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Identification and quantification of bioactive compounds suppressing SARS-CoV-2 signals in wastewater-based epidemiology surveillance

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ABSTRACT

Recent SARS-CoV-2 wastewater-based epidemiology (WBE) surveillance have documented a positive correlation between the number of COVID-19 patients in a sewersheds and the level of viral genetic material in the wastewater. Efforts have been made to use the wastewater SARS-CoV-2 viral load to predict the infected population within each sewershed using a multivariable regression approach. However, reported clear and sustained variability in SARS-CoV-2 viral load among treatment facilities receiving industrial wastewater have made clinical prediction challenging. Several classes of molecules released by regional industries and manufacturing facilities, particularly the food processing industry, can significantly suppress the SARS-CoV-2 signals in wastewater by breaking down the lipid-bilayer of the membranes. Therefore, a systematic ranking process in conjugation with metabolomic analysis was developed to identify the wastewater treatment facilities exhibiting SARS-CoV-2 suppression and identify and quantify the chemicals suppressing the SARS-CoV-2 signals. By ranking the viral load per diagnosed case among the sewersheds, we successfully identified the wastewater treatment facilities in Missouri, USA that exhibit SARS-CoV-2 suppression (significantly lower than $5 \times 10^{11}$ gene copies/reported case) and determined their suppression rates. Through both untargeted global chemical profiling and targeted analysis of wastewater samples, 40 compounds were identified as candidates of SARS-CoV-2 signal suppressors. Among these compounds, 14 had higher concentrations in wastewater treatment facilities that exhibited SARS-CoV-2 signal suppression compared to the unsuppressed control facilities. Stepwise regression analyses indicated that 4-nonylphenol, palmitelaidic acid, sodium oleate, and polyethylene glycol dioleate are positively correlated with SARS-CoV-2 signal suppression rates. Suppression activities were further confirmed by incubation studies, and the suppression kinetics for each bioactive compound were determined. According to the results of these experiments, bioactive molecules in wastewater can significantly reduce the stability of SARS-CoV-2 genetic material. Based on the concentrations of these chemical suppressors, a correction factor could be developed to achieve more reliable and unbiased surveillance results for wastewater treatment facilities that receive wastewater from similar industries.

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1. Introduction

Coronaviridae (Coronavirus) is a family of positive single stranded RNA viruses, responsible for various severe respiratory infections (Qu et al., 2020; Yeo et al., 2020). This family contains over 30 kinds of viruses and has a genome of approximately 30 kb, the largest reported genome of all RNA viruses (Amaoth et al., 2020; Woo et al., 2005). In the past 17 years, there have been three major outbreaks caused by human coronaviruses, including the severe acute respiratory syndrome coronavirus (SARS-CoV) that occurred in China in 2003 and affected 26 countries (Hemida, 2019; Lau and Chan, 2015). In 2012, the outbreak of the Middle East Respiratory Syndrome Coronavirus (MER-S-CoV) (Hemida, 2019; Zakì et al., 2012) affected 27 countries with over 2,400 cases (WHO, 2020). Recently, the ongoing Coronavirus Disease 2019 (COVID-19) pandemic caused by Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) that emerged in Wuhan, China, has affected the global community and individual daily function (Balogh et al., 2020; Liu et al., 2020; Zhu et al., 2020). Recent studies have revealed that both SARS-CoV-2 and SARS-CoV can recognize and bind to the angiotensin-converting enzyme 2 (ACE2) on the cell surface. Between the two viruses, subtle differences in the amino acid sequence in addition to conformation of the S protein in SARS-CoV-2 contribute to a significantly stronger affinity of SARS-CoV-2 to ACE2 (Li et al., 2003; Sternberg and Naujokat, 2020). ACE2 is not only highly expressed in lungs, but also in the gastrointestinal tract, including the small intestine and colon (Jiao et al., 2021).

Wastewater-based epidemiology (WBE) has been used as a surveillance tool for population-wide infectious diseases, featuring a proven track record for hepatitis A and polio (Asghar et al., 2014). Different studies in the United States, the Netherlands, Italy, and elsewhere have detected the presence of SARS-CoV-2 in domestic sewage and have found a positive relationship between the amount of viral material in sewage and the number of reported COVID-19 cases in the area that collects and treats wastewater for a community, called a “sewer-shed” (Agrawal et al., 2021; Hokajärvi et al., 2021; La Rosa et al., 2020; Sherchan et al., 2020). Although a majority of the SARS-CoV-2 viral loads in wastewater are introduced through the gastrointestinal tract, SARS-CoV-2 can also be introduced into wastewater (domestic and hospital) through several other sources, such as sputum, handwashing, and vomit (Haagmans et al., 2014; Han et al., 2020; Sung et al., 2016). However, the main source of SARS-CoV-2 viral loads to wastewater that has been reported is feces containing viral RNA shed by infected people (Chen et al., 2020; Ling et al., 2020; Xiao et al., 2020; Zhang et al., 2020).

Due to the documented positive correlation between the number of COVID-19 patients in a sewershed and the level of viral genetic material in the wastewater in recent SARS-CoV-2 WBE studies (McMahan et al., 2021; Weidhaas et al., 2021), efforts have been made to use the wastewater SARS-CoV-2 viral load to predict the infected population for each sewershed using a multivariable regression approach. However, reported clear and sustained variability among treatment facilities have made clinical prediction challenging. Specifically, wastewater at some facilities consistently exhibits higher genetic material per diagnosed patient, indicating a likely underestimate in the number of COVID-19 patients, while wastewater from other facilities has much lower levels of the genetic material per diagnosed case, suggesting suppression of the genetic material from the sewershed. Since it is quite common that wastewater treatment facilities receive some input from industries, several classes of molecules released by regional industries and manufacturing facilities, particularly the food-processing industry, could significantly suppress SARS-CoV-2 signals in wastewater by breaking down the lipid-bilayer of the membranes (Helenius and Simons, 1975; Kruzelnicka et al., 2019; Montalvo and Khan, 2002; Palmer and Hatley, 2018).

The active ingredients in detergents, surface-active agents (surfactants), emulsifiers, and disinfection products (e.g., pyrrolidones, sodium dodecylbenzinesulfonate, sodium xlenesulfonate, polyethylene glycol, sodium stearate and cocamidopropyl betaine), as well as bioconjugate and cross-linking agents (e.g., ethylenediaminetetraacetic acid) are commonly found in industrial wastewater (Barambu et al., 2020; Merret-Bruns and Jelen, 2009; Olkowska et al., 2013; Olsson et al., 2008; Zoller and Romano, 1983). Among these chemicals, surfactants are one of the main compounds that can be exist in wastewater (Scheibel, 2004). The surfactants consist of two major functional groups: one is hydrophilic (lipophobic) and the other is non-polar hydrophobic (lipophilic) (Olkowska et al., 2013). Generally, the two functional groups are referred as head and tail, respectively. The surface-active agents are usually classified based on the charge of the head, including anionic, cationic, non-ionic and zwitterionic compounds. Approximately 65% of the total world production of surfactants corresponds to the compounds classified as anionic surfactants (“Novel Surfactants,” 2002; Olkowska et al., 2013). Surfactants are mainly used in surface cleaners, household detergents, shampoos, dishwashing liquids, cosmetics, and laundry detergents (Lara-Martín et al., 2006). Moreover, different varieties of surfactants are used as starting materials in the production of pigments, catalysts, dyes, pesticides, pharmaceuticals, and plasticizers (Süttner et al., 2008). These compounds could significantly reduce the stability of SARS-CoV-2 genetic marker signals in wastewater by breaking down the lipid bilayer of SARS-CoV-2. Therefore, for facilities that receive wastewater from industries, a correction factor based on the concentrations of such bioactive molecules is needed to achieve more reliable and unbiased surveillance results.

As a result of recent advancements in mass spectrometry, metabolomics algorithm, computational capacity, and mass spectral reference databases, untargeted metabolomics has been widely applied to identify bioactive molecules in the complex and organic-rich matrices. Untargeted metabolomics is the global profiling of small molecules in a system without any bias. Although several analytical techniques can be employed to perform untargeted metabolomics, liquid chromatography coupled with high-resolution mass spectrometry (LC/HRMS) has been frequently used because of the large number of molecules that can be evaluated in a single analysis (Tautenhahn et al., 2012). For example, ten to thousands of features (a feature is defined as an ion with a distinctive m/z and retention time) can be detected by high resolution LC/HRMS in one extract. In general, the main purpose of untargeted metabolomics is to determine which of these features is dysregulated (upregulated and downregulated) between different sample groups or treatments. Due to the complexity and the number of features in a dataset, it is challenging to accomplish this comparison manually (Domingo-Almenara et al., 2018). Several software programs for automated processing of LC/HRMS data have been developed over the past decade. However, most of these programs have restrictions that limit their utility and applicability to different instrumentation. One widely applicable program for processing LC/HRMS data is XCMS Online, a web-based platform that contains all of the tools necessary for the entire untargeted metabolomic workflow, including signal detection, peak alignment, retention time correction calculations, raw data processing, statistical analysis, and metabolite assignment (Gowda et al., 2014; Lu et al., 2019; Vu et al., 2020). An untargeted metabolomic profiling approach that utilizes a comprehensive program like XCMS Online is well-suited to the identification of candidate compounds that suppress the SARS-CoV-2 genetic signal in complex wastewater matrices.

The objectives of this study are to (1) identify the wastewater treatment facilities in Missouri, USA that exhibit SARS-CoV-2 suppression and determine their suppression rates, (2) identify possible active compounds suppressing the SARS-CoV-2 genetic signal through a combination of stepwise regression and metabolomic profiling approaches, (3) confirm and quantify the identified bioactive molecules using targeted analysis, and (4) validate the suppression activities through incubation studies.
2. Materials and methods

2.1. Materials

High performance liquid chromatography (HPLC) grade methanol (MeOH), acetonitrile (ACN), and formic acid (FA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC grade ammonium acetate was purchased from Fisher Scientific (Pittsburgh, PA, USA). Analytical standards were purchased from Sigma-Aldrich unless otherwise mentioned. The TaqPath™ 1-Step RT-qPCR Master Mix and TaqMan Probes were purchased from Thermo Fisher Scientific. The primers and probes used in the qPCR assay were purchased from Integrated DNA Technologies, Inc. (Coralville, IA, USA).

2.2. Wastewater sample collection

From July-December 2020, more than 57 wastewater treatment facilities across the state of Missouri, USA were monitored weekly for SARS-CoV-2. The wastewater samples were gathered from the influent of the wastewater treatment facilities (i.e., prior to primary treatment) (Table S1). Once per week, triplicate 50 mL subsamples were collected in polypropylene centrifuge tubes from the 24-hour composite waste water samples. Subsamples kept chilled (between 0 and 3 °C) during transportation to the laboratory at the University of Missouri in Columbia. All the samples were stored at -20 °C until they were analyzed.
2.3. Quantification of SARS-CoV-2 in wastewater

2.3.1. RNA extraction from wastewater samples

Fifty mL of wastewater from the catchment were filtered through a 0.22 µm filter (Millipore cat# SCGPOO525). Thirty-six mL of filtered wastewater were mixed with 12 mL of 50% (W/V) polyethylene glycol (PEG, Research Products International, cat# P48080) and 1.2 M NaCl, wastewater were mixed with 12 mL of 50% (W/V) polyethylene glycol followed by incubation for 1 h at 4°C. Samples were further centrifuged at 12,000 rpm for 2 h. RNA was extracted from the pellet using Qiagen Viral RNA extraction kit following the manufacturer’s instructions after the supernatant was removed. RNA was eluted in a final volume of 60 μL. The samples were stored at -20°C if not processed immediately. Due to high inhibition rates found in the wastewater collected from the Macon sewershed, efforts have been made to validate the suppression rates, identify the responsible molecules, and track the possible sources. Therefore, to prove that the chemicals in the wastewater are suppressing SARS-CoV-2, wastewater from the influent of Macon WWTP was collected and used in this experiment. A20 mL of Macon wastewater was mixed with 20 mL wastewater with high SARS-CoV-2 concentration from one of the correction facilities (Prison) in Missouri. The control samples consisted of 20 mL ultrapure water and 20 mL of the same wastewater with high RNA copy number. Both sets of samples were mixed for 24 h and RNA was immediately extracted.

2.3.2. Plasmid standard and quantitative RT-qPCR assay

A plasmid carrying a unique puro resistance gene fragment along with a N gene fragment was constructed, purified from Escherichia coli, and used as standards for the RT-qPCR assay to ensure an equal molar ratio of puro and N gene detection. A standard curve was constructed at concentrations of 200,000 through 2 gene copies/μL and utilized to determine the copy number of the target puro gene in the wastewater samples that had puro control virus added prior to concentration as an internal control (Robinson et al., 2022). The Puro is non-infectious retroviral virus that contain an RNA genome with a unique engineered sequence (Puro) which have the same size and properties as SARS-COV-2. The Puro was used as the internal standard fortified to the wastewater samples to (1) determine the RNA extraction rates and efficiency, (2) evaluate the RT-qPCR efficiency, and (3) examine if there is RT-PCR inhibition due to the possible PCR inhibitors as quality assurance and quality control. The TaqMan probe and the primer pairs for N1 and N2 detection were purchased from Integrated DNA Technologies (IDT), based on the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel (Acceptable Alternative Primer and Probe Sets) https://www.cdc.gov/coronavirus/2019-ncov/downloads/List-of-Acceptable-Commercial-Primers-Probes.pdf. The TaqMan probe (VIC’-CGGTAAGGTTTGGTCGCCGAC 3’-QSY) and the primer pair (puro Forward: 5’-CCCGATGCCACATAGAGC 3’; puro Reverse: 5’-CCATTC-TAGGGCCAATTTCGTC 3’) were designed and used to target the puro RNA. More details regarding plasmid standard and quantitative RT-qPCR assay are provided in the sections SI. 1 and SI. 2 (Supplementary Information), respectively.

2.4. Determination of average wastewater SARS-CoV-2 viral load for each reported patient to identify the facilities exhibiting suppression

In order to predict the average SARS-CoV-2 gene copies produced by each patient contributing to the sewershed as the benchmark for assessing the suppression rate for each facility, 57 facilities were monitored from July 6, 2020, to December 7, 2020. Wastewater samples were collected in triplicate from each facility once a week during that period. Flow rates information was collected by the wastewater treatment facility operators, while the number of cases reported for each sewershed was provided by the Missouri Department of Health and Senior Services (DHSS). To establish the relationship between SARS-CoV-2 viral load and case number, the total viral loads were calculated according to Eq. (1):

\[
\text{Total viral load} = [N1, N2] \times F \times Q \times D
\]

where \([N1, N2]\) (copies/μL) is the average SARS-CoV-2 concentration in the wastewater samples, determined by RT-qPCR. \(F\) is the extraction factor (350), that converts the units from copies/μL to copies/L, \(Q\) is the flow rate (L/day), and \(D\) is the number of days (161 days). The average viral load per diagnosed case was calculated by developing a regression relationship between the viral load and diagnosed case numbers.

2.5. Identifying the facilities for chemical analysis

The facilities consistently showing low viral load per diagnosed case which are deviated from the established correlation between viral load and reported cases, suggests suppression of the viral genetic material from the sewershed, were identified. Thus, the viral load per diagnosed case for all the 57 tested facilities were ranked according to their standardized suppression rates.

To develop the relationship between suppression rates and the concentrations of each identified molecule, the facilities representing a gradient of suppression rates, including no suppression, moderately
suppression and severely suppression, were selected for further chemical analysis. The chemical analysis in combination of the stepwise regression analysis were integrated to help identify the bioactive compounds that suppressed the SARS-CoV-2 signals.

2.6. Sample preparation for chemical profiling and targeted analysis

Triplicate wastewater samples collected in 50 mL polypropylene centrifuge tubes were vortexed (Vortex Genie 2, Fisher, NY, USA) for 10 s before being transferred to smaller tubes. Then, 1.8 mL of the wastewater was transferred to 2 mL microcentrifuge tubes and centrifuged (Eppendorf 5415D, Hamburg, Germany) at 12,000 rpm for 15 min. After centrifugation, 1.5 mL of the wastewater supernatant and 1.5 mL MeOH were mixed in 5 mL glass tubes. The mixture was vortexed for 10 s and 1.5 mL was filtered through 0.2 \( \mu \)m syringe filter (Acrodisc with PTFE membrane, Waters, MA, USA). Extracts were stored at -20°C until analysis with the high-performance liquid chromatography-tandem mass spectrometry (LC-MS/MS).

2.7. Untargeted metabolomics global chemical profiling analyses

Ultra-High Performance Liquid Chromatography (UHPLC) system coupled to a maXis impact quadrupole-time-of-flight high-resolution mass spectrometer (Q-TOF) (Bruker Co., Billerica, MA, United States) was used to analyze the wastewater extracts. The system was operated in

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**Table 1**

SARS-CoV-2 gene suppression rates for the wastewater treatment facilities included in this study.

| No | Facility | Suppression rate (%) |
|----|----------|----------------------|
| 1  | MACON    | 94.1                 |
| 2  | JOPTC    | 58.1                 |
| 3  | INDRC    | 47.7                 |
| 4  | MSDFN    | 21.2                 |
| 5  | MSDMR    | 3.76                 |
| 6  | MSDBP    | 3.65                 |
| 7  | COILMB   | 0.912                |
| 8  | MSDGG    | -0.912               |

*Fig. 4. Location of wastewater treatment facilities included in the suppression study (N = 8).*
either negative or positive electrospray ionization modes. Each waste
water sample and methanol blank (control) were analyzed in triplicate.
All the details regarding operational parameters and column are pro-
vided in the section SI. 3 (Supplementary Information).

To identify the molecules of interest that exhibited statistically sig-
nificant differences in relative intensities among the wastewater treat-
ment facilities, the CDF files obtained from UHPLC-MS analysis were
uploaded and processed using XCMS Online (xcmsonline.scripps.edu).
XCMS is a cloud-based informatics platform that can process and visu-
alyze mass-spectrometry-based untargeted metabolomic data and
perform statistical analysis [22,23]. The data process includes spectra
alignment and visualize the differences in profiles of compounds among different
facilities, partial least squares-discriminant analysis (PLS-DA) was per-
fomed and heatmap was generated via the web-based tool Meta-
bon (Wishart Research Group, University of Alberta, Alberta, Canada) (Xia and Wishart, 2011). Finally, to determine if there is a
significant difference between the means of the controls and treatments in
kinetic experiments, paired samples t-test with a significance level of
0.05 was used. Statistical analyses were conducted using XLSTAT soft-
ware (XLSTAT 2018: Data Analysis and Statistical Solution for Microssoft
Excel. Addinsoft, Paris, France).

### 2.9. Statistical analysis

Stepwise linear regression models and least absolute shrinkage and
selection operator (LASSO) regression models were utilized to identify
the compounds that are positively correlated with the SARS-CoV-2
suppression rates. In all models, chemical signal intensities quantified by
UHPLC-MS in positive or negative ion mode were the predictor
variable and the viral suppression rate at selected WWTPs facilities was
the response variable. Four different statistical approaches were used to
determine the positive correlation between the relative intensities of
the compounds and suppression rate. The four approaches included: for-
ward stepwise regression, backward stepwise regression, best subset
linear regression, and LASSO (SI. 6, Supplementary Information). In
XCMS platform, pair comparisons were used for two groups (i.e.,
water extracts and MeOH control blanks). To further characterize and
visualize the differences in profiles of compounds among different
facilities, partial least squares-discriminant analysis (PLS-DA) was
performed and heatmap was generated via the web-based tool Metab-
on (Wishart Research Group, University of Alberta, Alberta, Canada) (Xia and Wishart, 2011). Finally, to determine if there is a
significant difference between the means of the controls and treatments in
kinetic experiments, paired samples t-test with a significance level of
0.05 was used. Statistical analyses were conducted using XLSTAT soft-
ware (XLSTAT 2018: Data Analysis and Statistical Solution for Microssoft
Excel. Addinsoft, Paris, France).

### 2.10. Suppression study

The suppression experiments were carried out to investigate the effect
of the identified molecules on SARS-CoV-2 genetic materials in the
wastewater. Stock solutions of each identified compound were prepared
with commercially available standards in 100% methanol at a concen-
tration of 10,000 mg/L. A 20 mL wastewater sample with verified high
SARS-CoV-2 concentrations was mixed with 20 mL ultrapure water
(Milliq system, 18.2 mΩ·cm at 25 °C, Synergy® Water Purification Sys-
tem, MA, USA). The mixture was stirred gently for 5 min and transferred
to 50 mL polypropylene tubes (SARSTEDT, Newton, NC, USA). Then,
the diluted wastewater samples were spiked with 200 µL of 10,000 mg/L of
each target compound to reach a final concentration of 50 mg/L.
Another set of the control samples were spiked with 200 µL of methanol.

Table 2: The XCMS data processing parameters are described in the section SI. 4.

| List of the compounds putatively identified in the wastewater extracts. |
|---------------------------------|-------------------------------|
| Putatively identified compound   | Abbreviation | Formula | Retention time (min) | Theoretical mass | Extracted mass | ppm | Adducts |
| --------------------------------|---------------|---------|----------------------|------------------|---------------|-----|---------|
| 4-Octylphenol                    | OCPH          | C8H10O  | 24.21                | 205.1599         | 205.1599      | 0.487 | [M-H+]  |
| Oleic acid                       | OACD          | C18H34O2| 32.09                | 281.256          | 281.2494      | 1.422 | [M-H+]  |
| Lauroyl peroxide                 | LAPE          | C12H24O4| 33.86                | 397.3232         | 397.3325      | 0.503 | [M-H+]  |
| Palmitic acid                    | PAAC          | C16H32O2| 36.03                | 255.2330         | 255.2331      | 0.392 | [M-H+]  |
| 2,4-Dichlorothioleone            | DITO          | C3H4Cl2 | 0.52                 | 158.9774         | 158.9785      | 0.619 | [M-H]   |
| Nitrilcin                        | NETI          | C9H10NO2| 4.98                 | 476.2979         | 476.3074      | -1.049 | [M-H]   |
| Trolamine                        | TROL          | C16H32O2| 0.56                 | 172.0944         | 172.0945      | 0.581 | [M-Na+] |
| 3-Chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone | CDHF | C8H8Cl2O | 36.02 | 216.9221 | 216.9229 | 3.687 | [M-H+] |
| Dimethicone                      | DIME          | C10H16S1| 1.51                 | 163.0696         | 163.0697      | -1.226 | [M-H+] |
| 4-Dodecylphenol                  | DOPH          | C12H24O  | 27.55                | 280.2635         | 280.2639      | 1.427 | [M-NH4+]|
| 2-Dodecylbenzenesulfonic acid   | DBS           | C21H18O1| 2.95                 | 344.2254         | 344.2261      | 0.033 | [M-NH4+]|
The tubes were sealed, shaken, and sit on the bench at ambient temperature for 24 h. After 24 h, RNA was extracted immediately from raw samples, and viral concentrations were quantified by RT-qPCR.

The suppression rates (SR) were calculated using Eq. (2):

$$SR(\%) = \left(\frac{[N1]_a - [N1]_b}{[N1]_a}\right) \times 100$$

(2)

where \([N1]_a\) and \([N1]_b\) (copies/µL) are the SARS-CoV-2 concentration in the control (no chemical added) and in the treatment respectively.

### 2.10.1. Suppression kinetics

The suppression of SARS-CoV-2 genetic material in wastewater over time was also investigated. The experiments were conducted at room temperature. The spiked wastewater samples (with 50 mg/L of each compound) were collected at times: 0, 3, 6, 12, 24, 48, and 96 h. The samples were immediately extracted and processed by RT-qPCR. The dissipation data were fit to the first and second-order kinetic models:

#### 2.10.1.1. First-Order rate law

If the rate of reaction exhibits first-order dependence on the concentration of one reactant \((C)\), the rate law is expressed in Eq. (3):

$$-\frac{d[C]}{dt} = k[C]$$

(3)

where \([C]\) is the concentration of reactant \(C\), \(k\) is the first-order rate constant, and \(t\) is time. Rearranging the rate law and solving the integral using initial conditions of \(t = 0\) and \(C = C_0\), the new expression can be found in Eq. (4):

$$\int_{t_0}^{C} \frac{d[C]}{[C]} = -k \int_{t_0}^{t} dt \implies [C] = [C_0] e^{-kt}$$

(4)

Subsequently, this expression can be written as \(\ln[C] = -kt + \ln[C_0]\). Plotting the natural logarithm of the concentration \([C]\) versus \(t\) for a particular reaction will, therefore, allow determination of whether the reaction is first-order. If the reaction is first-order, the slope of the resulting line yields the rate constant \(k\). The half-life \((t_{1/2})\) of the reaction is calculated by using Eq. (5):

$$t_{1/2} = \frac{\ln(2)}{k} = \frac{0.6931}{k}$$

(5)

#### 2.10.1.2. Second-Order rate law

If the reaction is greater than first-order, the rate law is expressed in Eq. (6):

$$-\frac{d[C]}{dt} = k[C]^n$$

(6)

After integrating, the Eq. (7) can be obtained:

$$\frac{1}{n-1} \left( \frac{1}{C} \right)^{n-1} - \frac{1}{C^n} = -kt$$

(7)

For the second-order reaction \((n = 2)\), both with respect to \(C\) and overall, the rate law is expressed in Eq. (8):

$$\frac{1}{[C]} = \frac{1}{[C_0]} + kt$$

(8)

The half-life \((t_{1/2})\) of the reaction is calculated using Eq. (9):

$$t_{1/2} = \frac{1}{k[C_0]}$$

(9)

For a second-order reaction involving a reactant, the rate constant \(k\) can be determined by plotting \(1/[C]\) versus \(t\) to yield a straight line with a slope of \(k\).

### 3. Results and discussion

#### 3.1. Identification of the facilities with high suppression rates

Between July 2020 and December 2020, more than 57 wastewater treatment facilities across the state of Missouri, USA were monitored weekly for SARS-CoV-2. This extensive testing of wastewater treatment facilities has provided a comprehensive overview of signal intensity from COVID patients in wastewater. The long-term monitoring showed a clear correlation between the number of COVID patients in a sewershed and the level of viral load in the wastewater (Fig. 1). However, there is also clear variability among treatment facilities (Ahmed et al., 2021). Specifically, some facilities consistently have lower recovery rates of SARS-CoV-2 load per diagnosed case, suggesting suppression of the genetic material in the sewershed.

With data available from Missouri Department of Natural Resources (MoDNR) and DHSS (including reported case numbers), flow rates, along with RT-qPCR results, the average quantity of SARS-CoV-2 load per patient that contributing to the sewershed was calculated (Fig. 1).
The results showed that on average, there are around $5 \times 10^{11}$ SARS-CoV-2 viral load per reported case with minimum and maximum values of $2.8 \times 10^{10}$ and $7.7 \times 10^{11}$ respectively. Although the amount of SARS-CoV-2 contributed per case varies among communities, there were clear outlier communities that produce little or no genetic material in the wastewater despite the presence of known outbreaks. For example, Troy Southeast WWTP (TRYSE), Macon WWTP (MACON), and Marston WWTP (MARST) (Fig. 2).

Fig. 2 presents the average SARS-CoV-2 viral load per diagnosed case among all the facilities included in this study. According to the results, sewersheds can be divided into three major zones based on SARS-CoV-2 signal suppression (Fig. 2): Zone 1 includes all the facilities with average viral load/case lower than $5 \times 10^{11} \pm 10\%$ variations. These facilities consistently have low recovery rates of viral load per diagnosed case, which suggests viral genetic material suppression in the wastewater. Suppression of viral genetic material in the wastewater could explain the results of Ahmed et al. (Ahmed et al., 2021), in which no correlation was found between viral genetic material and daily reported cases. Since SARS-CoV-2 is an enveloped virus, it is very likely to be sensitive to chemicals, especially detergent because of a disruption of the lipids composing the envelope. To test this hypothesis, Robinson et al. (2022) treated raw wastewater with 1% TritonX-100, which is a nonionic surfactant. The results showed that the treatment with TritonX-100 reduced SARS-CoV-2 signal about 100-fold.

Zone 2 consists of the facilities within the average SARS-CoV-2 load/case (no suppression or signal enhancement). Hence, the general trend (viral load/reported case) was first established by plotting copies vs. cases as demonstrated in the Fig. 1. Any facility’s viral load/reported case fall within the 10% variation of the trend was considered no suppression (Zone 2). However, any facility with a viral load/reported case falls beneath the trend was considered as the suppression (Zone 1) (Fig. 2). Finally, Zone 3 is comprised of the facilities that have higher numbers of average viral load/case than the predicted values, indicating a likely underestimate in the number of COVID patients. Unreported cases are considered one of the major reasons for average SARS-CoV-2 gene copies being higher than the corresponding case number. During
the early phase of the pandemic, clinical testing was limited to multiple criteria, including symptoms and close contacts with a positive case (Ahmed et al., 2021). The strong correction between the viral load in the wastewater and reported cases strongly suggests that the reported cases (even if the report rate is low) are representative of the infected population, and the underreported rates should have been quite constant, otherwise, it would have been impossible to have such strong correlation.

The significantly lower ratio of the viral load/case than the ratio expected could be attributed to two factors (1) higher reported case number due to more accessible to clinical testing, and/or (2) chemicals degrade/suppress the SARS-CoV-2 signals in the wastewater. In order to distinguish between the two hypothesis (chemical suppression vs unreported cases), wastewater samples from the facilities in the Zone 1, such as Macon WWTP, which showed low gene copies/reported cases (Fig. 2), were mixed for 24 h with wastewater collected from one of the correction facilities. The results showed that Macon wastewater quickly suppressed SARS-CoV-2 by 56% ($P \text{-value} = 1\times10^{-4}$) (Fig. 3). This finding confirms that the chemical suppression is the most likely predominant factor that leads to the significant lower gene copies/cases ratio. To further confirm our hypothesis, we implemented the sampling regime along the upstream (manholes and wastewater effluent) of the identified wastewater facilities (e.g., Macon) to track the sources of the chemical suppressors. We have identified several sources of the chemical suppressors (e.g., effluent from food processing plants and drinking water treatment plant). From these results, among the 57 ranked facilities according to their suppression rates, eight facilities with different suppression rates were chosen for untargeted and targeted analysis (Figs. 1, 4, and Table 1). Six facilities with a range of suppression rates were chosen, including Macon WWTP (MACON), MSD Missouri River WWTP (MSDMR), MSD Fenton WWTP (MSDFN), Independence Rock Creek WWTP (INDRC), Joplin Turkey Creek WWTP (JOPTC), and MSD Bissell Point WWTP (MSDBP). Furthermore, two other facilities with no suppression were included in this study and used as a control: Columbia WWTP (COLUMB) and MSD Grand Glaize WWTP (MSDGG).

### Table 4
Molecular and product ions, retention times, and polarity of the compounds identified in wastewater.

| Compound                          | Molecular Ion (m/z) | Product Ion (m/z) | RT (min) | ESI+ / ESI- |
|-----------------------------------|---------------------|------------------|----------|-------------|
| 1-Octadecanamine                  | 242.25              | 56.9             | 12.69    | ESI-        |
| Diethanolamine                    | 106.1               | 88               | 2.19     | ESI-        |
| 2-Diethylaminooctanol             | 118.2               | 72               | 2.13     | ESI-        |
| Dicyclohexylamine                 | 182.3               | 83               | 7.16     | ESI-        |
| Nonoxynol-9                       | 265.3               | 89               | 11.9     | ESI-        |
| Dodecylenzene sulfonic acid       | 325.2               | 183.15           | 10.73    | ESI-        |
| Oleic acid                        | 281.2               | -                | 12.11    | ESI-        |
| Lauryl peroxide                   | 255.3               | 237.5            | 12       | ESI-        |
| Palmitic acid                     | 255.1               | 254.4            | 11.9     | ESI-        |
| Stearic acid                      | 283.3               | -                | 12.02    | ESI-        |
| 4-Nonylphenol                     | 219.14              | 133.2            | 12.1     | ESI-        |
| Palmitoleic acid                  | 253.24              | 252.8            | 12.8     | ESI-        |
| Sodium oleate                     | 281.4               | -                | 11.72    | ESI-        |
| Polyoethyleneglycol dioleate      | 309.3               | 308.6            | 12.29    | ESI+        |
| Didecyldimethylammonium chloride  | 256.6               | 60               | 11.28    | ESI-        |
| Bisoctyl Dimethyl Ammonium Chloride| 271.3              | 159.3            | 10.9     | ESI+        |
| C12-C14-Alkyl(ethylbenzyl)dimethylammonium chloride| 332.7              | 119              | 11.78    | ESI+        |
| Linear alkylbenzenesulphonic acid | 325.1               | 183.4            | 10.74    | ESI-        |

Fig. 6. (A) Variable importance in projection (VIP), (B) Partial least squares-discriminant analysis (PLS-DA). In the VIP score plot, the colored boxes indicate the relative intensities of the corresponding compounds in the control and suppression samples. Red represents higher relative abundance, while blue represents lower relative abundance in the VIP score plot. In the PLS-DA plot, the same-colored circles represent replicates of metabolic profiles for each group. The colored ellipses indicate 95% confidence regions of each group.

3.2. Untargeted analyses for wastewater extracts

The total ion chromatograms as well as the spectra of active compounds in the wastewater extracts were captured from liquid chromatography-high resolution MS (LC-HRMS) studies. The raw data were processed with the XCMS online platform and the features were annotated using the METLIN library, which resulted in the putative identification of 30 compounds (Table 2). These compounds are used for a variety of products such as surfactants, bleaching agents, emulsifiers,
and stabilizers (Table 3). Heatmap visualization of the clustering of chemical profiles is based on the 30 most significant compounds identified by using a t-test ($p < 0.001$) (Fig. 5). Twenty-three compounds exhibited higher relative intensities in suppressed facilities compared to control facilities, contributing significantly to the distinction between control (non-suppression) and suppression facilities (Fig. 5). Contribution of the variables was determined by examining the variable importance in projection (VIP) score, calculated from the weighted sum of the square for each partial least squares (PLS) loading of each compound (Ammons et al., 2015). From the top ten compounds identified by VIP, palmitelaidic acid (PAMA), 4-octynophenol (OCPH), N-undecylenbensulfonic acid (NUDS), aluminium dodecanoate (ALDO), and 2-dodecylbenzenesulfonic acid (DCBS) were identified as important compounds that significantly contributed to both control and suppression facilities (Fig. 6A). To further characterize the differences in the relative intensities, partial least squares-discriminant analysis (PLS-DA), a supervised regression technique for classifying groups from multidimensional data, was performed using MetaboAnalyst. PLS-DA analysis with two principal components (PCs) covered 85% of the total variability of the data (Fig. 6B), indicating significant differences in chemical profiles in control and suppression facilities. The first principal component (PC1) explained 63.9% of the data variability, whereas the second principal component (PC2) accounted for 21.1% of the total variability of the data set.

### 3.3. Targeted analyses for confirmation and quantification

The molecules tentatively identified through global metabolomic profiling analysis were further confirmed and quantified by LC-MS/MS targeted analyses. Authentic reference standards were used for unambiguous confirmation of compounds and the absolute quantification of the concentrations for each compound identified in the untargeted analysis approach. Due to the limitations of the instrument and limited availability of chemical references standards, eighteen compounds out of thirty were detected and quantified (Table 4) and (Table 5). Table 4 summarizes the molecular ions, product ions, retention times and ionization modes for targeted LC-MS/MS analysis of these compounds. The results showed that most of the bioactive compounds had higher concentrations in the wastewater of facilities exhibiting SARS-CoV-2 signal suppression than the control facilities. Four compounds had much higher concentrations in the suppression facilities than the control facilities. In particular, 4-nonylphenol, palmitelaidic acid, sodium oleate, and polyethylene glycol dioleate exhibited concentrations that were 73.3%, 35.3%, 54%, and 58.8% higher in the suppression facilities than the control facilities, respectively (Fig. 7). These compounds are mainly used in the production of surfactants and detergents in various industries (Andrade et al., 2017; Jin et al., 2004).

The concentrations of 4-nonylphenol in the urban wastewaters were determined in Japan, China, and USA. The concentrations were about 190 μg/L (Isobe and Takada, 2004), 2 μg/L (Lian et al., 2009), and 400 μg/L (Bergé et al., 2012), respectively. In this study, average concentrations of 4-nonylphenol were 1169 ± 13.3 μg/L and 2025.7 ± 247 μg/L in the control and suppression facilities, respectively. No information was found regarding the concentrations of the other three compounds in wastewater. Palmitelaidic acid was reported to be used to produce cosmetics, soaps, and industrial mold release agents (Deaver et al., 2020), and the average concentrations were 353.4 ± 5.12 μg/L and 478.2 ± 62 μg/L in the control and suppression facilities, respectively. According to the Consumer Product Information Database (CPID), polyethylene glycol dioleate (PEGD) is used as surface active agent and lubricant additive in different kinds of household and commercial products (e.g., stainless steel cleaner & polish, wood polish) (CPID, n.d.). The average concentration of PEGD in the control facilities was 689.3 ± 58.4 μg/L, while the average concentration in the

### Table 5
Concentrations of the identified compounds (ppb= μg/L) in influent of each wastewater treatment facility.

| Compound                              | COLUMN a | MSDGG a | INDR C | MACON a | MSDBP a | MSDFN a | MSDMR a | JOPTC a |
|----------------------------------------|----------|---------|--------|---------|---------|---------|---------|--------|
| 1-Octodecanamine                       | 143±4.2  | 80.22±10.3 | 346±3.36 | 315±21.1 | 196.82±8.8 | 73.27±5.6 | 71±14.3 | 201.32±24.2 |
| Diethanolamine                         | 197.19±2.5 | 286±56.7 | 293.36±21 | 145.32 | 830.32 | 555.47 | 201±18.8 | 1515±114 |
| 2-Diethylaminoethanol                  | 43.54±1.8 | 34.78±6.8 | 44.46±5.6 | 570±28 | 56.15±8.2 | 36.4±1.2 | 35.92±9.2 | 36.48±2.6 |
| Dicyclohexylamine                      | 0.63±0.05 | 1.1±0.1 | 0.72±0.04 | 1±0.09 | 67.55±3.8 | 0.85±0.1 | 0.7±0.08 | 0.82±0.2 |
| Nonoxynol-9                            | 470.7±74.1 | 353.6±69.1 | 356.2±10.5 | 1120.7 | 548.6±65.2 | 617.16 | 532.31 | 323.77 |
| Dodecylbenzenesulfonic acid            | 1510.46 | 1647.18 | 1459.32 | 578±25.1 | 907.45 | 1563.43 | 1833.8 | 1198.93 |
| Oleic acid                             | 939.34±56.8 | 495.62 | 421.51 | 712.94 | 360.25 | 221.52 | 560.71 | 422.54 |
| Lauryleic acid                         | 4860.82 | 4333±151 | 5106±105 | 11017±111 | 7759.6±212 | 2176.29 | 2591.2 | 5383.6 |
| Palmitic acid                          | 3279±165 | 2142±64.2 | 3886.2 | 3769±214 | 3114±121 | 1667.8 | 1840±36.5 | 4041.3 |
| Stearic acid                           | 1621.7±89.4 | 1692.5 | 1751±36.5 | 1649.3±125 | 1541.6 | 1346.5 | 1536.8 | 1652.1 |
| 4-Nonylphenol                          | 1159.6 | 1432.5 | 2263.31 | 2220.28 | 1733±255.2 | 2095.5 | 1699.8 | 2142.4 |
| Palmitelaidic acid                     | 570±51.7 | 317.3±21.7 | 353.43±5.6 | 369.7±44.6 | 458±15.7 | 476±40.5 | 491.7±78.5 | 537.75 |
| Sodium oleate                          | 288.15±35 | 340.33±32 | 417.71 | 742.3 | 543.6 | 349.5±46.5 | 570.1 | 286.1±38 |
| Polyethylene glycol dioleate           | 1182.2 | 873.8±138 | 963.1±169 | 1014.86 | 1128.32 | 1162.42 | 863.64 | 1418.75 |
| Didecyldimethy lammonium chloride      | 284.6±35 | 12.8±4.3 | 22.4±5 | 18.2±7 | 29.5±5.2 | 23±3.5 | 22.5±7.7 | 32.4 |
| Butyric Dimethyl Ammonium Chloride     | 19.8±2.1 | 4.7±0.18 | 4.7±0.66 | 96.2±2 | 4.5±1.5 | 6.5±0.5 | 20.5±1.3 | 8.2±1 |
| C12-C14-Alkyl(phytylethyl)             | 2.1±0.8 | 3±0.6 | 2.5±0.2 | 6.1±0.6 | 2.1±0.6 | 2.2±0.1 | 4.3±0.2 | 3.6±0.6 |
| Linear alkylbenzenesulfonic acid       | 753±65.7 | 1863±58 | 312±9.6 | 323±138.9 | 3855±7.576 | 3073±825 | 3730.1 | 1286.5 |

### Notes

- Absolute concentrations were determined by LC-MS/MS with authentic standards.

### References

Ammons et al., 2015
Andrade et al., 2017
Bergé et al., 2012
Isobe and Takada, 2004
Jin et al., 2004
Lian et al., 2009
Deaver et al., 2020
suppression facilities was $1095.2 \pm 189.2 \, \mu g/L$. Finally, sodium oleate is one of the major ingredients of metal polishes and is also used as an emulsifier in the polymerization of different compounds, according to Hazardous Substances Data Bank (HSDB). The observed concentrations of sodium oleate were $314.2 \pm 37 \, \mu g/L$ and $485 \pm 183 \, \mu g/L$ in the control and suppression facilities, respectively.

The presence of different industries in the sewersheds served by the suppression facilities might be the reason behind the high concentrations of these surfactants in the wastewater (Table 6). For example, the majority of the sewersheds contain food processing, cleaning products, plastics, and fabrics, and metal finishing industries which can significantly contribute chemicals to the wastewater received by the investigated facilities. Several studies have been done on the monitoring of wastewater for different compounds used as surfactants and detergents (Ahmia et al., 2016; Devi and Chattopadhyaya, 2013; Kopiec et al., 2015; Kruszelnicka et al., 2019; Olsson et al., 2008; Ross and Liao, 2015). However, there was no study on the effect of these compounds on SARS-CoV-2 in the wastewater. Thus, in the next section, the stability of SARS-CoV-2 genetic material in wastewater in the presence of four compounds is discussed.

### 3.4. Identification of the bioactive molecules associated with suppression of SARS-CoV-2 signals

To further characterize the findings from the metabolomic approach, stepwise regression models and LASSO regression models were used to determine the significant predictor variables (i.e., compounds’ relative intensities) which are positively correlated with the response variable (i.e., SARS-CoV-2 suppression rate). Results from positive and negative ion modes were analyzed separately.

The relationships among chemical signal intensities (generated from UPLC-MS positive ion mode analysis) and SARS-CoV-2 RNA suppression rate were examined using four different statistical approaches. According to the forward and backward stepwise regression models, the signal intensities of 13 out of 21 compounds were positively correlated with the viral suppression rate (Table S2). Best subsets regression also identified the signal intensities of 13 out of 21 compounds as being positively correlated with the viral suppression rate (Table S3). The signal intensities of eight out of 21 compounds were kept in the lasso regression.
model and obtained positive estimated coefficients (Table S4). Palmitelaidic acid, 4-nonylphenol, dicyclopentadiene, tetrabutylammonium and sodium oleate signal intensities were positively correlated with the viral suppression rate among all four statistical approaches (Tables S2-S4). Furthermore, using the same statistical approaches, polyoxyethylene glycol dioleate and 4-nonylphenol appeared to be positive correlated to viral suppression rate among all four approaches when the signal intensities from negative ion mode were analyzed (Tables S5 and S6). In conclusion, only the signal intensity of 4-nonylphenol was positively correlated with the viral suppression rate for both positive and negative ion modes.

### 3.5. Suppression experiments

The results from the statistical approaches suggested that the signal intensities of 4-nonylphenol, palmitelaidic acid, sodium oleate, and polyethylene glycol dioleate are positively correlated with SARS-CoV-2 suppression rates (Tables S2-S6). Therefore, the suppression of these compounds on SARS-CoV-2 were tested in incubation studies using real wastewater. A wastewater with known high viral copy numbers from “non-suppressed” facilities was used in these experiments. Fig. 8 shows the suppression rates (SR) of the compounds tested. After reacting for 24 h, the SR (%) were 57.2%, 35%, 43.3%, and 78.2% when adding PEGD, NOPH, SOOE, and PAMA, respectively.

![Fig. 8. Chemical effect on the SARS-CoV-2 signals in the wastewater. Samples from different batches were treated with 50 mg/L PEGD (polyethylene glycol dioleate), NOPH (4-nonylphenol), SOOE (sodium oleate), and PAMA (palmitelaidic acid). Duplicate wastewater samples were reacted with each chemical individually for 24 h at room temperature (N = 16). Error bars represent standard deviation.](image)

Enveloped viruses like SARS-CoV-2 have a variety of sites on the lipid membrane/envelop embedded with proteins where surfactants (nonionic, anionic, and cationic surfactants) can bind and interact (Simon et al., 2021). In general, surfactants are well known to bind to proteins, with the main mechanisms being hydrophobic, electrostatic, and H-bonding. The binding of the surfactants often leads to denaturation of the protein, either by the formation of protein-surfactant complexes or by unfolding (Richieri et al., 2000; Simon et al., 2021).

For enveloped viruses, a major point of attraction to surfactant molecules is the lipid bilayer in which hydrophobic interaction may become the main driving force. In addition to hydrophobic interactions, electrostatics may also play a role, especially if the surfactant was oppositely charged (Simon et al., 2021). Some surfactants might be bound within the lipid bilayer and this binding will raise the chemical...
potential of the surfactant in the bilayer, leading to thermodynamic instability (Tan et al., 2002). The four compounds tested were considered hydrophobic because their partitioning coefficient (logP) ranges between 5.6 and 15, demonstrating that hydrophobic interaction plays an important role in the interaction between surfactants and lipid bilayers.

The suppression of SARS-CoV-2 RNA in wastewater over time was also investigated. The experiments were conducted at room temperature. Spiked wastewaters (with 50 mg/L of each compound) were collected at the following times: 0, 3, 6, 12, 24, 48, and 96 h. Samples were immediately extracted and processed by RT-qPCR (Robinson et al., 2022). Fig. 9 shows the kinetic experimental results for both palmitelaidic acid (PAMA) and polyethylene glycol dioleate (PEGD). For both figures (A and B), the data are normalized by the number of RNA copies/µL in the control samples at time zero. PAMA and PEGD suppressed 70% and 65% of SARS-CoV-2 RNA for the first 6 hrs of the experiment, respectively. Both experiments showed significant differences between the control and treatment (P-value = 1.4E-4 and 9.8E-7, respectively). From our observation, the two compounds immediately suppressed the genetic material in the wastewater, and as such, the existence of these two compounds at 50 mg/L will dramatically decrease the COVID-19 signals in wastewater. It is therefore critical to determine the real concentrations of the compounds that reduce the stability of the genetic material signals in wastewater. Based on the known concentrations, correction factors may be developed to achieve more reliable and unbiased surveillance results for wastewater treatment facilities receiving wastewater from industries.

In order to calculate the rate constant of the reaction (k) and the half-life of the viral RNA (t_1/2), the data from Fig. 9 was used to determine the order of the reaction. Zero-order, first-order, and second-order were tested and the results showed that all the data fit the second-order reaction (Fig. 10). This meant that the rate of the reaction increases by the square of the increased concentration of the SARS-CoV-2 RNA in the wastewater. Second-order kinetic was utilized to determine the degradation rate of bacterial antibiotic resistance genes (ARGs) during the exposure to free chlorine, monochloramine, chlorine dioxide, ozone, ultraviolet light, and hydroxyl radical (He et al., 2019). The calculated half-lives were compared to the results from 24 h (Fig. 8). The SARS-CoV-2 RNA were suppressed by 78.2% and 57.2% when adding PEGD and PAMA, respectively. The calculated t_1/2 (the time when SARS-CoV-2 concentrations drop to its half value) were 8.5 h and 2.2 h for PEGD and PAMA respectively (Fig. 10).

The effect of field concentrations on the stability of SARS-CoV-2 was also investigated. Eighteen compounds have been divided into two groups depending on their environmentally relevant concentrations in real wastewater. Eleven compounds had concentrations in µg/L range and seven in mg/L range. The mixture experiments were conducted by spiking wastewater samples with 100 µg/L of µg/L range compounds and 1000 µg/L of mg/L range compounds. Fig. 11 (A) shows the kinetic experimental results for eighteen compounds detected in wastewater samples from different locations. The mixed chemicals suppressed 78.2% of SARS-CoV-2 RNA in the first 24 h. The difference between control (no chemicals) and treatment was significant with P-value = 5E-4. According to the results from environmental relevant concentration, a correction factor of [100/(100-78.2)] = 4.59x can be utilized to achieve more reliable and unbiased surveillance results. This correction factor illustrates the utility of this concept and how it can be used to correct wastewater surveillance results. Further studies need to be conducted to correlate the suppression rates with chemicals’ concentration to generate reliable correction factor for each sewerhed. To confirm that the suppression of SARS-CoV-2 RNA comes from chemicals effect not PCR inhibition, puro control virus was spiked to each wastewater sample and RT-qPCR was conducted at the same time frames.
wastewater sample as an internal control. Fig. 11 (B) shows puro gene copies in control and treatment samples at each time point. No significant difference in the puro copy numbers was found between the two sample sets ($P$-value = 0.57). For the further confirmation, dilution was also performed to the selected samples. The results suggested that all PCR inhibition was insignificant at the concentration range of the studied chemicals. Finally, the rate constant of the reaction ($k$) and the half-life of the viral RNA ($t_{1/2}$) were also calculated (Fig. 11 (C)). The data from mix experiment fit the second-order reaction with $t_{1/2} = 8.6$ h and $k = 0.143$. This finding might explain the conflicted findings reported among different studies. For example, Robinson et al (Robinson et al., 2022) (Missouri team), the Ohio State (Ai et al., 2021), and the team at University of Notre Dame (Bivins et al., 2020) reported constant stability of SARS-CoV-2 in wastewater at room temperature for at least 5-7 days, while the findings reported by Weidhaas et al (Weidhaas et al., 2021) (team from Utah) suggest rapid degradation of the SARS-CoV-2 signal following a first order decay constant at both 4°C, 10°C, or 35°C within 24 h, with the virus signal not being detectable after 12 h of storage at 35°C. Similar susceptibility to decay and degradation of SARS-CoV-2 RNA by increasing temperature in wastewater were also reported by Ahmed et al. (Ahmed et al., 2020). Furthermore, when wastewater was spiked with SARS-CoV-2, linear decay at 4°C was observed by Hokajarvi et al. (2021) on the first 28 days, while no decay was visible within 58 days at -20°C or -75°C.

Finally, it is important to mention that one of the limitations of previous studies is not taking in consideration the presence of different compounds that degrade SARS-CoV-2 and lead to loss of viral RNA. Therefore, we recommend considering the following when studying the stability of SARS-CoV-2 in wastewater 1) the presence of industry within investigated sewershed, 2) industrial category, 3) The type and concentration of chemicals released by the industry.

4. Conclusions

Approximately 20% of our currently tested wastewater treatment facilities (WWTFs) in Missouri, USA receive some input from industries. Several classes of molecules released by these regional industries and manufacturing facilities, particularly the food processing industry, significantly suppressed the signals of SARS-CoV-2 in wastewater by breaking down the lipid-bilayer of viral membranes. By taking advantage of recent advancements in mass spectrometry, metabolomics algorithms, computational capacity and mass spectral reference databases, we have successfully identified 30 bioactive chemicals. These chemicals represent active ingredients in surfactants, detergents, lubricants, preservatives, degreasers, and disinfection products. Eighteen compounds out of thirty were detected and quantified. Incubation studies validated the suppression activities of mixture of eighteen compounds. The simulated mixture of the active chemicals with environmentally relevant concentrations suppressed 78.2% of SARS-CoV-2 RNA in the first 24 h. Thus, for wastewater treatment facilities receiving wastewater from industries, a correction factor could be developed to achieve more reliable and unbiased surveillance results for assessing the prevalence of COVID-19 in the sewersheds.

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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