruses in wastewater show clear seasonality in concentrations and are unlikely to be detected in wastewater at all times of year [69,71]. Human virus panels designed 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.

4.2. 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 [1]. 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 (Table 2). 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 [72]. 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 [17,73] suggest gut-associated bacteriophages beyond crAssphage as additional potential viral surrogates, with the advantage of adding human specificity over the more abundant plant viruses.

4.2.1. 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 [74]. 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 and they have higher persistence in treatment processes [75–77]. 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 [78]. 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 [79] but are now known to include viruses with DNA- or RNA-based genomes [80]. 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 [76,81,82]. 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 indicators in samples where they predominate, such as groundwater, clay sediments, and reclaimed waters [83]. 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 [82], 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 [75,84].

4.2.2. CrAssphage

CrAssphage is a group of dsDNA bacteriophages infecting Bacteroides spp. [85] 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 [86]. 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 [69,87–93]. 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 [70].

4.2.3. Pepper Mild Mottle Virus

Pepper mild mottle viruses (PMMoV) are non-enveloped, rod-shaped plant pathogens that contain a single-stranded RNA (ssRNA) genome [94,95]. 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 [96]. PMMoV virions are also stable over a range of environmentally relevant temperatures [96]. 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 [96]. Finally, PMMoV is rarely found in animal feces, limiting the potential for animal fecal contributions to bias PMMoV-based estimates of human fecal load [95]. 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.

4.3. 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 [97], 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 [98], while one study estimated more than 320,000 viral species infecting mammals alone [99]. 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 [100–103], 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) [99,101,104,105]. 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 [72]. 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 [106–108].

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 [109], was also shown to be the most abundant phage in wastewater virome [110]. 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 [100,104,111,112]. 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 [113]. Among many different plant viral families, the PMMoV was previously detected by metagenomic sequencing as the dominant RNA virus in human feces [114], which has also been suggested as a viral surrogate in fecal pollution [95], and may also be potentially suitable for water quality monitoring in water reuse.

5. Non-Viral Indicators of Viral Quality

5.1. 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 [115,116]. Other physicochemical parameters, such as pH, NH_4^+, turbidity, and adenosine triphosphate (ATP), also offer rapid and low-cost measures of water quality. In Section 6.2, we discuss new modeling approaches that could integrate diverse data inputs to determine which provide a meaningful indication of virus infectivity and removal.

5.2. Bacterial Surrogates

Bacterial surrogates for human viral pathogens are likely to provide an incomplete understanding of viral health risks 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* [117,118]. 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 [119], 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) [120], the 16s rRNA gene assay [118], and flow cytometry (FCM) [121]. Such approaches are especially useful for monitoring bacterial regrowth in drinking-water infrastructure [122] and generally for assessing water quality in highly treated waters where the concentration of any individual surrogate is expected to be low [123].

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 [124–127], and most recently, within temperature regions [128,129]. Alternative bacteria were identified as *Clostridium perfringens* [130] and enterococci (previously known as fecal streptococci). Enterococci include a group of 26 species of *Enterococcus* [131]. These alternative indicators of fecal contamination can potentially be used to supplement viral surrogates in water reuse.

5.3. 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 [132], 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 [133]. Ma et al. [134] and Huang et al. [133] 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 [121] 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 [135]. 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.

6. Modeling

6.1. QMRA

Quantitative microbial risk assessment (QMRA) is an important tool for determining fit-for-purpose water reuse applications. QMRA is a mathematical approach used to estimate the risk of illness when humans are exposed to microbes. QMRA requires identifying the hazard, assessing the exposure, and understanding the response or illness once the dose is estimated. The results provide a characterization of risk, which is typically expressed as the probability of illness. QMRA of viral pathogens for water reuse in irrigation and

recreational impoundments has been investigated since the 1990s [136,137]. In recent years, risk modeling evolved (1) from generating point-estimates of risk to characterizing its distribution, (2) from using hypothetically assumed water volumes retained in food crops to data collected through physical experiments, and (3) from simplifying assumptions about virus infectivity to considering relationships between infectious viruses and viral genomes in some cases [138–140]. However, nearly all food crop irrigation QMRAs were based on very old viral monitoring data from wastewater reclamation plants in Southern California (late 1970s data) that do not represent the current state of water reclamation practices. Moreover, early QMRA studies assumed that enterovirus results were equivalent to rotavirus results and used a rotavirus dose–response model for a conservative risk measure [141,142]. However, the risk of rotavirus infection does not appear on the top list for illness cases in the U.S. and is significantly lower than that of norovirus.

A norovirus dose–response model based upon PCR- detected viral genome was developed and adopted in the risk analysis of stormwater harvesting for household uses and food crop irrigation [143]. They also showed the risk of viral transport from irrigation water through plant roots to edible portions even without direct contact with irrigation water [144]. Moreover, previous research showed that pathogen dose is the most sensitive parameter in the risk outcome [144]. Therefore, to improve the accuracy of risk estimations, the data in gaps in viral concentrations in treated wastewater and dose–response models incorporating multiple pathogens should be addressed.

6.2. Modeling of Infectious Viruses Using Artificial Neural Network

A greater understanding of the complex and interdependent relationships between treatment performance, parameters that indicate viral quality, and the presence of infectious pathogenic viruses is needed to improve risk assessments. An intelligent systems approach, including models based on artificial neural networks (ANNs), offers a potential solution to this classic challenge. An early application of ANNs in wastewater treatment demonstrated the superiority of neural networks compared to conventional kinetic models of microbial inactivation during disinfection [145]. In the past quarter-century, there was an increase in the application of ANN to a myriad of contexts, including wastewater process control [146,147], constituent monitoring [148], treatment performance [149,150], and virus disinfection [151] or removal [152] to deal with scaling challenges associated with multi-dimensional data. Yet, applications of such data-driven models to assess viral risk are lacking. Here, we offer an example ANN model framework that incorporates treatment performance and viral quality parameters discussed in this paper to predict infectious enteric viruses (V_i) as follows (Figure 3):

$$[V_i] \in \left\{[Ent][Nor][Ade][Aic][s\varnothing][f\varnothing][PMMoV][CrA\varnothing][VLPs]\left[NH_4^+\right][TSS]\left[ClO^-\right][Temp][P]\right\},$$

where viral surrogates including PCR-detected viral pathogens (*Ent*, *Nor*, *Ade and Aic*), somatic and F-specific coliphage (*s*∅ *and f*∅), PMMoV, CrAssphage (*CrA*∅), and virus-like particles (*VLPs*) by flow cytometry, as well as water quality parameters including ammonia (NH_4^+), total suspended solids (*TSS*), free chlorine (ClO^-), temperature (*temp*), and a dummy variable (*P*) to differentiate different treatment operations and processes used in the reclamation plant operation are used as input variables in the model.

In this example, the presence of the infectious enteric virus in the treated wastewater relates to water quality parameters, virus surrogates, and treatment processes. ANN modeling can identify which parameters influence viral risk significantly and facilitate adaptive treatment strategies. Stable output predictions would require a multi-layer perceptron (MLP) ANN, composed of neurons, arranged into hidden layers, interconnected in parallel. In wastewater applications, ANNs with one hidden layer have the best structure to achieve accurate predictions without excessively increasing complexity and computational costs [153–155]. The number of neurons in the hidden layer is a fundamental parameter, which can be analyzed to obtain the desired accuracy of the infectious virus predictions.

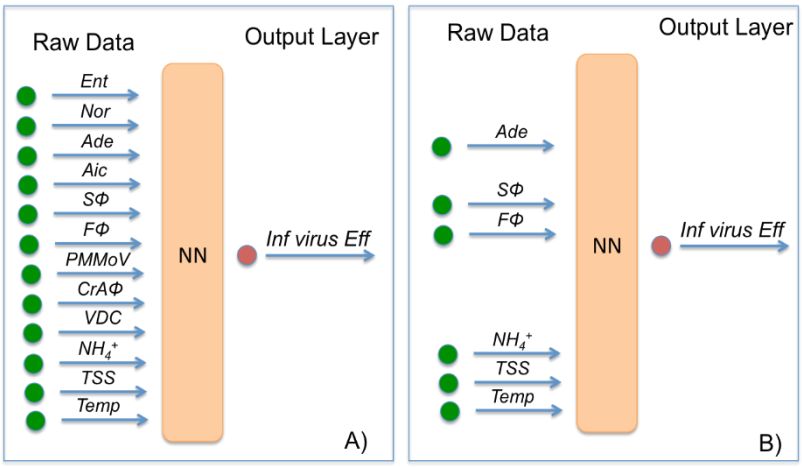

Figure 3. Illustration of ANN structure change based on sensitivity analysis of input variables. (**A**) Illustrate the initial scenario that includes all surrogates and parameters, (**B**) Illustrate the removal of a subset of input variables based on the sensitivity analysis.

In addition to using the main neural network to predict the infectious virus in the effluent by exploiting the information from all the viral surrogates and treatment conditions, networks for detecting faults in the input variables would facilitate the selection of appropriate model inputs. This involves the construction of a neural network for each input variable to predict the targeted variable using the other surrogates and water quality parameters as input variables. Sensitivity analysis would point to which input variables have a greater influence on the output prediction and facilitate a reduction in model complexity [156]. For example, the model structure can be simplified, as illustrated in Figure 3, from scenario (A), which includes all surrogates and water quality parameters in this example, to scenario (B), in which only a subset of surrogates and parameters are necessary. The surrogates with the greatest influence on the prediction outcome are the recommended surrogates for viral risk indication. Monte Carlo simulation can then be applied to quantify model uncertainty from model parameters, input data, or model structure [157].

7. Discussion and Limitations

Our inability to adequately monitor human viruses and understand their removal during wastewater reclamation processes present significant challenges to critical water reuse objectives. The direct monitoring of human viruses to estimate viral risk in water reuse is currently limited in the quantification of infectious viruses within a reasonable time. Cell culture assays for viral infectivity, including the Integrated Cell Culture (ICC)-PCR method, take days to weeks and require highly trained professional staff, which is not feasible for most wastewater reclamation utilities or regulatory agencies. PCR-based methods for virus detection are relatively fast and sensitive but lack the ability to directly indicate infectious viruses, and, therefore, the risk of infection. One possible solution is to integrate the presence of human viral genomes, or other viral genomes shed in human feces, as determined by PCR, with loss of viral infectivity as observed by viral surrogates alongside water quality parameters to predict infectious virus levels. Coliphage showed promise for evaluating the treatment efficacy for infectious viruses. Enumerating surrogates can help address challenges in direct monitoring of infectious human viral pathogens.

Although sophisticated measurement techniques can be developed, there will be practical limitations to their implementation. The integration of PCR detection technologies requires the purchase and maintenance of equipment and use of this equipment by trained personnel. The availability of equipment and trained personnel will represent a challenge for many water reuse operations. Even with PCR-based technologies, results may not be tied directly to infectivity, which would require additional techniques to address, such as culture-based coliphage assays and/or ICC-PCR or propidium monoazide (PMA) assisted

RT-qPCR. All infectivity protocols would require refurbishing traditional water quality laboratories, which typically measure only for bacterial surrogates. Coliphage infectivity assays can be conducted in fashions similar to fecal indicator bacteria quantification, but ICC-PCR would require viral culturing capabilities, which have become less common in recent years resulting in a smaller pool of trained personnel.

Additional limitations in direct viral surrogate or pathogen quantification are the need for measuring very low levels of viruses which translate to concentrating very large volumes of water. This is particularly true for direct potable reuse, which would dictate very low levels of viruses, at values of less than one per many liters of treated water. Filtering tens to hundreds (or even thousands) of liters of water would require on-site sample filtration processes, which are then to be eluted and concentrated for analysis. This preprocessing is time-consuming, and the filter cartridges can be expensive.

Given the technical challenges in directly measuring viral surrogates and pathogens, other simpler non-microbial measures should be considered along with viral detection technologies. The use of physical, chemical, and biological water quality measures such as conductivity, total organic carbon, and total bacteria and virus-like particles can be indicative of possible treatment inadequacies or breakthroughs and can be used to supplement direct specific viral detection programs. Potentially simpler measures of water quality should be integrated into risk-based models with the aid of artificial neural networks. Risk-based QMRA models in themselves are limited in that dose–response relationships are not available for all viruses of concern, and the synergistic effects of different viruses within a water sample are not well known. There is also uncertainty in the relationships between surrogates and viral pathogens. Ideally, the hazard characterization portion of the QMRA should be recalibrated occasionally for a specific site through PCR-based measures of viral pathogens and/or surrogates coupled with culture-based measures of coliphage.

The desired outcome of a proposed viral monitoring program is that the target is measurable and is technically simple for widespread implementation. The ongoing COVID-19 pandemic promoted the widespread implementation of wastewater-based surveillance of SARS-CoV-2 using genome-based approaches. These experiences suggest the feasibility of adopting molecular methods by wastewater treatment utilities. Moreover, the metagenome analysis of wastewater does not only provide information on the viral quality of treated water but also gives insight into the pandemic prediction and forecasting. The rapid advancement of sequencing technology, robotic liquid handling for sample concentration, and downstream target detection by ddPCR have already revolutionized the detection of diverse viral pathogens in wastewater. Looking into the future, streamlined sample collection, concentration, nucleic acid extraction, ddPCR detection of specific targets, or automatic bioinformatic programs for pathogen identification using metagenomic sequencing data are possible within the next 5 years. More research on an improved understanding of the best surrogates, their response to treatment relative to particular viral pathogens, and their relationship to risk are needed in water reuse scenarios. A considerable amount of additional investigation is needed to develop practical approaches to ensure safety in water reuse.

8. Summary and Conclusions

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. In this review, we describe viral surrogates, viral pathogens, and other surrogate measures that can be used to monitor the safety of reused waters. We recommend integrating all available treatment plant information (including unit operations utilized and physical–chemical water quality data) with artificial neural networks, which in turn assess the adequacy of treatment processes to remove viral pathogens. This information can then be combined with a QMRA to evaluate risks from viral pathogens on a real-time basis. In an ideal scenario, the reuse

plant would have their waters intermittently tested for viral pathogens directly, perhaps through sensitive metagenomics approaches, coupled with measures of targeted viruses by qPCR and possibly viral surrogates by culture, to assess vulnerability to specific viruses and to assess the suitability of viral surrogates. A model based upon simple measures and QMRA is envisioned to assess risk on a continuous basis.

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