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Normalization of viral loads in Wastewater-Based Epidemiology using routine parameters: One year monitoring of SARS-CoV-2 in urban and tourist sewersheds

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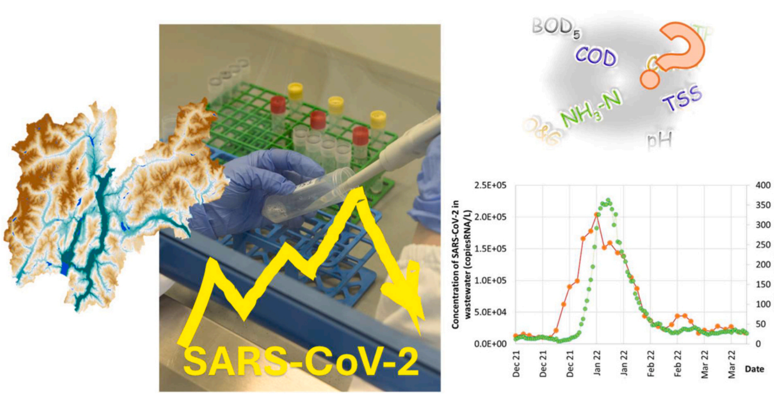
HIGHLIGHTS

- Shared parameters do not exist for normalization in wastewater-based epidemiology.
- Normalization performed with population size based on hydrochemical parameters.
- Normalization is not so relevant in urban areas with negligible fluctuating people.
- Population based on NH_4-N is effective for normalization of data in touristic areas.
- The monitoring of ammonium appears routinely and easy for decision-making in WBE.

GRAPHICAL ABSTRACT



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ABSTRACT

In wastewater-based epidemiology, normalization of experimental data is a crucial aspect, as emerged in the recent surveillance of COVID-19. Normalization facilitates the comparison between different areas or periods, and it helps in evaluating the differences due to the fluctuation of the population due to seasonal employment or tourism. Analysis of biomarkers in wastewater (i.e. drugs, beverage and food compounds, microorganisms such as PMMoV or crAssphage, etc.) is complex to perform, and it is not routinely monitored. This study compares the results of alternative normalization approaches applied to SARS-CoV-2 loads in wastewater using population size calculated with conventional hydraulic and/or chemical parameters (i.e. total suspended solids, chemical oxygen demand, nitrogen forms, etc.) commonly used in the routine monitoring of water quality. A total of 12 wastewater treatment plants were monitored, and 1068 samples of influent wastewater were collected in urban areas and in highly touristic areas (summer and/or winter). The results indicated that both census and population estimated with ammonium are effective and reliable parameters with which to normalize SARS-CoV-2 loads in wastewater from urban sewersheds with negligible fluctuating populations. However, this study reveals that, in

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the case of tourist locations, the population calculated using NH_4-N loads can provide a better normalization of the specific viral load per inhabitant.

1. Introduction

Wastewater-based epidemiology (WBE) has proven to be a valuable means with which to acquire epidemiological information and understand the circulation of pathogens and viruses (i.e. SARS-CoV-2 in recent years) spatially and temporally in the population [1-7]. Put briefly, the main advantages of the WBE applied for epidemic surveillance are the following: (i) coverage of a large population, because the results represent the entire sewershed; (ii) low cost, because the results are based on only a few wastewater analyses; (iii) quasi real-time analysis, because 24-h sample collection and analysis can be completed in less than 48 h; (iv) capacity to include presymptomatic and asymptomatic individuals. For example, WBE applied to COVID-19 surveillance made it possible to avoid underestimation of the actual circulation of the virus, as happened in the case of clinical testing in the post-vaccination periods.

In May 2023, more than three years after the outbreak of the COVID-19 pandemic, the WHO recommended the end of the state of emergency for COVID-19; but the risk of new emerging variants that may cause new waves of cases and deaths and the potential threat of other pandemics still remains. The experience acquired during the COVID-19 pandemic has produced tools and technologies with which to be better prepared for pandemics and recognize them sooner. The European Commission has recommended the surveillance of SARS-CoV-2 and its variants in wastewater [8] and the indications can be used to be prepared also in the future. This guidance on how to monitor, analyze and interpret wastewater data includes the need to normalize viral load results.

Normalization is one of the most crucial aspects of WBE, useful to compare prevalence in different areas and periods [9-12]. The purpose of normalizing SARS-CoV-2 concentrations is to account for changes in the amount of wastewater due to periods of precipitation and differences in the size of the population over time due to commuting, tourism, or other factors. The US CDC's guidelines recommend normalizing SARS-CoV-2 loads by using population size [13]. Keshaviah et al. [14] surveyed wastewater monitoring programs in 43 high- and low-income countries, and highlighted that many researchers are evaluating different approaches to normalization and standardization.

For population normalization in WBE, the effective size of the population served by the sewerage – called "de facto" population – must be used [15]. Instead, census data – called "de jure" population – do not furnish the adequate resolution required to understand ongoing population dynamics, because they do not include fluctuations in commuters, tourists,

or other demographic changes [15,16].

Certain endogenous or exogenous human biomarkers that occur diluted in wastewater, such as pharmaceuticals, personal care products, human metabolites or compounds in common beverages and food (among others acesulfame, creatinine, cholesterol, caffeine, nicotine, cortisol and many others) can be used for "de facto" population size estimation in WBE [16-18,12,19]. Biomarkers specifically proposed for normalization of SARS-CoV-2 data in wastewater are pepper mild mottle virus (PMMoV), cross-assembly phage (crAssphage), Bacteroides rRNA, and 18 S rRNA [20].

Monitoring biomarkers requires analytical chemistry skills, complex and expensive instrumentation, time-consuming procedures, and sometimes −80 °C sample storage, which is not always available in WWTPs laboratories. Furthermore, the level of biomarkers depends on population habits and on stability and sorption to particulate matter, factors that contribute to generating severe uncertainties [16,10,12]. Normalization using PMMoV does not improve the correlation between SARS-CoV-2 RNA in wastewater and clinical cases [21]. Moreover, some authors [20,22] have reported that normalization of SARS-CoV-2 load in wastewater using crAssphage, PMMoV and Bacteroides rRNA can deteriorate the correlation with the number of daily cases in comparison with the correlation before normalization.

In order to improve the population size estimation, a combination of multiple biomarkers has been proposed in the algorithm developed by Daughton [15], in the Bayesian model of O'Brien et al. [18], in the multi-parameter based approach of Hou et al. [10] or in the model proposed by Zheng et al. [23] who used an analytic hierarchy process and weighted factors.

Hydraulic (flow rate of influent wastewater) and chemical parameters such as Total Suspended Solids (TSS), Chemical Oxygen Demand (COD), forms of nitrogen and phosphorus are commonly used in the routine monitoring of water quality, and they have been widely used to estimate the so-called "population equivalent" (henceforth 'PE') [19,24,23]. These parameters can be used to calculate the population size with several advantages: (i) they are discharged by 100 % of the population; (ii) their analysis is standardized with official methods; (iii) there are no additional costs because they are already monitored in WWTPs for the quantification of removal efficiency and compliance with regulations; (iv) some parameters can be measured in real time with online probes; (v) they do not need mathematical models for the routine calculation of population size; (vi) they take less time, are less expensive, and are more affordable [25].

However, hydrochemical parameters are not only human-related, and they can be influenced by sources other than human metabolisms, i.e. industrial discharges, stormwater or agricultural runoff which can add further loads in the sewer network [10,15]. Compared with the other hydrochemical parameters, ammonium nitrogen (NH_4-N) is an anthropogenic biomarker that derives primarily from urine excretion and has been proposed for PE calculation in wastewater epidemiology [26,16,27,28]. Furthermore, the daily urinary excretion of NH_4-N varies only slightly among individuals.

Greenwald et al. [20] observed that the non-normalized SARS-CoV-2 RNA signal had a significantly positive correlation with clinical testing data, whereas normalization with various biomarkers had limited benefit for improving the correlation. Markt et al. [29] observed that the emergence of virus variants (e.g., B.1.1.7) might change the viral wastewater signal, probably due to different shedding patterns, which suggests the relevance of including viral variants when analyzing the correlation of the SARS-CoV-2 signal in wastewater and the incidences. Wade et al. [30] and Wilder et al., [31] emphasized that more work is needed to evaluate the most accurate biomarker for normalization in WBE. Furthermore, the impact of a chosen normalization method on the goodness of the relationship with active clinical cases has yet to be fully explored [32].

The research reported in this paper compared the results of alternative normalization approaches applied to SARS-CoV-2 loads in wastewater using the population size calculated with conventional hydrochemical parameters only. 12 WWTPs were monitored: 3 urban plants and 9 tourist plants characterized by an important presence of fluctuating inhabitants not known a priori. Time profiles of SARS-CoV-2 RNA in wastewater were acquired over a long period (1 year with a frequency of 3 samples/week) and then normalized and statistically compared with the active case of COVID-19 to evaluate the strength of the relationship. In particular, normalization was performed using population size calculated with a selected set of "ubiquitous" parameters (flow rate, TSS, COD, ammonium) routinely monitored and available in WWTPs.

This paper explores the following open issues [32]: (i) how normalization using an estimated *de facto* population compares to the use of census data; (ii) how normalization works with variable population dynamics; (iii) how normalized viral loads correlate when clinical cases decrease; (iv) the need for further research with larger datasets generated over a long period of time. The aim is to support the development of a more comprehensive understanding of the impact of normalization approaches in WBE applications.

2. Materials and methods

2.1. Wastewater treatment plants and sewersheds

The monitoring of SARS-CoV-2 in raw wastewater was carried out in 12 municipal WWTPs in the Province of Trento (Italy) which differed in population size and sewershed type: (i) urban areas in 3 plants, (ii) areas with a high tourist numbers in 9 plants (Table 1; the WWTPs are indicated with the numbers 1–12). The tourist period coincided with the summer in all the plants, while the ski districts (5 plants) also had tourism in the winter period (Table 1). The WWTPs sewersheds included one or more municipalities or parts of municipalities (Table 1). All the WWTPs considered in this study were connected to almost completely separate sewerage systems with a separate stormwater drainage system.

The census population ranged from 800 to 86,000 inhabitants (henceforth 'inh.'). The census population of each WWTP was calculated by adding up the entire municipalities or parts served by the plant as described in Cutrupi et al. [33]. It is very important to consider the entire extent of the sewage netwrk and the entire population connected to a WWTP in order to calculate the correct incidence rate of COVID-19 in the community [34].

To be noted is that the total population served by WWTP may be higher than the census data due to fluctuation in tourist numbers. Furthermore, additional inflows enter WWTPs and have to be treated, such as industrial wastewater, stormwater, or hauled wastewater. For these reasons, WWTPs are designed to deal with a load much greater than that produced by the mere census population (Table 1). In detail, the design capacity is usually expressed in terms of Population Equivalent (PE), where 1 PE is defined as the entity that would contribute an amount of 60 $gBOD_5/d$ (BOD_5 = Biochemical Oxygen Demand after 5 days of incubation). The design capacity of the 12 WWTPs ranged from 16,000 to 120,000 PE. The values of PE ranged from 1.4 (WWTP7) to 40 times (WWTP9; M. Campiglio is an extremely flourishing tourist area) higher than the census population.

The WWTPs treat wastewater discharged from several municipalities or parts of municipalities, so it is not easy to calculate the number of tourists for separate parts of the municipalities day per day. Moreover, many tourists only travel for 1 day or stay in private holiday homes. However, to give a rough idea, the recorded number of overnight stays in the entire Province of Trento was 9.2 million in summer 2021 and 6 million in winter 2021/22.

2.2. Period of monitoring

The wastewater monitoring period covered an entire year from the beginning of May 2021 to the end of April 2022. In particular, wastewaters entering WWTP1–2-3 were sampled continuously throughout the year. WWTP4–5-6–7 serving locations near lakes were monitored only in the summer period (June 2021-September 2021). WWTP8–9-10–11-12, characterized by mountain tourism (Alps), were sampled in the period June 2021-September 2021 (summer) and December 2021-April 2022 (winter + spring for skiing). Periods without tourism were not monitored because the areas were less likely to become COVID-19 hotspots.

2.3. Wastewater sampling and frequency

Overall, 1068 samples were collected from the WWTPs (Table 1), with an average of: (i) 152 samples in urban plants; (ii) 51 samples in plants with summer tourism only; (iii) 81 samples in plants with summer and winter tourism.

Raw wastewater was sampled at the WWTP inlet after sieving and degritting. Refrigerated (4 °C) autosamplers were used to collect 96 equal volume aliquots per day to form 24-hour composite samples. Then, the samples were collected in 250 mL bottles, without providing the acidification often applied to preserve their integrity for chemical analyses because the same samples were used for viral load quantification. The bottles were then transported to the laboratory and stored at 4 °C for a maximum of one week before analysis. For each composite sample, the influent daily flowrate (expressed in m^3/d) was measured onsite and recorded.

During the monitoring period, the sampling frequency was three times a week for all the WWTPs: two weekdays and one weekend. Consecutive sampling days were avoided. Samples were always collected with the same periodicity, so that they included both dry and wet weather. Sampling three times per week was considered optimal to perform accurate calculation of the 7-day moving average of SARS-CoV-2 concentrations and thus obtain good understanding of the COVID-19 trends according to literature [33,34].

2.4. Physico-chemical characterization of raw wastewater

The following physico-chemical parameters were analyzed in the influent wastewater in all the WWTPs using the American Public Health Association (APHA) standard procedures [35]: TSS (2540D using filtration and heating at 104 ± 1° C), COD (dichromate technique), Ammonium (NH_4-N, using Nessler method). Average concentrations and standard deviations measured during the monitoring period are summarized in Supplementary Material 1.

Table 1
List of the 12 WWTPs included in the study with their design capacity and characteristics of the sewersheds served. Key: S = tourism in summer; W = tourism in winter.

WWTP			Characteristics of the sewersheds served			No. of wastewater samples analysed
Acronym	Name	Design capacity (PE)	Type of area	No. municipalities served	Census population (inh.)	
WWTP1	$Trento_{North}$	120,000	Urban	6	86,000	156
WWTP2	$Trento_{South}$	110,000	Urban	1	47,000	157
WWTP3	Rovereto	96,000	Urban	9	55,000	142
WWTP4	Levico	100,000	Tourist (S)	14	36,000	48
WWTP5	Riva Arena	50,000	Tourist (S)	3	16,300	54
WWTP6	Riva S.N.	16,000	Tourist (S)	1	7,800	52
WWTP7	Arco	25,700	Tourist (S)	3	17800	52
WWTP8	Mezzana	30,000	Tourist (S,W)	5	4800	82
WWTP9	M.Campiglio	32,000	Tourist (S,W)	2	800	81
WWTP10	Tesero	50,000	Tourist (S,W)	5	10,300	81
WWTP11	P. Fassa	40,000	Tourist (S,W)	2	4100	80
WWTP12	Imer	30,000	Tourist (S,W)	2	6,000	83

2.5. Identification and quantification of SARS-CoV-2 in wastewater by RT-qPCR

The analysis of SARS-CoV-2 in wastewater was based on PEG precipitation combined with centrifugation, in compliance with the protocols of the Italian national program (SARI project) for wastewater surveillance [36]. The procedure is now briefly summarized:

1) Inactivation at 56 °C for 30 min and cooling at room temperature.
2) Addition of murine norovirus as process control.
3) Removal of solids by centrifugation at 4500g; addition of 4 g of PEG8000 and 0.9 g of NaCl to 40 mL of supernatant and mixing at a cold temperature for 15 min. Then a second centrifugation at 12,000g for 2 h was applied and the pellet was resuspended with 2 mL of lysis buffer containing guanidine thiocyanate (bioMerieux) for the extraction of nucleic acids for 20 min. Magnetic silica beads (50 µL) and a semi-automatic extraction platform (eGeneUp, bioMerieux) were used, obtaining a final volume of 100 µL. Samples were then cleansed of matrix inhibitors with the commercial One-Step PCR Inhibitor Removal Kit (Zymo Research, CA, USA).
4) Amplification was carried out by a real-time one-step qPCR reaction (Applied Biosystems™ 7500, ThermoFisher Scientific). The assay described in [37] was applied for the detection of a target gene of the virus, the *orf1b* (nsp14) with the mixture AgPath-ID One-Step RT-PCR (Life Technologies), primer 2297-CoV-2-F, primer 2298-CoV-2-R and probe 2299-CoV-2-P. The thermal cycling conditions were: reverse transcription for 30 min at 50 °C, inactivation for 5 min at 95 °C, and 45 cycles of 15 s at 95 °C and 30 s at 60 °C. All qPCR analyses were processed in duplicate.
5) Calculation of SARS-CoV-2 concentration, expressed in Genomic Units per Liter (GU/L), using a calibration curve produced with a dsDNA standard provided by the National Institute of Health (Italy).

Detection of SARS-CoV-2 can be performed down to concentrations of 10^2-10^3 GU/L. The assay limit of detection (ALOD) was calculated according to Cutrupi et al. [38]. Briefly, three different genomic concentrations were used (1, 2 and 4 GU/µL) and 24 replicate samples were run for each concentration. The concentration of SARS-CoV-2 identifiable with 95 % probability (ALOD95) resulted 0.92 GU/µL [38].

The analyses were carried out in the same laboratory, so that no inter-laboratory comparative assays were performed. However the laboratory used passed a national proficiency test in 2021.

The lab analyses of WWTP1–2-3, which are plants included in the national surveillance in accordance with the European Recommendation [8], were completed within 48 h after sampling. In the other plants, analyses were completed within 3–4 d after sampling.

2.6. Calculation of daily SARS-CoV-2 load in influent wastewater

The daily viral load (expresses as GU/d) was calculated using the influent flow rate (Q_{in} expressed in m^3/d) and the concentration of RNA copies (GU/L) in the wastewater:

$$\text{Viral Load} = Q_{in} \text{ x RNA copies/L x } 1000$$

Normalized viral load (expressed as GU inh^{-1} d^{-1}) was calculated by dividing by the population size, as follows (inter alia, [13]):

$$\text{Normalized Viral Load} = (Q_{in} \text{ x RNA copies/L x } 1000)/\text{Population} = \text{Viral Load/Population}$$

The population size can assume different values depending on the method used for its calculation, as indicated in Section 2.7.

2.7. Calculation of population equivalent

Use of hydraulic data – The population size using only hydraulic data (PE_{hydr}) was quantified according to this expression:

$$PEhydr = (Qin \text{ x } 1000)/D$$

where D (expresses as L inh^{-1} d^{-1}) is the average daily domestic wastewater production per capita. It is typically 80 % of the drinking water supplied by the network.

Use of physico-chemical parameters – The Population Equivalent (PE) was calculated taking into account the daily per capita excretion of a specific physico-chemical parameter. In detail, PE_{COD} was calculated with the following expression [39]:

$$PE_{COD} = (Q_{in} \text{ x COD x } 1000)/F_{COD}$$

where COD is the concentration in the influent wastewater (expressed in mg/L), Q_{in} is measured in the same sampling day, F_{COD} is the daily per capita excretion of COD, which was here assumed equal to the typical value of 110 gCOD $capita^{-1}$ d^{-1} [39].

Analogously, similar expressions considering TSS and NH_4-N concentrations can be used to calculate PE_{TSS} and PE_{NH4}, respectively, as follows:

$$PE_{TSS} = (Q_{in} \text{ x TSS x } 1000)/F_{TSS}$$

$$PE_{NH4} = (Q_{in} \text{ x } NH_4\text{-N x } 1000)/F_{NH4}$$

The coefficient F_{TSS} is typically 90 gTSS $capita^{-1}$ d^{-1} [39].

The coefficient F_{NH4} was assumed equal to 9 gNH_4-N $capita^{-1}$ d^{-1}. In the literature, various values have been proposed: 8.1 ± 0.37 gNH_4-N $capita^{-1}$ d^{-1} [16], 5.78–7.57 gNH_4-N $capita^{-1}$ d^{-1} [40], 6.4 gNH_4-N $capita^{-1}$ d^{-1} [41], 6 gNH_4-N $capita^{-1}$ d^{-1} [28], 9.4 ± 1.1 gNH_4-N $capita^{-1}$ d^{-1} [42], 6.5–10.7 gNH_4-N $capita^{-1}$ d^{-1} [43].

Use of a multi-parametric model – The Population Equivalent was calculated with a simple mathematical model (PE_{mod}) modified from the one proposed by Zheng et al. [23] and by Hou et al. [10]. A combination of hydraulic and physico-chemical parameters was considered. Each parameter was then weighted using a respective W factor:

$$PE_{mod} = W_{hydr} \text{ x } PE_{hydr} + W_{TSS} \text{ x } PE_{TSS} + W_{COD} \text{ x } PE_{COD} + W_{NH4} \text{ x } PE_{NH4}$$

where the weights were assumed equal to 0.1, 0.2, 0.3, 0.4 for W_{hydr}, W_{TSS}, W_{COD} and W_{NH4}, respectively (weights derived from our experience of the significance given to these parameters in routine management).

2.8. Epidemiological data

The publicly available data on "daily new cases" [44] in the municipalities belonging to the WWTP sewersheds were used. Then the "current active cases" were calculated according to Cutrupi et al. [33], taking into account the number of daily cases, deaths and recovered patients. In particular, current active cases account directly for the duration of the infection, because the virus remains in the stool during the entire period of positivity or even longer, and is therefore related to the SARS-CoV-2 load in wastewater.

COVID-19 cases were then scaled to 100,000 inhabitants, according to the protocols often used by health authorities to notify prevalence or incidence and based on the following expression:

$$\text{Prevalence} = \text{Current active cases} \times 100{,}000 \text{ / Population}$$

where the population is generally based on the census. Put briefly, prevalence is defined as the proportion of a population that has the disease at one time point. It therefore includes both new and existing cases [45]. The calculation of the prevalence of confirmed COVID-19 cases considers the population at risk (usually the census population) as the denominator.

2.9. Statistics

Descriptive statistics, boxplot representations, scatter plots, linear correlation analysis, t-tests, were applied to the datasets of physico-chemical parameters in wastewater, SARS-CoV-2 loads and COVID-19 current active cases. The Unequal Variance (independent) T-test was used with different amounts of data in the series and different variances of the dataset; a level of probability of 5 % ($p = 0.05$) was assumed as a criterion for acceptance. Linear correlation, expressed as Pearson's correlation coefficient (r, between 1 and −1) was used to evaluate the similarity between 2 series, and in particular between current active cases datasets and normalized/not normalized SARS-CoV-2 loads in wastewater. In our WWTPs, the datasets were quite long, so that a normal distribution of the data can be assumed. All statistical computations were performed using Microsoft Office Excel.

3. Results and discussion

3.1. Not-normalized profiles of SARS-CoV-2 RNA loads in wastewater and current active cases of COVID-19

Time profiles of daily SARS-CoV-2 loads in the influent wastewater of the urban WWTP1–2-3 are shown in Fig. 1: data were calculated using the procedure described in Section 2.6, expressed as GU/d and not-normalized. For better visualization and interpretation of the trends in Fig. 1, the 7-day moving average of SARS-CoV-2 loads is indicated in order to smooth the daily fluctuations (inter alia, [33,46]). Visually, the profiles of SARS-CoV-2 loads in Fig. 1, even if not yet normalized, well mirror the dynamic of the current active cases.

During the monitoring period (May 2021-April 2022), the epidemic situation evolved rapidly due to the emergence of new variants and waves of SARS-CoV-2. In particular, the Omicron wave that occurred over two months, from the end of December 2021 to February 2022, recorded the highest peaks of viral loads and active cases of COVID-19 (Fig. 1), thus providing better conditions in which to test normalization and correlations between the two datasets, as detailed in Section 3.3. To be noted is that the number of active cases depends on the coverage of clinical testing [47] and fewer active cases are expected when fewer tests are performed. Conversely, in the more recent period, due to the increase in the percentage of people vaccinated and the reduction of symptoms that make people less likely to get tested, it is more difficult to compare the datasets correctly due to the widening gap between confirmed cases and wastewater data. Moreover, the virus variants may have an important role [29,48] and the calculation of the variant-specific reproduction number deduced from changes in virus load in wastewater was proposed as a surrogate for the effective reproduction number [48].

The profile of SARS-CoV-2 loads in wastewater may exhibit a lag phase shortly before the peak of current active cases, because viral loads are able to anticipate the increase in the epidemic signal. The duration of the lag phase in published studies ranges from 2 to 28 days before Omicron and around 6 days during the Omicron wave [33,49]. However, this value depends on various factors such as incubation time and shedding duration, type of variant, demographic characteristics, hydraulic retention in the sewage network, solid deposits in the network, etc. In the research presented in this study, the correlation analyses were applied without considering the lag phase between the two datasets because there was not yet a single shared value able to represent the entire 1-year monitoring period.

3.2. Comparison of different calculations of population size for normalization

The hydrochemical parameters flow rate TSS, COD and ammonium nitrogen in the influent wastewater, routinely measured in WWTPs, and a multi-parametric model were used to calculate different values of PE according to the procedure set out in Section 2.7. The statistical box plots of PE in WWTP1–2-3 shown in Fig. 2 include PE fluctuations during the entire year. The PE values are also compared with the census inhabitants and the design capacity of the WWTPs (horizontal lines in Fig. 2).

A feature shared by the three WWTPs is the wide variability of PE_{TSS} and PE_{COD} as indicated by the large interquartile ranges (IQR) (Fig. 2). Another feature shared by plants is the lower median and average value of PE_{TSS} compared to other markers (Fig. 2); the PE_{TSS} box plots indicate that 50 % or more of the data always underestimate the census value. To be noted is that a meaningful estimate of PE should be close to or higher than the census value, but not much lower, because the potential presence of additional flows and loads – such as industrial discharges, commuters, etc. – increase PE. In a particular case, the estimated PE may be significantly lower than the census value: when the sewer network is associated with combined sewer overflows (CSO) spills during heavy rainfall, spills due to blockages, transport delay due to pump failures, or other operational problems that can cause losses in wastewater reaching the WWTP. In the area considered by the present study, WWTP1–2-3 were connected to separate sewerages and CSOs were absent or negligible during the year.

The estimate of PE_{hydr} (Fig. 2) provided median and average values

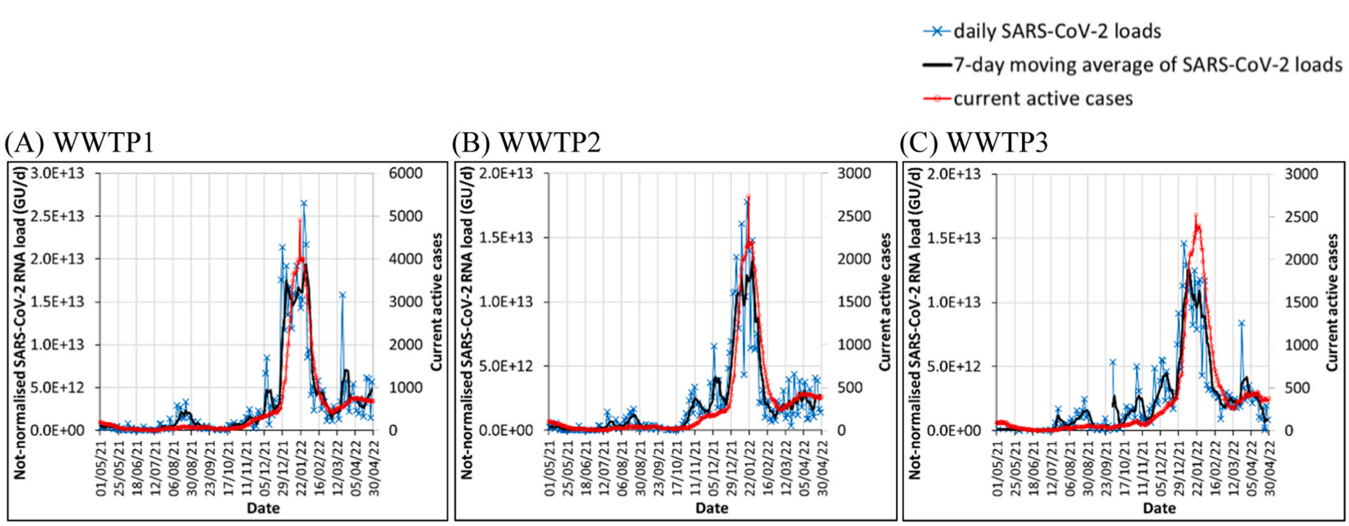

Fig. 1. Time profiles of not-normalized SARS-CoV-2 loads in influent wastewater of WWTP1–2-3 during the 1-year monitoring and current active cases of COVID-19.

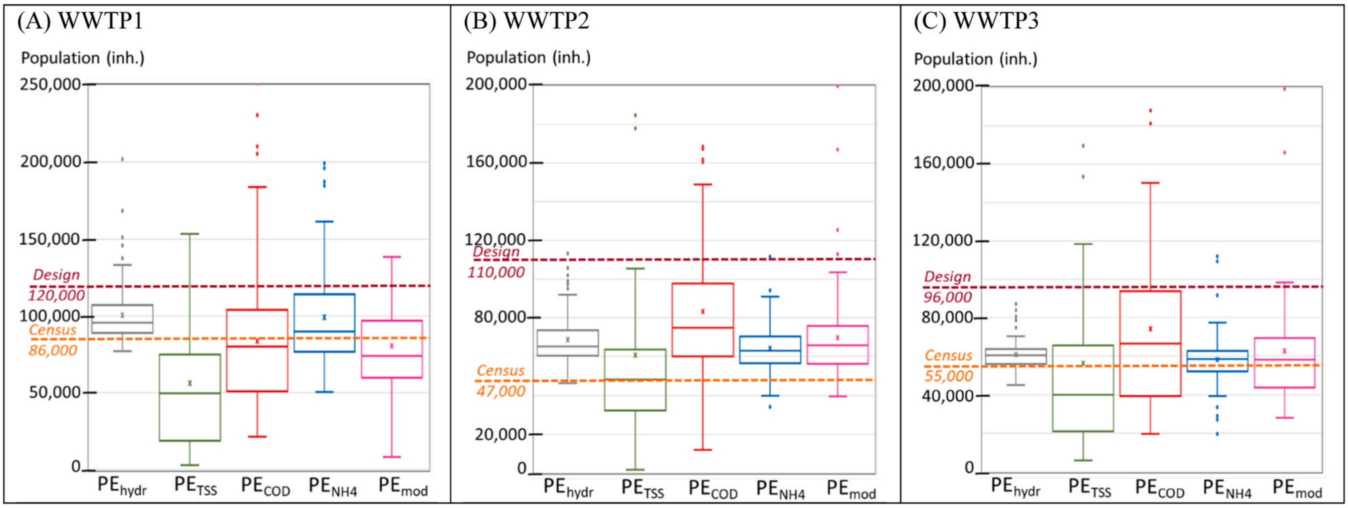

Fig. 2. Box plots of PE in WWTP1–2-3 calculated with different methods and compared with the census inhabitants and the design capacity (horizontal lines). Key: the horizontal bars in the boxes represent the median, the bottom and top of the box represent 1st and 3rd quartiles, the whiskers represent 10th and 90th percentiles. Symbols "× " indicate the average values. Outliers are plotted as individual points.

slightly higher but close to the respective census values. This is surprising because the PE_{hydr} estimate can be influenced by the presence of non-domestic waters (e.g. stormwater, infiltration of extraneous water along the network or industrial discharges) which may cause large uncertainties and a strong overestimation of PE_{hydr} compared to the census value. In this study, thanks to the separate sewer networks, the PE_{hydr} refers mostly to the average daily domestic wastewater production per capita (D expressed in L inh^{-1} d^{-1}; Section 2.7). This value depends on the region, and it may vary widely from 100 to 250 L inh^{-1} d^{-1}; in some cases it can be acquired from the local authorities. For example in the Netherlands, this value can be considered very stable at 120 L inh^{-1} d^{-1} [50].

Population size estimated using NH_4-N (Fig. 2) provided median and average PE_{NH4} values in agreement with PE_{hydr} and close to the respective census values. Furthermore, PE_{NH4} and PE_{hydr} exhibited the lowest IQR in all plants. This indicates that the magnitude of PE fluctuations is small when using NH_4-N with respect to COD or TSS. This difference is due to the fact that suspended solids and particulate organic matter in wastewater are affected by deposition and resuspension during dry or wet periods along the sewer network and their transport depends on the slope of the pipes and the flow rate. Conversely, soluble forms such as ammonium are markedly less affected by these factors. Therefore, the use of an anthropogenic marker, such as NH_4-N, can be extremely useful [16] also thanks to its simple analysis and the potential of being measured with affordable online probes.

Population size estimates with the multi-parametric model (PE_{mod}) did not yield a significant advantage over PE_{hydr} and PE_{NH4}: the IQR of the PE_{mod} box plot is higher, indicating results less reliable and consistent than those obtained from ammonium and flow rate.

Fig. 2 shows the design capacity of the WWTPs for comparison; it is a constant value, generally larger than the "de facto" population because the design takes into account future urban developments and intraday peaks of hydraulic and pollutants loads. Therefore, this parameter is considered not important for normalization here and consequently not used further.

Detailed comparison between PE estimated in WWTP1–2-3 with the various markers is presented in Table 2 with the main statistical results and p-values obtained from t-test. In particular, to evaluate the

Table 2
Statistical parameters of PE calculated with the various markers in WWTP1–2-3 and matrix of p-values obtained via t-test describing the significance of difference between PE series. Key: Asterisk and grey shading indicate p > 0.05.

Census ($inh.x10^3$)	PE marker	Median ($inh. x 10^3$)	Average ($inh. x 10^3$)	Matrix of p-values obtained via t-test: PE_{hydr}	PE_{TSS}	PE_{COD}	PE_{NH4}	PE_{mod}
WWTP1 86	PE_{hydr}	95	***100***	1				
	PE_{TSS}	49	56	2.3×10^{-7}	1			
	PE_{COD}	80	***84***	4.2×10^{-5}	0.002	1		
	PE_{NH4}	90	***100***	0.750	8.4×10^{-7}	0.001	1	
	PE_{mod}	74	***80***	5.5×10^{-8}	0.005	0.499	1.0×10^{-5}	1
WWTP2 47	PE_{hydr}	65	***69***	1				
	PE_{TSS}	48	***61***	0.243	1			
	PE_{COD}	75	83	3.2×10^{-5}	0.003	1		
	PE_{NH4}	63	***64***	0.003	0.589	1.1×10^{-7}	1	
	PE_{mod}	66	***70***	0.628	0.198	0.001	0.025	1
WWTP3 55	PE_{hydr}	60	***61***	1				
	PE_{TSS}	40	***56***	0.483	1			
	PE_{COD}	67	74	0.005	0.024	1		
	PE_{NH4}	58	***58***	0.074	0.757	0.001	1	
	PE_{mod}	58	***63***	0.523	0.361	0.037	0.165	1

similarity between two PE series calculated with two different markers, a p-value > 0.05 indicates not intrinsic differences and the average PE series appear similar, while a p-value < 0.05 indicates that the two averages are statistically different.

In WWTP1 (Table 2), the census value was 86,000 inh. and the closest estimates were the average PE_{COD} (84,000 inh.) and average PE_{mod} (80,000 inh.). However, the median of both PE_{COD} and PE_{mod} (80,000 and 74,000 inh., respectively) was lower than the census inhabitants. As previously mentioned, when the average and the median are lower than the census data, the estimates are considered to be of little significance. A good similarity was also established between average PE_{hydr} and average PE_{NH4} ($p < 0.05$), but in this case the population size was estimated at 100,000 inh., significantly higher than the census value.

Considering WWTP2 (Table 2), the averages of PE_{hydr}, PE_{TSS}, PE_{NH4} and PE_{mod} showed no significant differences ($p > 0.05$) and assumed values in the range 61,000–70,000 inh., which overestimated the census inhabitants (47,000 inh.) by 30–49 %. The parameter COD differs because it provides the highest median and average values of PE_{COD} (75,000 and 83,000 inh.). This result indicates that WWTP2 may receive additional sources of organic pollutants (e.g. industrial wastewater) able to increase COD loads in influent wastewater.

The PE results in WWTP3 (Table 2) were similar to those calculated in WWTP2. In detail, there were no statistical differences among the averages of PE_{hydr}, PE_{TSS}, PE_{NH4} and PE_{mod} (all in the range 56,000–63,000 inh.), which closely aligned with the census of 55,000 inh, and the overestimation was less than 15 %. Conversely, COD gave the highest average PE of 74,000 inh., which exceeded the census value by 34 %.

In conclusion, PE_{hydr} and PE_{NH4} yielded average values close to or slightly higher than the respective census values in all the WWTPs. Therefore both appear suitable for normalization. However, PE_{hydr} is significant for separate sewer networks (as in this study), while in combined sewers it is strongly affected by non-domestic sources. Consequently, the use of this parameter for normalization cannot be generalized. Finally, the PE_{mod}, introduced to reduce the PE variations generated by a single hydrochemical marker, did not give a significant improvement in the results.

3.3. Correlations between normalized SARS-CoV-2 loads and current active cases

The relationship between normalized SARS-CoV-2 loads in wastewater (with different normalization parameters) and the prevalence of active cases was evaluated with the linear statistical model using the Pearson's correlation coefficient r to estimate the strength of the correlation. The objective was to highlight one or more normalization methods that can provide the best match between normalized viral loads and COVID-19 prevalence. This aspect needs further research because the impact of alternative normalization methods on the relationship between normalized viral loads and current active cases has yet to be fully understood [32]. In this context, although the methods applied are not new, the monitoring of WWTPs characterized by a widely variable population, as those chosen in this work, offer original and innovative comparisons that can be useful for a shared approach to normalization.

The Pearson's r in WWTP1–2-3 is shown in Table 3; the results highlight the always positive correlation between the current active cases and the viral loads not normalized as well as normalized by census, PE_{hydr}, PE_{TSS}, PE_{COD}, PE_{NH4} and PE_{mod}.

The not-normalized dataset has high Pearson's correlation coefficients (Table 3), but there is a strong limitation in the use of viral loads without normalization (expressed as GU/d) because results can show higher prevalence in large urban areas than in small areas. In other words, normalization is needed to conduct comparison between different sewersheds and different communities and understand the spread of the epidemic.

Table 3
Pearson's correlation coefficients (r) of normalized as well as not-normalized SARS-CoV-2 loads against current active cases in WWTP1–2-3. Key: grey shading indicates high correlation coefficients.

Normalization metric of SARS-CoV-2 loads	WWTP1	WWTP2	WWTP3
Not-normalized	0.84	0.83	0.81
Normalized to census	0.84	0.83	0.81
Normalized to PE_{hydr}	0.83	0.81	0.81
Normalized to PE_{TSS}	0.72	0.68	0.57
Normalized to PE_{COD}	0.81	0.76	0.69
Normalized to PE_{NH4}	0.85	0.81	0.81
Normalized to PE_{mod}	0.84	0.82	0.76

Normalized viral loads to census correlated with active cases (Pearson's $r = 0.81–0.84$) exactly equal to the not-normalized viral loads (Table 3); in fact, on dividing the viral loads by a constant value (census) the variability of the dataset does not change, so that the correlation coefficients after normalization remain the same. In other words, the normalization with the "simple" census information provides useful normalized data but the correlation is comparable to the not-normalized dataset. This observation is in agreement with the results of Isaksson et al. [32] which indicated that the relationship between normalized viral loads and positive cases was relatively insensitive to whether census population or population size estimates were used. These authors suggested that the normalization to census inhabitants can be sufficient to detect temporal changes of SARS-CoV-2 viral loads during environmental surveillance [32]. To be noted is that normalization to census may not be representative in the case of tourist locations as highlighted in Section 3.4 relating to plants from WWTP4 to WWTP12.

Normalization with PE_{TSS} and PE_{COD} resulted in a decrease of correlation coefficients ($r < 0.8$) in some plants. Theoretically, PE_{TSS} is expected to be a good parameter for normalization, because higher SARS-CoV-2 concentrations would be associated with higher TSS concentration in wastewater due to attachment of viral particles to solids [9, 51]. Instead, here, the viral loads normalized to PE_{TSS} had the lowest Pearson's correlation coefficients in all the WWTPs. As said previously in Section 3.2, PE_{TSS} and PE_{COD} showed the largest IQR in the box plots (Fig. 2) for various reasons, including the high fluctuations of particulate matter and solids along the sewer network. An inappropriate marker used for normalization and high variability may deteriorate the correlation with active cases compared to the correlation before normalization, according to the observations of other authors [20,22].

Considering the hydrochemical parameters, only normalization of SARS-CoV-2 loads with PE_{hydr} and PE_{NH4} strongly correlated with active cases in all three WWTPs, always providing Pearson's $r > 0.80$ (Table 3). However, normalization to PE_{hydr} is not appropriate in the presence of combined sewerage. Conversely, PE_{NH4}-based calculation appears effective and reliable to normalize SARS-CoV-2 loads in wastewater, providing a good correlation with COVID-19 positive cases. Finally, normalization to PE_{mod} adds no significant advantage with respect to PE_{NH4}, while introducing an unnecessary complication.

These observations are in agreement with the findings of Amoah et al. [9], who used an Adaptive Neuro-Fuzzy Inference System model to demonstrate that the major physico-chemical parameter potentially having an association with the concentration of SARS-CoV-2 in wastewater is ammonium and, to a lesser extent, pH and TSS. In fact, ammonium in wastewater derives mainly from human urine and is therefore associated with the shedding of viral particles by infected individuals in their faeces. The use of NH_4-N as a reliable parameter for normalization of SARS-CoV-2 loads in WBE applications has also been proposed by Rauch et al. [27] and Arabzadeh et al. [26]. These studies once again confirm our conclusion that ammonium is a good parameter in the normalization of the viral load.

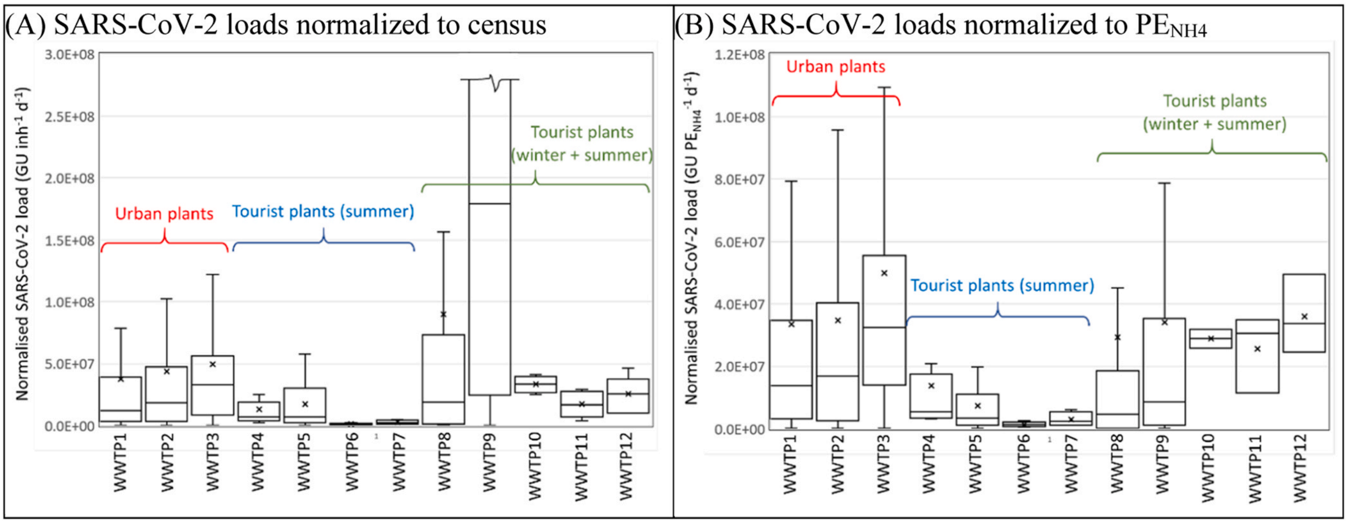

Fig. 3. Box plots of SARS-CoV-2 loads in urban and tourist WWTPs: (A) normalization of loads (expressed as GU inh^{-1} d^{-1}) to the census; (B) normalization of loads (expressed as GU PE^{-1} d^{-1}) to PE_{NH4}. Key: the horizontal bars in the boxes represent the median, the bottom and top of the box represent 1st and 3rd quartiles, the whiskers represent 10th and 90th percentiles. Symbols "× " indicate the average values.

3.4. Normalisation of SARS-CoV-2 loads in tourist locations using ammonium

The normalization of viral loads with PE_{NH4} becomes very important when the population fluctuates strongly with respect to the census value, as in the case of tourist locations characterized by high seasonal or weekly variations. The SARS-CoV-2 loads in the influent wastewater of the 12 WWTPs included in the monitoring (3 urban plants + 9 tourist locations) are compared in Fig. 3, showing the loads after normalization with census (Fig. 3A) and with PE_{NH4} (Fig. 3B).

The census population, indicated in Table 1, was extremely different among the 12 WWTPs: WWTP9 was characterized by the lowest census value of 800 inh., while WWTP1 registered the highest value of 86000 inh. Instead, the estimated PE_{NH4} increased significantly in all the WWTPs, ranging from 9000 ± 5300 inh. in WWTP11 to 99,200 ± 32,200 inh. in WWTP1, indicating the presence of a certain number of people (not known, but only estimated from influent wastewater) in addition to the census. For example, in WWTP9 the census value was about 800 inh., while the average PE_{NH4} was 13,700 ± 10,500 inh. This marked increase in population size affected the normalization of viral loads: in WWTP9, the average SARS-CoV-2 load normalized to census was $1.2 \pm 2.9 \times 10^9$ GU inh^{-1} d^{-1} (higher than the scale on the vertical axis in Fig. 3A), while the normalization to NH_4-N led to $3.5 \pm 6.5 \times 10^7$ GU PE^{-1} d^{-1} (Fig. 3B). Statistical analysis of this couple of values indicated a significant difference between the two normalization methods (p-value > 0.05). This example highlights that normalization to census can cause a significant overestimation of SARS-CoV-2 loads, even by orders of magnitude, especially in locations with a large number of fluctuating people and tourists.

Observing the plants WWTP8–9-10–11-12 in Fig. 3B, which are those with summer and winter tourism, the average viral loads with PE_{NH4} normalization (indicated with symbols "×" in the boxplots) are comparable and concordant with the average values in the urban WWTP1–2-3 belonging to same province. This could be interpreted as a meaningful normalization. Another observation derives from WWTP4–5-6–7 in Fig. 3B: these plants show significantly lower viral loads after PE_{NH4} normalization. This result is reasonable because the tourism only occurred in summer (June-August 2021) when the spread of the virus was low, with the absence of detectable SARS-CoV-2 genetic material in some samples, and the climatic conditions less favorable for virus transmission (open spaces, closed schools, etc.).

All these results demonstrate that, in the context of this study, the use of PE_{NH4} was a reasonable choice for normalization in tourist plants in order to follow the large seasonal fluctuations of population, while also ensuring the advantages of a simple, practical, cheap and repeatable parameter. In non-touristic plants, on the other hand, normalization with PE_{NH4} does not substantially change the overall patterns and trends of viral loads normalized to census. Indeed, the statistical analysis applied to compare the data of WWTP1–2-3 in Fig. 3A and B did not show any significant difference between the two normalization methods (using census and PE_{NH4}; p-value < 0.05).

4. Conclusions

None of the normalizations approaches substantially improved the strength of the correlation between COVID-19 active cases and normalized SARS-CoV-2 loads in wastewater. In fact, correlations with the non-normalized data showed Pearson's r-values equal to those with normalized data. However, normalization facilitates comparisons among different WWTPs in order to understand the circulation of the virus in different areas and periods. Both census and estimated population size by ammonium are effective and reliable parameters to normalize SARS-CoV-2 loads in wastewater from urban sewersheds with negligible population fluctuations.

However, this study reveals that in the case of tourist locations, since NH_4-N is an anthropogenic indicator, sensitive to population size fluctuations, PE_{NH4} can provide a better normalization of the specific viral load per inhabitant. This shows the benefit of including the simple monitoring of ammonium, without the need for other costly physicochemical parameters, in wastewater surveillance for WBE and decision-making.

Environmental Implication

Our research, focused on the WBE of SARS-CoV-2, belongs to the fields of environmental engineering and sciences. It respects the criterion of "environmental relevance" because SARS-CoV-2 is an environmental contaminant with hazardous effects on environment and humans. SARS-CoV-2 contaminates water and receiving bodies, reaching many surfaces and food products and remain infective for weeks/months. The study was conducted under environmentally relevant conditions since wastewater monitoring was performed during 12 months of pandemic and virus loads in wastewater were measured. The study has environmental relevance because aimed at early warning purposes, considering that virus contamination is an emerging environmental issue worldwide.

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CRediT authorship contribution statement

Paola Foladori: Writing – original draft, Methodology, Data curation, Conceptualization. **Francesca Cutrupi:** Writing – review & editing, Investigation, Data curation. **Maria Cadonna:** Writing – review & editing, Investigation, Data curation. **Mattia Postinghel:** Investigation.

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.

Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2024.135352.

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