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Detection of SARS-CoV-2 in urban stormwater: An environmental reservoir and potential interface between human and animal sources

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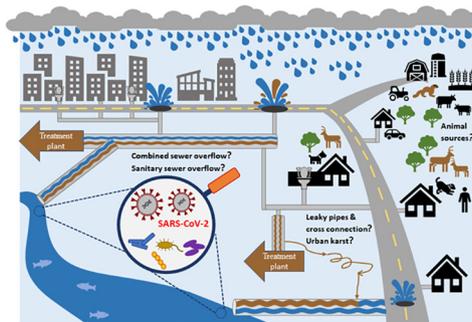
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HIGHLIGHTS

- 88% of the stormwater samples had detectable SARS-CoV-2 genes.
- Fecal contamination in stormwater was primarily from human sources.
- Future research must study stormwater & wastewater SARS-CoV-2 infection potential.
- SARS-CoV-2 gene significantly correlated with HF183 and *E. coli* concentrations.
- N2 gene concentrations correlated with time since previous rainfall.

GRAPHICAL ABSTRACT

Stormwater: An interface between human and animal waste in urban and semi-urban environments



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ABSTRACT

While wastewater has been found to harbor SARS-CoV-2, the persistence of SARS-CoV-2 in stormwater and potential transmission is poorly understood. It is plausible that the virus is detectable in stormwater samples where human-originated fecal contamination may have occurred from sources like sanitary sewer overflows, leaky wastewater pipes, and non-human animal waste. Because of these potential contamination pathways, it is possible that stormwater could serve as an environmental reservoir and transmission pathway for SARS-CoV-2. The objectives of this study are: 1) determine whether the presence of SARS-CoV-2 could be detected in stormwater via RT-ddPCR (reverse transcription-digital droplet PCR); 2) quantify human-specific fecal contamination using microbial source tracking; and 3) examine whether rainfall characteristics influence virus concentrations. To accomplish these objectives, we investigated whether SARS-CoV-2 could be detected from 10 storm sewer outfalls each draining a single, dominant land use in Columbus, Xenia, and Springboro, Ohio. Of the 25 samples collected in 2020, at minimum one SARS-CoV-2 target gene (N2 [US-CDC and CN-CDC], and E) was detected in 22 samples (88%). A single significant correlation ($p = 0.001$), between antecedent dry period and the US CDC N2 gene, was found between target gene concentrations and rainfall characteristics. Grouped by city, two significant relationships emerged showing cities had different levels of the SARS-CoV-2 E gene. Given the differences in scale, the county-level COVID-19 confirmed cases COVID-19 rates were not significantly correlated with stormwater outfall-scale SARS-CoV-2 gene concentrations. Countywide COVID-19 data did not accurately portray neighborhood-scale confirmed COVID-19 case rates. Potential hazards may arise when human fecal contamination is present in stormwater and facilitates future investigation on the threat of viral outbreaks

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via surface waters where fecal contamination may have occurred. Future studies should investigate whether humans are able to contract SARS-CoV-2 from surface waters and the factors that may affect viral longevity and transmission.

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1. Introduction

In December 2019, the novel coronavirus (COVID-19), an illness caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), was first detected in Wuhan, China (World Health Organization, 2020). The ongoing COVID-19 outbreak, declared a public health emergency by the World Health Organization (WHO) on March 11th, 2020, poses a significant risk to international public health with more than 187 million confirmed cases worldwide and 4.04 million deaths as of July 2021 (World Health Organization, 2020). At present, there are two known primary methods of transmission for COVID-19: person-to-person contact and respiratory droplets emitted during exhalation (Cui et al., 2019; Kang et al., 2020). Contraction of this disease is not limited to humans, as it can also infect other mammals including domesticated felines and canines, commodity animals such as mink, and wildlife species such as deer and non-human primates (Kitajima et al., 2020; Munnink et al., 2021; Newman et al., 2020; Palmer et al., 2021; U.S. Center for Disease Control and Prevention, 2021).

To date, little is known about whether water is a possible method of transmission, including transmission via contact with or accidental ingestion of wastewater or stormwater. Studies have shown that SARS-CoV-2 can be shed in fecal matter of infected individuals (Aguilar-Oliveira et al., 2020; Ahmed et al., 2020a). Because this virus can continue to be intact and viable in water under specific conditions (e.g., turbidity, temperature, and time in solution impact viability), research is needed to determine whether SARS-CoV-2 can be found in stormwater, its potential as a source of infection, and what variables in water influence its viability (Sayess et al., 2020). Published studies currently show that SARS-CoV-2 is detectable and viable for up to two weeks in sewage, and thus could cause COVID-19 infections (Zanetti et al., 2020). Zheng et al. (2020) found that SARS-CoV-2 was detectable in human stool for a median 22 days. Another study found that fecal shedding of SARS-CoV-2 could continue up to seven weeks past the cessation of COVID-19 symptoms (Kitajima et al., 2020). This is concerning since the virus can potentially survive for multiple days in wastewater and be transmissible once the original host is no longer contagious (Orive et al., 2020; Zheng et al., 2020). Even less is known about the ability of SARS-CoV-2 to persist in stormwater. Additional research is necessary to determine SARS-CoV-2 transmissibility in waters, as current data only gives insight into whether it is culturable from fecal matter (Wang et al., 2020).

Since the outset of the COVID-19 pandemic, efforts to understand how the virus spreads, infects, and persists have been substantial. Recent studies found that the virus sheds in stool in sufficiently high levels for successful detection and analysis (Gao et al., 2020; Holshue et al., 2020; Wurtzer et al., 2020). Stormwater exists as a potential conveyance mechanism for SARS-CoV-2 due to sanitary sewer overflows; fecal matter may interact with stormwater through animal feces or water in separate sanitary and storm sewers through leaks or otherwise accidental cross-connection. Herein, we define stormwater as runoff discharging from separated storm sewers into streams, lakes, and rivers (i.e., not combined sewers which convey both waste and stormwaters); since recreation often occurs in these locations, there is a potential public health risk if interaction with viable SARS-CoV-2 occurs (King, 1995). Population density has been positively correlated to higher transmission rates of SARS-CoV-2, making urban environments more likely hotspots for the virus (Liu, 2020). It is well known that infection rates grouped by county cannot account for the wide disparities of diseases

between adjacent neighborhoods; evidence is mounting that infection rates in the US may be significantly higher in impoverished neighborhoods than their higher income counterparts (Adhikari et al., 2020).

Parks and lakes are common summertime destinations and have increased in popularity as worldwide shutdowns reduce the number of available indoor activities (Venter et al., 2020; Geng et al., 2021). Surface waters near stormwater outfalls may pose a potential risk of exposure of SARS-CoV-2 to humans. Monitoring microbial quality at storm sewer outfalls has been used in the U.S. to determine whether downstream waters are safe for swimming and other recreation (Dorevitch et al., 2015). This is typically done through fecal indicator bacteria (FIB) as a proxy for estimating recreational waterborne disease risk (Marion et al., 2010). Fecal coliforms are routinely found in stormwater at relatively high concentrations (Lee et al., 2020; Sauvé et al., 2012; Mallin et al., 2016), suggesting that stormwater may harbor and convey SARS-CoV-2. Infiltration and inflow between the sanitary and storm sewer via the 'urban karst' phenomenon (Bonneau et al., 2017; Shepley et al., 2020), fecal matter from wild animals, and accidental or illicit wastewater connections to the storm sewer may result in the transport of SARS-CoV-2 by stormwater. Wastewater sewage leaking into storm sewers is an established issue where aging infrastructure conveying human sewage may leak into the storm sewer, discharging the contaminated stormwater with minimal treatment to surface waters, potentially at a location where the public may interact with the contaminated water (Ahmed et al., 2020b). Human and animal fecal contamination in stormwater collected from urban areas has already been confirmed, possibly making stormwater a new SARS-CoV-2 transmission pathway (Lee et al., 2020). One study in Spain reports SARS-CoV-2 infections in two free-ranging mink and posits that the mink were exposed to SARS-CoV-2 via surface waters and gives insight into the potential threat that is SARS-CoV-2 transmission via surface waters (Aguiló-Gisbert et al., 2021). Mink are semi-aquatic and highly susceptible to SARS-CoV-2, and this report provides preliminary and plausible support for the potential threat that is SARS-CoV-2 transmission via surface waters (Aguiló-Gisbert et al., 2021). A recent study conducted in the United States also highlighted a previously undocumented phenomenon – wide-spread evidence of SARS-CoV-2 in free-ranging white tailed-deer; 33% of the wild deer sampled in Pennsylvania, Michigan, New York, and Illinois harbored SARS-CoV-2 antibodies in their serum (APHIS, 2021b). There is no current evidence for deer-to-human SARS-CoV-2 transmission, but it is possible that deer may be a reservoir for the virus, potentially along with bats, mink, and/or some non-human primates.

To date, few conclusions have been drawn about how serious a risk SARS-CoV-2 may pose in water, particularly stormwater where concentrations may be dilute. Closely related coronaviruses, including Severe Acute Respiratory Syndrome (SARS-CoV), and Middle East Respiratory Syndrome (MERS-CoV), have been reported to persist in water with SARS-CoV confirmed to have strong survivability in water (Duan et al., 2003). Some enveloped viruses have demonstrated to be stable in water environments, and both coronaviruses MERS-CoV and SARS-CoV are enveloped (Wigginton et al., 2015). Given its status as an enveloped coronavirus, it is important to investigate the mechanics of potential water-based presence, survival, and transmission of SARS-CoV-2, which are at present poorly understood. Studies on the persistence of SARS-CoV-2 in wastewaters have identified many possible parameters that affect the viability of virus transmission in water including temperature, duration of time in water, the presence of other chemicals, pH,

and virus concentration (Liu, 2020; World Health Organization, 2020). Lab studies estimated that SARS-CoV-2 could survive under ideal conditions outside the human body for as little as three days to multiple weeks (World Health Organization, 2020; Tran et al., 2020). Data suggests that the likelihood of contracting SARS-CoV-2 from treated wastewaters is low (World Health Organization, 2020; Tran et al., 2020), but this does not account for stormwater that is subject to minimal treatment that may directly discharge to surface waters during wet weather.

The main objective of this study was to determine if SARS-CoV-2 was detectable in stormwater to lay the foundation for determining whether stormwater could be a potential transmission pathway. To this end, we collected stormwater samples from three communities with varying population densities in central Ohio, USA. To measure the extent of human and animal fecal contamination in stormwater, we conducted microbial source tracking (MST) by targeting host-specific fecal bacterial genetic markers. In addition, stormwater-related parameters were compared against SARS-CoV-2 target gene concentrations to examine their potential relationships. This study highlights the importance of the One Health paradigm as urban stormwater provides a connected and tight interface between humans, animals, and the environment, while addressing the need for managing this possible transmission route now and in the future.

2. Methods and materials

2.1. Sewershed descriptions and stormwater sample collection

Ten storm sewer catchments (hereafter sewersheds) were monitored from May 10th to July 24th of 2020 in Ohio for the presence of SARS-CoV-2 in stormwater runoff discharging from their respective separate storm sewer network and are summarized as follows: Columbus (high density) in Franklin County (population 1.317 million), Xenia (low density) in Greene County (population 168,937), and Springboro (moderate density) in Warren County (234,602) (Ohio Development Services Agency, 2019; U.S. Census Bureau, 2019). Sewersheds are defined as a portion of land that drains to the same storm sewer to a single, defined outfall and were characterized by distinctive land use, sewershed area, and imperviousness (Table 1). All sewersheds were representative of a single, dominant land use (i.e. residential, commercial, industrial, etc. covering $\geq 75\%$ of the sewershed area) in an urban or suburban setting. Land use and sewershed boundaries were defined in GIS using aerial imagery and LiDAR data. A total of 25 stormwater samples were collected for SARS-CoV-2 analysis from single family residential (18 samples), light industrial (3 samples), commercial (2 samples), and multi-family residential land uses (2 samples). 14 of the samples were collected in Columbus, two in Xenia, and nine in Springboro. Urban sewersheds were the focus of this work and water quality samples were only collected during wet weather events.

The Columbus sewersheds were developed in the 1920s–1940s and are known to have leaky sewers with cross connection between the sanitary and storm sewers (City of Columbus, 2015). Portions of the neighborhood (C4 and C5) were retrofitted with green stormwater infrastructure in 2018 (City of Columbus, 2021), resulting in treatment of typical stormwater pollutants (i.e. nutrients, sediments, metals, and

oil and grease) from runoff in the C4 and C5 sewersheds (Table 1). The other three sewersheds in Franklin County (C1, C2, and C3) discharged untreated stormwater to the Olentangy River. The Xenia (X) sewershed was developed in the 1960s–1970s, while the Springboro (S) sewersheds have all been constructed since 1990. All sewersheds in Xenia and Springboro lacked green stormwater infrastructure and thus stormwater discharge was untreated at each monitoring point. Sewershed area ranged from 7 to 154 ha (mean 51 ha) and sewershed imperviousness ranged from 22 to 87% (mean 44%). Sewersheds were grouped for analysis by land use and county. Land use was classified by the predominant type within a sewershed: single family residential (SFR) land use was populated mostly by separate houses, multi-family residential (MFR) was populated mostly by single structures housing many families, light industrial land use (LI) was populated by warehousing operations and manufacturing, and commercial land (Comm) was populated by businesses and commercial properties.

Rainfall and runoff were monitored at the outfall of each sewershed. A rain gauge cluster consisting of a manual rain gauge and a tipping bucket rain gauge were deployed adjacent to each outfall in an area clear of overhead obstructions. Rainfall data were collected using 0.25-mm resolution Davis Rain Collector tipping bucket rain gauges (Cat. No. 7852.804, Davis Instruments, Hayward, CA, USA) and stored on Hobo Pendant data loggers (Cat No. UA-002-08, Onset Computer Corporation, Bourne, MA, USA). Manual rain gauges were used to check rainfall depth on-site in order to calibrate runoff sample pacing on the automated samplers. Hobo loggers recorded the time of each 0.25-mm tip which allowed for calculation on storm duration, intensity, depth, and antecedent dry period. Storm events were separated by a minimum 6-h dry period.

Most outfalls were fitted with a Teledyne ISCO 750 (Cat. No. 60-9003-465, Teledyne ISCO, Lincoln, NE, USA) area velocity meter paired with an ISCO 6712 (Cat. No. 69-9003-588, Teledyne ISCO Lincoln, NE, USA) automated sampler. One outfall was fitted with an ISCO 2150 flow module (Cat. No.68-2050-001, Teledyne ISCO Lincoln, NE, USA) and an ISCO 2105 interface module (Cat. No. 69-2003-588, Teledyne ISCO Lincoln, NE, USA) paired with an ISCO 3700 automated sampler (Cat. No. 60-3703-267, Teledyne ISCO Lincoln, NE, USA). The area velocity meters measured flow depth and velocity. Given the known cross-sectional area of each sewer outfall, flow rate was calculated as the product of flow area and velocity. During runoff, the automated samplers were programmed to take runoff-volume proportional sample aliquots. The sample trigger was set such that up to 50 sample aliquots were captured during a 50 mm rain event of variable duration; each aliquot was 350 mL allowing for a maximum collection volume of 17.5 L. Aliquots were suctioned using the automated sampler's peristaltic pump into an 18.9 L composite bottle. Samplers were programmed with an enable such that baseflow was disregarded and only wet weather flows were sampled; when conditions returned to baseflow after the cessation of flow, the sampler ceased collecting samples.

Composite samples selected for analysis described greater than 80% of the pollutograph (Quigley et al., 2002). To prevent degradation of the virus, samples were collected within 24 h of cessation of rainfall. Upon collection, the 18.9 L containers were vigorously shaken and subsampled into sterile Nalgene bottles (Nalgene, Fisher Scientific, USA). Samples were immediately placed on ice in a cooler during transit to the laboratory. Twenty-five composite samples were collected across the ten sewershed outfalls between May 10th and July 24th, 2020.

Data concerning the daily new confirmed COVID-19 cases for the counties where stormwater sampling occurred were obtained from the Ohio Department of Health COVID-19 dashboard (ODH, 2021) for the day of and week immediately preceding each sample collection. These data were collected using the Ohio Disease Reporting System and case numbers were reported using date of illness onset when known or earliest known date of symptoms if unknown.

Table 1

Characteristics of the ten sewersheds sampled for SARS-CoV-2 in stormwater runoff.

| Sewershed | X | S1 | S2 | S3 | S4 | C1 | C2 | C3 | C4 | C5 |
|-------------|-----|----|-----|------|-----|-----|-----|-----|-----|-----|
| Land Use | SFR | LI | MFR | Comm | SFR | SFR | SFR | SFR | SFR | SFR |
| Area (ha) | 149 | 11 | 8 | 7 | 9 | 22 | 23 | 111 | 48 | 12 |
| Imperv. (%) | 40 | 46 | 49 | 87 | 22 | 37 | 36 | 38 | 40 | 31 |
| County | G | M | W | W | W | F | F | F | F | F |

X: Xenia, S: Springboro, C: Columbus.

SFR: Single family residential, LI: Light industrial, MFR: Multi-family residential, Comm: Commercial.

G: Greene, M: Montgomery, W: Warren, F: Franklin.

2.2. *Escherichia coli* enumeration

To quantify FIB in water samples, samples collected from the sewersheds in Columbus (i.e., 14/25 samples) underwent *Escherichia coli* (*E. coli*) analysis. Funding for this protocol extended only to the samples taken in Franklin County, thus samples from Warren, Montgomery, and Greene counties were not subject to *E. coli* analyses. Analyses were conducted within 6 h of sample collection, utilizing the U.S. Environmental Protection Agency (US EPA) Method 1603 (U.S. Environmental Protection Agency, 2002). Briefly, three dilutions (1/100, 1/1000, and 1/10000) were prepared in duplicate with PBS (1×) and filtered through sterile 0.45 µm membrane filters (Cat. No. HAWG047S6, Millipore Sigma, Burlington, MA, USA). Filters were placed on modified mTEC agar (Difco, Detroit, MI, USA) microplates and incubated at 35 °C for 2 h, followed by 44.5 °C for 22 h. Colored colonies were counted, with final results reported as colony forming units (CFU) per 100 mL after considering the dilution factors and filtration volumes.

2.3. Microbial source tracking processing and gene quantification

To further determine possible fecal indicator bacteria sources, all sample water was processed for MST analyses. Two host markers, human fecal (HF183) and ruminant (Rum2Bac), were targeted for downstream analyses. These genes were used due to previous studies from the Columbus sewer outfalls that confirmed the dominance of human- and ruminant-associated fecal bacteria in stormwater (Lee et al., 2020). For microbial filtration, 100 mL of stormwater sample was filtered in triplicate through a sterile 0.22 µm membrane filter (Cat. No. GTTP04700, Millipore Sigma, Burlington, MA, USA). The membranes were folded, placed in a sterile screw-cap microcentrifuge tube and stored at -20 °C for approximately 1–2 weeks until further analysis could be undertaken. Microbial DNA extractions were conducted using a DNeasy PowerSoil Kit (Cat. No. 12888-100, QIAGEN, Germantown, MD, USA), following the manufacturer's protocol. Quantification and quality of the extracted DNA were determined using a Qubit 3.0 fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) prior to further analyses.

Two individual monoplex assays were conducted to target HF183 and Rum2Bac, with primers and probe sets previously used in stormwater studies (Lee et al., 2020). Droplet digital PCR (ddPCR) was employed for gene quantification. Gene amplifications were conducted using 20 µL reactions containing ddPCR supermix for probes (Cat. No. 1863024, Bio-Rad), DNase- & RNase-free water, 900 nM of forward and reverse primers, 250 nM of probe, and DNA templates. Following droplet generation using the QX200 Droplet Generator (Bio-Rad), a Bio-Rad C1000 Touch Thermal Cycler (Bio-Rad, Hercules, CA, USA) was used to amplify the targets with the following conditions: 94 °C for 10 min, 40 cycles of denaturation and annealing/extension at 94 °C for 30 s and 60 °C for 60 s, respectively, followed by 98 °C for 10 min and then a final hold of 4 °C. Following amplification, target gene concentrations were determined using a QX200 droplet reader (Bio-Rad) and QuantaSoft (V 1.7; Bio-Rad).

2.4. Viral concentration procedure

The viral concentration protocol was modified from the USEPA Method 1615 (Fout et al., 2014). Briefly, 600–800 mL of stormwater sample was passed through a positively charged ViroCap filter (Scientific Methods, Inc., Granger, IN, USA) using a peristaltic pump at a rate of 0.5 L/min. 150 mL of 1.5% beef extract (Cat. No. 211520, Becton, Dickinson and Company, USA) containing 0.05 M glycine (pH 9) (Cat. No. G8898, Sigma-Aldrich, USA) was added to the filter for elution. The eluent was soaked for 30 min then circulated using the peristaltic pump for 5 min at room temperature prior to elution. Secondary concentration was performed via organic flocculation. The eluent was pH adjusted to 3.5 ± 0.1 using small additions of 1.2 M hydrochloric acid (HCl) while slowly mixing at room temperature, followed by a 30-min slow mixing period. Next, the adjusted eluent was centrifuged for 15 min at 2500 ×g at 4 °C and then the pellet containing the flocculated virus was resuspended in 30 mL of 0.15 M sodium phosphate (pH 9) (Cat. No. 255793, Sigma-Aldrich, USA). For complete dissolution, the precipitate was then shaken at room temperature at 160 rpm for 10 min on an orbital shaker. The sample was centrifuged again at 4000 ×g for 10 min at 4 °C to remove impurities, and the virus-containing supernatant was pH adjusted to 7.0–7.5 using 1.2 M HCl. Lastly, the virus-containing solution was filtered through a 0.22 µm sterile filter (Cat. No. SLGPM33RS, Millipore, USA) and transferred to a Vivaspin 20 unit (30,000 MWCO, Sartorius Stedim, Cat. No. VS2022, Germany) for tertiary concentration. The filtrate was centrifuged at 4000 ×g at 4 °C until the final volume was less than 400 µL. The solution was washed with 1 mL of sterile 0.15 M sodium phosphate (pH 7–7.5) and centrifuged at 4000 ×g at 4 °C for a final volume of 200 µL (Ijzerman et al., 1997). The concentrated virus filtrate was used for RNA extraction or stored at -80 °C until further analysis.

2.5. SARS-CoV-2 RNA extraction and viral quantification

RNA extraction of the concentrated viral filtrate was conducted using an AllPrep PowerViral DNA/RNA Kit (Cat. No. 28000-50; QIAGEN, Germantown, MD, USA) following the manufacturer's protocol. 10 µL of total RNA was then reverse transcribed using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, cat. No. 4368814). The resulting cDNA was then used for SARS-CoV-2 gene quantification with ddPCR assays.

By targeting the nucleocapsid (N) gene and the envelope (E) gene of SARS-CoV-2 (Table 2), the N gene assays employed two primer and probe sets, one from the China Centers for Disease Control and Prevention (CN-CDC) (Suo et al., 2020) and one from the United States CDC (US-CDC) (Hirotsu et al., 2020; Jung et al., 2020; Wu et al., 2020). The ddPCR assay used for the E gene is based on the E_Sarbeco primers and probe set (Corman et al., 2020) recommended by the World Health Organization (WHO). Gene amplifications were conducted using 20 µL reactions containing ddPCR supermix for probes (Bio-Rad, cat. No. 1863024), DNase- & RNase-free water, 900 nM of forward and reverse primers, 250 nM of probe, and cDNA templates.

Table 2
Primers and probes used in the SARS-CoV-2 ddPCR assays.

| Target gene | Oligonucleotide | Sequence | Reaction conc. | Reference |
|-------------------------------|-----------------|--------------------------------|----------------|--|
| Envelope protein (E) gene | E_Sarbeco_F | ACAGGTACGTTAATAGTTAATAGCGT | 900 nM | Corman et al., 2020 |
| | E_Sarbeco_R | ATATTGCAGCAGTACGCACACA | 900 nM | |
| | E_Sarbeco_P | FAM-ACACTAGCCATCCTTACTGCGCTTCG | 250 nM | |
| Nucleocapsid protein (N) gene | N_CNCDC_F | GGGGAACCTTCTCTGCTAGAAT | 900 nM | Suo et al., 2020 |
| | N_CNCDC_R | CAGACATTTTGCTCTCAAGCTG | 900 nM | |
| | N_CNCDC_P | HEX-TTGCTGCTGCTTGACAGATT | 250 nM | |
| | N2_USCDC_F | TTACAACATTGGCCGCAAA | 900 nM | Wu et al., 2020; Jung et al., 2020; Hirotsu et al., 2020 |
| | N2_USCDC_R | GCGCGACATCCGAAGAA | 900 nM | |
| | N2_USCDC_P | FAM-ACAATTGCCCCAGCGCTTCAG | 250 nM | |

Table 3

Summary statistics of gene concentration per liter (GC/L) of SARS-CoV-2 N2 and E genes, MST (Rum2Bac and HF183) and *E. coli* colony forming units (CFU) per 100 mL for the 25 stormwater samples.

| | SARS-CoV-2 gene concentrations (GC/L) | | | Microbial source tracking (GC/L) | | Fecal indicator (CFU/100 mL) |
|--------|---------------------------------------|--------------------|--------------------|----------------------------------|--------------------|------------------------------|
| | US-CDC N2 | CN-CDC N2 | E | HF183 | Rum2Bac | <i>E. coli</i> |
| Mean | 2.38×10^2 | 1.57×10^2 | 1.06×10^2 | 8.58×10^3 | 3.56×10^2 | 1.48×10^5 |
| Median | 0 | 0 | 0 | 3.50×10^3 | 0 | 4.75×10^4 |
| Min | 0 | 0 | 0 | 0 | 0 | 5.00×10^2 |
| Max | 1.23×10^3 | 8.89×10^2 | 1.83×10^3 | 1.43×10^5 | 8.00×10^3 | 1.05×10^6 |

Parallel to the gene quantifications of the MST targets, droplet generation using the QX200 Droplet Generator (Bio-Rad) was followed by amplification of SARS-CoV-2 genes using a Bio-Rad C1000 Touch Thermal Cycler (Bio-Rad, Hercules, CA, USA) with the following conditions: 94 °C for 10 min, 40 cycles of denaturation and annealing/extension at 94 °C for 30 s and 60 °C for 60 s, respectively, followed by 98 °C for 10 min and then a final hold of 4 °C. Following amplification, target gene concentrations were determined using a QX200 droplet reader (Bio-Rad) and QuantaSoft (V 1.7; Bio-Rad). The limit of detection (LOD) for all assays conducted in this study was 667 GC/L. For a sample to be considered SARS-CoV-2 positive, a single gene, either E or one of the two detection pathways for the N2 gene, must be detected in the samples with concentrations greater than the LOD.

2.6. Data analysis

Statistical analyses were performed using R statistical software (R Core Team, 2021). Normality was assessed using the Shapiro-Wilk test for all datasets. All data were non-normally distributed except for the *E. coli* data. Because most data were nonparametric, the Spearman correlation analysis was used to determine relationships between SARS-CoV-2 genes and indicator bacteria. Instances where the SARS-CoV-2 E and N2 genes were not detected were included as zero concentrations in analyses. A correlation analysis was used to assess relationships between gene concentrations, rainfall patterns (depth, duration, and antecedent dry period), and indicator bacteria concentrations.

Sewersheds were grouped by county in accordance with the highest resolution data from the ODH (2021) COVID-19 dashboard. Sewershed S1 (Table 1), in Montgomery County, was located approximately 0.6 km north of the Warren County border; due to the minimal distance from Warren County and for simplicity of analysis, this sewershed was grouped with Warren County. Specific COVID-19 infection data at the county level were obtained from the ODH dashboard (ODH, 2021) for the day of and week immediately preceding the stormwater sample collection date. These data were normalized by calculating the number of infections per 100,000 people within each county. Multiple regression analyses were used to determine trends between SARS-CoV-2 target gene concentrations and county-specific COVID-19 infection data for the day of sample collection, the seven-day average in the week prior to sample collection, and the seven-day cumulative infections for the week prior to sample collection. This analysis was repeated for each gene in each county for all sampling windows except for Greene County, which was excluded from these analyses due to the low number of samples. Because the data were non-parametric, the Wilcoxon rank sum test was conducted for the sampling window in Warren County and both

Table 4

Spearman's correlation coefficients for SARS-CoV-2 and microbial source tracking genes. Statistically significant values (p -values < 0.05) are indicated by *.

| | N2 (CN-CDC) | N2 (US-CDC) | E | Log HF183 | Log Rum2Bac | Log <i>E. coli</i> |
|--------------------|-------------|-------------|-------|-----------|-------------|--------------------|
| N2 (CN-CDC) | 1 | 0.18 | 0.41* | 0.23 | -0.13 | 0.35 |
| N2 (US-CDC) | | 1 | -0.05 | 0.22 | 0.23 | 0.63* |
| E | | | 1 | 0.42* | -0.18 | 0.25 |
| Log HF183 | | | | 1 | 0.17 | 0.06 |
| Log Rum2Bac | | | | | 1 | 0.39 |
| Log <i>E. coli</i> | | | | | | 1 |

sampling windows in Franklin County comparing the SARS-CoV-2 gene concentrations and infection data normalized by county population.

The Kruskal-Wallis test, a non-parametric analysis of variance (ANOVA), was used to determine if the SARS-CoV-2 gene concentration datasets significantly varied when grouped by distinct land use or by city location (i.e., sewersheds located in Springboro and Xenia were grouped as Dayton and sewersheds located in Columbus were grouped). Since four distinct land uses were present (SFR, MFR, LI, and Comm), Dunn's test was used as a multiple comparison post-hoc test to determine significant differences in gene concentrations between each of the land uses. The Wilcoxon ranked sum test was used to determine significant differences of gene concentrations between the Columbus and Dayton sewersheds. Significant relationships were determined at the $\alpha = 0.05$ level.

3. Results

3.1. Presence of SARS-CoV-2 in stormwater

Out of 25 analyzed stormwater samples, 22 samples (88%) were found to have detectable levels of SARS-CoV-2. *E. coli* was found present in all 14 samples analyzed, ranging from 5.00×10^2 to 1.05×10^6 CFU/100 mL; these concentrations were consistent with previous studies exploring fecal contamination of stormwater (Lee et al., 2020; Sidhu et al., 2012; Schoen et al., 2017). Mean human-specific fecal markers (HF183) (8.58×10^3 GC/L) were more than twenty times greater than mean Rum2Bac (3.56×10^2 GC/L), the fecal marker associated with ruminant fecal material, suggesting that a sizeable portion of the fecal contamination of stormwater from these 10 sewersheds is from human sewage (Table 3).

3.2. SARS-CoV-2 gene-to-gene, MST, and *E. coli* correlation analyses

The SARS-CoV-2 (E gene) and log HF183 concentrations were significantly correlated ($p = 0.03$; Table 4). A significant correlation was also observed between the SARS-CoV-2 (US-CDC N2) gene and log *E. coli* concentrations, with a correlation coefficient of 0.63 (Table 4). When comparing between the SARS-CoV-2 genes, there was a significant correlation between the CN-CDC N2 and E genes with a correlation coefficient of 0.41.

3.3. SARS-CoV-2 and FIB concentrations in land use

The target gene (E, US-CDC N2, CN-CDC N2) concentrations were compared to predominant land use for each sewershed (Fig. 1).

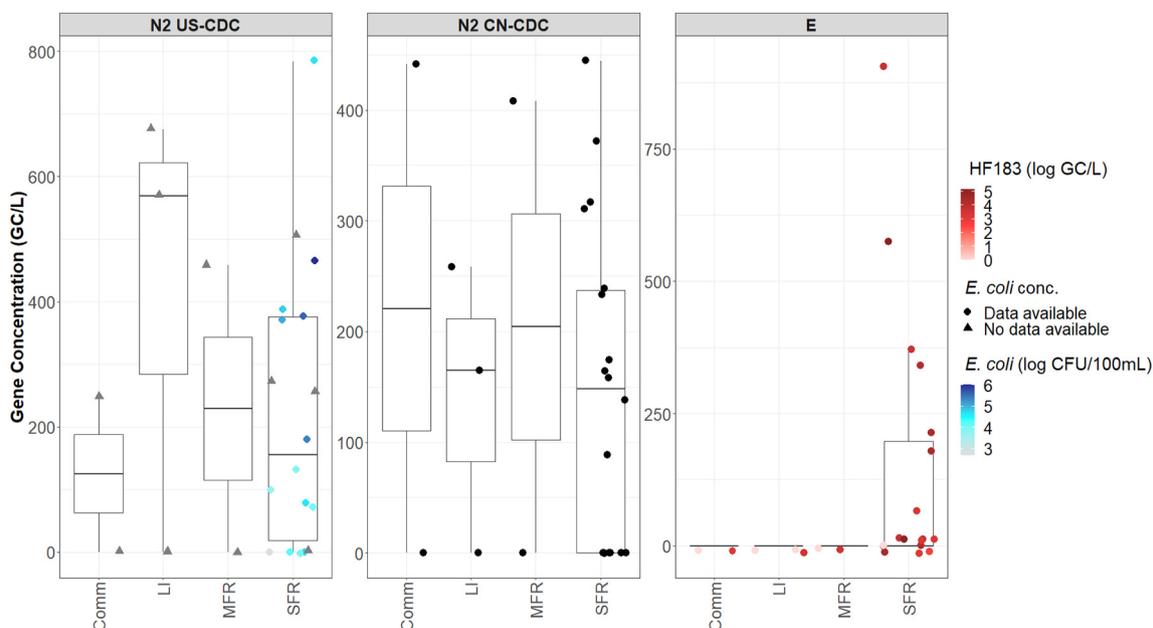


Fig. 1. SARS-CoV-2 target gene concentrations grouped by land use. *E. coli* concentration data availability noted by circular datapoints and non-availability by triangular datapoints. Color ramps indicating concentrations are included in for *E. coli* in the N2-US-CDC graph and HF183 in the E graph due to significant correlations (Table 4) between these data and the genes in the plots they are depicted in.

Commercial and light industrial land uses had the highest concentrations of both N gene amplifications with a mean value of 1.95×10^2 GC/L (Comm) and 2.53×10^2 GC/L (LI) for the US-CDC N2 gene and 1.70×10^2 GC/L (Comm) and 2.21×10^2 GC/L (LI) for the CN-CDC N2 gene. The highest concentration of the SARS-CoV-2 E gene was present in the SFR land use (mean 1.77×10^2 GC/L). Of the 16 samples taken from the SFR land use, 15 (94%) had detectable levels of SARS-CoV-2.

3.4. SARS-CoV-2 concentration and county infection data analyses

No significant trends emerged between the county infection data and the SARS-CoV-2 gene concentrations in stormwater samples in any of the counties or sampling windows. Multiple linear regression of the FIB data from the Franklin County sites failed to yield significant relationships with county infection data. Wilcoxon ranked sum tests for each of three SARS-CoV-2 gene amplification pathways were conducted for both sampling windows in Franklin County and the sampling window for Greene County (Fig. 2). *P*-values for these *t*-tests were above 0.05.

3.5. SARS-CoV-2 concentration and rainfall characteristic analyses

A significant positive correlation ($\rho = 0.6$, $p = 0.001$) was observed between antecedent dry period (ADP) and the US-CDC N2 gene. Combinations of all other SARS-CoV-2 genes or indicator bacteria with rainfall characteristics were not significant. Likely, the lack of significant correlation between rainfall characteristics and SARS-CoV-2 data is the result of the low concentration of the virus in stormwater, high LOD, and relatively small sample size in this study. Improved extraction processes of viral RNA and increased sample size may help elucidate these connections in future studies.

3.6. SARS-CoV-2 concentration data analysis grouped by county and city data

Utilizing the Kruskal-Wallis test, no significant differences were observed between the SARS-CoV-2 genes and the following groupings: single land uses, SFR grouped against all other land uses, and SFR and MFR grouped against LI and Comm land uses. The Kruskal-Wallis test

showed significant differences between the following groupings and the SARS-CoV-2 E gene but not the N2 genes: Columbus, Xenia, and Springboro grouped against each other ($p = 0.049$), and Columbus grouped against Dayton ($p = 0.014$). A Dunn's test post-hoc analysis on the Columbus, Xenia, and Springboro groupings showed that Columbus had a significantly higher ($p = 0.01$) concentration of the SARS-CoV-2 E gene than Springboro. Both Dunn and Wilcoxon post-hoc tests showed that Columbus had a significantly higher concentration ($p = 0.007$ and $p = 0.016$, respectively) of the SARS-CoV-2 E gene than Dayton.

4. Discussion

Of the samples collected between May 10th and July 24th, 88% had detectable levels of at least one SARS-CoV-2 gene. Of the samples taken from SFR land uses during this same sampling window, 94% had detectable levels at least one SARS-CoV-2 gene. The concentrations of SARS-CoV-2 genes in these stormwater samples were lower than what was detected in wastewater samples collected during the same period of the pandemic in northeastern United States, likely due to the dilute nature of fecal matter in stormwater (Peccia et al., 2020).

The presence of human-specific fecal contamination in stormwater is a potential cause for concern as a vehicle of transmission for SARS-CoV-2. Given that only two of the 25 samples collected for SARS-CoV-2 analysis had detectable levels of the Rum2Bac gene while 21 of the 25 samples tested had detectable levels of HF183, we concluded that the majority of fecal bacteria detected in this study were likely human-associated, though these data were not significant in our results ($\rho = 0.06$, $p = 0.85$), likely due at least in part to the small sample size. Further, data from Csiszar et al. (2020) suggests that animals, including ruminants, make up a minority of SARS-CoV-2 infections (Center for Disease Control, 2020). Based on this, it is unlikely that non-human mammals were the source of the SARS-CoV-2 genes detected in this study. However, whole genome sequencing would need to be completed to confirm these findings.

Theoretically, all genes within the SARS-CoV-2 genome should correlate with one another; this was not the case herein. It was determined through correlation analysis that only the CN-CDC N2 and E genes correlated with one another. The employed detection and quantification

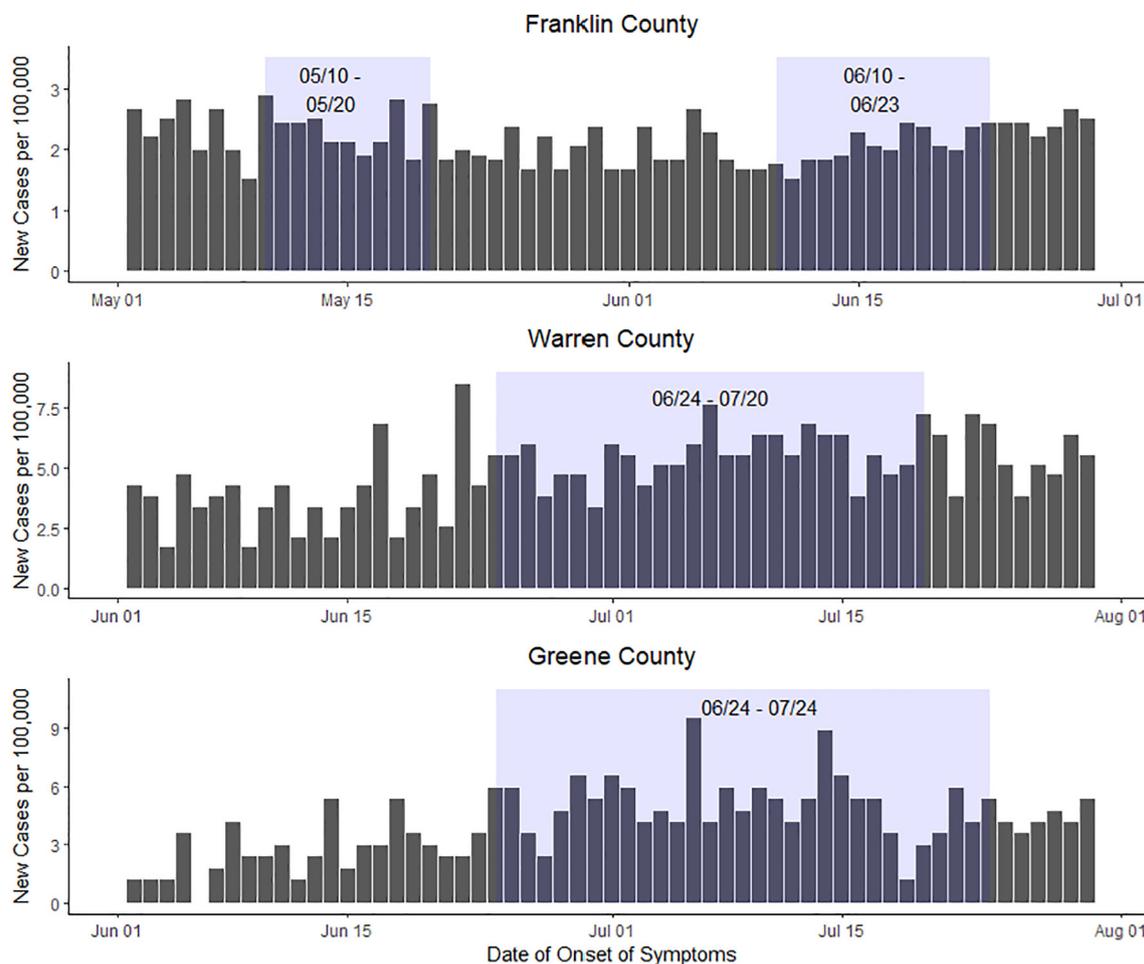


Fig. 2. Per 100,000 new caseload counts of COVID-19 infections in sampled counties in 2020 with sampling date ranges highlighted.

methods were less sensitive to low levels of SARS-CoV-2 present in these samples. As the limit of detection is lowered and gene copies per liter of SARS-CoV-2 in solution increases, gene-to-gene and gene-to-HF183 correlations should increase past the threshold of significance. This study was conducted in the early months of the SARS-CoV-2 outbreak and utilized less sensitive concentrating methods (Ai et al., 2021) since few published methodologies existed at the time. Although concentrating methods varied, it was clear within the scientific community at the time what primers and probes proved sufficient for SARS-CoV-2 detections, supported by both WHO and CDC scientists around the world, and we ruled out poor primer and probe specificity as a possible cause for lack of detection of SARS-CoV-2 genes in the collected samples (Ai et al., 2021; Center for Disease Control, 2020; Corman et al., 2020). Moreover, increased sample sizes could also improve potential correlations which should be explored in future studies.

As we detected SARS-CoV-2 and other fecal markers in stormwater, albeit with low sensitivity, this study unveils the possible strength in utilizing wastewater-based viral concentration methods for other water types, such as stormwater. As the science around viral concentration in water developed throughout the pandemic, concentration methods improved to better process larger volumes of dirtier water and to better fit the needs of SARS-CoV-2 surveillance research (Ai et al., 2021; LaTurner et al., 2021). With the relatively low viral concentrations observed in the collected stormwater, it would be possible to process larger volumes of water using better-understood methods for the same purpose of viral surveillance moving forward. Stormwater as a matrix of interest during current and future public health crises may grow, and confirming feasible methods for this research is important. Knowing what we now understand as more appropriate concentrating

methods, and confirming that these methods do overlap for both wastewater and stormwater, will support best practice in the future.

A single correlation between rainfall characteristics (ADP) and SARS-CoV-2 genes (US-CDC N2) was present in our data. Rainfall depth, duration, and intensity influenced concentrations of SARS-CoV-2 in stormwater less than ADP in this study because as time between storms increases, pollutant loads accumulate in the sewersheds, including genetic material from SARS-CoV-2. The very strong correlation between the US-CDC N2 gene and ADP ($\rho = 0.6$, $p = 0.001$) suggests that buildup and wash-off processes may be at play. Further research is required to elucidate further relationships between viral genes and rainfall characteristics.

The surface of the SARS-CoV-2 envelope is positively charged; however, the spike proteins which protrude from the envelope and are responsible for binding net an overall negative charge (Pawłowski, 2021; Hassanzadeh et al., 2020). The charge of particles strongly influences sorption to sediment surfaces (Björklund and Li, 2018; Chen et al., 2013), with negatively charged particles typically repelled by sediment in stormwater. First flush events are a phenomenon observed in stormwater runoff where early stages of the hydrograph contain disproportionately high pollutant loads compared to the remainder of the hydrograph (Perera et al., 2021). High sediment loads are commonly documented in first flush events (Chow and Yusop, 2014; Hathaway and Hunt, 2011). Other pollutants are present in first flush events as well, and of these, most are positively charged and are bound to the negatively charged sediment surfaces (Holzmann et al., 2021; Taebi and Droste, 2004). Rainfall characteristics often significantly impact the first flush, especially intensity, duration, and depth of rainfall (Tiefenthaler and Schiff, 2001; Zuraini and Alias, 2020). Given that

SARS-CoV-2 has negatively-charged spike proteins that are unlikely to bind to negatively-charged sediment surfaces, concentrations of the virus in stormwater might not be significantly influenced by rainfall characteristics that cause the first flush. This could explain why SARS-CoV-2 did not correlate with rainfall depth, duration, or intensity.

The results of the Kruskal-Wallis tests showed significant differences between the SARS-CoV-2 E gene and the groupings of Columbus, Xenia, and Springboro and the grouping of Columbus and Dayton. These differences may be related to the differences in caseloads by county. Franklin County had more COVID-19 infections but lower per capita infections than of the other counties for the entire sampling duration (ODH, 2021). With higher caseloads and higher density housing, we expect higher viral loads in wastewater and subsequently stormwater. The lower population densities present in Xenia and Springboro explains the low concentration of SARS-CoV-2 in stormwater, especially when compared to the relatively high population density in Columbus. Again, we did not see this same pattern emerge for either N2 gene quantified herein.

Of the samples obtained in Franklin County, 100% had detectable human-specific fecal marker and 100% had *E. coli* concentrations present. *E. coli* concentrations were comparable in number to Lee et al. (2020), where it was concluded that stormwater could contain what the authors refer to as "high" concentrations of enteric bacteria, including ruminant- and human-associated enteric microorganisms. Given the results from APHIS (2021b) identifying SARS-CoV-2 antibodies in white-tailed deer from two US states that border Ohio, and the recent report of SARS-CoV-2 virus in Ohio deer (APHIS, 2021a), continued investigations of both human and deer SARS-CoV-2 infections and surface water contamination are warranted. Our data are too sparse to draw any definitive conclusions. Evidence is mounting for white-tailed deer as a potential reservoir for SARS-CoV-2, and if these deer shed virus into the environment (e.g. fecally), they may also be a feasible source for the virus in stormwater. Experimental studies confirm that deer are highly susceptible to SARS-CoV-2 and can transmit the virus vertically and horizontally (Palmer et al., 2021; Cool et al., 2021; Center for Microbiome Science, 2021). Furthermore, active research across the state of Ohio has brought together wildlife, microbiome, and veterinary scientists to study and test various species that may be susceptible to or high risk for SARS-CoV-2 contraction. Hypothetically, deer could be contaminating the stormwater, although we did not find strong support for ruminant fecal contamination in stormwater (i.e., Rum2Bac) but this warrants further investigation and contradicts a previous study that did see ruminant contamination (Lee et al., 2020). Further analyses should include other MST markers for other animal sources of interest, especially those that are found to have had a SARS-CoV-2 variant, to better understand the interaction between the virus, humans, animals, and the environment.

Stormwater can serve as a key matrix of study for future exploration of pathogens in the environment. The severity and frequency of emerging infectious disease outbreaks are expected to increase in the future due largely to the effects of increasing extreme weather events (Redding et al., 2019; Hertig, 2019; Sanderson and Alexander, 2020) and increased population density (Liu, 2020; Aabed and Lashin, 2021). Given the lack of data surrounding the potential transmission pathway of SARS-CoV-2 via contaminated surface waters, the increasing risks of outbreaks because of climate change, continued urbanization worldwide, and aging sewer infrastructure, it is imperative that stormwater be explored as a real and present reservoir of SARS-CoV-2 and other potential pathogens and contaminants in the future.

5. Conclusion

This study was one of the first to detect SARS-CoV-2 in stormwater from the early waves of the COVID-19 pandemic in the United States, between May 10th and July 24th, 2020. This study confirmed the presence of SARS-CoV-2 in stormwater. The MST data collected suggests that the majority of fecal contamination present in the samples did

not come from ruminant sources but from human sources. The viability of the virus in surface water and wastewater is still poorly understood, and further analysis is necessary to better understand the relationship between the virus and the water it may contaminate.

With respect to how viral load relates to rainfall characteristics, given the small data set, wide variation in land use, and relatively high LOD, it is possible that any of the SARS-CoV-2 genes could correlate with a wide array of rainfall characteristics, though this is speculation. Likely, the lack of significant correlation between rainfall characteristics and SARS-CoV-2 data is the result of the low concentration of the virus in stormwater, the high LOD, and the relatively small sample size in this study. Improved extraction processes of viral RNA and increased sample size may help elucidate these connections in future studies. A larger, more robust data set is required to fully investigate the relationship of viral load and rain characteristics.

This study makes no claims about transmissibility or the likelihood of contracting SARS-CoV-2 from surface waters, only investigating whether it is detectable. Follow up studies should investigate whether SARS-CoV-2 is intact, viable, infectious, and transmissible through fecal-oral (enteric) routes. Stormwater is a conveyance mechanism for a variety of pathogens and is one cause of increased risks associated with waterborne diseases in increasingly populated urban areas. This study showed that urban stormwater is subject to contamination from SARS-CoV-2, among other pathogens, and should be considered as a potential public health threat. Future work should focus on strategies to reduce bacterial and viral contamination of stormwater prior to discharge to surface waters.

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CRedit authorship contribution statement

Kay Bernard: Writing – original draft, Formal analysis, Data curation, Visualization. **Angela Davis:** Writing – original draft, Methodology, Investigation, Visualization. **Ian M. Simpson:** Writing – original draft, Data curation, Visualization. **Vanessa L. Hale:** Funding acquisition, Project administration. **Jiyoung Lee:** Conceptualization, Project administration, Funding acquisition, Supervision, Writing – review & editing. **Ryan J. Winston:** Project administration, Funding acquisition, Supervision, Writing – review & editing.

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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