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Enhanced decay of coronaviruses in sewers with domestic wastewater

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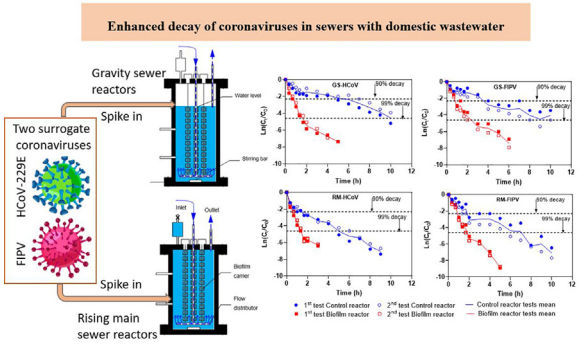
HIGHLIGHTS

- First study investigating impacts of sewer biofilms on the decay of coronaviruses in wastewater.
- Decay of infectious coronaviruses was enhanced greatly in sewers with domestic wastewater.
- 99% of HCoV-229E and FIPV decayed within 2 h in wastewater with sewer biofilms.
- In-sewer decay of coronaviruses conforms to a biphasic first-order kinetic model.

GRAPHICAL ABSTRACT



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ABSTRACT

Recent outbreaks caused by coronaviruses and their supposed potential fecal-oral transmission highlight the need for understanding the survival of infectious coronavirus in domestic sewers. To date, the survivability and decay of coronaviruses were predominately studied using small volumes of wastewater (normally 5–30 mL) in vials (in-vial tests). However, real sewers are more complicated than bulk wastewater (wastewater matrix only), in particular the presence of sewer biofilms and different operational conditions. This study investigated the decay of infectious human coronavirus 229E (HCoV-229E) and feline infectious peritonitis virus (FIPV), two typical surrogate coronaviruses, in laboratory-scale reactors mimicking the gravity (GS, gravity-driven sewers) and rising main sewers (RM, pressurized sewers) with and without sewer biofilms. The in-sewer decay of both coronaviruses was greatly enhanced in comparison to those reported in bulk wastewater through in-vial tests. 99% of HCoV-229E and FIPV decayed within 2 h under either GS or RM conditions with biofilms, in contrast to 6–10 h without biofilms. There is limited difference in the decay of HCoV and FIPV in reactors operated as RM or GS, with the T_{90} and T_{99} difference of 7–10 min and 14–20 min, respectively. The decay of both coronaviruses in sewer biofilm reactors can be simulated by biphasic first-order kinetic models, with the first-order rate constant 2–4 times higher during the first phase than the second phase. The decay of infectious HCoV and FIPV was significantly faster in the reactors with sewer biofilms than in the reactors without biofilms, suggesting an enhanced decay of these surrogate viruses due to the presence of biofilms and related processes. The mechanism of biofilms in virus adsorption and potential inactivation remains unclear and requires future investigations. The results indicate that the survivability of infectious coronaviruses detected using bulk wastewater overestimated the infectivity risk of coronavirus during wastewater transportations in sewers or the downstream treatment.

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1. Introduction

Over the last decade, outbreaks caused by different types of coronaviruses have posed serious threats to human health and the economy. The outbreak of severe acute respiratory syndrome coronavirus (SARS-CoV) occurred in 2002–2003 and Middle East respiratory syndrome coronavirus (MERS-CoV) in 2012. Both outbreaks caused thousands of infections and death. In particular, the current ongoing outbreak of COVID-19, caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has led to more than 225 million infections, 4.6 million deaths by September 2021 (Dong et al., 2020), and placed millions of people under lockdown or stay-at-home orders.

The primary transmission of coronaviruses is through the direct contact or respiratory aerosols or droplets of infected patients. However, infectious coronaviruses have been detected in the feces of infected individuals, leading to the potential of oral-fecal transmission (Goh et al., 2013). During the SARS outbreak, high concentrations of SARS viral aerosols were detected in sewers of a 50-storied building in Hong Kong, which resulted in a rapid spread of the disease (342 cases in the building) (McKinney et al., 2006). In the SARS-CoV-2 outbreak, one similar transmission that occurred in China was believed to be linked to the leakage of wastewater generated by COVID-19 patients in the vicinity of the residence property (Liu et al., 2020). Hwang et al. (2021) also reported such a case in one apartment building in Seoul, South Korea. There were a total of 15 sewer stack aerosol-related outbreaks of COVID-19 in different high-rise buildings of Hong Kong and Guangzhou, China (Wang et al., 2021). Despite the reports of wastewater-related outbreaks of COVID-19, they occurred in the most upstream of the urban sewer systems. To date, although SARS-CoV-2 RNA has been detected in wastewater samples collected from wastewater treatment plants (WWTPs), sewer manholes, and other sewer compartments (Li et al., 2021), infectious SARS-CoV-2 has not been successfully detected or isolated from wastewater (Anand et al., 2022; Rimoldi et al., 2020; USCDC, 2021; Westhaus et al., 2021; WHO, 2020). This leads to great research needs to understand the fate of infectious SARS-CoV-2 or other coronaviruses during transportation in sewer systems to help minimize the transmission risks.

Due to the safety challenges in handling highly infectious human coronaviruses, surrogate coronaviruses were used in research. Human coronavirus 229E (HCoV-229E), a respiratory virus, and feline infectious peritonitis virus (FIPV), an enteric virus, are within the *Coronaviridae* family and have been commonly used as surrogates for human coronaviruses, given their similar size, composition, and morphology (Silverman and Boehm, 2020). Gundy et al. (2008) investigated the survival of HCoV-229E and FIPV in water and wastewater using plaque assay or median tissue culture infectious dose ($TCID_{50}$) techniques, where a shorter survival time was observed in wastewater than the tap water. Similarly, another study also found a faster decay of infectious SARS-CoV-2 in wastewater than in tap water (Bivins et al., 2020). Apart from HCoV-229E and FIPV, other surrogate coronaviruses such as murine hepatitis virus (MHV), transmissible gastroenteritis virus (TGEV) and the enveloped bacteriophage Phi6 have also been used for investigating the fate of coronaviruses in wastewater. A meta-analysis summarized the decay rate of HCoV-229E, FIPV, MHV, TGEV and bacteriophage Phi6 under water and wastewater conditions, where a constant higher decay rate was observed for all of them in wastewater than in water (i.e. river water, lake water, and tap water) under 4–56 °C (Silverman and Boehm, 2020). These studies suggest that wastewater might accelerate the decay of infectious coronaviruses. However, these former studies used small volumes (5 or 30 mL) of bulk wastewater (either raw or filtered) as the testing environment. In real applications, sewers are more complicated systems than bulk wastewater alone.

Sewer systems collect and transport wastewater from households and commercial areas through piped connections, which can be further divided into rising mains and gravity flow regions (Hvitved-Jacobsen et al., 2013; Li et al., 2017). Rising main sewers that pump wastewater to higher altitudes are fully filled with wastewater and operated under anaerobic conditions. Gravity regions of sewers include gravity pipes, manholes, and pumping stations, where the wastewater flows to lower altitudes due to gravity. Gravity sewers are normally operated with partially filled wastewater, thereby combining both aerobic and anaerobic conditions (Hvitved-Jacobsen et al., 2013). With the broad range of nutrients and microorganisms, diverse types of biofilms form on the surface of sewers, which function as 'microbial reactors' (Jiang et al., 2015; Li et al., 2020). Previous studies have identified that sewer operational conditions and wastewater properties affect the transformation of different illicit drugs, pharmaceutical compounds, and health and lifestyle biomarkers in wastewater (Choi et al., 2020; Gao et al., 2018; He et al., 2021; Li et al., 2021a; O'Brien et al., 2019; Thai et al., 2014b). To date, the impact of sewer conditions and corresponding biofilms on the fate of coronavirus remains unclear.

Recent studies in membrane reactors and drinking water systems found that biofilm facilitated the virus removal (Skraber et al., 2005; Tobias and Bérubé, 2020). Furthermore, a study found a close correlation between dissolved oxygen (DO) concentration and the SARS-CoV-2 RNA concentration in wastewater, suggesting the potential impact of DO on the adsorption and decay of SARS-CoV-2 in wastewater (Petala et al., 2021). More importantly, former studies found that 10% of infectious coronaviruses could still be detected after 1–3 days in raw wastewater and 1–5 days in sterilized (autoclaved or pasteurized) wastewater under 10–25 °C (Silverman and Boehm, 2020). Infectious SARS-CoV-2 was detected in bulk wastewater for up to 7 days (Bivins et al., 2020). However, to date, no infectious coronaviruses have been isolated from wastewater collected from sewers or WWTPs (Anand et al., 2022). It is thus hypothesized that the sewer conditions, especially the presence of sewer biofilms and its operational conditions, enhanced the decay of coronavirus in wastewater.

This study investigated the survivability of two surrogate coronaviruses (i.e. HCoV-229E and FIPV) in laboratory-scale sewer reactors operated under rising main or gravity sewer conditions with different biofilms. Control reactors without sewer biofilms were also employed to determine the infectivity and decay of HCoV-229E and FIPV in wastewater alone. Batch tests were conducted using raw domestic wastewater with infectious HCoV or FIPV. Concentrations of HCoV-229E and FIPV were monitored at different time points during a period of 10 h, which is of relevance to wastewater residence time in sewers, after being spiked into the reactors. The results obtained delineate the survivability of surrogate coronaviruses in sewer conditions and will help the risk management of current or future coronavirus outbreaks.

2. Materials and methods

2.1. Surrogate coronaviruses

2.1.1. Cell lines

All the cell lines and viruses were purchased from American Type Culture Collection (ATCC®) (In Vitro Technologies, Australia). All the media ingredients and chemicals were obtained from Life Technologies, Australia. Human MRC-5 lung fibroblast cells (ATCC® CCL-171™) and Crandell-Rees Feline Kidney (CRFK) cells (ATCC® CCL-94™) were cultured in T-75 or T-175 flasks (Interpath Services, Australia) in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) at 37 °C in a humidified incubator with 5% atmospheric CO_2. In order to passage and plate cells, they were gently washed twice with phosphate buffered saline (PBS) to remove dead cells as well as the serum in culture media which might inactivate trypsin in the next step. The cells were then dissociated with 0.05% Trypsin-EDTA and pelleted via centrifugation at 300 × *g*, 5 min. The cells were resuspended in pre-warmed DMEM, supplemented with 10% FBS.

2.1.2. Virus preparation

Human coronavirus 229E (HCoV-229E) (ATCC® VR-740™), a respiratory virus, was propagated and assayed in MRC-5 cells. Feline infectious peritonitis virus (FIPV) (ATCC® VR-990™), an enteric feline coronavirus, was propagated and assayed in CRFK cells. Virus preparation process followed well-established methods suggested by ATCC as well as reported by (Montazeri Aliabadi et al., 2021; Vabret et al., 2001). Before inoculating viruses, the host cells were cultured to 80% to 90% confluency within 24 to 48 h for best infection results. HCoV-229E and FIPV viruses were then seeded and propagated in individual host cell lines with DMEM and 2% FBS at 35 °C and 37 °C respectively with 5% atmospheric CO_2 as suggested by ATCC. The cytopathic effect (CPE) shown by infected cells was monitored regularly under Leica DMi1 inverted microscope (Leica, Germany). When 90% of the cells showed cytopathic effect (CPE), the culture medium containing majority of the viruses was collected from the flasks and centrifuged at 1000 ×*g* for 5 min. The clarified supernatant (s1) and pellet (p1) were kept in separate tubes on ice. Meanwhile, the attached cells in the culture vessels were dissociated with 0.05% Trypsin-EDTA and pelleted via centrifugation at 300 ×*g* for 5 min (p2). The two pellets (p1 and p2) were resuspended and combined with 5 mL serum-free DMEM and quickly went through three freeze and thaw cycles with liquid nitrogen and 37 °C water bath to release viruses. The cell lysates were centrifuged at 300 ×*g* for 5 min, and the supernatant (s2) was combined with the clarified supernatant (s1). The stock virus (s1 and s2) was then filtered through a 0.45 μm polyethersulfone (PES) membrane (Sarstedt, Germany) and carefully transferred to high-speed centrifuge tubes with sealing caps (Thermo Fisher, Australia). Centrifugation was undertaken at 85,000 ×*g* for 4 h at 4 °C with Sorvall LYNX 6000 superspeed centrifuge (Thermo Scientific, Australia). Virus pellets were carefully resuspended with cold PBS to 5% of the original virus suspension volume. The coronaviruses were titered and aliquoted in sterile tubes at −80 °C until further use.

2.1.3. Virus titration

The virus concentration of virus stock as well as in wastewater samples were titered by the Reed–Muench method (Payment and Trudel, 1993) and indicated as median tissue culture infectious dose ($TCID_{50}$). Briefly, this technique worked by adding a serial dilution of virus sample to host cells in 96 well plates and calculated the dilution at which 50% of the wells show a CPE. Specifically, MRC5 and CRFK cells were plated into flat-bottom 96-well plates (Interpath Services, Australia) and cultured in DMEM medium supplemented with 10% FBS at a 37 °C incubator with 5% atmospheric CO_2. When the cells were grown to 80% to 90% confluency within 24 to 48 h, the culture medium was changed to fresh DMEM with 2% FBS and ready for virus inoculation and propagation. The viral stock or wastewater sample was taken out from a −80 °C freezer and quickly melted in a 37 °C water bath. Each sample was transferred to individual well in round-bottom 96-well plates (Interpath Services, Australia) and diluted in series with DMEM medium supplemented with 2% FBS. 10 μL of each diluted sample was then added to respective host cells in flat-bottom 96-well plates. Eight replicates were performed for each serial dilution. The samples aiming for HCoV-229E virus titration were inoculated to MRC5 cell plates and cultured at a 35 °C incubator with 5% atmospheric CO_2. The diluted samples aiming for FIPV virus titration were plated in CRFK cell plates and cultured at a 37 °C incubator with 5% atmospheric CO_2. Pure viral stock of HCoV-229E and FIPV was diluted in series and inoculated in respective host cells to serve as a positive control. Cells with medium only and no virus inoculation were served as a negative control. The CPE was regularly monitored under a microscope within 7 days. Numbers of wells showing CPE were then recorded and the dilution at which 50% of the wells show a CPE was calculated. The dilution was multiplied by one hundred to reveal virus titer per mL as the inoculation volume was 10 μL. The inverse log was taken for this dilution as the virus titer, which was expressed as $TCID_{50}$/mL in wastewater.

2.2. Laboratory-scale sewer reactors

Considering the sewer operational conditions, two types of laboratory-scale reactors (i.e. rising main (RM) and gravity sewer (GS) reactors) were employed in this study. These reactors have been previously demonstrated to represent the typical sewer conditions (Choi et al., 2020; Jiang et al., 2011; Li et al., 2018; Li et al., 2021a; Thai et al., 2014a; Thai et al., 2019). The RM reactor, made of Perspex™, with a volume of 0.75 L (a diameter of 80 mm and a height of 150 mm), was operated under air-tight conditions to mimic the anaerobic sewer conditions in rising main sewers. Plastic biofilm carriers of 1 cm diameter (Anox Kaldnes, Norway) were clustered on three stainless-steel rods to provide additional surfaces for biofilm growth in the reactor (Fig. S1). The total biofilm area in each reactor, including both the reactor wall and carrier surfaces, was approximately 0.05 m^2. The area to volume ratio (A/V) was therefore 70.9 m^2/m^3. GS reactors were also made of Perspex™ with a dimension of 80 mm diameter, 200 mm height. To mimic the gravity sections of sewers, GS reactors were operated with partially filled wastewater (the highest flow depth of 150 mm, working volume of 0.75 L), allowing a gas phase in each reactor (Fig. S1).

To investigate the impact of sewer biofilms and operational conditions on the fate of infectious coronaviruses, one RM reactor (RM_B) and one GS (GS_B) reactor were fed with domestic wastewater for 6 months for the biofilm cultivation, while the control reactors (RM_C and GS_C) received no wastewater feeding. The domestic wastewater was collected fortnightly from the inlet of a wastewater treatment plant (raw wastewater) in Wollongong (Australia). The wastewater (at pH around 7.2) typically contained sulfate at 10–45 mg-S/L, sulfide at <3 mg-S/L, dissolved oxygen (DO) around 0.7 mg/L, methane at <5 mg-COD/L, total suspended solids (TSS) 200–600 mg/L, volatile suspended solids (VSS) at 100–400 mg/L, total chemical oxygen demand (TCOD) at 350–600 mg/L and soluble chemical oxygen demand (COD) at 50–150 mg/L. The wastewater was stored at 4 °C and warmed through titanium heating coils in a water bath to room temperature (22 °C). It was then pumped by a peristaltic pump (Masterflex L/S) every 6 h (a typical wastewater hydraulic retention time (HRT)). Each feed pumping event lasted for 2 min, providing one reactor working volume wastewater (0.75 L) into each reactor. Magnetic stirrers (MLS8, VELP Scientifica, Italy) were used to provide continuous mixing at 250 rpm for the wastewater in the reactors (Fig. S1).

Prior to the surrogate virus survivability test, batch tests were performed to determine the biofilm activities (i.e. sulfate reduction rate and COD reduction rate) in RM_B and GS_B reactors to confirm that they reached the semi-steady states as described previously (Jiang et al., 2011; Li et al., 2018).

2.3. Survivability tests for surrogate coronaviruses in sewer reactors

Batch tests of surrogate coronavirus survivability were conducted in each of the four sewer reactors RM_B, RM_C, GS_B, and GS_C. The survivability of infectious coronaviruses in sewer reactors was measured by the loss of their infectivity over time. Prior to the batch test, fresh raw wastewater was collected from the inlet of a WWTP in Wollongong, Australia, heated up to 22 °C using a water bath, and then pumped into both control and biofilm reactors within 1 h of collection. Ten liters of wastewater were pumped into each reactor to ensure the full replenishment with fresh wastewater. Then, a reactor working volume wastewater (0.75 L) was further pumped into each reactor. Before spiking surrogate viruses, 1 mL aliquot of wastewater was taken from each reactor to ensure the absence of infectious HCoV-229E and FIPV in the background wastewater.

Surrogate coronaviruses (i.e. HCoV-229E and FIPV) as described in Section 2.1, were spiked into four sewer reactors RM_B, RM_C, GS_B, and GS_C to a final concentration of 1.5×10^5 $TCID_{50}$/mL wastewater for HCoV-229E and 1.2×10^6 $TCID_{50}$/mL wastewater for FIPV. The

reactors were stirred with magnetic stirrers at 250 rpm immediately after virus spiking and maintained during the batch test. Wastewater samples were taken at 20 min intervals for the first 2 h, then every hour until 10 h after spiking. At each sampling event, an aliquot of 1 mL wastewater was collected from each reactor and immediately filtered into sterilized 1.5 mL tubes using 0.2 μm PES membrane filter (Sarstedt, Germany) which was pretreated with 3% beef extract (Becton Dickenson, USA) as suggested by Gundy et al. (2008). All the samples were stored at −80 °C and titered within 48 h with the Reed–Muench method ($TCID_{50}$ technique) as indicated in Section 2.1.3. For each reactor, a single $TCID_{50}$ measurement was performed at each time points as described in Section 2.1.3. The survivability batch tests of each virus were conducted in duplicate.

2.4. Wastewater and bioreactor analysis

The pH was monitored by ST310 pH electrode (Ohaus, USA). DO was monitored by HI98196 multiparameter (Hanna Instruments, Australia). TSS and VSS of the samples were determined within 48 h after wastewater collection. Sulfate was measured by SulfaVer® 4 sulfate reagent powder pillows with complimentary 10 mL sample cells (Hach, Australia) following US-EPA Standard Method 375.4. TCOD and SCOD were analyzed with TNTplus® COD test kit following the US-EPA Standard Method 5220 D. Hach DR3900 spectrometer (Hach, Australia) was used for the determination of COD concentrations.

2.5. Kinetic modelling and statistical analysis

Monophasic first-order decay kinetics have been widely used to assess the survivability of coronaviruses such as murine hepatitis virus (MHV), SARS-CoV-2, HCoV-229E, and FIPV, and achieved appropriate fitting in wastewater environment (Bivins et al., 2020; Gundy et al., 2008; Ye et al., 2016). Thus, the survival of HCoV-229E and FIPV in reactors was linearized using the natural log (ln)-transformation of the titer of the values at each sampling point as shown in Eq. (1), where C_t and C_0 are the concentrations (based on $TCID_{50}$/mL) of HCoV-229E or FIPV at time t and time 0, respectively, and k is the decay rate constant.

$$ln\left(\frac{C_t}{C_0}\right) = -k \times t \tag{1}$$

The monophasic first-order decay rate constant along with the associated 95% confident interval (CI) was estimated by linear regression using GraphPad Prism Version 9.0.0 (GraphPad Software, La Jolla, CA, USA). The runs test was used to evaluate the appropriateness of the linear model and fit was assessed by the coefficient of determination (R^2) and root mean square error (RMSE). The time required to achieve 90% (T_{90}) and 99% (T_{99}) decay of the spiked coronaviruses based on the monophasic first-order decay kinetics were further estimated using k values as Eqs. (2) and (3).

$$T_{90} = \frac{\ln(0.1)}{k} \tag{2}$$

$$T_{99} = \frac{\ln(0.01)}{k} \tag{3}$$

The biphasic first-order decay model contains one fast decay phase (Eq. (4)), followed by one slow decay phase (Eq. (5)), with a turning point from the first phase to the second phase at time t_1 (h). k_1 and k_2 are the decay rate constant of the first phase and second phase, respectively. The decay rate constant and turning point (t_1) along with the associated 95% confident interval (CI) was estimated using GraphPad Prism Version 9.0.0. T_{90} and T_{99} values were estimated by considering both k_1 and k_2.

$$ln\left(\frac{C_t}{C_0}\right) = -k_1 \times t, \ when\ t \le t_1 \tag{4}$$

$$ln\left(\frac{C_t}{C_0}\right) = -k_1 \times t_1 - k_2 \times (t - t_1), \ when\ t > t_1 \tag{5}$$

Two-way analysis of variance (ANOVA) with Tukey's multiple comparison tests were used to evaluate the impact of sewer operational conditions (i.e. gravity sewer or rising main sewer), biofilm impact, and types of coronavirus (i.e. HCoV-229E or FIPV) on the decay characteristics ($\alpha = 0.05$) using GraphPad Prism.

3. Results

3.1. Survivability of surrogate coronaviruses in sewer reactors

No infectious HCoV-229E and FIPV were detected in the wastewater before the spiking. After the spiking, the initial concentration of infectious HCoV-229E and FIPV was around 10^5 and 10^6 $TCID_{50}$/mL, respectively at time 0. The spiked viral concentrations were increased over expected possible infectious SARS-CoV-2 concentrations in wastewater to enable the determination of inactivation kinetics using $TCID_{50}$ technique. In comparison to the control reactors (wastewater only condition), it is clear that the decay of infectious HCoV-229E and FIPV was accelerated under both gravity and rising main conditions in the reactors with sewer biofilms (Fig. 1). T_{90} of infectious HCoV-229E reduced from c.a. 6 h in the control reactor (GS_C) to about 1.5 h in the biofilm reactor (GS_B) of gravity conditions, and from c.a. 2 h in the control reactor (RM_C) to 1 h in the biofilm reactor (RM_B) of rising main conditions. Similarly, T_{90} of infectious FIPV reduced from c.a. 3.5 h in GS_C to 1 h in GS_B and from c.a. 2 h in RM_C to 1 h in RM_B (Fig. 1). More importantly, 99% of HCoV-229E and FIPV decayed within 1.5–2 h under either gravity or rising main sewer conditions in presence of sewer biofilms, in contrast to 5–10 h without sewer biofilms (Fig. 1), suggesting an enhanced decay of HCoV-229E and FIPV due to the presence of sewer biofilms. Regardless of the sewer conditions (i.e. gravity or rising main sewers), the decay of HCoV-229E or FIPV was significantly enhanced by the presence of sewer biofilms ($p = 0.006$–0.03, Table 1).

With the presence of sewer biofilms, the decay characteristics of HCoV-229E and FIPV were not significantly different in rising main sewer reactors ($p = 0.999$) or gravity reactors ($p = 0.879$) (Table S1). Also, the different operational conditions of biofilm reactors, i.e. rising main or gravity sewer, didn't show significant impact on the decay of HCoV-229E or FIPV ($p > 0.9$, Table 1). This suggests that sewer operational conditions have negligible impacts on the decay of HCoV-229E or FIPV. More importantly, rising main sewer biofilms and gravity sewer biofilms provided the same or similar inactivation effect on HCoV-229E and FIPV in wastewater.

In reactors without biofilms, faster decay of HCoV-229E or FIPV was observed in RM_C reactor than GS_C reactor (Fig. 1), but not statistically significant ($p = 0.071$ and 0.433 for HCoV-229E and FIPV, respectively, Table 1). In addition, under the same conditions, there was no statistically significant difference between the fate of HCoV-229E and FIPV (p values range: 0.84–1.0, Table 1), suggesting that in all the experimental conditions, the decay pattern of both coronaviruses was comparable.

3.2. Decay kinetics of surrogate coronaviruses

The monophasic first-order decay kinetics well described the decay of HCoV-229E and FIPV in control reactors (GS_C and RM_C), with no significant deviations of replicates from the curve ($p = 0.07$–0.33, Table 2). For HCoV-229E, the mean monophasic first-order decay rate constants (k) were 0.45 h^{-1} in GS_C and 0.86 h^{-1} in RM_C. Similar k was observed for FIPV at 0.50 h^{-1} in GS_C and 0.73 h^{-1} in RM_C (Table 2). The slightly faster decay in RM_C was consistent with the observations as mentioned in Section 3.1. Through monophasic first-order

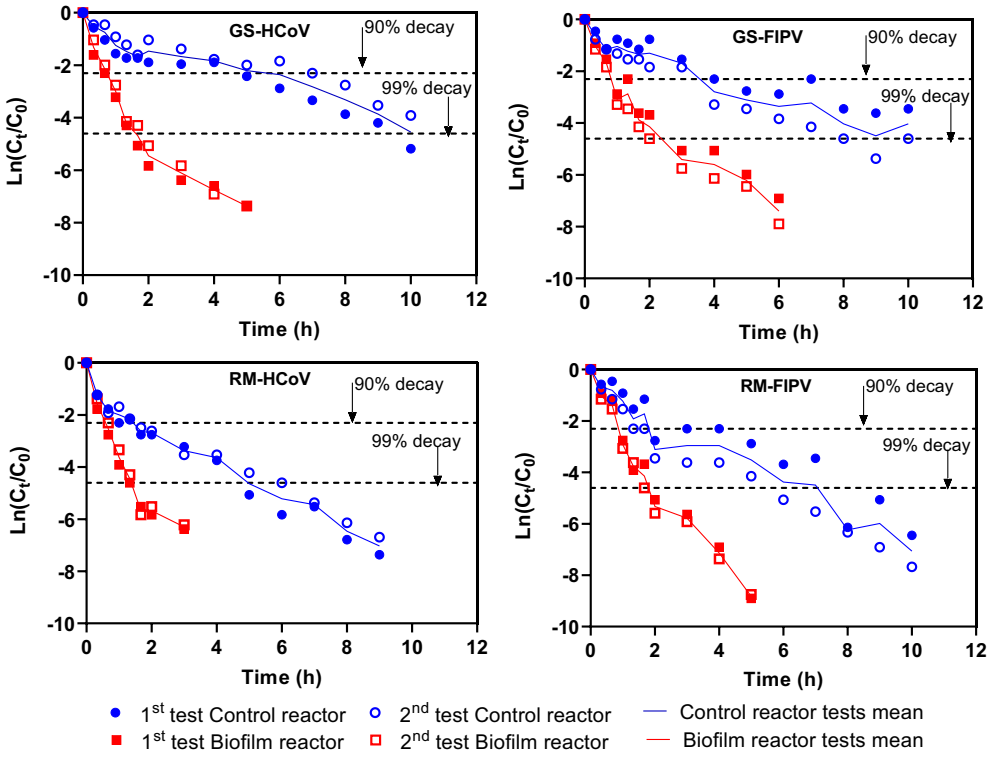

Fig. 1. Decay of infectious HCoV-229E and FIPV in gravity sewer reactors (GS) and rising main sewer reactors (RM). Two duplicate tests (1st and 2nd test) were performed on two different days. The middle line represents the mean of the duplicate tests under the same conditions.

decay kinetics analysis, higher k values were determined for reactors with biofilms than the control reactors. The k was 1.85 h^{-1} in GS_B and 2.69 h^{-1} in RM_B for HCoV-229E, and 1.34 h^{-1} in GS_B and 1.82 h^{-1} in RM_B for FIPV. As one k value was determined based on the duplicate tests for each virus under each condition (i.e. gravity or rising main sewers, with or without biofilms), the statistical difference between k values was not analyzed. It is clear that the presence of sewer biofilms increased the k by 1.5–4.2 times in comparison to the control reactors for each virus, which is consistent with the significantly higher decay observed in reactors with biofilms (Table 1) as discussed in Section 3.1. The k values observed in GS_B and RM_B were similar for both viruses, which is consistent with the non-significant difference observed for the decay rates between GS_B and RM_B (Table 1). However, further studies are encouraged to perform more replicates to evaluate the statistical significance of k values measured between different sewer conditions. The difference of k for RM and GS sewer reactors is likely due to the different sewer biofilms, i.e. anaerobic and mixed anaerobic/aerobic condition in RM and GS reactors, respectively.

Although the monophasic first-order decay model achieved reasonable overall R^2 (0.70–0.86) and RMSE (0.77–0.90) for the reactors with biofilms, some deviations of data and fitted curve were evident (p<0.001, Table 2) as shown in Fig. S2. Thus, monophasic first-order kinetics cannot adequately represent the decay characteristics of HCoV-229E and FIPV in the presence of sewer biofilms. The decay of infectious HCoV-229E and FIPV in RM_B and GS_B was further analyzed using biphasic first-order decay model and the results are summarized in Table 3 and Fig. S3.

Compared with monophasic first-order decay models, biphasic first-order decay models showed better performance with higher R^2 and lower RMSE. Biphasic first-order decay significantly improved the fit of the model to the observed data ($p < 0.001$) for both HCoV-229E and FIPV. Through biphasic first-order model analysis, it is clear that the decay of HCoV-229E and FIPV started with a faster decay phase, followed by a slower decay phase with the t_1 (turning point from faster phase to slower phase) ranged from 1.51–2.19 h. The k_1 estimated in biphasic first-order decay models ranged from 2.29 h^{-1} to 3.56 h^{-1}, which was about 2–4 times higher than the k_2 values. Based on the biphasic first-order decay model, 99% of the HCoV-229E and FIPV decayed in the first phase. T_{99} estimated through the biphasic first-order decay model ranged from 1.30–2.01 h for HCoV-229E and FIPV, which are more comparable to the experimental results (Fig. 1) than the monophasic first-order decay model.

Table 1
Summary of p values for the statistical difference of decay of HCoV-229E and FIPV between different sewer conditions using two-way analysis of variance (ANOVA) with Tukey's multiple comparison test.

	GS_B vs GS_C	RM_B vs RM_C	GS_B vs RM_B	GS_C vs RM_C
HCoV-229E	0.0012, **	0.046, *	>0.999	0.071
FIPV	0.0079, **	0.026 *	0.932	0.433

Note: Significance codes represent p values: 0–0.001: ***; 0.001–0.01: **; 0.01–0.05: *.

4. Discussion

Previously, the decay of infectious coronaviruses such as MHV, TGEV, Bacteriophage Phi6, SARS-CoV-2, HCoV-229E and FIPV, have been widely investigated in bulk wastewater (Table 4). Ye et al. (2016) investigated the decay of MHV in raw and pasteurized wastewater, where 99% of MHV decayed after 30 h. In pasteurized settled wastewater (raw wastewater that has undergone an initial settling step after entry into the plant to separate large solids from the liquid), infectious MHV and TGEV was detected for more than 7–9 days (Casanova et al., 2009). Bacteriophage Phi6, another common surrogate for enveloped viruses, decayed by 90% in pasteurized or autoclaved wastewater in 0.5–3.5 days (Aquino de Carvalho et al., 2017; Casanova et al., 2009). Similarly, infectious SARS-CoV-2, HCoV-229E and FIPV were also detected in wastewater for days, with T_{90} and T_{99} ranging from 0.8–2.1 days and 1.7–4.3 days, respectively (Bivins et al., 2020; Gundy et al., 2008). All

Table 2
Decay rate k (h^{-1}), T_{90} and T_{99} values (h) of HCoV-229E and FIPV in rising main and gravity sewer reactors with and without biofilms based on the monophasic first-order decay models.

Reactors		GS_B	GS_C	RM_B	RM_C
HCoV-229E	k (h^{-1}) [95% CI]	1.85 [1.60–2.10]	0.45 [0.41–0.49]	2.69 [2.35–3.04]	0.86 [0.78–0.93]
	R^2	0.70	0.77	0.78	0.82
	RMSE	1.30	0.61	1.00	0.86
	Runs test[a]	S[b], $p < 0.001$	NS[b], $p = 0.13$	S, $p < 0.001$	NS, $p = 0.07$
	T_{90} (h) [95% CI]	1.24 [1.10–1.44]	5.17 [4.71–5.74]	0.86 [0.76–0.98]	2.69 [2.48–2.94]
	T_{99} (h) [95% CI]	2.49 [2.19–2.87]	10.35 [9.42–11.49]	1.71 [1.52–1.96]	5.38 [4.95–5.88]
FIPV	k (h^{-1}) [95% CI]	1.34 [1.20–1.47]	0.50 [0.45–0.56]	1.82 [1.65–2.00]	0.73 [0.67–0.79]
	R^2	0.79	0.76	0.86	0.84
	RMSE	1.17	0.72	1.04	0.86
	Runs test[a]	S, $p < 0.001$	NS, $p = 0.33$	S, $p < 0.001$	NS, $p = 0.31$
	T_{90} (h) [95% CI]	1.72 [1.56–1.92]	4.58 [4.15–5.12]	1.26 [1.15–1.39]	6.33 [5.82–6.93]
	T_{99} (h) [95% CI]	3.45 [3.13–3.85]	9.16 [8.30–10.23]	2.53 [2.31–2.79]	7.36 [6.45–8.55]

[a] Run test was used to check whether the model was chosen correctly, and all scatter is Gaussian.
[b] S: significant deviations from the model; NS: non-significant deviation from the model.

Table 3
Decay parameters including rate constants k_1 and k_2 (h^{-1}) and transiting time point t_1 (h) of HCoV-229E and FIPV in rising main and gravity sewer reactors with biofilms based on the biphasic first-order decay model.

Viruses	Reactors	k_1 (h^{-1}) [95% CI]	k_2 (h^{-1}) [95% CI]	t_1 (h) [95% CI]	R^2	RMSE	T_{90} (h) [95% CI]	T_{99} (h) [95% CI]
HCoV-229E	GS_B	2.99 [2.80–3.42]	0.64 [0.43–0.92]	1.78 [1.33–2.04]	0.98	0.30	0.77 [0.67–0.82]	1.54 [1.35–1.64]
	RM_B	3.54 [3.30–3.78]	0.70 [0.41–0.99]	1.54 [1.36–1.73]	0.99	0.27	0.65 [0.61–0.70]	1.30 [1.22–1.40]
FIPV	GS_B	2.29 [2.06–3.79]	0.40 [0.13–1.07]	2.19 [0.73–2.73]	0.95	0.47	1.01 [0.61–1.12]	2.01 [1.22–2.24]
	RM_B	2.76 [2.42–3.14]	1.27 [0.94–1.47]	1.51 [1.10–2.18]	0.98	0.36	0.83 [0.73–0.95]	1.67 [1.47–1.90]

these previous studies suggest that infectious coronaviruses could persist in wastewater for days.

In our study, the T_{90} and T_{99} in control reactors (wastewater only, without sewer biofilms) of HCoV-229E and HIPV were estimated as 3–6 h and 6–11 h, respectively, which were shorter than previous reports. This is likely related to the experimental scale, wastewater properties, and experimental conditions. Previous studies normally spike viruses into a small volume of wastewater (5–45 mL) without external mixing during the decay tests. Our study utilized larger well-controlled laboratory reactors (a working volume of 0.75 L), with proper mixing, which avoided the biases caused by the sampling volume of wastewater, providing better representatives of the real sewer conditions. Some previous studies also used the primary or secondary effluent in WWTP (effluent from the primary or secondary sedimentation units) (Bivins et al., 2020, Gundy et al., 2008), old wastewater (being stored at −80 °C) (Bivins et al., 2020, Gundy et al., 2008), pasteurized or autoclaved wastewater (Aquino de Carvalho et al., 2017; Casanova et al., 2009; Casanova and Weaver, 2015), and filtered wastewater (Gundy et al., 2008). The sedimentation, filtration, pasteurization, autoclaving, and storage of wastewater would change the characteristics of wastewater such as suspended solids content, microbial and enzyme activity etc., which largely affect the decay of coronaviruses in wastewater (Silverman and Boehm, 2020).

Table 4
Comparing the survivability of coronaviruses in wastewater of different studies.

Virus	Testing condition	T_{90}	T_{99}	Decay ratio[a], 2 h	Decay ratio[a], 6 h	Decay ratio[a], 10 h	Decay ratio[a], 24 h	Reference
MHV	Wastewater 25 °C, starting concentration: 10^4 PFU/mL	10 h	30 h	37%	75%	90%	97%	(Ye et al., 2016)
	Pasteurized wastewater 25 °C, starting concentration: 10^4 PFU/mL	15 h	30 h	26%	60%	78%	97%	
	Pasteurized settled wastewater 25 °C, starting concentration $10^{5.8}$ MPN/mL	3 days	7 days	5%	15%	24%	48%	(Casanova et al., 2009)
TGEV	Pasteurized settled wastewater 25 °C, starting concentration $10^{6.6}$ MPN/mL	4 days	9 days	4%	12%	19%	40%	
Bacteriophage Phi6	Autoclaved wastewater influent, room temperature, starting concentration: 10^9–10^{11} PFU/mL	2.5 days	5 days	7%	21%	32%	60%	(Aquino de Carvalho et al., 2017)
	Pasteurized wastewater 22 °C	3.5 days	6.5 days	6%	16%	26%	51%	(Casanova and Weaver, 2015)
	Pasteurized wastewater 30 °C	12 h	1 day	32%	68%	85%	99%	
SARS-CoV-2	Wastewater 20 °C, starting concentration: 10^5 $TCID_{50}$/mL	1.6 days	3.2 days	11%	30%	44%	75%	(Bivins et al., 2020)
	Wastewater 20 °C, starting concentration: 10^3 $TCID_{50}$/mL	2.1 days	4.3 days	9%	24%	37%	67%	
HCoV-229E	Wastewater 23 °C, starting concentration: 10^5 $TCID_{50}$/mL	1.2 days	2.4 days	15%	39%	56%	86%	(Gundy et al., 2008)
	Reactors with biofilms, 22 °C, starting concentration: 10^5 $TCID_{50}$/mL	<1 h	<2 h	99.6–99.7%	>99%	>99%	>99%	This study
	Reactors without biofilms, 22 °C, starting concentration: 10^5 $TCID_{50}$/mL	3–6 h	6–11 h	56–72%	88–98%	>98%	>99%	
FIPV	Wastewater 23 °C, starting concentration: 10^5 $TCID_{50}$/mL	0.9 days	1.7 days	20%	49%	67%	93%	(Gundy et al., 2008)
	Reactors with biofilms, 22 °C, starting concentration: 10^6 $TCID_{50}$/mL	c.a. 1 h	c.a. 2 h	98–99%	>99%	>99%	>99%	This study
	Reactors without biofilms, 22 °C, starting concentration: 10^6 $TCID_{50}$/mL	3–6 h	7–10 h	36–64%	91–98%	>98%	>99%	

[a] Estimated from figures or decay kinetics.

In comparison to the control reactors and previous studies, faster decay of HCoV-229E and FIPV was observed in reactors with sewer biofilms. In the presence of sewer biofilms, more than 98% of HCoV-229E and FIPV decayed within the first 2 h, while only 4–70% of coronaviruses decayed in the control reactors or other previous studies using bulk wastewater (Table 4). More importantly, after 6–10 h (normal HRT of sewers), >99% of HCoV-229E and FIPV decayed in reactors with sewer biofilms. In contrast, 10–76% of coronaviruses remained active in bulk wastewater after 6-10 h, and 3–60% of infectious coronaviruses can still be isolated from bulk wastewater even after 24 h in previous reports (Table 4), which conflicts with the current observations during COVID-19 and SARS outbreaks. During the COVID-19 and SARS outbreaks, the disease transmission from wastewater to humans was only reported inside the same residence building, which has a much shorter HRT (several minutes) than sewers (Hwang et al., 2021; Liu et al., 2020; McKinney et al., 2006). For sewers or WWTPs (with HRT longer than 2 h generally), infectious SARS-CoV-2 or SARS has not been isolated from wastewater so far (Anand et al., 2022; Rimoldi et al., 2020; USCDC, 2021; Westhaus et al., 2021; WHO, 2020). A recent review also stated that infectious risks of SARS-CoV-2 from wastewaters are likely to be low compared to well-documented person-to-person transmission via respiratory droplets/aerosols, based on the failure to isolate infectious SARS-CoV-2 from wastewater in current studies (Ahmed et al., 2021b). Thus, it is likely the sewer biofilms enhanced the decay of infectious coronaviruses in wastewater, which prevented the disease transmission from wastewater to humans. Using the results obtained from in-vial tests with wastewater would overestimate the transmission risks in sewers. However, extra cautions should be taken while handling the wastewater in sewers or WWTPs with short HRT (<2 h), where infectious coronaviruses might persist at high concentrations.

Kinetics analysis revealed that the decay of HCoV-229E and FIPV in reactors with sewer biofilms followed the biphasic first-order decay with the turning point (t_1) from the first phase to the second phase at <2 h. In contrast, the monophasic first-order decay model describes the decay of coronaviruses in bulk wastewater without sewer biofilms (control reactors and previous studies) better (Aquino de Carvalho et al., 2017; Bivins et al., 2020; Casanova et al., 2009; Gundy et al., 2008). The k_2 achieved in the biofilm reactors was comparable to the decay constant k of control reactors without biofilms. Considering the short t_1 and the same wastewater characteristics and operational conditions between the control and biofilm reactors, the enhanced decay of HCoV-229E and FIPV is likely related to the initial adsorption of HCoV-229E and FIPV to biofilms. In the outbreak of SARS-CoV-2, within the same sewer in the vicinity of an isolation center, higher quantities of the SARS-CoV-2 RNA were detected in the downstream of the drain than the upstream, suggesting the potential accumulation of SARS-CoV-2 within the sewer (Ahmed et al., 2021a). The accumulation of SARS-CoV-2 RNA observed is likely related to adsorption of viral particles to sewers biofilms. Additionally, in membrane reactors and drinking water systems, enhanced removal of viruses was observed due to the biofilm attached (Skraber et al., 2005; Tobias and Bérubé, 2020). The attached viruses are likely subject to longer residence time in wastewater and thus higher inactivation to mitigate the transmission. To date, the mechanism of biofilms in virus adsorption and potential inactivation remains unclear and requires future investigations.

5. Limitations and future research recommendations

This is the first study investigating the decay of infectious coronaviruses in domestic wastewater in the presence of sewer biofilms. Enhanced decay of HCoV-229E and FIPV in wastewater was observed due to the presence of sewer biofilms, which was likely caused by the adsorption of HCoV-229E and FIPV to the biofilm. Due to the methodological constraints, the infectivity of HCoV-229E and FIPV inside the biofilm was not determined. A previous study revealed that viral indicators (somatic coliphages and F-specific phages) persisted longer in biofilms than in the corresponding wastewaters at 4 °C as well as at 20 °C (Skraber et al., 2007). To date, the persistence of coronaviruses in sewer biofilms has not been reported, which requires future investigations.

The decay of infectious coronaviruses was monitored for 10 h in laboratory rising main and gravity sewer reactors, which mimic the real sewer conditions. However, in real sewers, the rising main sewers and gravity sewers are usually connected (Hvitved-Jacobsen et al., 2013; Li et al., 2017). The pumps for rising main sewers are regularly turned on and off, causing intermittent flow, varying mixing conditions, and turbulence of wastewater in the rising main sewers and also downstream gravity sewers (Sharma et al., 2008). In this study, the sewer reactors were operated with fixed flow height (i.e. full-filled for rising main reactors and half-full with gravity reactors), constant mixing, and no turbulence. Furthermore, depending on the pipe length, the HRT varies from several minutes to several hours in each gravity and rising main pipe (Sharma et al., 2008; Short et al., 2017). A catchment generally contains multiple gravity and rising main sections, leading to an overall HRT of several minutes to 6–10 h (Li et al., 2021b; McCall et al., 2017). In this study, we monitored the decay of infectious coronaviruses in each reactor for 10 h to cover the range of HRT under each condition (i.e. gravity and rising main). In addition, the gravity reactor was built in a vertical format, which may not represent the varying degrees of aeration (different dissolved oxygen in wastewater) in actual horizontal gravity sewers. The impact of intermittent flow patterns, mixing, aeration, and flow velocity on the decay of infectious coronaviruses requires future investigations.

Furthermore, the coronaviruses usually enter the sewers in the form of feces, sputum, and other bodily fluids (Pan et al., 2020; van Doorn et al., 2020), the decay characteristics of which may differ from the exogenous viruses spiked into the reactors (Bivins et al., 2020). Additionally, due to the detection limit, HCoV-229E and FIPV were seeded at a concentration of 10^5–10^6 $TCID_{50}$/mL, allowing the proper detection during the decay process. The concentration of coronaviruses in real sewers is likely to be much lower and the observed decay characteristics might differ due to the density effect. Thus, future studies are encouraged to explore the decay kinetics of coronaviruses in sewers at lower concentrations.

In addition, although evidence suggests that HCoV-229E and FIPV are good representatives of SARS-CoV-2 RNA decay kinetics in wastewater (Chik et al., 2021), the similarity in infectivity during in-sewer transportation between SARS-CoV-2 and these surrogates remains unclear. Thus, the findings from this study should be interpreted with caution while quantifying the transmission risks of infectious SARS-CoV-2 in sewers.

6. Conclusions

This study investigated the decay of infectious HCoV-229E and FIPV in laboratory sewer reactors mimicking the gravity and rising main sewers with and without sewer biofilms. The results lead to the following conclusions:

- The decay of infectious coronaviruses in domestic wastewater at ambient temperatures was significantly enhanced in sewers. HCoV-229E and FIPV decayed rapidly in wastewater reactors with sewer biofilms, reaching a 99% reduction within 2 h, in comparison to 8–10 h in the control reactors.
- The decay of HCoV-229E and FIPV in wastewater showed very limited difference in sewers with different operational conditions, i.e. rising main or gravity sewer, with the T_{90} and T_{99} difference of 7–10 min and 14–20 min, respectively.
- This study demonstrated that sewer biofilms significantly reduced the transmission risks of coronaviruses from wastewater to humans. Using the decay results obtained from bulk wastewater would overestimate the transmission risk in urban wastewater systems.

CRediT authorship contribution statement

Jiahua Shi: Conceptualization, Methodology, Data curation, Writing – review & editing. **Xuan Li:** Conceptualization, Data curation, Formal analysis, Writing – original draft. **Shuxin Zhang:** Data curation. **Elipsha Sharma:** Data curation. **Muttucumaru Sivakumar:** Writing – review & editing. **Samendra P. Sherchan:** Writing – review & editing. **Guangming Jiang:** Conceptualization, Methodology, Writing – review & editing, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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