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Emerging investigators series: the source and fate of pandemic viruses in the urban water cycle

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Abstract

Several recent high profile outbreaks such as SARS, MERS, Ebola and avian influenzas draw attention to the continued risk of a deadly viral pandemic. In general, these enveloped viruses are not considered a major threat for the wastewater and water industries due to their assumed low concentrations in municipal wastewater and high susceptibilities to degradation in aqueous environments. A number of clinical reports, however, suggest that certain enveloped viruses are excreted in human feces during infection. Furthermore, survivability studies show that many enveloped viruses are capable of retaining infectivity for days to months in aqueous environments. Here, we examine the potential presence and fate of enveloped viruses in the urban water cycle, with emphasis on coronaviruses (*e.g.*, SARS and MERS) and avian influenza viruses. We identify a number of pressing research questions that must be answered before the water and wastewater industries can confidently assure the public, through the dissemination of evidence-based guidance, that irrigation waters, recreation waters, and drinking water sources are safe during a viral outbreak or pandemic event.

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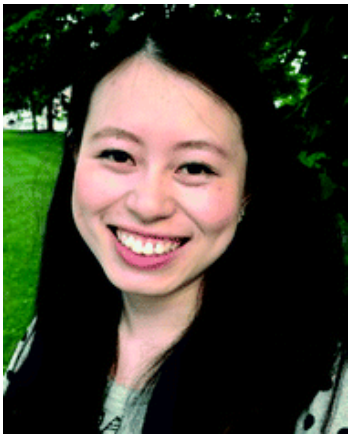

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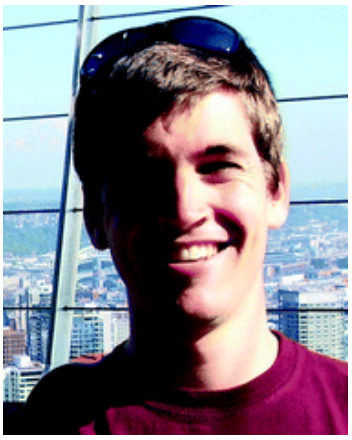

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Water impact

Enveloped viruses are generally considered unstable in the aqueous environment and have therefore not been emphasized in waterborne virus research, methods, or regulations. Certain enveloped viruses, however, do survive for long periods of time in wastewaters and surface waters. Avian influenza viruses and some coronaviruses, for example, are relatively stable in water and also occasionally cross over to humans to cause outbreaks of severe disease. This review examines the potential presence and fate of enveloped viruses in municipal wastewater and drinking water. We conclude that future outbreaks or pandemics could involve enveloped viruses that are transmitted *via* the urban water cycle.

Introduction

High-profile viral outbreaks in recent years have heightened concerns of a deadly virus pandemic. These include the severe acute respiratory syndrome coronavirus (SARS-CoV) in 2003, the Middle Eastern respiratory syndrome coronavirus (MERS-CoV) since 2012, the highly pathogenic avian influenzas H5N1 and H797 in 2003 and 2013, respectively, and the recent Ebola outbreak in 2014 (Table 1). The public fear is understandable, as viral pandemics can be devastating; the 1918 influenza pandemic, for example, killed an estimated 50 million people in two years, and one-third of the world contracted the illness.[1] Although the number of cases and deaths caused by SARS, MERS, avian

influenzas H5N1 and H7N9, and Ebola are orders of magnitudes lower than those caused by the human pandemic influenzas (Table 1), scientists and public health officials carefully monitor for signs that these viruses re-emerge or become more easily transmitted from person to person.

Table 1 Examples of viral diseases that rapidly emerged in humans, likely from an animal source. Viral diseases are listed chronologically with respect to the initial outbreak date

Outbreak/pandemic	Years	Most likely animal source	Deaths	Approximate case f
"Spanish" pandemic influenza H1N1	1918–1920	Unresolved	>40 million	2–3%
Ebola virus (EBOV)	1976–present	Unresolved	10 353[a]	50%
Avian influenza H5N1	1997–present	Birds	398[b]	60%
SARS-CoV	2002–2003	Bats	774	10%
Pandemic influenza H1N1 2009	2009–2010	Unresolved	>284 500[c]	Up to 0.03%
MERS-CoV	2012–present	Unresolved	456[d]	40%
Avian influenza H7N9	2013–present	Poultry	177[e]	40%

[a] Data for 2014–15 outbreak only, as of March 24, 2015. [b] As of December 4, 2014. [c] In first 12 months of circulat

December 10, 2014.

Infectious diseases caused by viruses emerge or re-emerge every year, although most do not lead to public health threats as significant as familiar viral pathogens like measles or human seasonal influenza.[2] Of the estimated 87 novel pathogens that were first reported in humans over a twenty-five year period (1980–2005), two-thirds were due to viruses.[2] RNA viruses, such as SARS and influenza H5N1, made up 85% of the emerging human viruses, primarily due to the high mutation rate of single-strand RNA viruses. Emerging or re-emerging human viruses tend to have animal reservoirs—a phenomenon called zoonosis—which may be exacerbated by increased global travel, deforestation, factory farming, animal markets, and bush meat hunting.[3] Climate change may facilitate the spread of some viruses into new geographical regions in the coming years.[4]

Should a major virus pandemic occur, wastewater and drinking water treatment industries would be under increased scrutiny for serving as a potential means of transmission (Fig. 1). Utilities need to respond rapidly and make decisions that minimize occupational and public health risks based on the cumulative available evidence. Treated wastewater is a common source of recreation, irrigation, and drinking waters, and although wastewater treatment does reduce virus levels, infective human viruses have frequently been detected in wastewater treatment plant effluent.[12–14]

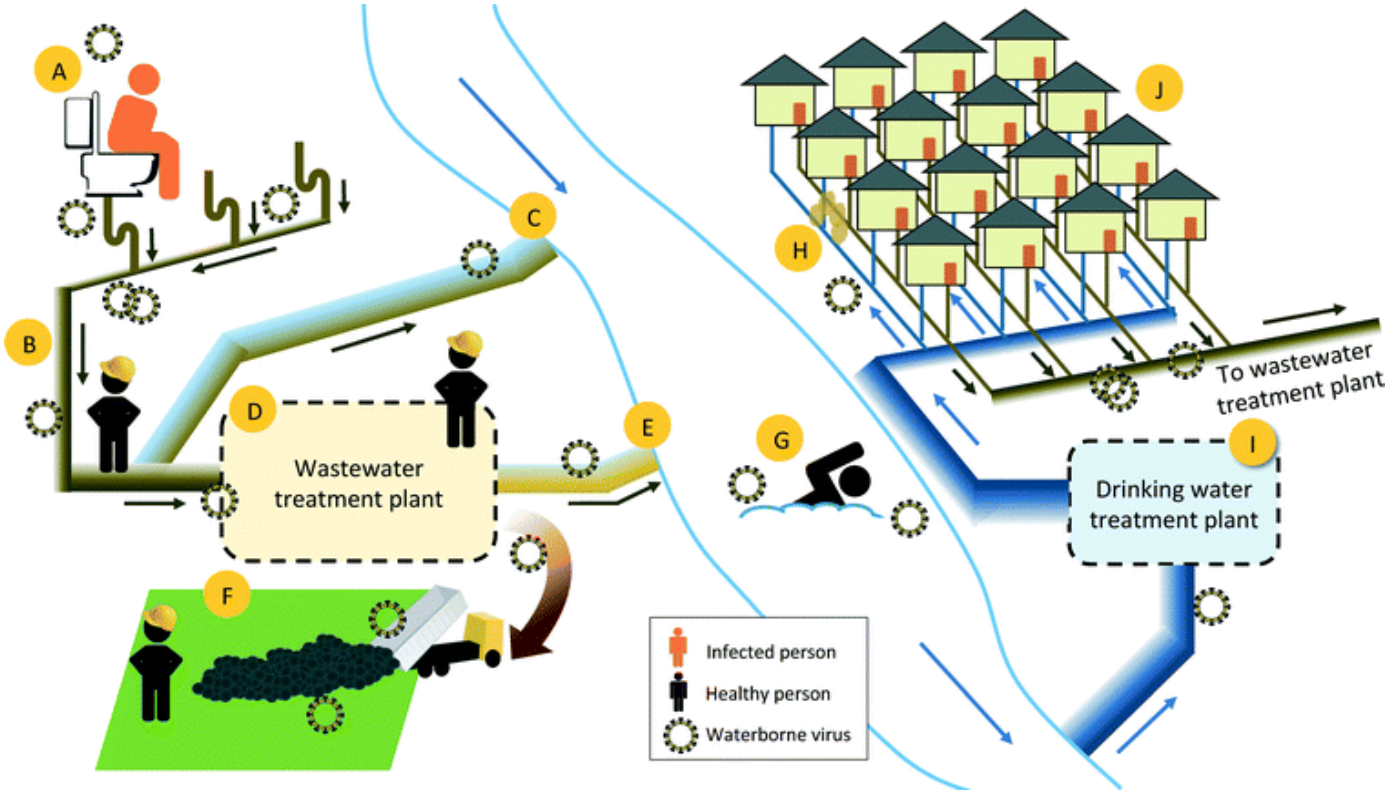

Fig. 1 The fate of infective viruses in the urban water cycle and locations of potential human exposure. A) Viruses that are excreted in feces, urine, and vomit enter the sewage system. Toilet flushing or problems with indoor plumbing systems may form virus-laden aerosols that could result in human exposure. B) Viruses are transported through the municipal sewage system to the wastewater treatment plant (WWTP). Workers servicing sewage systems could be exposed to infective viruses. C) Combined sewage overflow events lead to the release of infective viruses in untreated sewage to surface waters. D) Viruses that enter the municipal WWTP are exposed to physical, biological, and chemical treatment processes. WWTP employees may be exposed to infective viruses present in the untreated and treated wastewater, as well as residual biosolids. E) Wastewater effluent can carry viruses that have survived treatment to surface waters. F) Residual biosolids from WWTP are disposed, often *via* land-application. Workers or others in close contact with the biosolids may be exposed to infective viruses that have survived the solids treatment processes. G) Recreational activities can lead to exposure to infective viruses present in surface waters. H) Leaky sewage pipes can lead to contamination in the underground drinking water distribution systems. I) Intake water at drinking water treatment plants can contain infective viruses. The water is treated with a range of physical and chemical treatment processes to remove contaminants, including viruses. J) Municipal drinking water consumers are exposed to viruses that either maintain infectivity through drinking water treatment and distribution or enter the distribution system through leaks in underground pipes.

If the novel human viruses were shed in the feces, urine, or vomit of infected individuals, it would enter the municipal wastewater system and be transported to the municipal wastewater treatment plants (WWTPs). The 2014 Ebola cases in the U.S. highlighted the lack of data on the presence and fate of emerging viruses in human waste and

sewage.[15] In this case, government agencies communicated that the Ebola virus was rapidly inactivated outside of the human host and thus municipal wastewater and finished wastewater treatment products did not carry significant occupational and public health risks. At the time, however, there were very few survivability studies of Ebola virus outside of the human host and none in municipal wastewater. Studies on the fate of Ebola virus and Ebola virus surrogates in the environment have since been initiated and results are beginning to appear in the literature.[16,17] At this time, the assumptions that were made about the rapid inactivation of Ebola virus outside of the human host have not been validated.

The vast majority of research on the presence and fate of viruses in the urban water cycle has focused on a relatively small set of enteric viruses (*i.e.*, viruses that replicate in the gastrointestinal tract and are readily transmitted *via* the fecal-oral route; Table 2). Enteric virus particles consist of an RNA or DNA genome that is protected by a protein shell (*i.e.*, capsid). Enteric viruses are considered resistant to heat, acids, and oxidants, and thus survive for long periods of time in the environment. Enveloped viruses, such as influenza viruses, coronaviruses, and Ebola virus, have an additional outer envelope that consists of lipids and proteins. Enveloped viruses are generally not associated with fecal routes of transmission in humans and are considered more susceptible to inactivation in aqueous environments. Recently, however, viral metagenomes of sewage have revealed a large diversity of human viruses including some enveloped viruses.[18,19] Although the presence of genes in wastewater does not equate with the presence of infective viruses, the recent wastewater metagenomics studies do prompt a closer look at the preparedness of water industries and government agencies for a pandemic or outbreak event.

Table 2 Human viruses of interest to water and wastewater industries

Virus common name	Associated illnesses	Taxonomy
Norovirus	Gastroenteritis	Family *Caliciviridae* Gen
Sapovirus	Gastroenteritis	Family *Caliciviridae* Gen
Adenoviruses	Respiratory disease, pneumonias, gastroenteritis, keratoconjunctivitis	Family *Adenoviridae*
Astrovirus		Family *Astroviridae*
Rotavirus	Gastroenteritis	Family *Reoviridae*
Hepatitis A virus	Hepatitis	Family *Picornaviridae* Ge
Enteroviruses	Respiratory illness, meningitis, flaccid paralysis, *etc.*	Family *Picornaviridae* Ge
Torque Teno virus	Unclear	Family *Anelloviridae*

Virus common name	Associated illnesses	Taxonomy
Hepatitis E virus	Hepatitis	Family: *Hepeviridae* Gen
Aichi virus	Gastroenteritis	Family: *Picornaviridae* G
Klassevirus/Salivirus	Unclear	Family *Picornaviridae*
Polyomavirus	Unclear	Family *Polyomaviridae*
Ebolavirus	Ebola	Family *Filoviridae*
SARS coronavirus	Severe Acute Respiratory Syndrome	Family *Coronaviridae* Ge
MERS coronavirus	Middle Eastern Respiratory Syndrome	Family *Coronaviridae* Ge
Avian influenza	Influenza	Family *Orthomyxovirida*

Here, we examine the literature on emerging viruses in wastewater with the intention of informing and preparing wastewater and drinking water treatment industries for future virus pandemics. We apply a definition of pandemic based on the World Health Organization guidelines for identifying an influenza pandemic, or the emergence of a new virus that causes serious illness in humans and experiences easy and sustained human-to-human transmission.[20] This is in contrast to an outbreak event, wherein the incidence of a disease rises above levels considered normal for a specific area. Two types of enveloped viruses are particularly important for wastewater and drinking water communities to consider—coronaviruses and avian influenza viruses—due to their confirmed presence in feces and their survival characteristics in aqueous environments. Coronaviruses include the viruses responsible for the recent SARS and MERS global outbreaks. Avian influenza viruses usually cause illness in birds, but occasionally cross over to humans and result in serious illnesses. Although typically considered respiratory viruses, certain coronaviruses (*e.g.*, SARS) and avian influenza viruses can be transmitted *via* fecal matter in water; they have also been responsible for recent outbreaks with particularly high mortality rates (Table 1). As mentioned above, these enveloped viruses are structurally dissimilar to the enteric viruses that have historically been the focus of waterborne virus studies and are thus believed to behave differently in aqueous environments.

Potential pandemic viruses in wastewater

Human viruses do not replicate in the environment; therefore, for a virus to be transferred *via* the urban water cycle, it must be introduced to water through human bodily fluids and then retain its infectivity until another person comes into contact with the water (Fig. 1). Studies on the presence and fate of viruses in aqueous environments are often limited to enteric viruses, as are regulations, such as those specified by the USEPA Surface Water Treatment Rule and Groundwater Treatment Rule.[21] Wastewater can contain a number of human viruses outside of the common enteric viruses; untreated wastewater sludge and Class B biosolids, for example, contained genes from coronaviruses in more that 80% of the samples, and rubella virus at lower frequencies.[19]

Human viruses enter municipal wastewater when they are shed in the feces and urine of infected individuals. A number of human enteric viruses have been quantified in municipal wastewater by qPCR and culture methods, with reported concentrations as high as 10^9 genome copies per liter (Table 2). For those viruses that have not been studied in wastewater, existing information on their presence in human fecal and urine samples may shed light on their potential importance in the water cycle. Feces and urine samples are rarely collected for illnesses perceived as non-enteric, but genes of the respiratory viruses RSV, human rhinovirus, coronaviruses, and seasonal influenza have been detected in stool samples.[22–25] In many cases, the presence of respiratory virus genes in feces is thought to stem from a patient swallowing virus-laden nasal secretions. Unfortunately, the PCR techniques commonly used to detect viruses in human samples do not relay infectivity information. To be of concern in the urban water cycle, an entire infective virus particle, and not just pieces of viral genome, would need to be present in wastewater. Indeed, infective SARS-CoV and avian influenza virus particles have been detected in fecal or intestinal tract samples of infected individuals.[26–28]

Influenza viruses

Influenza viruses are enveloped viruses with segmented, single-stranded RNA genomes. They infect humans and other animals, such as birds and pigs. Influenza viruses have been responsible for four human pandemics in the last century, including the 1918 H1N1 “Spanish Flu” pandemic, the 1957–58 H2N2 “Asian Flu” pandemic, the 1968–69 H3N2 “Hong Kong Flu” pandemic, and the 2009 H1N1 “Swine Flu” pandemic. These pandemics were instigated by the emergence of novel strains to which humans lacked immunity. Novel strains emerge in humans when animal influenza viruses either mix with human viruses or cross directly to humans (*e.g.*, avian influenza viruses).

Human influenza viruses

Seasonal human influenza viruses constantly circulate in human populations around the globe causing seasonal epidemics. The viruses slowly mutate as they circulate *via* a phenomenon called antigenic drift. The illnesses associated with the seasonal influenza viruses typically involve respiratory tract symptoms and fever. Gastrointestinal symptoms are occasionally experienced, especially in children.[29] Transmission of seasonal human influenza occurs

through droplet, airborne, and contact transmission modes; however, the relative importance of these three modes of transmission remains unclear.[30] As for the potential transmission of human seasonal influenza *via* wastewater, viral RNA has been detected in human stool samples[24,31–33] and has also been detected in municipal wastewater.[34,35] Levels in feces ranged from 4.9×10^3 to 8.0×10^7 PCR copies per gram of stool. In one rare case, infective human influenza virus was cultured from human feces,[36] but this was suspected to be due to the patient swallowing virus-laden nasopharyngeal secretions, or the spillover of viruses in the blood to other organs.[32] Influenza virus receptors are not present on normal human intestinal cells, thus it is unlikely that the viruses infect and replicate in gut cells.[32] Based on the available evidence in the literature, high concentrations of infective human seasonal influenza viruses are unlikely in municipal wastewater, even during large outbreaks.

Avian influenza viruses

Birds can be infected by either low pathogenic influenza or highly pathogenic influenza. Whereas low pathogenic avian influenza viruses cause minor illness in birds and are rarely fatal, highly pathogenic avian influenza viruses spread rapidly in birds and are highly virulent. On rare occasions, the highly pathogenic avian influenza viruses have crossed directly over to humans, often when humans were in close proximity to large numbers of domesticated birds. Avian influenza virus infections in humans have had high associated fatality rates (Table 1), but recent avian influenza viruses in humans have not undergone sustained human-to-human transmission. There are serious concerns that an avian influenza will cross over to humans in the future and mutate in a way that allows the virus to spread easily from human to human. This hypothetical event could lead to a deadly avian influenza pandemic in humans like the Spanish influenza pandemic in the early 20th century.

Unlike seasonal human influenza, the highly pathogenic avian influenza strains are transmitted *via* the fecal-oral route in birds and bind with receptors on avian gut tissues (*i.e.*, gastrointestinal tract infections).[52] When the highly pathogenic H5N1 virus crossed over from birds to humans in 2004, many patients experienced severe diarrhea,[28,53] viruses were present in stool samples,[28] and the viruses infected and replicated in human gut tissues.[54] Genes of the novel avian influenza H7N9 virus that emerged in humans in 2013 were also frequently detected in stool samples.[8,55] Although not identified as an avian influenza, the H1N1 illness in humans also exhibited unusually high gastrointestinal symptoms,[36,56] infective virus particles in feces,[57] and efficient replication in intestinal cells.[58]

Taken together, the frequency of gastrointestinal symptoms, the prevalence of virus particles in the patient stool samples, and the ability of the viruses to replicate in gastrointestinal tract cells, highlights the potential importance of the urban water cycle in the control of avian influenza viruses in humans. Thus far, humans infected with avian influenza viruses, such as H5N1 and H7N9, have been limited in their ability to transmit the illness to other humans. Public health officials, however, fear that a new strain will emerge in humans that spreads easily from person to person; should this occur, humans would not have immunity to the illness and effective vaccines would take time to prepare.[59] It seems likely that under potential outbreak or pandemic scenarios, avian influenza virus particles would enter municipal wastewater, possibly at high concentrations.

Coronaviruses

SARS-CoV emerged in Hong Kong in 2003 and caused severe lower respiratory illness that exhibited high mortality rates. During one cluster of 319 cases at an apartment complex in Hong Kong,[8,60] the outbreak spread *via* aerosolized fecal particles in the air ducts of the apartment complex. Despite being associated primarily with respiratory illness, SARS-CoV RNA was often detected in the feces of infected individuals, with the detection frequencies in different cohorts ranging from 16% to 97%.[26,61] Diarrhea symptoms were experienced in 23% to 73% of patients, with higher frequency during the first week of the illness.[26,62,63] SARS-CoV RNA levels peaked in fecal samples at 9 to 14 days after the onset of the illness,[61] and in one case, fecal samples tested positive for up to 73 days following the illness onset.[26] The presence of SARS-CoV in feces may be due to its ability to replicate in the small and large intestines, as evidenced by infective viruses in lower intestine samples.[26]

In addition to SARS-CoV, there are five other known human coronaviruses. MERS-CoV emerged in Saudi Arabia in 2012, and at the time of this writing, has resulted in 1110 confirmed cases and 456 deaths.[7] MERS-CoV RNA was detected at low levels in the stool and urine of one infected individual (~10^3 gene copies per mL),[64] but absent in stool samples from two other infected individuals.[65] Likewise, human coronavirus HKU1 (HKU1-CoV) RNA has also been detected in stool samples,[66] in some cases from patients exhibiting gastrointestinal symptoms.[67] In a recent metagenomic study of wastewater, HKU1-CoV was identified in sludge samples collected from different wastewater treatment plants in the U.S., although confirmation with PCR was not conducted.[19] Unfortunately, the infectivity state of the HKU1 viruses in stool and wastewater samples cannot be assessed, as there is no tissue culture system compatible with HKU1. The remaining three human coronaviruses (HCoVNL63, HCoV-OC43, and HCoV-229E), have been detected in stool samples[25,67,68] but are not believed to play a significant role in infections of the gastrointestinal tract. Based on the available data, the human coronaviruses that are circulating at this point are unlikely to pose significant threats in the urban water cycle. That being said, it is quite plausible that a highly virulent new coronavirus like SARS-CoV will emerge in the future and pose challenges for the water and wastewater industries.

Fate of enveloped viruses through the urban water cycle

The major components of the urban water cycle include drinking water supply, wastewater disposal, and stormwater runoff. Viruses excreted in feces, urine, or vomit enter the urban water cycle in human sewage. We therefore focus most of our discussion on enveloped virus fate in the urban water cycle on wastewater and wastewater treatment. We also include a discussion on the very limited data on enveloped virus fate in natural waters and in drinking water treatment processes.

Virus concentrations in wastewater

In a pandemic scenario, virus concentrations in wastewater would depend on the number of people infected in the community and the rate at which infected individuals shed the viruses. Most data on human virus titers in wastewater

are based on enteric viruses and qPCR measurements (Table 2). Concentrations as high as 10^8–10^9 genome copies per liter (gc L^{-1}) have been reported in wastewater influent. Relatively few studies have assessed the levels of infective virus particles in wastewater, likely due to limited cell culture techniques and issues with virus extraction methods. Enteroviruses, adenoviruses, and rotaviruses have been monitored in wastewater influent, and reported concentrations are generally less than 10^4 plaque forming units (PFU) or infective units (IU) per liter.[13,46,69] Viruses released in feces tend to be in aggregated states and virus extraction methods have notoriously low recovery rates;[51,70] therefore, the reported PFU L^{-1} values may greatly underestimate the number of infective viral particles in wastewater.

When data on virus loads in wastewater are absent, virus loads in human stool or urine samples may help predict levels in wastewater during an outbreak event. As expected, viruses associated with enteric disease have higher viral loads in human stool samples than those associated with respiratory disease. Norovirus concentrations in human fecal samples can be greater than 10^{10} gc g^{-1},[71,72] compared to concentrations as high as 10^9 gc L^{-1} in sewage during outbreaks.[39] Human polyomaviruses (JCPyV and BKPyV) in human urine reach concentrations higher than 10^{10} gc L^{-1} and are present in wastewater at levels up to 10^8 gc L^{-1}.[44] Influenza viral loads as high as 8.0×10^7 gc g^{-1} stool[33,57] and SARS virus loads of 10^7 gc mL^{-1} in diarrhea[73,74] and 2.5×10^4 gc mL^{-1} in urine[73] have been reported. As these are the highest reported levels, they could be used to predict worst-case scenarios for wastewater concentrations.

Survival in municipal wastewater

To be of concern in the urban water cycle, a virus that is excreted in feces or urine must retain its infectivity in sewage until it comes into contact with humans (Fig. 1). Human waste that enters the sewage system is carried through a complex underground pipe system to the municipal wastewater treatment plant. Although the majority of municipal wastewater produced in urban settings in developed countries reaches the wastewater treatment plants, a portion of the wastewater leaks into the subsurface or is released untreated to surface waters during major wet weather events. The untreated wastewater released from leaky pipes to the subsurface can potentially penetrate drinking water distribution pipes and thus result in drinking water exposure. Untreated wastewater released to surface waters can lead to exposure through recreational activities and can contaminate groundwater that is under the direct influence of surface water.

The hydraulic residence times of sewage networks and wastewater treatment plants are typically less than one day, combined.[82,83] Outside of the human body, viruses exhibit a range of susceptibilities to environmental factors, with T_{90} values (*i.e.*, time until 90% of viruses inactivated) in aqueous environments ranging from minutes to years (Table 3). Data on survivability is limited to viruses that can be assayed *in vitro*; the survivability of nonculturable viruses is often predicted based on the survivability of similar viruses that can be cultured.

Table 3 Time for enveloped viruses to reach 90% inactivation (T_{90}) in aqueous environments compared to time for non-enveloped poliovirus to reach 90% inactivation

Virus	T_{90} (days)	Temp. (°C)	Matrix	Ref.
Avian influenza virus H5N1	84	20	Distilled water	75
Avian influenza virus H5N1	508	10	Distilled water	75
Avian influenza virus H5N1	19	20	Surface water	75
Avian influenza virus H5N1	61	10	Surface water	75
SARS-CoV	9	RT	Serum-free culture media	76
HCoV 229E	<1	RT	Serum-free culture media	76
HCoV 229E	2	RT	Dechlorinated tap water	77
FIPV (feline coronavirus)	<1	RT	Primary wastewater effluent	77
TGEV (swine coronavirus)	11	25	Reagent-grade water	78
TGEV	110	4	Reagent-grade water	78
TGEV	4	25	Pasteurized settled wastewater	78
TGEV	24	4	Pasteurized settled wastewater	78
MHV (murine coronavirus)	9	25	Reagent-grade water	78
MHV	>365	4	Reagent-grade water	78
MHV	3	25	Pasteurized settled wastewater	78
MHV	35	4	Pasteurized settled wastewater	78
Hantavirus	3	20	Cell culture media	79
HIV	<1	25	Primary wastewater effluent	80
Poliovirus	4	23	Primary wastewater effluent	77
Poliovirus	56	23	Mineral water	81
Poliovirus	342	4	Mineral water	81

Viruses with lipid envelopes are often assumed to readily lose infectivity in aqueous environments. Indeed, viruses such as Human Immunodeficiency Virus (HIV) do rapidly lose their infectivity in aqueous environments, with reported T_{90} values in water at room temperature in the range of 1–2 hours.[84] For comparison, the enteric non-enveloped poliovirus has a T_{90} value of 56 days in water at room temperature.[81] Interestingly, not all enveloped viruses rapidly lose their infectivity (Table 3). Avian influenza H5N1, for example, has a T_{90} value of approximately 100 days at room temperature in distilled water[75,85] and SARS-CoV has a T_{90} value of 9 days in culture media at room temperature. Inactivation rates are highly dependent on the water temperature and matrix. Higher temperatures are associated with higher inactivation rates,[86] as is increased salinity.[85] In two studies of human coronaviruses in water,[77,78] temperature was found to have the most pronounced effect of a range of variables on survivability, with up to an order of magnitude difference in decay rates between samples at 4 °C and room temperature. The water composition, such as the presence of proteins or microorganisms, also influences virus survivability. Viruses in sterilized wastewater, for example, are inactivated at different rates than viruses in non-sterilized wastewater.[87] The presence of suspended solids and organic material increased the survivability of enteric viruses in aqueous environments.[88]

Likewise, coronaviruses survived longer in unfiltered primary effluent than in filtered primary effluent.[77] The enhanced survival in the presence of sediment and organic material may be due to protection from chemical or biological inactivating agents in the water. Extraneous material can also cause faster inactivation, as was the case for coronaviruses in pasteurized settled sewage *versus* distilled water.[78] Based on the collective data, matrix effects on virus inactivation are complex and vary significantly amongst different viruses and environmental samples.

Highly related strains can have significantly different environmental stabilities (Table 3). SARS-CoV, for example, lost its infectivity in culture media at a much slower rate than human coronavirus 229E under the same conditions (Table 3).[76] Likewise similar avian influenza viruses had T_{90} values ranging from 58 to 171 days under the same experimental conditions.[85] A mechanistic understanding of this difference in susceptibility is unknown. The virus lipid membranes may play a role, as seasonal influenza virus grown in different cell types, and thus having different sources of their lipid membranes, had varied stabilities in water.[89] Based on the cumulative survivability data, it is plausible that an enveloped virus excreted in human feces or urine could survive in aqueous environments for periods of time that are relevant to the wastewater and drinking water treatment fields. Higher levels of infective viruses would be expected in a wastewater treatment plant influent when there is a large incidence rate in the community and when wastewater temperatures are cooler.

Wastewater treatment

To our knowledge, there are no reports on the fate of enveloped viruses through wastewater treatment trains. The extent of non-enveloped human virus removal and inactivation depends on the type of virus and on the treatment processes employed at a particular WWTP. Enteroviruses, adenoviruses, and reoviruses are poorly removed from primary wastewater treatment (*i.e.*, settling), with less than 1-log reduction reported.[90,91] Enteroviruses and adenoviruses are more effectively removed in secondary treatment, with 1–4 log reduction reported,[90–92] but reovirus reduction was less than 1 log.[91] The mechanism of removal in activated sludge has been attributed to adsorption to solids that settle in secondary clarifiers, followed by inactivation of the adsorbed viruses.[90,93] The final disinfection step is critical for reducing the number of infective viruses in effluent,[94] but even disinfected effluent can contain infective viruses.[91,95] A survey of ten plants of various size and treatment processes found that wastewater treatment lead to between 0- and 2-log reduction of infective enteroviruses and 2- to >3-log reduction of infective adenoviruses, although effluent samples were collected prior to disinfection.[51] A different survey of five WWTPs found that the log reduction from the influent to the disinfected effluent ranged from 1.9 to 5.0 and infective viruses were detected in the effluent of every plant.[42]

In solids treatment, digestion has varying effects on non-enveloped viruses, with thermophilic aerobic digestion being much more effective than mesophilic digestion.[96] Infectious enteroviruses and adenoviruses were frequently detected in Class B biosolids treated with mesophilic digestion.[42,97] Concentrations of enteroviruses and adenoviruses in the mesophilic digestion biosolids were in the range of 10^1–10^3 and 10^2–10^4 most probable number

(MPN) per gram, respectively. Treating biosolids with heat pelletization and composting processes resulted in significantly lower indicator virus levels in final products compared to mesophilic and thermophillic digestion processes.[98] Once applied to land, viruses adsorbed to the biosolids can leach out.[99] There is also evidence that viruses in biosolids are aerosolized during land-application, leading to a significant risk of airborne exposure.[100] It should again be noted that all of these studies on virus fate in wastewater treatment have focused on non-enveloped viruses.

Survival in natural waters

Infective viruses present in WWTP effluent or in combined sewage overflow are usually released into surface waters. In surface waters, viruses are exposed to a number of potentially inactivating stresses, including sunlight, oxidative chemicals, predation by microorganisms, *etc.*

Research on enveloped viruses in natural waters has focused primarily on avian influenza viruses, due to the likelihood that natural waters serve as environmental reservoirs for influenza viruses. Avian influenza virus survival times in surface waters samples collected from Lake Constance were in the range of 10–100 days at temperatures above freezing (Table 3).[75] In this case, the samples were not exposed to sunlight. Avian influenza viruses survived several months to years in frozen natural water samples.[75,101] The viruses are most stable between pH 7.4 and 8.2, with rapid inactivation below pH 6.5 and above pH 10.[102] There remains a lack of published data on avian influenza inactivation by sunlight.[102]

Drinking water treatment

Studies exploring the fate of enveloped viruses in drinking water treatment demonstrate the need for additional experiments. The coagulation-flocculation-settling process was not effective or consistent in removing influenza H5N1.[31] Ultrafiltration was effective at removing influenza H5N1, and bacteriophage MS2 was a conservative surrogate for this process.[31] In general, enveloped viruses are more susceptible to common drinking water disinfectants than non-enveloped viruses. Influenza H5N1 was readily inactivated with UV_{254} treatment, with greater than 5-log inactivation observed after a dose of 25 mJ cm^{-2}; for comparison, bacteriophage MS2, a surrogate for enteric viruses, underwent less than 2-log inactivation at the same dose.[31] Low pathogenicity influenza H5N2, a surrogate for high pathogenicity influenza H5N1, was also very susceptible to UV.[34] SARS-CoV, on the other hand, was persistent in experiments with UV disinfection.[103] Chlorination readily inactivated influenza in aqueous samples.[31,34] With monochloramine disinfection, influenzas H1N1 and H5N1 required monochloramine CT values (*i.e.*, products of disinfectant dose and contact time) of less than 60 mg L^{-1} min^{-1} for 4-log inactivation at ambient temperature;[31] EPA recommends a monochloramine CT value higher than 700 mg L^{-1} min^{-1} for 4-log virus inactivation at 20 °C. More experiments are needed on enveloped viruses in water quality engineering processes before major conclusions can be drawn on their fate in wastewater and drinking water treatment.

Detecting enveloped viruses in water

Research on enveloped virus presence and fate in water is hindered by the lack of proven detection methods. Detecting and quantifying viruses in environmental samples requires first concentrating the viruses in the sample into a smaller volume to improve detection limits. Most available methods were designed and optimized for non-enveloped enteric viruses, and despite efforts, there remains no consensus concentration method.[104] The published enteric virus concentration methods focus on combinations of filtration,[14,70] ultracentrifugation,[70] and PEG precipitation,[105] with a range of pH values, chemicals added, filter types, and centrifuge speeds. Envelopes render the viruses more sensitive to organic solvents, temperature, and pH;[76] therefore many extraction and purification methods used for non-enveloped enteric viruses are not optimal for enveloped viruses. Protocol steps with chloroform or cesium chloride solutions, for example, destroy the lipid outer layer and should therefore be avoided.[106] A mere 1.02% recovery of SARS coronaviruses was achieved from hospital sewage with an electropositive filtration and aluminum hydroxide precipitation method.[107] In comparison, non-enveloped viruses have been recovered from wastewater at rates as high as 80%,[108] although recovery rates are often much lower. In one of the few attempts to optimize enveloped virus recovery from environmental samples, Deboosere *et al.* achieved an 8% recovery for influenza A viruses in lake water using glass wool filtration and polyethylene glycol (PEG) precipitation.[109] Even when purification steps are included, virus concentrates often contain material that interferes with the detection methods, but these can be minimized by dilution or the addition of quenching agents.[110–112] Future successful environmental monitoring efforts will depend on the development and wide acceptance of effective extraction and purification techniques.

Viruses present in sample concentrates are typically identified or quantified by genomic- and culture-based methods. PCR methods detect virus genes and are presently used most often for detecting specific viruses in environmental and clinical samples. The prevalence of PCR methods is due to the fact that they tend to be much faster than culture methods, they provide species and strain-specific identification, and they detect viruses that are not presently culturable *in vitro*. Drawbacks of PCR methods are that they do not relay virus infectivity and they require the development of appropriate primers; consequently, viruses may go undetected if primers are improperly designed or if the virus has mutated in the targeted genome region. Metagenomic methods, which aim to sequence all of the viruses present in a sample, are increasingly employed for virus detection and discovery, and do not require *a priori* knowledge of the viruses present. Both RNA and DNA viruses have been sequenced in fecal and environmental samples.[113] RNA viruses are sequenced by first reverse transcribing the RNA with random primers, and then sequencing the cDNA. At this time, high-throughput sequencing is not appropriate for routine virus measurements, due to high sample costs, large data sets, and the lack of absolute quantification. Additionally, the detection limits for a specific virus using metagenomic analyses is typically poorer than PCR due to the high abundance of genetic material in samples.[114] Following sequencing, PCR is often used to confirm the presence of the

detected viruses of interest.[113,114] It should be noted that the methods used to concentrate the viruses and separate them from other organisms can influence the viral metagemonic dataset.[115]

Virus culture methods, such as plaque assays or cytopathic effect assays, continue to play a critical role in environmental virology due to their ability to characterize the infectivity state of the virus—this is not possible with PCR or sequencing techniques. For example, when the fate of human viruses through environmental or engineering processes is of interest, or when the risk a sample poses to human health needs to be assessed, culture methods remain the only reliable detection approach. Unfortunately, a number of important human viruses cannot be cultured *in vitro*, such as MERS-CoV. For those viruses, molecular detection methods remain the best and only option.

PCR-based and culture-based methods are both employed to detect influenza viruses in human or environmental samples. The matrix (M) gene is often targeted in PCR to detect all influenza subtypes simultaneously, whereas the hemaglutinin (HA), neuraminidase (NA), and other genes are targeted to detect specific influenza subtypes.[116,117] Potential novel strains are detected by a positive influenza A assay result followed by negative subtype-specific assay results; this approach led to the identification of the 2009 H1N1 pandemic influenza.[118] Influenza A viruses are readily cultured and enumerated in embryonated chicken eggs and a number of cell lines, with the Madin-Darby canine kidney (MDCK) cell line preferred.[119] Viruses cultured in MDCK cells can be enumerated *via* plaque assay or 50% tissue culture infectious dose ($TCID_{50}$) assays.

For PCR detection of coronaviruses, primers that target conserved regions in the polymerase gene can detect most, if not all coronaviruses, with a limit of detection on the order of 5×10^1–5×10^3 gene copies.[120–122] Degenerate primers are also used to detect a range of coronaviruses.[68] Specific coronavirus primers have targeted the S glycoprotein or nucleocapsid protein genes.[68,123,124] Due to their varied cell tropisms, human coronaviruses cannot be cultured in the same type of cell systems. SARS-CoV and HCoV-NL63, for example, are readily culturable in common cell lines such as CaCO-2 (SARS) and LLC-MK2 (HCoV-NL63).[125,126] HCoV-229E and HCoV-OC43 are also culturable *in vitro*, but are much more fastidious, and replicate in a narrow range of hosts. The remaining two known human coronaviruses, HCoV-HKU1 and MERS-CoV, are not readily culturable in available cell lines. Consequently, their infectivity state and ultimate fate in environmental samples cannot be easily assayed.

Conclusions

At this time, major knowledge gaps exist on the potential role of the urban water cycle in the spread of enveloped viruses, particularly for avian influenza viruses and coronaviruses. In order to address this, we suggest environmental engineers, virologists, and public health researchers work together to address the following research needs:

(1) Methods need to be developed and optimized for extracting and purifying enveloped viruses from complex sample matrices such as wastewater, residual biosolids, and surface waters. During testing, samples should be spiked with a range of enveloped viruses, as well as with enteric viruses or enteric virus surrogates, in order to develop a better understanding of how extraction recoveries vary with virus physical properties.

(2) Research should address the variability of enveloped virus survivability in aqueous environments. What structural characteristics of certain avian influenza strains, for example, allow them to remain infective in water for longer periods of time than other enveloped viruses?

(3) Research should address the presence and fate of enveloped viruses during wastewater and drinking water treatment processes, as well as when they are released into the environment *via* wastewater effluent or land-applied biosolids. Whenever possible, these should focus on viruses that are culturable *in vitro* so that infective virus concentrations, as well as gene copy concentrations, are reported.

(4) More research should focus on the environmental fate of the highly pathogenic viruses, such as avian influenza strains. As demonstrated by the data in Table 3, similar viruses can exhibit very different survivability characteristics and surrogates may not accurately predict the environmental fate of a highly pathogenic virus. This type of work requires access to BSL3 and BSL4 facilities. As most environmental virologists do not have access to or training for this level of biosafety, collaborative efforts will be required.

(5) Quantitative risk assessments should be conducted for highly pathogenic enveloped viruses in wastewater, recreational waters, and drinking waters.

These research questions are critical to prepare the environmental engineering and public health communities in the event that enveloped viruses causing a deadly outbreak or pandemic enter the urban water cycle.

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