Environmental Science & Technology Letters

pubs.acs.org/journal/estlcu

Letter

Complementing RNA Detection with Pharmaceutical Monitoring for Early Warning of Viral Outbreaks through Wastewater-Based Epidemiology

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Cite This: *Environ. Sci. Technol. Lett.* 2022, 9, 567−574

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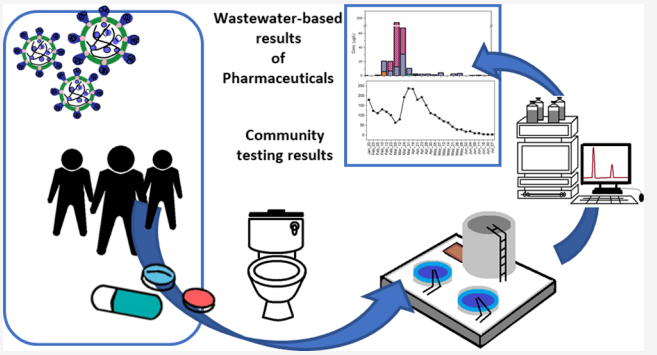
ABSTRACT: Wastewater-based epidemiology using viral nucleic acids to predict community viral outbreaks has many challenges, including differences in viral shedding of infected individuals and interference from the wastewater matrix. In this study, we demonstrate that monitoring pharmaceutical residues in untreated sewage provides complementary information that correlates with future occurrences of viral outbreaks. We monitored 63 pharmaceutically active compounds, including antivirals used to treat COVID-19 and influenza and over-the-counter drugs commonly used to relieve the symptoms of infection. Weekly sampling was conducted at four municipal sewage treatment plants in Western New York. Residues of drugs associated with managing COVID-19 symptoms were detected, including azithromycin (1.99−5.00 μg/L), chloroquine (0.01−33.00 μg/L), hydroxychloroquine (0.05−30.54 μg/L), and lopinavir (13.75−181.20 μg/L). A significant correlation ($p < 0.001$) was observed between the total COVID-19-related drugs detected and the 5-day rolling averages of reported cases. Acetaminophen concentrations spiked approximately 2.5 weeks before a spike in SARS-CoV-2 RNA copies in all wastewater treatment plants sampled. The results suggest over-the-counter analgesic concentrations, in particular, acetaminophen in raw sewage to be used to complement viral RNA data as an early warning system for effective management of viral outbreaks at the community level.



KEYWORDS: *COVID-19, wastewater-based epidemiology, LC-MS/MS, RT-qPCR, antiviral drugs, pandemic, analgesics, over-the-counter drugs*

INTRODUCTION

In the recent decade, infectious diseases such as coronavirus 2019 (COVID-19) and other high-threat diseases have surged to pandemic levels. The immense uncontrollable negative impact posed by these diseases has underscored the importance of tracking early trends of viral outbreaks to prevent the occurrence of catastrophic situations. For instance, COVID-19 has led to an unprecedented global public health crisis and the death of more than 6.2 million people to date.[1] Depending on the severity, hospitalization rates can increase and intensify the shortages of clinical supplies, hospital beds, public health services, and reagents and kits for clinical tests.[2,3] Prediction of viral outbreaks is critically important to combating resource shortages and requires robust and sensitive techniques to effectively track a broad range of viral infections.

Several disease monitoring strategies have been implemented, such as sentinel surveillance,[4] clinical-based surveillance,[5] prescription rates,[6] and surveys.[7] Each of these techniques has advantages and limitations,[8] and the results can vary on the basis of resources and the sophistication of public health facilities in a country.[9] One of the main pitfalls of currently available systems is that most fail to identify critical locations and sufficient early warning to predict disease outbreaks. Wastewater-based epidemiology (WBE) has attracted attention as an emerging outbreak surveillance tool due to its advantages, including flexibility, cost-effectiveness in sample collection, near real-time data, and capacity to provide a snapshot of an entire community's health compared to traditional surveillance systems.[10−12] Wastewater-based epidemiology is based on collecting relevant information from untreated sewage, through biological and/or chemical analyses

Received: April 18, 2022
Revised: May 13, 2022
Accepted: May 13, 2022
Published: May 18, 2022

to detect biomarkers associated with infectious diseases or environmental risk factors.[13,14] Because human excreta contain biomarkers indicative of multiple aspects of risks, raw sewage is an ideal source of information.[12] As a result, WBE has been widely implemented for the detection of SARS-CoV-2,[15] as well as illicit drug usage[16,17] and consumption of pharmaceuticals.[13]

Current WBE efforts to predict disease outbreaks, including COVID-19, are based on monitoring viral RNA using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) techniques.[18−25] However, the presence of PCR inhibitors in wastewater,[26,27] bias among different commercially available PCR kits,[28] and nondetection of trace concentrations of viral RNA in wastewater limit the utility of this technique.[26] Recently, allele-specific RT-qPCR[29] and sensitivity- and selectivity-enhanced RT-qPCR assays have been developed to address sensitivity problems for the determination of variants of SARS-CoV-2.[30] Nevertheless, detection of viral RNA in wastewater remains a critical challenge.[31] Therefore, complementary monitoring approaches using antiviral or other therapeutic drugs used to manage viral infections could strengthen RNA-based methods in WBE early warning systems.

An infected person will typically show common symptoms such as fever, cough, and headache during the viral incubation period leading to self-medication using over-the-counter (OTC) drugs.[11] When symptoms persist, patients acquire prescription medication. A major portion of the ingested pharmaceuticals' active ingredients are excreted in urine.[32] Most active ingredients of pharmaceuticals are not readily degraded in raw sewage, and the degradation during treatment will depend on the design and operation of the wastewater treatment plant (WWTP).[33] Thus, tracking active ingredients of OTC drugs in influent wastewater can provide early information that may signal potential disease outbreaks. The occurrence of pharmaceuticals in sewage depends on factors such as regional medical needs, living standards, and temporal aspects.[34] Consequently, selection of the most relevant biomarkers remains a challenge for complementary WBE analyses. Analysis of pharmaceuticals to complement WBE studies can be challenging due to pharmaceutical treatment overlap across multiple diseases,[35] while some diseases require a range of pharmaceuticals during the course of treatment depending on the severity of the stage.[36] For instance, many of the antiviral drugs approved or under investigation for COVID-19 by the U.S. Food and Drug Administration (FDA) are repurposed drugs used in other viral infection treatments.[37] In such cases, monitoring of OTC pharmaceuticals used in treating symptoms of infection could facilitate the development of an accurate predictor of disease outbreaks and may be combined with monitoring of prescription drugs to validate the predictive model.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods are widely used to analyze trace-level pharmaceuticals due to their high water solubility, low vapor pressure, and the thermally labile nature of most pharmaceutical residues. The objectives of this study are (1) to quantify targeted antiviral drugs and other related pharmaceuticals in untreated sewage using LC-MS/MS, (2) to investigate correlations between drug concentrations and SARS-CoV-2 viral RNA copies detected in wastewater, and (3) to evaluate the potential use of drug residues in community-based WBE analysis to complement RNA data to predict community-level outbreaks of viral infections.

MATERIALS AND METHODS

Sample Collection. Untreated wastewater (450 ± 50 mL) was collected twice each week from January 20, 2021, to July 9, 2021, at four municipal WWTPs in Erie County, NY. Post-outbreak sampling was performed from January 20, 2022, to March 3, 2022. Samples were collected using 24 h composite samplers and stored at 4 °C during transportation. Time-weighted composite samplers were operated for 24 h at 4 °C to collect 100 mL of influent wastewater every 30 min. The location and service population of each WWTP are detailed in the Supporting Information.

Sample Extraction and Analysis. Samples were concentrated and cleaned using solid-phase extraction (SPE) according to Singh et al.[38] upon arrival at the laboratory to minimize analyte degradation. Samples were analyzed on an Agilent 6410 triple quadrupole mass spectrometer equipped with a model 1100 high-performance liquid chromatography system (Palo Alto, CA). The autosampler was operated at 4 °C. All LC-MS/MS analyses were performed using multiple-reaction monitoring in positive electrospray ionization mode. Chromatographic separation was achieved on an XSelect Charged Surface Hybrid (CSH) C18 (2.1 mm × 150 mm, 2.5 μm) analytical column (Waters, Milford, MA). Quantification was performed using isotope dilution for analytes with an available isotopically labeled analogue or standard addition for all others. Acetaminophen and caffeine were quantified using a matrix-matched external calibration due to the very high concentrations in sample extracts. A detailed description of targeted analytes, the LC-MS/MS method, and the RT-qPCR technique of SARS-CoV-2 viral load determination are available in the Supporting Information.

Correlation Analysis. Estimated COVID-19 clinical case data were obtained from the Erie County, NY, SARS-CoV-2 wastewater monitoring dashboard.[39] Reported positive COVID-19 cases are classified as "estimates" because the sewersheds do not necessarily follow zip code boundaries. For each location, locally weighted scatter plot smoothing (LOWESS) was used to plot the estimated clinical cases of the populations contributing to each WWTP, copies of SARS-CoV-2 RNA, and population-normalized concentrations of acetaminophen in wastewater (frac. = 0.25). Population normalization was performed with the detected concentrations of caffeine in each sample collected.[40,41] Spearman correlation was performed for the correlation analysis of normalized concentrations of detected antivirals used for COVID-19 treatment and the clinical cases of COVID-19 in each location using the RStudio Statistical Computing Environment.[42] RStudio was also used for the LOWESS smoothing and calculation of rolling averages of the clinical cases[43] with the use of the ggplot2 package.[44]

RESULTS AND DISCUSSION

Mild COVID-19 infections generally can be managed through self-administered analgesics.[45] Severe COVID-19 infections can result in secondary infections[46] that require treatments with antimicrobials.[47] Thus, to determine a relevant set of pharmaceuticals that can serve as biomarkers of community viral infections, including COVID-19, we monitored several

https://doi.org/10.1021/acs.estlett.2c00259
Environ. Sci. Technol. Lett. 2022, 9, 567−574

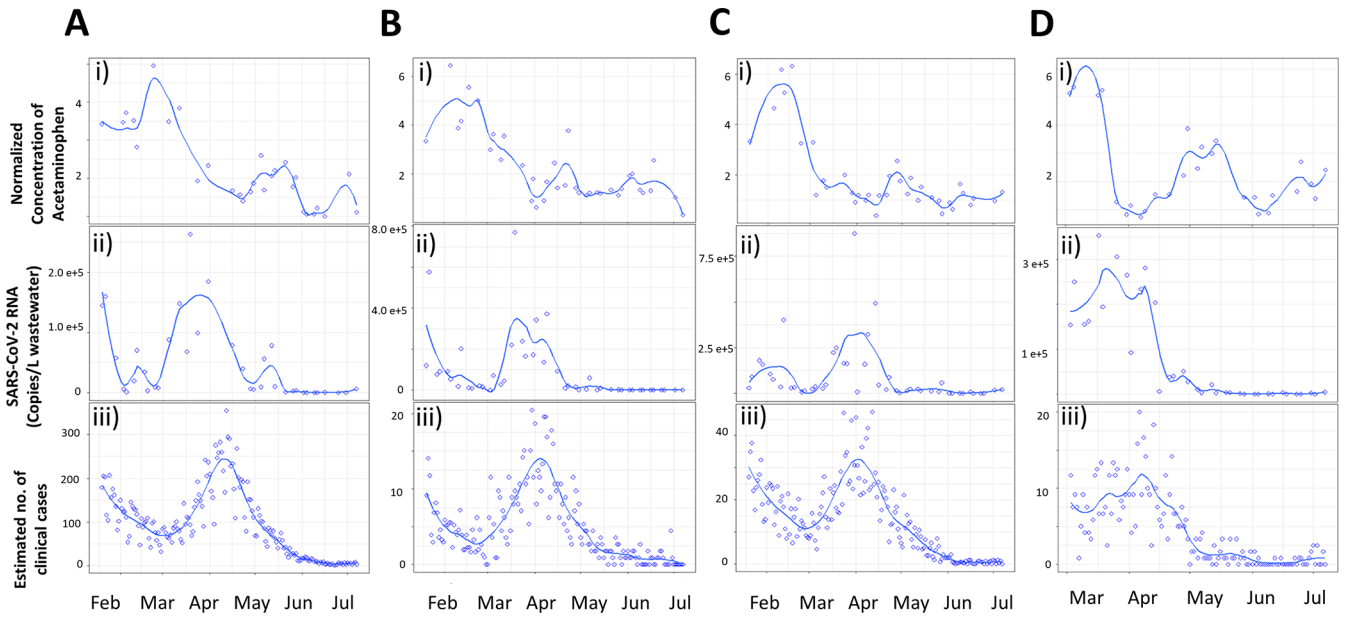


Figure 1. Population-normalized concentrations of detected acetaminophen (i), wastewater severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) RNA concentrations (ii), and estimated clinical cases of coronavirus 2019 (COVID-19) (iii) in (A) Bird Island, (B) City of Tonawanda, (C) Kenmore-Tonawanda, and (D) Lackawanna sampling sites. Concentrations of caffeine in each location were used for population normalization. Locally weighted scatter plot smoothing (LOWESS) was used to show the trends (blue lines). Estimated numbers of clinical cases and RT-qPCR data were obtained from the wastewater monitoring dashboard of Erie County, NY. The study was performed in 2021.

commonly used OTC drugs, anti-COVID-19 drugs, and other drugs used to treat multiple prominent viral infections.

In this study, we detected pharmaceuticals from different classes of drugs, including analgesics, antibiotics, antidepressants, antiepileptics, and antivirals. Acetaminophen, an active ingredient of OTC analgesics, was detected in high frequencies and high concentrations at all four WWTPs. Acetaminophen is widely used as a first-choice treatment for mild pain[48] and is excreted at an average of 4% as the unchanged parent compound via the urinary tract.[49] Additionally, O'Brien et al. have shown that acetaminophen is stable in wastewater for ≤12 h.[50] Hence, the high concentrations of acetaminophen residues observed in wastewater can be related to the increased use of analgesics during the pandemic.[51]

Trends in population-normalized detections of acetaminophen in wastewater are shown in Figure 1 in comparison to SARS-CoV-2 RT-qPCR data and the estimated clinical cases of COVID-19 for each sewershed. In this study, a spike in acetaminophen concentrations for the Bird Island WWTP (Figure 1Ai) was observed between the end of February and early March 2021, which is 2−2.5 weeks earlier than the observed spike in SARS-CoV-2 RNA copies (Figure 1Aii). This gap in the maximum detection of acetaminophen and number of RNA copies may be attributed to the period of incubation of the virus in the human body, the inability of genome determination techniques to quantify trace concentrations of viral genomes, and the lag in the significant community spread of COVID-19. Moreover, the incubation period of SARS-CoV-2 ranges from 2 to 14 days,[52] with 8 days post-onset of symptoms as the expected median duration of viral RNA shedding.[53] The clinical data (Figure 1Aiii) in the community served by the Bird Island WWTP show that the number of reported cases spiked in early April, which corresponds to approximately one week after the observed maximum SARS-CoV-2 RNA copies. This gap is not surprising because COVID-19 testing is typically performed a few days after infection occurs. Similar trends in acetaminophen concentration spikes were observed in the other studied WWTPs (Figure 1Bi−Di). The acetaminophen concentrations spiked approximately prior to 2.5 weeks compared to the spikes in copies of SARS-CoV-2 RNA and approximately 5.5 weeks before the estimated number of clinical cases spiked, for all of the study locations. Because acetaminophen is not specific to mitigating COVID-19 symptoms, spikes in acetaminophen concentrations may also be observed when other pathogen outbreaks occur. Hence, the second acetaminophen spike that occurred around April to May 2021 in each sewershed that did not correlate with COVID-19 RNA data may be a result of another underlying infection, including those caused by other variants of SARS-CoV-2 within the area. The latter infections would not have been indicated in either viral RNA data from wastewater or estimated numbers of clinical cases. Additionally, we analyzed post-outbreak levels of sewage acetaminophen concentrations (Table S4). Figure S7 details the post-outbreak fluctuations of normalized concentrations of acetaminophen, SARS-CoV-2 RNA data, and estimated clinical cases of the study locations. Accordingly, one can see that the post-outbreak acetaminophen concentrations ranged from 93.90 to 6.88 μg/L within the study locations, which is very low compared to spikes detected up to 204.12 μg/L in 2021. These baseline data suggest that the spikes in normalized acetaminophen concentrations during the outbreaks in 2021 can be due to COVID-19 infections within the area.

The incubation period for SARS-CoV-2 virus is estimated to be ≤14 days prior to the onset of symptoms.[54] Predominant symptoms of COVID-19 include fever, headaches, fatigue, dizziness, and abdominal pain, which can be easily mistaken for ordinary flu symptoms.[37,55] Analgesics, in particular, acetaminophen, is widely used for mild symptoms of COVID-19.[56] Thus, OTC drugs become the go-to choice of the general public to address symptoms.[51]

https://doi.org/10.1021/acs.estlett.2c00259
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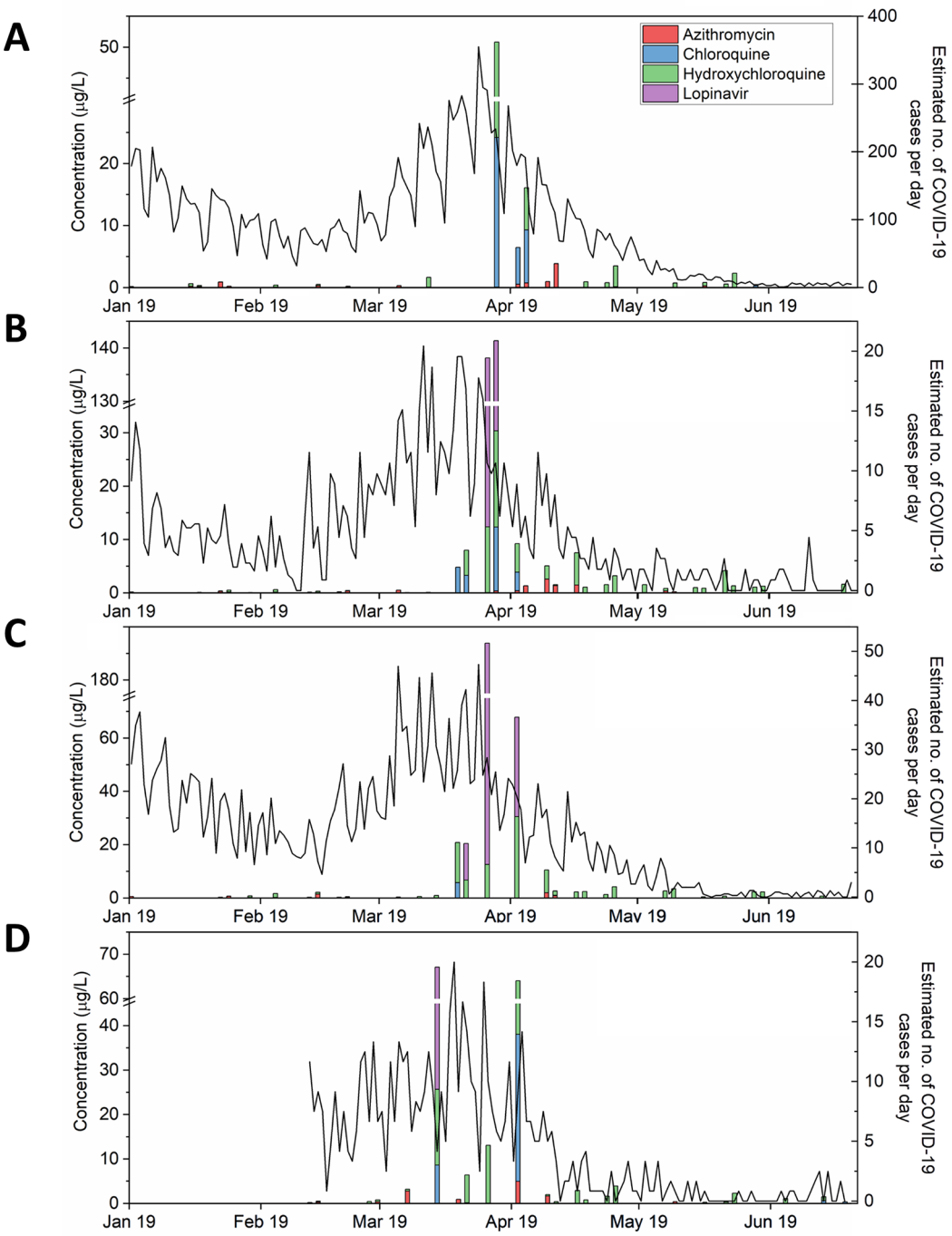

Figure 2. Fluctuations of detected coronavirus 2019 (COVID-19)-authorized drugs azithromycin, chloroquine, hydroxychloroquine, and lopinavir within the sampling period and the estimated clinical cases of COVID-19 in (A) Bird Island, (B) City of Tonawanda, (C) Kenmore-Tonawanda, and (D) Lackawanna sampling sites. The study was performed in 2021.

Population normalization is vital in WBE studies; in fact, it aids in better representation of data for temporal or spatial comparisons. However, population estimation is a challenge mainly because of population mobility during a given time period. Techniques for population estimation relevant to chemical analyses include sewershed receiving water flow rate, the number of connected dwellings to the sewersheds, projected plant capacities, counts of municipal bins, and the sum of the population within suburbs served from census data.[57] Collectively, these methods fail to accurately interpret population mobility. As an alternative approach of establishing a "good" population marker, small molecules that persist in wastewater have been introduced and validated as candidate population markers for WBE.[57] Among these, caffeine, a stimulant, is a good qualitative population marker due to its diverse usages, and it has also been shown previously by Senta et al. that caffeine is stable during 24 h storage at 4 °C.[40] Approximately 1−3% of consumed caffeine is excreted unchanged via urine and is stable at −20 °C for ≤4 weeks.[40] Another advantage of having a solid population marker is that it displays seasonal changes that might cause dilution or concentration of analytes such as melting of ice and high and/or low precipitation periods. Caffeine concentrations (Figures S5 and S6) and caffeine mass loads (Figures S3 and S4) in

https://doi.org/10.1021/acs.estlett.2c00259
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each study location are shown in the Supporting Information. It can be seen that the trends in terms of concentrations and mass loads follow more or less the same for all of the sampling locations, except for the Bird Island WWTP.

We also determined concentrations of antiviral drugs in sewage. In this study, we incorporated seven authorized anti-COVID-19 drugs: dexamethasone, lopinavir, ritonavir, azithromycin, chloroquine, hydroxychloroquine, and remdesivir, Dexamethasone, a corticosteroid, is found to reduce mortality in critical patients on oxygen or ventilation therapy.[47] As reported, the most promising antiviral agent, remdesivir, is used to inhibit viral RNA replication.[58] Chloroquine and hydroxychloroquine were found to potentially block viral entry by altering the structural configuration of cell receptors or by competitively binding with cell receptors.[59] Another effective combinatory treatment approach for COVID-19 was observed for hydroxychloroquine with azithromycin, which has the potential to inhibit viral replication.[60]

The targeted analytes in this study also included non-COVID-19 antivirals that are associated with several viral infections, including human immunodeficiency virus (HIV) and influenza virus. Non-COVID-19 drugs were included because of the prevalence of viral infections based on the Erie County Department of Health's regional data.[61] It should also be noted that co-infections with other diseases could increase the severity of viral infections.[62] Consequently, it is important to incorporate other high-threat pathogenic disease conditions in building pharmaceutical-based models to predict viral outbreaks.

Drugs used in managing COVID-19 infections were detected at various concentrations: azithromycin (min. 1.89 μg/L), chloroquine (min. 0.01 μg/L), hydroxychloroquine (min. 0.05 μg/L), and lopinavir (min. 0.64 μg/L). Antiviral drugs used in the treatment of HIV and hepatitis B infections, including abacavir (min. of 2.64 μg/L), efavirenz (min. 2.57 μg/L), emtricitabine (min. 7.39 μg/L), lamivudine (min. 0.97 μg/L), and zidovudine (min. 15.33 μg/L), were also detected at lower frequencies.

Due to the low frequency of detection and concentrations largely below the limits of detection (LODs) for non-COVID-19 antivirals in sewersheds, only azithromycin, chloroquine, hydroxychloroquine, and lopinavir were further analyzed to examine wastewater concentration trends. Additionally, detected COVID-19-related drugs in this study concur with the contemporary studies performed in other countries.[63] Figure 2 shows the fluctuations of the normalized concentrations of detected COVID-19-related drugs in the targeted WWTPs over the study period. The results showed a spike in their total concentrations during mid-April in samples collected from the Bird Island (Figure 2A), City of Tonawanda (Figure 2B), and Kenmore-Tonawanda WWTPs (Figure 2C); spikes were observed in mid-March and mid-April for the Lackawanna WWTP (Figure 2D). A significant correlation was observed for the Bird Island WWTP ($p < 0.001$) and City of Tonawanda WWTP ($p < 0.05$) with 5-day and 7-day rolling averages of clinical cases, respectively. However, no significant correlations were observed for the remaining sampling sites. Correlation coefficients and p values for the total concentrations of detected COVID-19-authorized antivirals in wastewater and the rolling averages of the clinical cases are listed in Table S3. The correlation between the detected COVID-19-related drugs and clinical cases is stronger in the Bird Island WWTP catchment (population of 437 357) than in the City of Tonawanda WWTP catchment (population of 14 875), the Kenmore-Tonawanda WWTP catchment (population of 70 470), or the Lackawanna WWTP catchment (population of 17 859). These results suggest that the potential utility of pharmaceuticals in WBE analysis will be more reliable for sewersheds that serve larger populations.

Antibiotics such as sulfamethoxazole and its metabolite, acetylsulfamethoxazole, sulfadiazine, sulfamerazine, sulfamethazine, sulfathiazole, and trimethoprim were detected frequently in all WWTP influents. Sulfamethoxazole was detected at $\leq$10.46 μg/L, and its metabolite, acetylsulfamethoxazole, was detected at $\leq$10.48 μg/L. The high excretion rate of sulfamethoxazole ($\leq$98%)[64] and its stability in wastewater[65] can explain its presence at high concentrations in untreated sewage. Trimethoprim, which is typically combined with sulfamethoxazole in therapeutic formulations, was detected at concentrations of $\leq$3.38 μg/L. Trimethoprim has an excretion rate range between 40% and 69%.[66] Other antibiotics, sulfadiazine (minimum of 2.44 μg/L), sulfamerazine (minimum of 0.25 μg/L), sulfamethazine (minimum of 0.56 μg/L), and sulfathiazole (minimum of 0.40 μg/L), were also detected in some WWTP influents but at lower frequencies. Anticonvulsants [carbamazepine (minimum of 6.16 μg/L) and lamotrigine (minimum of 0.49 μg/L)] and antidepressants [bupropion (minimum of 3.06 μg/L), citalopram (minimum of 1.49 μg/L), sertraline (minimum of 1.58 μg/L), and amitriptyline (minimum of 0.35 μg/L)] were infrequently detected at some WWTPs.

Figure S2 shows the fluctuation of each class of pharmaceuticals (total antibiotics, antidepressants, and anticonvulsants) detected in the influent WWTP samples from each sewershed. Except for detected analgesics (in particular acetaminophen), monitored total antibiotics, antidepressants, and anticonvulsants showed no trend related to clinical diagnostics of COVID-19 or copies of SARS-CoV-2 RNA in wastewater.

Because acetaminophen is widely used for other nonviral related symptoms, simultaneous monitoring of antivirals with acetaminophen and other OTC analgesics can be an effective strategy for increasing the degree of confidence of WBE studies by validating early spikes in acetaminophen concentrations with subsequent spikes in antiviral drugs in wastewater. The combination of data for the concentrations of OTC analgesics and antiviral drugs in influent wastewater can be particularly useful when new variants of viruses appear and escape detection by molecular techniques using WBE. While drug concentrations alone will not be able to single out the specific disease that causes the increased consumption of pharmaceuticals, these data can provide a much earlier warning than clinical data. WBE may serve as a surrogate for diseases in which viral RNA detection in wastewater is limited or not possible due to trace concentrations. A surveillance system that provides an early warning for a range of potential community viral outbreaks at the same time could be beneficial for the health care systems and providers in preparing for future catastrophic events.

In this study, we analyzed and monitored trends of various drugs including antivirals, antibiotics, and OTC drugs used to treat and manage symptoms of viral infections to track community outbreaks using WBE. The data obtained demonstrated the potential utility of acetaminophen, a nonprescription analgesic, to predict a viral outbreak approximately 2.5 weeks prior to SARS-CoV-2 RNA detections

https://doi.org/10.1021/acs.estlett.2c00259
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in untreated wastewater. In general, the concentrations of drugs detected in sewage can complement RNA data obtained by RT-qPCR techniques, especially when the concentrations of RNA are below the LODs or if variants of the viruses occur. The advantage offered by analysis of pharmaceutical residues in WBE also includes providing information for a broad spectrum of infections that may cause potential community outbreak, rather than being specific to a particular viral outbreak. This study utilized caffeine, a stimulant, as a population-normalization factor. Future work is required to explore other population markers, such as widely used artificial sweeteners, to apply the technique for a global scale implementation. Further epidemiological studies will be necessary in the future.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.estlett.2c00259.

Methods of pharmaceutical analysis, including chemicals and reagents, the sample extraction procedure, chromatographic conditions, and quality control, method of RNA extraction and RT-qPCR analysis, details of sampling locations, pre- and post-outbreak fluctuations of wastewater-based targeted drug concentrations, RT-qPCR data, and clinical data of COVID-19 infections (PDF)

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This research was funded by the SUNY Prepare Innovation and Internship Seed Grant Program (Grant 21002) and National Science Foundation PIRE-HEARD Grant 1545756. The authors thank Jonathan P. Antle and Md Mahbubul Alam for the technical assistance and the Erie County Department of Health and Division of Sewerage management for the publicly available wastewater monitoring dashboard of RT-qPCR data and estimated number of clinical cases of COVID-19 used in the study locations.

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https://doi.org/10.1021/acs.estlett.2c00259
Environ. Sci. Technol. Lett. 2022, 9, 567−574