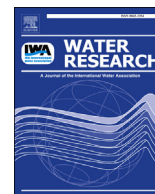




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Review

How much reduction of virus is needed for recycled water: A continuous changing need for assessment?

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ABSTRACT

To ensure the safety of wastewater reuse for irrigation of food crops and drinking water pathogenic viruses must be reduced to levels that pose no significant risk. To achieve this goal minimum reduction of viruses by treatment trains have been suggested. For use of edible crops a 6-log reduction and for production of potable drinking water a 12-log reduction has been suggested. These reductions were based on assuming infective virus concentrations of 10^5 to 10^6 per liter. Recent application of molecular methods suggests that some pathogenic viruses may be occurring in concentrations of 10^7 to 10^9 per liter. Factors influencing these levels include the development of molecular methods for virus detection, emergence of newly recognized viruses, decrease in per capita water use due to conservation measures, and outbreaks. Since neither cell culture nor molecular methods can assess all the potentially infectious virus in wastewater conservative estimates should be used to assess the virus load in untreated wastewater. This review indicates that an additional 2- to 3-log reduction of viruses above current recommendations may be needed to ensure the safety of recycled water. Information is needed on peak loading of viruses. In addition, more virus groups need to be quantified using better methods of virus quantification, including more accurate methods for measuring viral infectivity in order to better quantify risks from viruses in recycled water.

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Contents

1. Introduction	00
2. Factors which influence the concentration of viruses in wastewater	00
3. Estimate of infective virus levels in wastewater	00
4. Concentration of viruses in wastewater as determined by cell culture	00
5. Concentration of viruses determined by qPCR	00
6. Impact of time of and type of sampling i.e. composite vs. grab	00
7. Impact of outbreaks	00
8. Emerging viruses	00
9. Discussion	00
10. Conclusions	00
Acknowledgements	00
References	00

1. Introduction

Reuse of wastewater is practiced for augmenting water supplies that are subsequently used for both irrigation and potable purposes

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(NRC, 2012). Since domestic wastewater always contains pathogens capable of infecting humans they must be reduced to levels that do not pose a threat to populations that may be exposed. Various guidelines have been suggested for the needed reductions of pathogens by the treatment process to ensure minimal risk to the exposed population. In the case of recycled water used for irrigation a 6- to 7-log reduction of viruses by the treatment process has been suggested (WHO, 2006; Sano et al., 2016). In the Groundwater Replenishment Reuse Project of the state of California a 12-log reduction of virus is required when treated wastewater is used for groundwater recharge intended for indirect potable reuse (Title 22 and 17 California Code of Regulations State Board, 2015). The Texas Commission on Environmental Quality (Texas, 2015) has established a minimum baseline target for virus reduction of 8 logs although this is subject to collection of additional data (Sano et al., 2016). These reductions are designed to produce recycled water that results in a yearly risk of infection of 1:10,000 or less to persons who may ingest the irrigated food or water. These reductions were based on observations that infectious virus concentrations in untreated domestic wastewater were no greater than 5- to 6-log per liter. The data used for these assumptions were based on studies conducted before 1996 when virus quantification was based on animal cell culture assays (Asano et al., 2007). In the last 25 years major advances in molecular methods have greatly expanded our knowledge on both the quantity and types of viruses present in domestic wastewater and we believe a reassessment of the types and numbers of viruses present in wastewater and the factors which may influence this in the future is needed.

2. Factors which influence the concentration of viruses in wastewater

Many factors may influence the concentration and types of viruses in wastewater (Table 1). The incidence of infection within the population is the major factor. Basically, all viruses which infect humans are likely to end up in domestic wastewater. Incidence includes persons with both symptomatic and asymptomatic infections. Persons with symptomatic infections may excrete viruses for many weeks after a clinically observable infection. Other viruses may largely cause asymptomatic infections and be excreted throughout the lifetime of the individual (e.g. polyomavirus). Greater concentrations are usually released from infected individuals during clinical infections and then decrease over time. As shown in Table 2, the concentration of enteric viruses during diarrhea may be as high as 10^{10} – 10^{12} viral particles per gram of feces (Haas et al., 2014). Thus, an infected person with even a normal bowel movement of 100–300 g per day may excrete 10^{12} to 10^{14} per day (Feachem et al., 1983). Children tend to excrete greater numbers of viruses during an infection (Ayukekbong et al., 2011). New types and strains of viruses have evolved over time and the levels of these viruses will increase when they enter a new population that has not been previously exposed (La Rosa et al., 2012). Viral infections can also vary greatly seasonally. For example, norovirus infections in North America peak in the winter months

Table 1
Factors which influence the concentration of viruses in wastewater.

Incidence of infection within a community
Social economic status of the community
Season
Per capita water use
Time of day
Age distribution within the community
Chronic infections

Table 2
Concentration of enteric viruses in feces.

Enteric viruses	Per gram of feces ^a
Enteroviruses	10^3 – 10^7
Rotavirus	10^{10}
Adenovirus	10^{11}
Norovirus	10^{11}

^a Enteroviruses are based on infectivity assay in cell culture and data for the other viruses are from electron micrographs.
Adapted from Haas et al., 2014.

resulting in greater concentrations in the wastewater during this time of year (Kitajima et al., 2014). Other viruses appear to remain at high levels throughout the year i.e. adenoviruses, Aichi viruses (Kitajima et al., 2014). The socioeconomic status of the community is also a factor because of supposed greater spread of a virus through a population with poor hygiene.

Water use per capita will result in greater concentrations of pathogens in the wastewater. Usually domestic water usage is greatest in the morning and the evening and this will also influence the concentration of virus that enters a wastewater treatment facility (Almeida et al., 1999). Water use in the United States has been decreasing significantly since the requirement for use of low-flush toilets and of more efficient washing machines which use less water. Usage per single family homes has decreased 22% from 1999 to 2016 (DeOreo et al., 2016). Water use for cloth washing machines has decreased in the United States by 46% and toilet flushing by 37%. These two home devices have had the biggest impact on the decrease in per capita water use. Since both of these devices will carry the largest pathogen load to sewers the virus concentration can be expected to continue to increase as these new devices come into widespread use. In addition, since these devices are used more commonly during certain times of the day or days (i.e. morning or evening) this can also be expected to influence maximum concentrations (Butler, 1993). Finally, the increasing use of cold water laundry washes can be expected to increase the number of infectious viruses to sewers as water temperature is a major factor in inactivating viruses in laundry (Gerba and Kennedy, 2007). Decreasing use of chlorine in washer loads would also be expected to have the same effect.

3. Estimate of infective virus levels in wastewater

Estimates on the concentration of viruses in wastewater before the development of molecular methods were based on detection of growth of the viruses in cell culture (Fig. 1). These are infectivity assays and indicate that the virus is capable of reproducing in susceptible hosts. Infectivity is measured by the production of plaques (areas of cell destruction by the virus) in cell monolayers (plaque forming units or PFU) or a dilution assay in which cell destruction is observed and is referred to as tissue culture infectious dose which infects 50% of the inoculated cultures (TCID₅₀). It is important to recognize that these methods are not capable of detecting all the viruses in wastewater and always underestimate the true number of infectious viruses present (Table 3). Many factors influence the ability to detect viruses in cell culture. Different cell lines will give a wide range of values for the same type of virus. Most cell lines used for virus detection in wastewater have only been capable of detecting enteroviruses, reoviruses or adenoviruses. Primary kidney monkey or human primary cell culture are more sensitive for virus detection and have generally yielded greater numbers in wastewater than continuous cell lines (immortal cell lines) (Table 4). Primary cell lines are no longer used for virus testing of environmental samples and probably explain

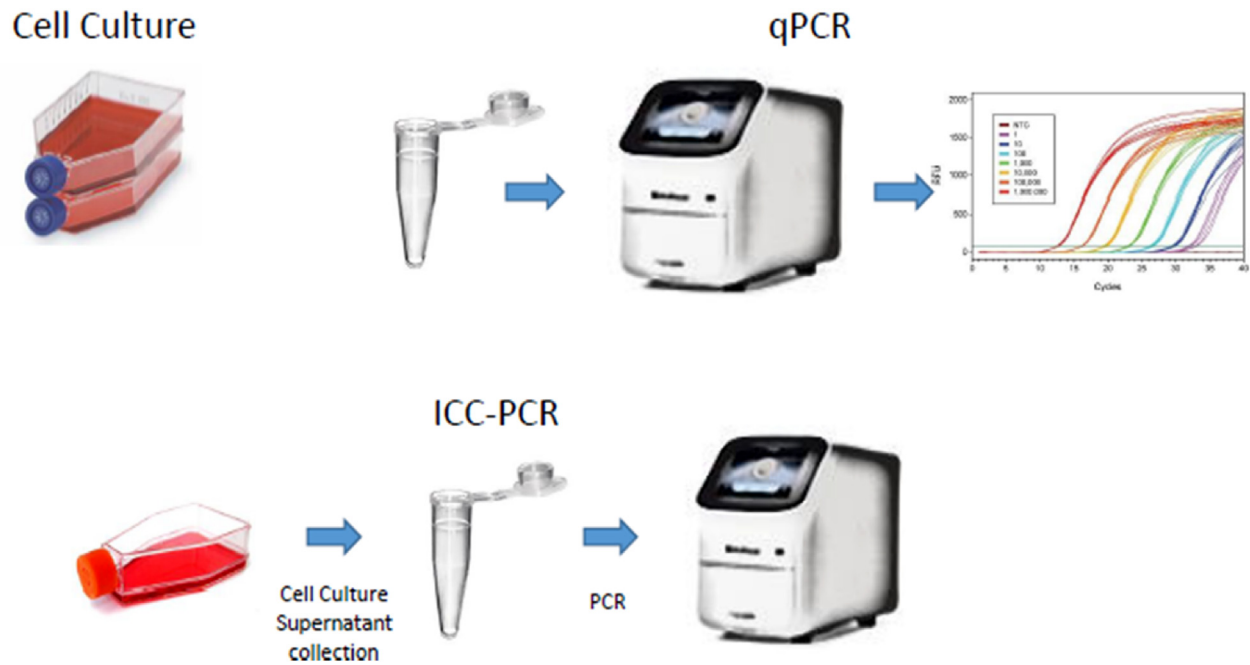


Fig. 1. Methods of virus quantification and detection in wastewater.

Table 3

Factors influencing infectivity assays for viruses.

Primary vs. continuous cell lines	Generally primary cell lines are more sensitive
Specific cell line	Numbers of viruses detected and types of viruses are dependent on the specific cell line; how many passages of the cell line have been performed
Assay conditions	Rocking cells, addition of certain enzymes and polyelectrolytes (e.g., trypsin), PFU vs TCID ₅₀ ; monolayer vs. suspended cells.
Ratio of viral particles to infectivity	Much lower for viruses directly isolated from human feces vs. laboratory grown viruses
Number of passages of the sample in cell culture	Passage of negative samples on first passage onto a second passage results in greater numbers of virus detected in a sample

PFU = plaque forming units; TCID₅₀ = Tissue culture infectious dose.

why lower numbers of virus have been reported since their use was discontinued. Also, cell culture is fairly insensitive in detecting all potentially infectious viruses, especially by those excreted in the feces. Generally enteric viruses grown in the laboratory will produce one plaque of cytopathogenic effect for every 10 to 100 observed under an electron microscope (Mahalanabis et al., 2010). In the case of rotaviruses from a human stool this ratio was

observed to be 1:46,000 (Ward et al., 1984). The difference is that laboratory-grown viruses have been selected for those that grow well in cell culture. Ward et al. (1984) observed that by passing human rotavirus from a stool specimen and this ratio of cell culture infective to infective virus decreased from 1:46,000 to 1: 6000. The assay method requires the virus to come in contact with the cell monolayer and the receptor on the cell must be exposed for virus attachment. In addition, virus present in a stool may be in the form of aggregates, which will only produce one plaque or one TCID₅₀ in cell culture, yet may represent many infectious virions (Wallis and Melnick, 1967; Kahler et al., 2016; Galasso et al., 1964; Langlet et al., 2007). It has also been found that the number of infective viruses observed in cell culture is related to how long samples are exposed to the cells, the presence of enzymes, polyelectrolytes, incubation temperatures, inoculum volume, and passage number of the cell line (Spendlove and Schaffer, 1965; Benton and Ward, 1982; Benton and Hurst, 1986; Dahling, 1991). The ratio of viral particles to infective virions in cell culture for reovirus has been found to range from 1 infective virion per every 2 to 2.5 viral particle (Wallis et al., 1964; McClain and Spendlove, 1966). Blackmer et al. (2000) found that poliovirus 1 could be detected by integrated cell culture PCR for up to 6 min after chlorine exposure, but only 1 min by observation for cytopathogenic effects if only one passage was performed. This indicates that without additional passage of viruses in cell culture infective virus levels would be underestimated.

Table 4

Concentrations of viruses in untreated wastewater as determined by cell culture assay (greatest values known).

Maximum Concentration of virus per liter	Method of assay	Cell line	Location	Reference
276,000	PFU; 80–100% efficiency	PMK; human amnion	San Diego, California, United States of America	England et al., 1964
210,000	TCID ₅₀	PMK	Johannesburg, South Africa	Malherbe and Strickland-Cholmley, 1967
1,106,000	PFU; direct inoculation	BGM	Haifa, Israel	Buras, 1976
95,000	TCID ₅₀	PMK	Worcester and Pietermaritzburg, South Africa	Grabow and Nupen, 1972
210,000	IF; reovirus only	Mouse L929	Dugway, UT, United States of America	Adams et al., 1982
463,500	TCID ₅₀	PMK	Windhoek, Namibia	Nupen et al., 1974

IF = infectious foci; PMK = primary monkey kidney; BGM = Buffalo green monkey.

Table 5
Concentration of viruses detected in untreated wastewater by qPCR.

Genome copies per liter	Virus	Location	Remarks	Reference
51,000,000	Norovirus GI, GII	Tucson, Arizona, United States of America	Sample collected the same day; composite	Schmitz et al., 2016
15,500,000	Adenovirus		sample; 24% efficiency	
1,100,800	Norovirus GI, GII	Tucson, AZ, USA	105.3% efficiency	Kitajima et al., 2014
5,191,200	Adenovirus		73.8% efficiency	
31,000,000	Rotavirus	Yungas region, Bolivia	>10% efficiency	Symonds et al., 2014.
158,000	Norovirus GI	Kyoto, Shiga, Saitama, Osaka, Tokyo, Ibaraki,	19% efficiency	Katayama et al., 2008
316,227	Adenovirus	Japan		
5,700,000,000	Norovirus GII	Central Italy	35% efficiency	La Rosa et al., 2010
1,600,000,000	Norovirus GI			
9,800,000,000	Adenovirus			
1,258,925,412	Adenovirus	Traverse City, Michigan, USA	1 MDS method for conc. 30–50% efficiency	Simmons et al., 2011
398,107,170	Norovirus GII			
1,000,000,000	Norovirus GI	Northwestern France	Composite; efficiency >10%	Da Silva et al., 2007
60,000,000	Norovirus GII			
1,000,000,000	Norovirus GI	Northwest France	Grab; >10% efficiency	Da Silva et al., 2008
39,810,717	Norovirus GII			
2,200,000	Adenovirus	Japan	Not given	Hata et al., 2012
510,000	Sapovirus			
416,686,938	Adenovirus	Several treatment plants across New	Beef extract flocculation –eff. Not given	Hewitt et al., 2011
4,677,351	Enterovirus	Zealand		
63,095,734	Adenovirus	Edmonton, Canada	30 to 50% efficiency	Qiu et al., 2015
19,952,623	Reovirus			
10,000,000	Norovirus			
10,000,000	Sapovirus			
12,589,254	Norovirus GII	New Orleans, Louisiana, USA	Composite; eff. Not provided;	Montazeri et al., 2015
12,589,254	Norovirus GI		ultracentrifugation	

Few studies have ever looked at cell culture assays and quantitative polymerase chain reaction (qPCR) on the same samples in untreated wastewater. Using several cell lines Francy et al. (2011) found a ratio of enterovirus detected by qPCR vs. infectivity in cell culture of 10.7:1 in untreated wastewater. Using only one cell line each for the detection of enteroviruses and adenoviruses with a concentration method with less than a 10% efficiency for infectious virus, Hewitt et al. (2011) found a maximum ratio of virus detected by qPCR to infectivity that ranged from 25:1 to 794:1 in wastewater. Since untreated wastewater represents recently excreted feces it can be expected that most of the viruses detected by qPCR are infectious. In a study of norovirus outbreaks related to consumption of contaminated shellfish it was found that one norovirus detected by qPCR in shellfish had an average probability of infection of 29–40% in persons consuming the oysters (Thebault et al., 2013). These oysters were contaminated by waste discharges into the ocean, requiring the virus to be transported via water currents to the shellfish and then harvested and brought to market. This indicates that even one norovirus detected by qPCR in an environmental sample has a significant probability of causing infection.

Thus, estimating ratios of infectious virus to genome copies detected by qPCR will probably never be known with certainty in the foreseeable future.

In summary determination of virus infectivity by cell culture can underestimate virus infectivity levels in wastewater by at least 2 to 3 orders of magnitude.

4. Concentration of viruses in wastewater as determined by cell culture

The greatest levels observed for viruses in wastewater detected by cell culture are shown in Table 4. Most of the viruses detected were either enteroviruses or reoviruses. The greatest value reported was by Buras (1976), who inoculated the wastewater directly into cell culture, without first attempting to concentrate the virus. Most of the other studies first concentrated the virus from a few 100 mL to a few liters. A review by Irving (1982) of the concentration of viruses in wastewater indicated that most studies have found a range of a

few hundred viruses to 10,000 viruses per liter by cell culture assays. However, we feel that for a risk assessment for water recycling the greatest value should be considered rather than an average because of uncertainty in the estimates can be significant.

5. Concentration of viruses determined by qPCR

The advantage of qPCR is that it is capable of detecting viruses which will not grow in cell lines and is more efficient in detection of the virus (Fig. 1). Viral nucleic acids degrade rapidly (within a few minutes) in wastewater and detection is likely limited to intact virions (Limsawat and Ohgaki, 1997). In addition, the concentration of naked virus RNA is much less efficient by membrane filters, which are often employed for primary concentration of viruses (Haramoto et al., 2007). However, both infectious and non-infectious viruses can be detected by qPCR. While different techniques have been developed for differentiating infectious vs. non-infectious virions, these techniques tend to be dependent upon the method of inactivation (protein capsids vs. nucleic acid) and are virus-type specific (Rodriguez et al., 2009).

Table 5 is a selection of studies showing the greatest levels of viruses detected by PCR. Only virus groups/virus with the greatest levels reported for a virus are shown. Studies usually involved a selection of enteric viruses or only one group of viruses (e.g. noroviruses). Not all studies used the same methods for the concentration of viruses, nucleic extraction, or primers and hydrolysis probes. Still, overall adenoviruses appear to occur in the greatest concentrations in most studies in which they were included. The same could be said for noroviruses. In most studies enteroviruses occur at levels 100 to 1000-fold less. Few studies have included reoviruses, but at least one study reported significant numbers (Table 5). Several studies have reported finding peak levels of adenoviruses and noroviruses at or above 10^9 /liter. Peak levels of virus groups/types at 10^8 /liter were reported by several studies. If one considers that the methods for concentrating the virus are less than 100% then values of 10^{10} /liter can occur (La Rosa et al., 2010). It should be pointed out these are only for groups of viruses or individual virus types. If all the enteric viruses which could be

assayed in an individual sample are included, then levels of virus would be greater. Thus, these values should be considered conservative estimates of the virus load in untreated wastewater. The median values for adenoviruses, noroviruses and Aichi viruses are in the 10^6 genome copies per liter range.

6. Impact of time of and type of sampling i.e. composite vs. grab

Another factor to consider is sample collection. Most studies have involved grab samples, likely often collected at the convenience of the laboratory performing the analysis (Tables 4 and 5). The volume of wastewater received by a treatment plant varies throughout the day depending on when bathing, toilet usage and laundry washing takes place (Asano et al., 2007). These activities can influence the peak load of viruses into the sewer system. Using composite samples collected over a 24-h period is designed to catch the peak flows, but only represents an average concentration.

7. Impact of outbreaks

The level of a given virus in wastewater is dependent upon the incidence of infection within the community (Sinclair et al., 2009). Seasonal peaks of noroviruses and enteroviruses are clearly seen in studies in temperate climates indicating differences in the number of infected individuals (Sinclair et al., 2009). Introduction of a new virus type or one without a significant amount of herd immunity could result in dramatic spikes or peaks of the virus in community sewage.

8. Emerging viruses

Within the last two decades' new viruses have been identified in fecal specimens and in sewage using conventional and highly-sensitive genome sequencing technologies (Ng et al., 2015; Kapoor et al., 2008, 2009, 2010). Novel viruses may also occur/appear as a result of virus mutations and genetic recombinations among virus types of the same or different species within the same genus (i.e., inter- and intra-typic recombination events), which play an important role on the evolution and epidemiology (e.g., spread, emergence and disappearance) of these viruses (Robinson et al., 2013; Tapparel et al., 2013). Frequent recombinations and mutations in enteroviruses have been recognized as the main mechanisms for the observed high rate of evolution, thus enabling them to rapidly respond and adapt to new environmental challenges (Kyriakopoulou et al., 2015). Table 6 lists new viruses recently identified in sewage, feces or urine.

9. Discussion

Because of difficulties in assessing the levels of human pathogenic viruses in water, wastewater treatment technology has been relied upon to prevent waterborne transmission. This approach depends upon accurate knowledge of the concentration of infectious virus in the water to be treated. In the United States, it is assumed that a 4-log reduction of viruses is needed for drinking water treatment plants which obtain their untreated water from surface water sources (Regli et al., 1991). This is based on the assumption of a likely level of virus in surface waters in the United States to result in a risk of infection of less than 1:10,000 per year. This same approach has been suggested for treatment designed for wastewater intended for potable reuse. Each treatment process in the treatment train is given a value or credit for a log removal of virus (Sano et al., 2016). A 12-log removal requirement has been suggested and used in California for this purpose (Title 22 and 17

Table 6

New viruses and new virus types excreted in feces or urine identified since 1996.

Virus	Reference
EV73	Oberste et al., 2001
EV 76, 89, 90, 91	Oberste et al., 2005
EV 74-75	Oberste et al., 2004
EV 77-78	Norder et al., 2003; Bailly et al., 2004
EV 79–88, 97, and 100-101	Oberste et al., 2007
EV 105 and EV 116	Grard et al., 2010, Lukashev et al., 2012
HAdV-G52	Jones et al., 2007
HAdV-D53	Engelmann et al., 2006
HAdV-D54	Ishiko et al., 2008
Human Astrovirus AstV-MLB1	Finkbeiner et al., 2008
Human Polyomavirus-9 (HPyV9)	Scuda et al., 2011
MW Polyomavirus (MWPyV) HPyV10	Siebrasse et al., 2012; Buck et al., 2012
Merkel cell polyomavirus (MCPyV)	Feng et al., 2008
Severe acute respiratory syndrome-related coronavirus SARS coronavirus (SARS-CoV)	Peiris et al., 2003; Drosten et al., 2003; Ksiazek et al., 2003
Small circular, Rep-encoding, ssDNA (CRESS-DNA) genomes (CRESS-DNA viruses) characterized in fecal or environmental samples	Ng et al., 2015
Human cyclovirus 1, 2 and 3	Li et al., 2010; Biagini et al., 2012
Human feces associated circovirus (HufacV)	
Human Bocavirus (HBoV) [HBoV-1, HBoV-2, HBoV-3, HBoV-4]	Allander et al., 2005; Arthur et al., 2009; Kapoor et al., 2009; Kapoor et al., 2010
Human Cosavirus (HCosV-A)	Kapoor et al., 2008
Human Salivirus	Greninger et al., 2009; Li et al., 2010
Human Cardiovirus [Saffold cardiovirus]	Jones et al., 2007
Human picobirnavirus D strain CDC23 (HuPBV-D-CDC23) and Human picobirnavirus E strain CDC16 (HuPBV-E-CDC16)	Ng et al., 2014

California Code of Regulations State Board, 2015). This level of removal was based upon an assumption of the presence of 10^5 to 10^6 infectious viruses per liter. This level of virus was also based upon levels detected in untreated wastewater determined with cell culture. The cell lines used would largely only detect enteroviruses and, if additional effort were made, reoviruses. In addition, the methods have not always been optimized to detect all of the potentially infectious viruses. Generally, enteroviruses and reoviruses have been detected in untreated wastewater by cell culture at levels from 10^3 to 10^4 per liter, although 10^5 and 10^6 have been documented (Table 5). Given the known limitation in cell culture detection for enteric viruses greater levels of infectious virus are likely present. The ratio of virion to infectious virus in cells has been reported as low as 1 to 1.2 depending on assay and pretreatment conditions (McClain and Spendlove, 1966).

10. Conclusions

Determining the numbers of viruses in untreated wastewater will be a moving target for the near future as many factors interact

to influence our assessment. Changing water-use patterns in households, emergence of new viral pathogens, better technology for the concentration of viruses and detection by PCR will influence our knowledge on the presence of viruses in wastewater. Our assessment at present indicates that the 12-log removal goal required when treated wastewater is used for groundwater recharge should probably be reassessed given the significant increase of our knowledge on viruses present in untreated wastewater. This review indicates that an additional 2- to 3-log reduction of viruses above current recommendations may be needed to ensure the safety of recycled water.

To better understand the significance of enteric virus levels in wastewater we recommend:

- the concentration efficiency of every sample be documented by use of a model virus
- collection of samples at peak flows into the wastewater treatment facility
- use of methods which could assess infectivity by qPCR or other methods
- peak values of viruses should be considered rather than average values of virus if untreated wastewater is to be used when determining viral removal requirements
- the ratio of infective virus to virions (as detected by qPCR) should be considered to be less than 1:10 unless proven otherwise.

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