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Surveillance of SARS-CoV-2 in nine neighborhood sewersheds in Detroit Tri-County area, United States: Assessing per capita SARS-CoV-2 estimations and COVID-19 incidence

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HIGHLIGHTS

• Occurrence of SARS-CoV-2 RNA in the neighborhood level in Detroit Tri-County area, United States was investigated.
• Application of population markers including water quality markers and human biomarkers in sewage surveillance was assessed.
• Per capita level of SARS-CoV-2 was estimated and correlated with COVID-19 incidences.

ABSTRACT

Wastewater-based epidemiology (WBE) has been suggested as a useful tool to predict the emergence and investigate the extent of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). In this study, we screened appropriate population biomarkers for wastewater SARS-CoV-2 normalization and compared the normalized SARS-CoV-2 values across locations with different demographic characteristics in southeastern Michigan. Wastewater samples were collected between December 2020 and October 2021 from nine neighborhood sewersheds in the Detroit Tri-County area. Using reverse transcriptase droplet digital polymerase chain reaction (RT-ddPCR), concentrations of N1 and N2 genes in the studied sites were quantified, with N1 values ranging from 1.92 × 10^2 genomic copies/L to 6.87 × 10^3 gc/L and N2 values ranging from 1.91 × 10^2 gc/L to 6.45 × 10^3 gc/L. The strongest correlations were observed between cumulative COVID-19 cases per capita (referred as COVID-19 incidences thereafter), and SARS-CoV-2 concentrations normalized by total Kjeldahl nitrogen (TKN), creatinine, 5-hydroxyindoleacetic acid (5-HIAA) and xanthine when correlating the per capita SARS-CoV-2 and COVID-19 incidences. When SARS-CoV-2 concentrations in wastewater were normalized and compared with COVID-19 incidences, the differences between neighborhoods of varying demographics were reduced as compared to differences observed when comparing non-normalized SARS-CoV-2 with COVID-19 cases. This indicates when studying the disease burden in communities of different demographics, accurate per capita estimation is of great importance. The study suggests that monitoring selected water quality parameters or biomarkers, along with RNA concentrations in wastewater, will allow adequate data normalization.
1. Introduction

It has been more than two years since the start of the coronavirus disease 2019 (COVID-19) pandemic which is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). During this period, scientists from multiple disciplines including epidemiology, evolutionary biology and environmental engineering have come a long way in understanding the distribution, transmission, genomic characteristics and risks of SARS-CoV-2 (Ahmed et al., 2022a; Ahmed et al., 2022c; Koelle et al., 2022; Laborde et al., 2020; Li et al., 2022; Lu et al., 2020; Sills et al., 2020; Subbaraman, 2021). Although our understanding about SARS-CoV-2 has improved, challenges ahead remain, with new emerging variants of concern, high transmissibility and COVID-19 risks, and developing control strategies in rural areas and the developing world. Wastewater-based epidemiology (WBE) is suggested to be an important and useful tool to investigate the prevalence and spatial distribution of viruses such as hepatitis virus, poliovirus, noroviruses in the environment. The corresponding wastewater surveillance system to provide scientific evidence for public health decision-making (Ahmed et al., 2022b; Ahmed et al., 2021b; Bibby et al., 2021; Hovi et al., 2012; Lodder et al., 2012; McCall et al., 2021; Miyani et al., 2020; Xagoraraki and O’Brien, 2020; Zhao et al., 2022). Wastewater surveillance systems have been launched to monitor SARS-CoV-2 RNA concentrations and transmission in the various environments, when coping with the COVID-19 pandemic (Ahmed et al., 2021a; Gerrity et al., 2021; Medema et al., 2020; Wu et al., 2021; Xie et al., 2022). Studies associated with SARS-CoV-2 vary in scales, ranging from small campuses (Betancourt et al., 2021; Gibas et al., 2021) to the large national scale programs (Bivins and Bibby, 2021; Gonzalez et al., 2020; Li et al., 2021b; Miyani et al., 2020; Miyani et al., 2021; Scott et al., 2021; Wu et al., 2021; Wu et al., 2022; Zhao et al., 2022). When conducting WBE coupled with targeted clinical testing in 13 dorms in the University of Arizona, both SARS-CoV-2 RNA in the wastewater and COVID-19 cases in the community were investigated, and the results provide evidence for the application of WBE in defined communities (Betancourt et al., 2021). A similar building-level SARS-CoV-2 surveillance study was implemented and found that identification of positive COVID-19 cases can be indicated by WBE (Gibas et al., 2021). All these instances validate the application of WBE as a promising and cost-effective tool to assist in public health responses to disease outbreaks. However, a critical factor associated with successful application of WBE is the accurate population estimation (Sims and Kasprzyk-Hordern, 2020). It is highly recommended by the United States Centers for Disease Control and Prevention (CDC) that normalizing the viral concentrations by the number of people served by the sewer system enables the viral levels comparisons across different sampling locations (CDC, 2022). The variability of population size within a catchment area is dynamic due to many factors such as commuting and tourism. This contributes to various uncertainties in real-time WBE. Additionally, if WBE were to be applied in rural areas or the developing world, where information on the characteristics of catchment areas and populations may not be available, estimating the contributing population will be of great importance.

Although both viral (e.g., pepper mild mottle virus, PMMoV) and human biomarkers have been suggested to normalize the SARS-CoV-2 RNA in the wastewater, however, it is still debated which marker is the most ideal to perform the normalization (Xie et al., 2022). In a most recent study, using coprostanol as a potential biomarker, the strongest correlation between normalized SARS-CoV-2 RNA concentrations and COVID-19 incidences was observed, which demonstrated the promising application of population biomarkers in wastewater surveillance studies (Reynolds et al., 2022). Additionally, 5-HIAA was selected to assess population size in an exploratory study, in which surveillance of SARS-CoV-2 RNA in wastewater from two municipalities in Latvia were discussed (Gudra et al., 2022). To evaluate the application of population biomarkers in normalizing SARS measurements, one can review the efforts and conclusions drawn in the study of drug abuse estimation using WBE, where biomarkers have been widely studied all over the world (Choi et al., 2018).

Two approaches were commonly used to estimate population sizes and compare with census data: measurement of water quality parameters in sewage (biochemical oxygen demand [BOD], nitrogen, etc.) (Tscharke et al., 2019; Zheng et al., 2019) and measurement of population biomarkers (Chen et al., 2014; Gracia-Lor et al., 2017). The advantage of using water quality parameters is that they can be easily measured and tracked, which makes them easily applicable especially in rural areas and the developing world. However, water quality parameters, such as BOD, total nitrogen (TN) or total phosphorous (TP) levels may be influenced by both human and non-human contributions (e.g., industrial discharges, agricultural activities) (Been et al., 2014; Daughton, 2012; Rico et al., 2017). However, population biomarkers in sewage, which are either endogenous compounds, such as creatinine, 5-HIAA, or exogenous substances like caffeine and its metabolites, are contributed by human metabolism only and, therefore, may be surrogates for estimating sewershed populations (Chen et al., 2014; Chiaia et al., 2008; Choi et al., 2018). Compared with the census approach, they may reflect the real-time fluctuation of population.

For example, a breakdown compound of muscle tissues, creatinine was proposed as a potential population biomarker and is widely used clinically (Chen et al., 2014; Chiaia et al., 2008). However, degradability of creatinine in sewer systems was reported, and this affects its potential as a population biomarker (Thai et al., 2014). Neurotransmitter metabolite 5-HIAA was suggested as an eligible population biomarker that is cultural independent and may be reliable for comparisons of population sizes among different countries (Chen et al., 2014). Caffeine is one of the most ubiquitous micro-contaminants found in the untreated wastewater due to its wide usage in many globally popular products (e.g., tea, cola drinks or coffee) as a stimulating agent. It has been suggested as a human biomarker for assessing the real-time fluctuations of population (Daughton, 2012; Froehner et al., 2010). Metabolism of caffeine is extensive and at least 17 urinary metabolites of caffeine are identified (Gracia-Lor et al., 2017). Occurrences of caffeine metabolites in wastewater are still scarce, which limit their application in population estimation. Meanwhile, concentrations of the metabolites depend on human habits, it was found that the average loads of caffeine and its metabolites were slightly lower during the weekend, which may result from the relatively lower consumption of coffee (Senta et al., 2015).

This study focuses on the evaluation of the relationship between the COVID-19 incidences and the measured and estimated per capita SARS-CoV-2 RNA concentrations. It is hypothesized that population normalization with water quality markers and population biomarkers could be a reliable approach to calculate the per capita SARS-CoV-2 RNA concentrations. To test the hypothesis, we collected 486 samples from nine sewersheds of different demographics in the Detroit Tri-County area, MI from December 2020 through October 2021, and quantified SARS-CoV-2 RNA concentrations at a neighborhood level. The population of each community was assessed using water quality parameters (BOD, TKN, total suspended solids [TSS], volatile suspended solids [VSS]) and human biomarkers (creatinine, 5-HIAA, caffeine and its metabolites), which was further validated with the census-reported population in the catchment area.
areas. Correlations between COVID-19 incidences and per capita SARS-CoV-2 RNA concentrations were performed to identify relatively identifiable population biomarkers that can be applied in sewage surveillance studies.

Moreover, using normalized SARS-CoV-2 concentration and COVID-19 incidences, spatial differences between nine sewersheds of different demographics were assessed. Previously, SARS-CoV-2 RNA concentrations in wastewater collected from the Great Lakes Water Authority (GLWA) Water Resource Recovery Facility (WRRF) influent in Detroit, Michigan were quantified and ranged from $10^3$ to $10^5$ gc/L (Miyani et al., 2020). Considering sewage surveillance can capture the presence of virus before the onset of symptoms, an "early warning" system was proposed to try and forecast the second COVID-19 wave in the Detroit metropolitan area by combining the sewage surveillance methodology with local public health records (Miyani et al., 2021; Richardson, 2021). However, sewage surveillance research of SARS-CoV-2 incorporating the contributed population of the studied sewersheds is still limited. To our knowledge, a spatial comparison across the Detroit neighborhoods sewersheds has not been performed. Such spatial investigations of SARS-CoV-2 RNA that take into consideration the social-demographic characteristics of various communities within the Detroit Tri-County would provide important information for understanding county-level comparisons of COVID-19 incidences as well as inform the public health decision-making process.

The study suggests that monitoring selected water quality parameters or biomarkers along with RNA concentrations in wastewater will allow adequate data normalization, especially in areas where detailed sanitary sewage flows in the catchment areas are not available. This opens the possibility of using WBE to assess community infections in rural areas or the developing world where the contributing population of a sample could be unknown.

2. Material and methods

2.1. Sampling sites and sample collection

The Detroit metropolitan area, often referred to as Metro Detroit, is a major metropolitan area in the U.S. and the largest in the state of Michigan. It is known for its developed economies and cultural diversity. The City of Detroit serves as the metropolitan area’s core, and the metropolitan area extends into three adjacent counties: Macomb, Oakland, and Wayne. This area is also referred as the Detroit Tri-County area. Social and demographic characteristics of communities in this area are varied. Although the underlying mechanisms of disparities are unknown, significant racial/ethnic differences in COVID-19 cases in the U.S. were revealed and social-demographic factors including economic status, racial/ethnic status, household composition should be included when assessing the COVID-19 burdens (Karmakar et al., 2021; Kim and Bostwick, 2020).

Sewer wastewater samples from three locations in Macomb County (EP, MT, SH), three locations in the City of Detroit, located in Wayne County (D1, D2, D3) and three locations in Oakland County (SF, WB, OP) were collected to conduct the neighborhood sewershed surveillance of SARS-CoV-2 in the Detroit Tri-County area (Fig. 1). Sampling locations were selected in the Detroit Tri-County area to ensure data from neighborhoods with varying demographics. Census tract level population and demographic information obtained from the Southeast Michigan Council of Governments (SEMCOG) website (https://semcog.org/) were evaluated along with sample location catchment areas to define sewershed level demographics (Table 1). Sampling locations in the City of Detroit (D1, D2 and D3) have relatively higher population densities and higher poverty rates and relatively lower household income than those in Macomb County (EP, MT and SH) and Oakland County (SF, WB and OP). Sample locations MT and SH in Macomb County have the largest catchment areas and populations. The nine sample locations include a demographic variety in the Detroit Tri-County area, and represent areas with differing racial makeups, education levels, poverty, and income levels (Table 1).

Sewer wastewater samples were collected during 18 sampling events from December 2020 to October 2021. For each site, triplicate samples were collected each sampling event for a total of 54 samples collected for each site. Dates for each sampling event are shown in the supplementary Table S1. Sewer flow values for each sampling event were measured by existing flow meters at two sites in Macomb County (MT and SH) and two sites in Oakland County (SF and WB). A hydraulic model [GLWA's Regional Wastewater Collection System (RWCS) Model] was updated with rainfall data for the sampling period and applied to estimate flow values for sewersheds D1, D2 and D3 in the City of Detroit (Wayne County) for each sampling event. There was no available flow meter data or hydraulic model that could be used to estimate flows for sites EP in Macomb County and OP in Oakland County for the study period.

Viruses were collected and isolated from wastewater using electrophoresis NanoCeram column filters (Argonide, Sanford, FL, USA) based on the EPA Virus Adsorption-Elution (VIRADEL) method (Miyani et al., 2020; Miyani et al., 2021; Xagoraraki et al., 2014; Zhao et al., 2022). Specifically, depending on the quantity of suspended solids in the wastewater, approximately 20 to 50 L of raw wastewater were passed through NanoCeram electropositive cartridge filters at a rate not $>11$ L/min. Flow meter readings were recorded at the beginning and termination of each sampling event to measure the total volume raw wastewater that passed through the filter. After sampling, the NanoCeram column filters were placed in sealed plastic bags on ice and transported to the laboratory for elution and downstream molecular analysis within 24 h. In addition, for each sampling event, triplicate grab samples of raw sewage were collected using 1 L autoclavable polyethene plastic bottles. To prevent degradation of biomarkers, the pH in the bottles was adjusted to 2. The bottle was transported to the laboratory within 24 h for the biomarkers analysis. Solid phase extraction for biomarkers analysis was performed within two weeks of sample collection. Another set of samples were collected for each sampling event and transported to Paragon Laboratories, Inc. in Livonia, MI for the analyses of water quality markers (BOD, TKN, TSS and VSS). Standards methods were applied to perform the analyses as follows: BOD: SM5210B; TKN: SM 4500-NH4 B; TSS: SM 2540D and VSS: SM 2540 E.

2.2. Quantification of SARS-CoV-2 in the wastewater samples

2.2.1. Virus elution and RNA extraction

Virus elution from the cartridge filters was conducted within 48 h of each sampling event. Viruses were eluted based on a previously described method (Miyani et al., 2020; Miyani et al., 2021) using 1.5 % beef extract (0.05 M glycine). Following elution, each sample was aliquoted into multiple 2 mL Corning tubes. Subsequently, 140 μL of sample was used from one of the corning tubes for RNA extraction. Viral RNA was extracted using QIAGEN QIAamp Viral RNA QIAGEN kits (QIAGEN, Hilden, Germany), following the manufacturer’s protocol with the volume of final eluting reagent (buffer AVE) modified from 60 μL to 140 μL as in the previous study (Miyani et al., 2021; Zhao et al., 2022). Bacteriophage Phi6 was spiked to estimate the losses of virus in elution and concentration. And the recoveries were decided from 10.37 % to 58.96 %, with a mean recovery of 24.91 % ($\pm 22.89 \%$). RNA extracts were stored at $-80 \degree C$ and RT-ddPCR were performed within 24 h after the extraction.

2.2.2. Reverse transcriptase droplet digital PCR (RT-ddPCR)

A QX200 AutoDG Droplet Digital PCR system (Bio-Rad, Hercules, CA, USA) was applied to perform RT-ddPCR and the One-step RT-ddPCR Advanced Kit was used for Probes (Bio-Rad, Hercules, CA, USA). Primers and probe targeting N1 and N2 of SARS-CoV-2 were used were summarized in Table S2. The N1 N2 gene Duplex Assay Reaction Mixture was prepared with 5.5 μL of One-step RT-Supermix (20 ×) (final volume ratio: 0.25), 2.2 μL of Reverse Transcriptase (RT) (final volume ratio: 0.1), 1.1 L 300 mM DTT (final volume ratio: 0.05), 3.3 μL of N1 primer probe mix (final volume ratio: 0.15), 3.3 μL N2 primer probe mix (final volume ratio: 0.15), and 1.1 μL of PCR-grade water (final volume ratio: 0.05) in a final volume of 16.5 μL per reaction. Amounts of the mixture were prepared according to the sample number. After mixing thoroughly, the reagents
The Detroit Tri-County Area

| Site | County   | City/Township               | ZIP Code          |
|------|----------|-----------------------------|-------------------|
| EP   | Macomb   | Eastpointe                  | 48021             |
| MT   | Macomb   | Macomb Township             | 48044,48042,48049,48095 |
| SH   | Macomb   | Sterling Heights            | 48310,48312,48313 |
| D1   | Wayne    | Detroit                     | 48205             |
| D2   | Wayne    | Detroit                     | 48210             |
| D3   | Wayne    | Detroit                     | 48235             |
| SF   | Oakland  | Southfield                  | 48076             |
| WB   | Oakland  | West Bloomfield             | 48322             |
| OP   | Oakland  | Oak Park                    | 48237             |

United States

City/Township and ZIP code of the selected locations

The Detroit Tri-County Area

Fig. 1. Locations of nine sewersheds sites selected from the Detroit Tri-County area in Michigan in the United States. Decisions were made with local health departments and sewersheds selected represent different demographic characteristics from County Macomb (EP, MT and SH), Wayne (D1, D2 and D3) and Oakland (SF, WB and OP) in the Detroit Tri-County area MI.

Table 1
Demographic characteristics of the catchment areas for the nine sampling sewersheds in the Detroit Tri-County area MI.

| Site | Sample tributary catchment | Area (acres) | Population | Density a | Asian (%) | Black (%) | Hispanic (%) | White (%) | Poverty (%) | 65 + b (%) | Degree c (%) | Total household income ($) |
|------|-----------------------------|--------------|------------|-----------|-----------|-----------|-------------|-----------|-------------|------------|---------------|-----------------------------|
| EP   |                             | 278          | 2400       | 8.6       | 0         | 37        | 5           | 54        | 5           | 16         | 17            | 56,450                      |
| MT   |                             | 29,264       | 99,970     | 3.4       | 3         | 5         | 3           | 88        | 4           | 14         | 34            | 102,850                     |
| SH   |                             | 6246         | 37,560     | 6.0       | 10        | 5         | 2           | 80        | 4           | 12         | 27            | 65,700                      |
| D1   |                             | 135          | 1690       | 12.5      | 0         | 95        | 0           | 4         | 19          | 11         | 5             | 39,200                      |
| D2   |                             | 372          | 5190       | 14.0      | 0         | 8         | 76          | 14        | 32          | 6          | 5             | 35,900                      |
| D3   |                             | 127          | 1300       | 10.2      | 0         | 95        | 0           | 2         | 44          | 17         | 14            | 22,100                      |
| SF   |                             | 717          | 3080       | 4.3       | 0         | 61        | 3           | 31        | 10          | 18         | 56            | 92,100                      |
| WB   |                             | 1218         | 5800       | 4.8       | 15        | 19        | 0           | 63        | 5           | 27         | 55            | 100,520                     |
| OP   |                             | 286          | 2270       | 7.9       | 2         | 85        | 3           | 6         | 15          | 16         | 20            | 51,680                      |

a: Unit of population density, people per acre.
b: Percent of population older than 65.
c: Percent of population with bachelor's degree or higher.
were pipetted into each well of a 96-well plate. Then, 5.5 μL of RNA product was added to each well reaching a total reaction volume of 22 μL.

The 96-well plate was sealed on a PX1 PCR Plate Sealer (Bio-Rad, Hercules, CA, USA), subsequently centrifuged and annealed at 1000 rpm for 30 s. Oil droplets were generated using a Automated Droplet Generator (Bio-Rad, Hercules, CA, USA). Samples were then run on a C1000 Touch Thermal Cycler (Bio-Rad, Hercules, CA, USA) using the following conditions for the N1 N2 Duplex: 25 °C for 3 min, 50 °C for 60 min, 95 °C for 10 min, following 40 cycles of 95 °C for 30 s and 55 °C for 1 min with a ramp speed of 2 °C/s, 98 °C for 10 min, and finally 4 °C until the next step. Plates were transferred to a QX200 Droplet Reader (Bio-Rad, Hercules, CA, USA) for a measurement of fluorescence in each droplet.

For each RT-ddPCR run, three positive controls (PTCs) and three negative controls (NTCs), and process negative controls (including virus elution and RNA extraction process controls) were included. Twist Bioscience Twist Synthetic SARS-CoV-2 RNA Control 2 (MN908947.3) with a concentration of 10^2 gc/μL was used for PTCs. Nucleic-acid-free water was used for NTCs. Nano-pure water was used as a substitute for 1.5 % beef extract in virus elution, as process negative control. Sterile nuclease-free water was used as a substitute for 140 μL of sample for RNA extraction, as process negative control. All samples were run in triplicate.

Determination of Limit of Blank (LOB) and Limit of Detection (LOD) was based on the methods described in the manufacturer’s (Bio-Rad) guidelines for testing analytical sensitivity and validation of RT-ddPCR (Bio-Rad, Hercules, CA, USA). The Limit of Blank (LOB) was determined by testing three types of samples using RT-ddPCR, across four consecutive days including the prior-to-COVID-19 pandemic samples collected from the same interceptors, nuclease-free water, and negative process control samples from elution and extraction processes. The LOB for N1 gene ddPCR was determined as 0.09 gc/μL, and the LOB for N2 gene ddPCR was 0.08 gc/μL. Furthermore, an LOD of 0.1 gc/μL with 72.9 % confidence for the N1 gene and 0.1 gc/μL with 81.3 % confidence for the N2 gene were determined.

2.3. Population biomarkers analysis

Population biomarkers were extracted from wastewater using solid-phase extraction (SPE). Specifically, 19.95 mL of wastewater sample passed through pre-conditioned 200 mg/6 cc Waters Oasis HLB cartridges connected to a 12-port vacuum manifold. The SPE cartridges were conditioned by sequentially washing with 5 mL of methanol and 5 mL of deionized (DI) water. The flow rate was controlled at approximately 2 mL/min. After the wastewater sample passed through the cartridge, the sample vial was rinsed with 3 mL of DI water and the rinse water was loaded to the SPE cartridge. Clean vials were placed in the vacuum manifold beneath each SPE cartridge to collect the eluate. The cartridge was eluted with 5 mL of methanol at a rate of 1 mL/min. The samples were analyzed within two weeks using a Shimadzu instrument; gc/C2

VPCR: Volume of RNA product used for RT-ddPCR; 5.5 μL.

VEX: Volume of RNA product used for each PCR reaction; 140 μL.

VPCR: Final reaction volume of RT-ddPCR; 22 μL.

3. Results and discussion

3.1. Prevalence of SARS-CoV-2 and clinical COVID-19 cases in the communities

SARS-CoV-2 concentrations were quantified using N1 and N2 RT-ddPCR assays for samples collected from Macomb County Sites (EP, MT and SH), City of Detroit (Wayne County) (D1, D2 and D3) and Oakland County sites (SF, WB and OP) in the Detroit Tri-County area. Concentrations of both N1 and N2 in all the studied sites were found to be > 1.91 × 10^5 gc/L and up to 6.87 × 10^6 gc/L. Average concentration of N1 for the nine sample sites ranged from 348 ± 14.2 gc/L in D1 to 937 ± 110 gc/L in MT. The average concentration of N2 ranged from 372 ± 17.0 gc/L in D1 to 879 ± 161 gc/L in SH. Concentrations of SARS-CoV-2 in MT and SH were relatively higher than those at other sites, which may relate to the larger population in these sewersheds. Tukey’s post hoc analysis support this observation and indicated concentrations of SARS-CoV-2 in MT and SH
are significantly higher than those in the remaining sites (Fig. 2A & B). Meanwhile, N1 and N2 measurements were strongly correlated ($r > 0.76$) for every site except site SF, where more high outliers of N2 measurements were found compared with N1 measurements.

Flow rates of seven sewersheds [MT and SH in Macomb County, D1, D2 and D3 in the City of Detroit (Wayne County), and SF and WB in Oakland County] were available and used to investigate SARS-CoV-2 loads in the study period. Flow data for the four sites located at existing flow meters (MT, SH, SF, and WB) were available in 5-minute intervals. The hydraulic model used to estimate flows for the three City of Detroit sites (D1, D2, and D3) had a 15-min reporting time step. Due to meter flow variability, either 30-min or 1 h moving average flow values were applied for the analyses. Averages of the flow rates for the seven sewersheds ranged from $7.88 \times 10^5$ L/day in D3 to $3.16 \times 10^7$ L/day in MT (Table S4). The average SARS-CoV-2 loads using N1 gene assay ranged from $3.64 \times 10^8$ gc/day in D3 to $2.94 \times 10^{10}$ gc/day in MT, while for N2 gene assay, they ranged from $3.53 \times 10^8$ gc/day in D3 to $2.60 \times 10^{10}$ gc/day in MT (Fig. 2C & D). Further Tukey’s post hoc analysis indicated SARS-CoV-2 loads in MT and SH were significantly larger than those in the remaining sites.

Cumulative COVID-19 cases in the nine sewersheds for the entire study period are presented in Fig. 2E. COVID-19 cases for MT and SH sewersheds (6770 and 7305 cases, respectively) were larger than those in the other seven sewersheds. Confirmed cumulative cases in the SF sewershed (635) were the lowest reported during the study period. Cumulative COVID-19 cases per capita was calculated (Fig. 2F) using the population data for the nine sewersheds. On a per capita basis, COVID-19 incidences in MT and SH remain to be the highest; however, the difference between these two sewersheds and the remaining seven sewersheds decreased compared to the cumulative cases without normalizing to population.

Both concentrations and loads of SARS-CoV-2 RNA in MT and SH sewersheds were higher than the other sewersheds, which is consistent with their high cumulative clinically confirmed cases. Relationships
between SARS-CoV-2 RNA loads (targeting at N1 and N2) and cumulative confirmed cases were explored using spearman correlation analysis (Fig. 3A&C). Also, linkages between SARS-CoV-2 RNA loads and populations that the sewersheds served were investigated through spearman correlation analysis (Fig. 3B&D). The results suggest SARS-CoV-2 RNA loads are correlated with both the cumulative clinical COVID-19 cases and population served by the sewersheds and agrees with previous published work (Bertels et al., 2022; Wilder et al., 2021; Wu et al., 2021). A linear relationship between SARS-CoV-2 RNA concentration and population size in the catchment area in New York, US was identified (Wilder et al., 2021).

Table 2
Concentrations of water quality markers and population biomarkers in the wastewater samples collected from nine sewersheds in the Detroit Tri-County area MI. Values are described as average ± standard deviation.

| Markers            | Sites | EP    | MT    | SH    | D1    | D2    | D3    | SF    | WB    | OP    |
|--------------------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| BOD (mg/L)         | 228 ± 61.0 | 208 ± 47.0 | 134 ± 28.0 | 120 ± 43.0 | 68.0 ± 19.0 | 79.0 ± 25.0 | 84.0 ± 37.0 | 215 ± 44.0 | 125 ± 59.0 |
| TKN (mg/L)         | 40.0 ± 9.00 | 58.0 ± 8.00 | 45.0 ± 8.00 | 25.0 ± 7.00 | 25.0 ± 7.00 | 30.0 ± 10.0 | 31.0 ± 10.0 | 34.0 ± 4.00 | 40.0 ± 16.0 |
| TSS (mg/L)         | 97.0 ± 50.0 | 126 ± 78.0 | 99.0 ± 21.0 | 39.0 ± 20.0 | 24.0 ± 12.0 | 37.0 ± 17.0 | 56.0 ± 30.0 | 107 ± 27.0 | 67.0 ± 49.0 |
| VSS (mg/L)         | 85.0 ± 41.0 | 106 ± 56.0 | 91.0 ± 17.0 | 35.0 ± 17.0 | 18.0 ± 4.00 | 33.0 ± 15.0 | 43.0 ± 19.0 | 98.0 ± 24.0 | 58.0 ± 41.0 |
| Creatinine (μg/L)  | 32.6 ± 15.9 | 34.1 ± 10.9 | 22.5 ± 10.9 | 24.8 ± 10.0 | 22.2 ± 9.1 | 25.6 ± 8.4 | 20.5 ± 12.3 | 24.1 ± 9.4 | 37.4 ± 20.3 |
| 5-HIAA (μg/L)      | 8.10 ± 4.80 | 11.1 ± 5.80 | 4.90 ± 5.10 | 5.10 ± 3.90 | 5.30 ± 3.90 | 6.50 ± 4.20 | 6.60 ± 3.90 | 6.60 ± 3.70 | 9.50 ± 6.30 |
| Caffeine (μg/L)    | 137 ± 60.4 | 182 ± 77.0 | 113 ± 50.9 | 55.4 ± 24.8 | 46.7 ± 18.4 | 33.6 ± 11.6 | 106 ± 64.1 | 156 ± 78.3 | 91.3 ± 36.4 |
| Xanthine (μg/L)    | 35.5 ± 21.2 | 33.4 ± 20.6 | 23.9 ± 16.0 | 20.8 ± 12.9 | 17.7 ± 11.5 | 21.3 ± 14.4 | 22.6 ± 14.7 | 26.3 ± 20.7 | 35.8 ± 23.6 |
| Methylxanthine (μg/L) | 64.6 ± 56.2 | 103 ± 74.2 | 63.0 ± 50.2 | 32.4 ± 24.8 | 30.5 ± 24.5 | 36.3 ± 27.0 | 49.0 ± 40.4 | 49.7 ± 42.0 | 67.6 ± 50.6 |
| Theophylline (μg/L) | 35.2 ± 11.3 | 60.0 ± 17.3 | 40.9 ± 12.8 | 16.5 ± 5.30 | 23.0 ± 9.30 | 19.3 ± 7.20 | 30.8 ± 9.40 | 34.4 ± 13.3 | 35.2 ± 11.1 |
| Theobromine (μg/L) | 37.4 ± 17.8 | 67.2 ± 25.4 | 41.5 ± 18.6 | 21.2 ± 7.20 | 24.3 ± 10.0 | 22.9 ± 9.00 | 31.6 ± 13.6 | 40.6 ± 19.6 | 38.0 ± 17.6 |
| Paraxanthine (μg/L) | 28.1 ± 8.10 | 46.6 ± 14.1 | 31.8 ± 10.2 | 13.5 ± 4.10 | 18.6 ± 7.00 | 15.8 ± 5.40 | 25.0 ± 7.60 | 27.0 ± 10.2 | 28.0 ± 8.70 |
3.2. Assessment of the potential population markers in wastewater-based surveillance

In addition to the census approach, contributing population to a sewershed can be assessed using water quality parameters (BOD, etc.) and population biomarkers (creatinine, etc.). In this study, both water quality and population biomarkers were studied to evaluate potential population markers in per-capita SARS-CoV-2 assessments and cross-site comparisons. Water quality constituent and population biomarker concentrations from the nine sites are shown in Table 2.

The highest concentrations of BOD were found in EP (228 ± 61.0 mg/L) and WB (215 ± 44.0 mg/L), which have populations of approximately 2400 and 5800, respectively, based on the census estimate. The two sites with the highest concentrations of TKN were MT (58.0 ± 6.00 mg/L) and SH (45.0 ± 8.00 mg/L), which also have the largest catchment level populations (99,970 and 35,560, respectively). High levels of TSS and VSS were found in site MT (TSS: 126 ± 78.0 mg/L; VSS: 106 ± 56.0 mg/L) and WB (TSS: 107 ± 27.0 mg/L; VSS: 98.0 ± 24.0 mg/L), however, the population estimated for the MT sewershed (99,970) is much larger than the WB sewershed (5800).

The highest concentration of creatinine was detected in sewer samples collected from the OP site (37.4 ± 20.3 μg/L), followed by the MT site (34.1 ± 10.9 μg/L). The estimated population for the MT sewershed is the largest (99,970), which is consistent with its high creatinine concentration. However, the population estimated for the catchment area of the OP site is relatively small (2270). Similar results were found for 5-HIAA, where concentrations were found to be very high at sites MT and OP (MT:11.1 ± 5.80 μg/L; OP:9.50 ± 6.30 μg/L), even though the estimated populations of the two sites vary significantly. Concentrations of caffeine and its metabolites were identified in microgram per liter, and concentrations of the metabolites were found to be slightly lower than the parent compound (Chen et al., 2014; Choi et al., 2018; Gracia-Lor et al., 2017).

Correlations analysis between the loads (g/day) of population markers and the population sizes obtained from census approach were performed (Fig. 4). The results showed that BOD, TKN, creatinine, 5-HIAA and three

![Correlation analysis between loads of potential population markers (including water quality parameters and human biomarkers) and population sizes obtained from census. Numbers in the shapes are the correlation coefficients. Unit of all the population markers is kept consistent and is g/day.](image)

With the wastewater surveillance study of SARS-CoV-2 across 40 states in the U.S. from February to June 2020, a positive correlation between SARS-CoV-2 RNA detection rates and population sizes were found (Bertels et al., 2022; Wu et al., 2021). However, as mentioned above, difference of COVID-19 incidences between sewershed MT and SH, and the remaining seven sewersheds decreased when normalized to population, which signifies the importance of assessing COVID-19 burden to per capita level.

![Normalized SARS-CoV-2 (A: N1; B: N2) by population markers to assess the COVID burdens to the per capita level. Correlation analyses were performed between normalized SARS-CoV-2 and COVID-19 cases per 1000-person. Events (8 events for each sewershed, from 1/18/21 to 3/22/21, dates in red in Table S1) with water quality markers, creatinine, 5-HIAA, and caffeine and its metabolites available were included to do the correlation. For each sewershed and normalizing factor, sum of the normalized SARS-CoV-2 for the 8 events were considered to do the correlation. COVID-19 cases rates were calculated for the periods corresponding to those 8 events. Unit of all the normalized SARS-CoV-2 concentrations is kept consistent and is gc/g population marker.](image)
of caffeine's metabolites are correlated strongly with the population sizes obtained from census ($r > 0.90$) (Fig. 4).

Concentration of the wastewater RNA may increase with the larger population sizes, as in our study, concentrations of SARS-CoV-2 RNA in MT and SH wastewater are much higher than those in other sites (Figs. 2A & 2B). To understand the per capita viral contribution, it is critical to have an accurate estimate of population to be able to normalize measured SARS-CoV-2 concentrations in wastewater.

### 3.3. Normalized SARS-CoV-2 and the clinical cases rate

To understand the per capita viral contribution and perform spatial comparisons, SARS-CoV-2 RNA concentrations were normalized to population served in order to relate with COVID-19 incidences in the nine sampling locations. Descriptive characteristics of the normalized SARS-CoV-2 were summarized in Table S5. Clinical case per 1000-person per site were calculated using the cumulative clinical cases divided by the population size served by each sewershed. Clinical cases per 1000-person for the nine sites ranged from 29 cases to 65 cases per 1000-person (Fig. 2F). Even though SH and MT have a relatively higher confirmed case rate, the deviation of these two sites to the remaining seven sites has reduced significantly compared to cumulative clinical cases in the study periods (Fig. 2E).

Correlations between normalized SARS-CoV-2 and the clinical cases were used to evaluate the application of the population markers. Results indicated that SARS-CoV-2 RNA normalized on the basis of TKN, creatinine, 5-HIAA and xanthine were correlated strongly with the clinical cases per 1000-person (Fig. 5). Furthermore, when TKN and xanthine were used to normalize the SARS-CoV-2 RNA concentrations, no significant differences were found among the nine sites. When creatinine and 5-HIAA were used, SH still stood out from the nine sampling locations (Fig. S1 & S2). Findings in this study may promote the per capita viral assessment and benefit the public health, especially in rural areas and the developing world, since factor like TKN can be measured easily onsite.

Concentrations of SARS-CoV-2 RNA in wastewater are affected by several factors including shedding-related factors, sewershed population, in-sewer factors (e.g., load and physiochemical properties of solid particles and organic matters, influx of rainwater/stormwater/groundwater), and sampling strategies (Bertels et al., 2022). Adjusting for these factors can help reduce uncertainties of the wastewater data. Among these factors, population normalization is crucial for accurate wastewater surveillance and confident viral assessment. By evaluating various water quality parameters and human biomarkers as the normalizing factors, SARS-CoV-2 RNA normalized by TKN, creatinine, 5-HIAA and xanthine correlated strongly with the clinical cases per 1000-person.

### 3.4. Demographic characteristics that may be associated with COVID-19 risks

Varying human behaviors and activities may influence the viral transmission and thus the COVID-19 burdens in the communities. According to a report of CDC, social determinants including educational and income gap in areas where racial minority groups live, learn, work, play and worship are associated with more COVID-19 cases (Kim and Bostwick, 2020; Webb Hooper et al., 2020). By synthesizing data for 3142 counties in the U.S., it was found that counties with higher fractions of residents with no

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**Fig. 6.** Principal component analysis (PCA) confirmed the demographic differences of the nine sampling locations in the Detroit Tri-County area, MI. A: PCA includes population sizes, total household income, proportion of different races, percent of poverty population, unemployed population and population with bachelor's degree or higher. B: PCA conducted with population markers (water quality parameters including BOD, TKN, TSS and VSS, human biomarkers including creatinine, 5-HIAA, caffeine and its metabolites). C: PCA conducted with parameters from wastewater surveillance (SARS-CoV-2 RNA concentrations targeting at N1 and N2, water quality parameters including BOD, TKN, TSS and VSS, human biomarkers including creatinine, 5-HIAA, caffeine and its metabolites). D: PCA conducted with SARS-CoV-2 RNA concentrations (targeting at both N1 and N2 genes) normalized by TKN, creatinine, 5-HIAA and xanthine.
high school diploma were associated with higher cumulative COVID-19 case and death rates (Li et al., 2021a). As mentioned previously, social and demographic characteristics of communities in the Detroit Tri-County area are varied, which is confirmed by the PCA (Fig. 6). Potential influences of factors besides population are explored further.

Analysis of PCA in Fig. 6A includes factors like population sizes, total household income, proportion of different races, percent of poverty population, unemployed population, and population with bachelor’s degree or higher in the sewersheds. There are no clear clusters identified, which may indicate the diverse demographic characteristics. While PCA was conducted based on the different population biomarkers, distribution of these nine sewersheds was found to be dispersive (Fig. 6B), which confirms the varied population characteristics of the nine studying sites. For both methods, the top two dimensions contributed >70% of the disparities, with the first dimension contributed >50% of the disparities. Variables that contributed to dimension 1 in Fig. 6 include total household income, percent population of white, and percent population in poverty, which indicates sewersheds WB, MT and SH may be separated with others due to their relatively high total household income, high percent population of white and low percent population in poverty (Fig. S3A1 & Table 1). Meanwhile, sewersheds of D2 may be separated with others due to their relatively low percent population of black, high percent population of Hispanic and low percent population with age older than 65 (Fig. S3A2 & Table 1).

Additionally, when SARS-CoV-2 RNA were involved in PCA (Fig. 6C), no obvious clusters could be identified, and distribution of the studying sites became more dispersive compared with that in Fig. 6B. However, when normalized SARS-CoV-2 RNA concentrations (by TKN, creatinine, 5-HIAA and xanthine) were used to conduct the PCA (Fig. 6D), it seems to form two clusters and all nine sites converged together except SH, which was consistent with the previous analysis after calculating the confirmed clinical cases per 1000-person (Fig. 2E). Considering the uncertainties in population normalization, complexities in quantifying SARS-CoV-2 RNA in wastewater, and the potential influences of other demographic characteristics like income and education, further studies are needed to investigate the potential linkages between social economic factors and viral risks.

4. Conclusions

Average concentrations of N1 (SARS-CoV-2) in wastewater samples collected from nine sites ranged from 3.48 × 10^2 to 9.37 × 10^2 gc/L, and for N2, averages ranged from 3.72 × 10^2 to 8.79 × 10^2 gc/L. Both levels and loads of SARS-CoV-2 RNA in wastewater in two neighborhoods (MT and SH) were found to be higher than that in the other seven sites. The differences were statistically significant. Comparisons between normalized SARS-CoV-2 by population markers and COVID-19 incidences, indicated that normalization of SARS-CoV-2 RNA concentrations with TKN, creatinine, 5-HIAA and xanthine correlated positively with the COVID-19 incidences. Analysis of PCA indicates the potential influences of demographic characteristics on the COVID-19 risks and further studies are needed to elaborate the linkages between social economic factors and viral risks.

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

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2022.158350.

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