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Review

# Reducing uncertainty in estimating virus reduction by advanced water treatment processes

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ABSTRACT

Treatment of wastewater for potable reuse requires the reduction of enteric viruses to levels that pose no significant risk to human health. Advanced water treatment trains (e.g., chemical clarification, reverse osmosis, ultrafiltration, advanced oxidation) have been developed to provide reductions of viruses to differing levels of regulatory control depending upon the levels of human exposure and associated health risks. Importance in any assessment is information on the concentration and types of viruses in the untreated wastewater, as well as the degree of removal by each treatment process. However, it is critical that the uncertainty associated with virus concentration and removal or inactivation by wastewater treatment be understood to improve these estimates and identifying research needs. We reviewed the critically literature to assess to identify uncertainty in these estimates. Biological diversity within families and genera of viruses (e.g. enteroviruses, rotaviruses, adenoviruses, reoviruses, noroviruses) and specific virus types (e.g. serotypes or genotypes) creates the greatest uncertainty. These aspects affect the methods for detection and quantification of viruses and anticipated removal efficiency by treatment processes. Approaches to reduce uncertainty may include; 1) inclusion of a virus indicator for assessing efficiency of virus concentration and detection by molecular methods for each sample, 2) use of viruses most resistant to individual treatment processes (e.g. adenoviruses for UV light disinfection and reoviruses for chlorination), 3) data on ratio of virion or genome copies to infectivity in untreated wastewater, and 4) assessment of virus removal at field scale treatment systems to verify laboratory and pilot plant data for virus removal.



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## 1. Introduction

Since domestic wastewater will always contain microbial pathogens, it is important that when intended for reuse applications, pathogens must be reduced to levels that do not have an impact on public health. Microbial risk assessments are useful to provide guidance for the needed reductions for treatment process to minimize risks of infection (NRC, 2012). Among the pathogen groups found in wastewater, viruses present the greatest risk because they generally occur in much greater concentrations and have a much greater infectivity (i.e. higher probability of infection with a given exposure), than bacteria and parasitic protozoa. With close to 200 species of enteric viruses, which can occur in wastewater, they represent the greatest number of different species of enteric pathogens (Gerba et al., 2017).

Minimum log reduction values of viruses by treatment trains designed for recycling of wastewater has been suggested. Recycled water intended for irrigation of edible crops requires a 6–7 log reduction (WHO, 2006) and for potable reuse applications (i.e., groundwater recharge and augmentation of surface water supply reservoirs) a 12-log reduction has been suggested (Title 22 and 17 California Code of Regulations. 2015). These reductions are based on assuming infective virus concentrations of $10^5$ to $10^6$ per liter in raw wastewater based on datasets collected in previous studies (Harwood et al., 2005; Rose et al., 2005). Recent application of molecular methods in wastewater and recycled water settings, suggests that some pathogenic viruses may be occurring in concentrations of upwards of $10^7$ to $10^9$ genome copies per liter (Gerba et al., 2017; Eftim et al., 2017). However, it is still unknown the relative proportions of infectious to non-infectious virus in these sample types. Viruses in raw sewage are more likely to be infectious due to direct excretion with feces. Moreover, their survival in sewage is facilitated by organic debris of the clinical matrix in which the virus is shed (e.g., feces or vomit) and virus aggregation formation, offering protection in the route to new human hosts (Rusiñol and Girones, 2017).

Several studies have attempted to estimate the impact of risk reduction by different treatment processes for pathogens present in untreated wastewater and at the same time quantifying the risks from viruses (NRC, 2012; Olivieri et al, 2014; WHO, 2017; Soller et al., 2018). However, most have not addressed the uncertainty in these estimates associated with the factors listed in Table 1. Exposure usually presents the greatest amount of uncertainty in risk estimation (Haas et al., 2014). Here we review those factors which exert the greatest influence on uncertainty in risk assessment for viruses in recycled treatment systems.

## 2. Factors influencing uncertainty in risk assessment for viruses in recycled water

### 2.1. Estimating virus concentrations in water

Knowing the concentration of infectious viruses in raw sewage entering a treatment facility is critical in assessing the needed efficacy of the entire processes in reducing viruses to acceptable levels. Recent advances in molecular biological methods have revealed that levels of viruses in untreated raw sewage are much greater than previously thought (Gerba et al., 2017). Applications of these advanced molecular based methods to raw sewage indicate that enteric virus levels can reach levels of 9,800,000,000 per liter. It has been documented that some viruses, such as adenoviruses, are much more abundant in wastewater and occur at higher concentration (1000 fold or more) than other common enteric viruses (Kitajima et al., 2014). In addition, real world data on the removal of naturally occurring viruses through wastewater treatment needs further assessment. However, determining the number of infectious viruses in water is challenging because no single method can detect all of the infectious viruses that may be present. Molecular methods, which detect the nucleic acids of viruses, do not inform us as to their infectivity. Methods for determining the infectivity of human viruses depend on documenting their replication in cell culture. Enteroviruses (e.g. poliovirus) were among the first viruses grown in animal cell culture and have been the most studied in water/wastewater. Numerous methods for detecting virus replication in cell culture have been developed (Payment and Trudel, 1993). However, no single cell culture system can be used for all enteric viruses. The propagation of viruses in cell culture followed by detection by the polymerase chain reaction (PCR) assay, termed integrated cell culture (ICC)–PCR, provides a new procedure for monitoring infectious viruses that do not induce cytopathic effect or plaques in cell culture (Reynolds et al., 1996; Chapron et al., 2000). This method also has the advantage of reducing the time for virus detection and increasing detection sensitivity. Unfortunately, only small number of the enteric virus types found in wastewater can replicate in routine cell culture. Even then, different virus types require different cell culture lines and the susceptibility of the cell line to a particular virus may change over time in the laboratory (Payment and Trudel, 1993; Chapron et al., 2000; Condit, 2013). In addition, the cultivation of naturally occurring viruses in wastewater may be less efficient than cultivation of laboratory-adapted strains which have been selected for rapid growth. For example, Ward et al. (1984) found that only one virion of rotavirus in 46,000 in stool resulted in observable growth in cell culture. Adaptation of the virus by two passages in cell culture resulted in a decrease in that ratio to 1:6600. In addition, one virus may mask the presence of other viruses in cell culture because of different growth rates or other factors (Calgua et al., 2002; Carducci et al., 2002). The method selected for assay can also affect the results i.e. suspended cell culture methods usually give a greater number of isolates versus the commonly used monolayer method (Slade et al., 1984). Given the variety of factors influencing viruses known to grow in cell culture the efficiency may range from 0.01% to perhaps 50% (Ikner et al., 2012).

To overcome the limitations encountered with cell culture methods, intercalating dyes such as propidium monoazide (PMA) in conjunction with real time PCR (RT-qPCR or qPCR for RNA or DNA viruses, respectively (PMA-RT-qPCR/qPCR) have been used to determine the potential infectivity of enteric RNA and DNA viruses in water and other environmental matrices (Parshionikar et al., 2010; Karim et al., 2015; Leifels et al., 2015; Fongaro et al., 2016). However, current methods are still limited in this assessment (Rodriguez et al., 2009). Success of such methods depends on knowledge of the mechanism of inactivation of a particular virus and the site of action of a particular disinfectant (Rodriguez et al., 2009; Coudray-Meunier et al., 2013; Gall et al., 2015; Prevost et al., 2016). In addition, complicating this approach is that some viruses such as adenoviruses rendered non-infectious by ultraviolet light can use host cell enzymes to repair DNA damages on their

**Table 1**
Variability and uncertainty introduced in molecular methods.

| Cause of variability | Factors introducing variability |
|---|---|
| Sample matrix | Amount of inhibitors |
| | Target DNA/RNA amount |
| | Non-target ("background") DNA/RNA amount |
| | DNA/RNA Purity |
| Sample processing | Virus concentration method |
| | Multiple nucleic acid extraction methods/kits |
| | Wide range in equivalent sample volume examined (representative sample size) |
| Molecular detection | cDNA synthesis process |
| | Individuals carrying out the experiment (human variability) |
| | Numbers of replicates |
| | qPCR assay design (or optimization) |
| | Primer/probe sequence & cycling conditions |
| | qPCR instrument |
| | Enzymes, plate/tube, seal/cap |
| | Between-run variation in qPCR experiment |
| | Process controls |
| | Standard DNA/RNA of "known" copy numbers |
| | Internal amplification controls |
| | Equivalent sample volume |
| Data analysis | qPCR data analysis software |
| | Statistical analysis |
| | Limit of detection and/or quantification |
| | Interpretation of process control data |
| | Automatic threshold setting by instrument |

genome (Day et al., 1975). Inactivated viruses can still cause infection in cells through multiplicity reactivation (McClain and Spendlove, 1966). This occurs when two viruses with their nucleic acids damaged in different regions of their genomes infect the same host cell resulting in a complete genome capable of replication. Multiplicity reactivation (MR) involves the infection of a cell by the cooperative effort of two or more UV and Gamma irradiated-damaged viruses, none of which is completely functional alone (Luria, 1947; Sharp and Dunlap, 1966; Barry, 1961). Compliment reactivation was first described by Luria to account for infection of *Escherichia coli* by two or more bacteriophages exposed to ultraviolet light, which resulted in increased titer compared to infection by a single phage. MR has been demonstrated in cells of an organized host, including vaccinia virus, influenza virus, poliovirus, adenovirus and reovirus (Yamamoto and Shimojo, 1971; McClain and Spendlove, 1966). The different reovirus types (T1L, T2J, T3D) have been demonstrated to undergo compliment reactivation among each other after exposure to UV light (McClain and Spendlove, 1966). MR was also observed in hydrogen-peroxide damages to the DNA of phage T4, thus MR may occur with chemical disinfectants as well as irradiation (Chen and Berstein, 1987).

### 2.2. Molecular methods for virus detection

Methods based on qPCR have become the standard for the detection and quantification of viruses in water especially difficult-to-culture ones such as human noroviruses (Girones et al., 2010; Rodríguez-Lázaro et al., 2012). Nevertheless, it is also true that the results obtained from qPCR are somewhat considered limited due to uncertainties introduced by a variety of environmental factors and method variability (Wu et al., 2013). Data on concentration of viruses in water based on molecular detection methods are influenced by a number of factors. Variability and uncertainty in virus concentration efficiency and qPCR quantification needs to be taken into account when estimating virus occurrence and concentration in aquatic environments (Girones et al., 2010; Rajal et al., 2007).

There are several major causes of variability in qPCR quantification of viral genomes and they are summarized in Table 1. Virus detection results for a specific sample can vary according to the following factors: (1) Sample matrix: environmental samples contain variable amounts of inhibitory substances (e.g., humic and fulvic acids, heavy metals), target DNA/RNA, and non-target DNA/RNA, which largely depends on the type of sample matrix (sewage, surface water, groundwater, etc.); (2) Sample processing: a variety of virus concentration methods (size exclusion, adsorption-elution, flocculation, or combination of methods) have been used (Ikner et al., 2012; Pang et al., 2012; Gentry-Shields et al., 2013; La Rosa and Muscillo, 2013; Hata et al., 2015; Gibson and Borchardt, 2016), which introduces substantial variability in virus recovery efficiencies and amount of inhibitory substances in the virus concentrate. Additionally, some virus concentration methods require the use of elution buffers such as beef extract that exhibit inhibitory effects on downstream molecular detection (Rock et al., 2010). Nucleic acid extraction is considered a critical step in accurate detection and quantification of viral genomes by qPCR (Burgener et al., 2003; Iker et al., 2013). Different nucleic acid extraction methods/kits result in various amounts and purity of the extracted nucleic acid (Burgener et al., 2003; Iker et al., 2013; Sidhu et al., 2013); (3) Molecular detection: this can also be referred as measurement error or variability due to PCR inhibition. The factors introducing variability include number of replicates, qPCR assay design, primer/probe design, thermal cycling conditions, and PCR amplification efficiency. Different qPCR assays may not be equally susceptible to inhibitory effects by substances co-extracted with viral nucleic acids. In addition, each investigator has a choice of distinct qPCR instruments based on slightly different technologies as well as a wide range of commercial enzymes that are refined by unique reaction buffer components depending on manufacturer. Viral RNA detection requires cDNA synthesis process prior to qPCR, which introduces additional factors affecting variability in an RT-qPCR assay (e.g., one-/two-step RT-qPCR, RT primers, enzyme, temperature profile). The investigator can choose and mix the components mentioned above, but overall details of their performance remain dependent on the instrument/reagent manufacturer. Nonetheless, it is also true that noise introduced into the assay can be inherent in any qPCR experiment. In order to address these issues, and problems with the resulting body of literature a set of

"guidelines" have been published that describe the minimum information necessary for evaluating qPCR experiments (Bustin et al., 2009).

### 2.3. Methods for virus concentration from water/wastewater

The detection of viruses usually requires their concentration from large volumes of water/wastewater. In the case of untreated wastewater, only a liter is often needed, but when assessing water/wastewater treatment processes volumes of 10 to 1000 L are often processed to reach appropriate equivalent sample volumes. Again, the type of virus and its physicochemical properties play a role in the efficiency of the concentration process. Manipulation involved in the process, such as changes in pH, addition of adsorbents and eluents have differing effects on different viruses (Ikner et al., 2012; Pang et al., 2012; Gibson and Borchardt, 2016). Most methods were originally developed for the concentration of enteroviruses with the vaccine strains of poliovirus more commonly used as a model (Gerba and Goyal, 1982; Berg, 1987). More recently, the efficiency of other groups of viruses have also been assessed (Haramoto et al., 2009; Dong et al., 2010; Ikner et al., 2012; Cashdollar et al., 2012; Cashdollar and Wymer, 2013). Water quality properties such as turbidity, pH, dissolved organic matter, inorganic compounds, harness, presence of divalent cations and other physicochemical properties such as salinity can affect the recovery of viruses (Rajal et al., 2007; Lukasik et al., 2000; Gibson and Borchardt, 2016; Alum et al., 2014).

With the advent of molecular methods, there has been an increase in the use of non-target animal viruses added to samples during processing to measure the efficiency of detection for every sample (Rajal et al., 2007). Such an approach to assess the efficiency of concentration for samples in which infectivity is to be determined by cell culture assays would be useful, but currently is seldom conducted.

### 2.4. Virus aggregation

Virus aggregation plays a role in both the survival of viruses in the environment and resistance to disinfectants (Gerba and Betancourt, 2017). The number of aggregated viruses may be as much as 90% of the total virus population (Narang and Codd, 1981), depending upon how they are formed. Viral aggregates form within living cells during infection, but may also form due to changes in water quality, the presence of particulates or induced by residual disinfectants. Aggregates may range from simple pairs to up to thousands of virions. Aggregates may be permanently stable or disaggregate upon changes in water quality (i.e. lower or increase in ionic strength) (Gerba and Betancourt, 2017). Thus, the potential exists that disaggregation may significantly increase the number of infectious virions in solution after a treatment process that effects pH or the types and concentrations of inorganic salts in solution.

In addition, aggregated viruses in cell culture are often only counted as one infectious virus i.e. results in only one countable plaque in a cell monolayer (Galasso et al., 1964). However, they may represent thousands of potentially infectious viruses and have a greater probability of infection when ingested and may affect probability of infection (Gerba and Betancourt, 2017).

### 2.5. Disinfection

In addition to the type of disinfectant and its concentration (dose), numerous factors intrinsic to the nature and behavior of viruses affect the efficacy of disinfection processes (Gall et al., 2015). Disinfectants may have different sites of action on the target virus (capsid vs. nucleic acid). Specific sites of the proteins in the capsid or nucleic acid may also be involved (Wigginton and Kohn, 2012) in the disinfection efficiency. In addition, various doses of the same disinfectant may have different modes of action against viruses, as in the case of chlorine on poliovirus (O'Brien and Newman, 1979). The resistance of viruses to a disinfectant may also vary among the same type of virus. Payment et al. (1985) reported isolation of Coxsackievirus from chlorinated drinking water that had Ct values more than 10 times greater than laboratory strains of the same virus type. Zhong et al. (2017) has shown that changes in the protein responsible for inactivation of echovirus 11 can result in increased resistance to chlorine dioxide.

Much of our knowledge on concentration (dose) of disinfectants to inactivate viruses is based on laboratory studies under highly controlled conditions, with monodispersed viruses often suspended in demand-free containers and solutions (Sobsey, 1989; Gerba et al., 2003). This is very much unlike full-scale treatment plants where aggregates and particulate associated viruses may be present (Hejkal et al., 1981). In addition, the presence of soluble organic matter and other chemicals may exert a demand on the disinfectant (Gerba, 2015). There is also uncertainty on how well laboratory data reflects removal by a full-scale treatment process. Laboratory studies indicate that reoviruses are the most sensitive viruses to chlorine disinfection (Liu et al., 1971), yet they are among the most common viruses detected in chlorinated wastewater (Betancourt and Gerba, 2016). Questions remain on what information is needed to extrapolate laboratory studies to plant operations with respect to virus inactivation by disinfection processes.

### 2.6. Physical removal by filtration processes

Membranes such as microfiltration or ultrafiltration and reverse osmosis are often used in advanced treatment processes to further reduce particulates, including microorganisms and chemical constituents. Studies have shown that while effective, neither filtration method are absolute barriers to all viruses. Although the size of the majority of pores (i.e. nominal pore size) is much smaller than most viruses, there can still remain pathways by which viruses can pass through these membranes including o-ring breakages, membrane perforations, etc. California reuse criteria currently do not give credit for virus removal for ultrafiltration and allow 1–2 log removal credit for reverse osmosis (NRC, 2012). Virus removal appears not to be affected as much by size of the virus as by its hydrophobicity and electrostatic surface properties including those of the membrane. For example, MS2 bacteriophage is removed to a greater degree than phiX-174 by reverse osmosis (Wu et al., 2016). This may be due the greater hydrophobicity of MS2 over phiX-174. MS2 is the most hydrophobic non-lipid virus known (Shields and Farrah, 2002). Jin et al. (2000) used this phenomenon to explain the difference in the greater removal of MS2 by unsaturated soil columns, with much greater retention of the MS2 at the air interface than phiX-174. Virus retention by reverse osmosis membranes may vary significantly based on the type of membrane and virus type (Wu et al., 2016).

## 3. Reducing uncertainty

Since, regulations for the reduction of viruses are required in California to ensure the safety of recycled water destined for potable reuse is important that the uncertainty in any calculations involving log reductions be taken into consideration to ensure these objectives are met (Title 22 and 17 California Code of Regulations. 2015). Goals for virus reduction or inactivation depends upon the concentration of the virus in the wastewater and the effectiveness of each treatment process (Ito et al., 2016). Table 2 attempts to quantify the range of uncertainty that may be

**Table 2**
Factors introducing uncertainty in estimating the concentration and removal/inactivation of viruses by treatment processes.

| Factor | Uncertainty | Remarks | References |
|---|---|---|---|
| Method for recovery from matrix | varies from 0.09 to 100% | Efficiency depends on virus type and the physical/chemical quality of the matrix (water or wastewater); volume of water processed | Ikner et al., 2012; Rock et al., 2010. |
| Method for assay of infectious virus, i.e., cell culture | 0.01–50% efficient | Dependent on cell lines and assay method; number of passages of sample in cell culture; Type and strain of virus; indigenous vs. laboratory strain; subjective nature of assessment | Gerba and Goyal, 1982 |
| Aggregation of the virus | 0 to near 100% can be in an aggregated form | Aggregates are more resistant to disinfectants; degree of aggregation depends on the type of virus and water quality conditions | Betancourt and Gerba, 2016 |
| Disinfection | 10-fold or greater | Efficacy varies greatly depending on the type and stain of virus and physical state (aggregates, association with particulate matter). Laboratory data may not reflect resistance of indigenous viruses due to matrix effects. | Pepper et al., 2015 |
| Physical removal by membrane processes | 0.1 log to 6.0 removal | Size, shape, hydrophobicity of the virus and membrane may impact removal | Pepper et al., 2015 |
| Concentration of the virus in the raw wastewater | The concentration of different types of virus may vary 1000-fold or more | Viruses vary greatly depending on the incidence of infection within a community, time of year (seasonality) | Gerba et al., 2013 |

**Table 3**
Approaches to reduce uncertainty in virus removal by treatment processes.

| |
|---|
| Use of seeded internal controls for each sample to determine efficiency of concentration |
| Use of seeded internal controls to determine efficiency of molecular methods (both concentration and detection) |
| Development of data on infectivity in cell culture versus genome detection by molecular methods |
| Evaluation of when peak concentrations (both daily and annual) of enteric viruses occur – what is the range of peak values for the different enteric viruses |
| Assessment of the occurrence of aggregated viruses in water and impact on disinfection, survival, and detection |
| Field scale studies to assess removal by physical processes (reverse osmosis) and disinfection of naturally occurring viruses |
| Indicators/surrogates of full-scale treatment plant performance for virus removal, for individual processes (i.e. ultrafiltration) or entire treatment system using the virus most resistant to removal by the process |

associated with some of the important factors in assessing virus reduction by treatment processes. As can be seen, the range in estimates may vary by orders of magnitude. However, approaches are available to reduce this uncertainty (Table 3).

While unable to be completely resolved, it is suggested that uncertainty can be reduced by using positive controls to assess the efficiency of concentration and assay methods for every sample that is processed. Such controls, internal amplification controls (IAC) have been developed for molecular methods such as qPCR that take into consideration both concentration method and detection by qPCR (Rajal et al., 2007; Hata et al., 2011; Kitajima et al., 2014). While more challenging for cell culture methods, attempts should be better made by combining both molecular and cell culture methods such as ICC-PCR (Reynolds et al., 1996) to better understand the impact of the matrix on the viral target as well as the method performance itself and the relationship between both culture and molecular based methods. Information on the concentration(s) and variability of emerging enteric pathogens in relation to known viral pathogens in untreated raw wastewater is needed to ensure that levels of viruses in the treated water meet an acceptable risk of infection for its' intended purpose.

Additionally, more comprehensive data are needed on the resistance of naturally occurring viruses to treatment processes in full-scale or pilot plant settings. This is especially needed for disinfection processes since laboratory data, with limited number of virus types in demand free buffers, may not always reflect the resistance of naturally occurring virus types or their physical state (e.g. aggregation). Consideration should also be given to developing indicators (or surrogates) for virus removal/inactivation for the various processes in an advanced treatment train based on the most difficult to remove based on size, hydrophobicity, isoelectric point or most resistant virus to the specific treatment process. For example, adenoviruses are the most resistant known viruses to ultraviolet light, and could be considered an indicator of performance for this process (Eischeid et al., 2011; Gerba et al., 2002). While reovirus may be considered for disinfection processes due to its resistance to chlorine (Betancourt and Gerba, 2016). Still yet, other viruses could be considered for assessing the performance of ultrafiltration, reverse osmosis and advanced oxidation processes based on their retention or resistance to these processes.

## 4. Conclusions

In conclusion, enhanced efforts are needed to better quantify and account for uncertainties with the removal/inactivation of viruses by treatment processes designed for producing purified water. This is essential to better ensure public confidence that risk targets are met and human health is protected. Until we fully address these uncertainties related to viral detection/removal, it is prudent to uses RT-qPCR viral data in microbial risk assessments as documented for norovirus (Van Abel et al., 2017). Dose-response models that account for virus aggregation and non-aggregation are also recommended.

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