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

Charles P. Gerba a, Walter Q. Betancourt a,*, Masaaki Kitajima b

a The Water, Energy and Sustainable Technology Center, Department of Soil, Water and Environmental Science, University of Arizona, Tucson, AZ, United States
b Division of Environmental Engineering, Faculty of Engineering, Hokkaido University, Sapporo, Hokkaido, Japan

Article history:
Received 20 August 2016
Received in revised form 2 November 2016
Accepted 3 November 2016
Available online 5 November 2016

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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1. Introduction

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

* Corresponding author.
E-mail addresses: gerba@ag.arizona.edu (C.P. Gerba), wbetancourt@email.arizona.edu (W.Q. Betancourt), mkitajima@eng.hokudai.ac.jp (M. Kitajima).
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 Replenishments 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 $10^{12}$ 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 $10^{13}$ per day (Peachem et al., 1983). Children tend to excrete greater numbers of viruses during an infection (Ayukekong 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 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

| 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 |

| Enteric viruses | Per gram of feces |
|-----------------|------------------|
| Enteroviruses   | $10^7$–$10^9$    |
| Rotavirus       | $10^{10}$        |
| Adenovirus      | $10^{11}$        |
| Norovirus       | $10^{11}$        |

* Enteroxviruses are based on infectivity assay in cell culture and data for the other viruses are from electron micrographs.

Adapted from Haas et al., 2014.
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 infectivity much lower for viruses directly isolated from human feces vs. laboratory grown viruses.

### 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 TCID50; 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.

### 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                            | PFU; 80–100% efficiency | PMK; human amnion | San Diego, California, United States of America | England et al., 1964 |
| 210,000 TCID50                         | TCID50          | PMK       | Johannesburg, South Africa | Malherbe and Strickland-Cholmley, 1967 |
| 1,106,000 PFU; direct inoculation      | BGM             | PMK       | Haifa, Israel | Buras, 1976 |
| 95,000 TCID50                         | PMK             | Worcester and Pietermaritzburg, South Africa | Grabow and Nupen, 1972 |
| 210,000 TCID50                         | Mouse I929      | PMK       | Dugway, UT, United States of America | Adams et al., 1982 |
| 463,500 TCID50                         | PMK             | Windhoek, Namibia | Nupen et al., 1974 |

IF — infectious foci; PMK — primary monkey kidney; BGM — Buffalo green monkey.
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 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 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-infections 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 10⁶/liter. Peak levels of virus groups/types at 10⁹/liter were reported by several studies. If one considers that the methods for concentrating the virus are less than 100% then values of 10⁹/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

| 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 sample; 24% efficiency | Schmitz et al., 2016 |
| 15,500,000              | Adenovirus  |                          |                                      |                                |
| 1,100,800               | Norovirus GI, GII | Tucson, AZ, USA         | 105.3% efficiency                     | Kitajima et al., 2014 |
| 5,191,200               | Adenovirus  |                          | 73.8% efficiency                      |                                |
| 3,000,000               | Rotavirus   |                          | >10% efficiency                       | Symonds et al., 2014, Katayama et al., 2008 |
| 158,000                 | Norovirus GI |                          | 19% efficiency                        |                                |
| 316,227                 | Adenovirus  |                          |                                      |                                |
| 5,700,000,000           | Norovirus GI | Central Italy            | 35% efficiency                        | La Rosa et al., 2010 |
| 1,600,000,000           | Norovirus GI | Northwestern France      | Composite; efficiency >10%           | Da Silva et al., 2007 |
| 9,800,000,000           | Adenovirus  | Traverse City, Michigan, USA | 1 MDS method for conc. 30–50% efficiency | Simmons et al., 2011 |
| 1,258,925,412           | Adenovirus  | Northwest France         | Grab; >10% efficiency                 | Da Silva et al., 2008 |
| 398,107,170             | Norovirus GI |                          |                                      | Hata et al., 2012 |
| 1,000,000,000           | Norovirus GI |                          |                                      | Hewitt et al., 2011 |
| 60,000,000              | Norovirus GI |                          |                                      | Qiu et al., 2015 |
| 1,000,000,000           | Norovirus GI |                          |                                      | Montazeri et al., 2015 |
| 39,810,717              | Norovirus GI |                          |                                      |                                |
| 2,200,000               | Adenovirus  | Japan                     | Not given                             |                                |
| 510,000                 | Sapovirus   |                          |                                      |                                |
| 416,686,938             | Adenovirus  | Several treatment plants across New Zealand | Beef extract flocculation – eff. Not given | Hewitt et al., 2011 |
| 4,677,351               | Enterovirus | Edmonton, Canada         | 30 to 50% efficiency                 |                                |
| 63,095,734              | Adenovirus  |                          |                                      |                                |
| 19,952,623              | Reovirus    |                          |                                      |                                |
| 10,000,000              | Norovirus   |                          |                                      |                                |
| 3,000,000               | Sapovirus   |                          |                                      |                                |
| 12,589,254              | Norovirus GI | New Orleans, Louisiana, USA | Composite; eff. Not provided; ultracentrifugation |                                |
| 510,000                 | Norovirus GI |                          |                                      |                                |
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 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 $10^5$ per liter, although $10^6$ and $10^7$ 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

Table 6

| 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 | Engelman 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 cycloivirus 1, 2 and 3 | Li et al., 2010; Biagini et al., 2012 |
| Human feces associated circovirus (HuCAV) | |
| 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 Cardioivirus [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 |
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.

Acknowledgements

We wish to thank Mr. Jeffrey R. Bliznick for providing the graphic in Fig. 1. This review was supported in part by the United States Department of Agriculture-National Institute of Food and Agriculture. Grant number 20166800725064, that established CONSERVE: A Center of Excellence at the Nexus of Sustainable Water Reuse, Water, and Health.

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