SARS-CoV-2 wastewater surveillance in the Czech Republic: Spatial and temporal differences in SARS-CoV-2 RNA concentrations and relationship to clinical data and wastewater parameters

Kateřina Sovová [a,*], Petra Vašíčková [b], Vojtěch Valášek [c], David Výravský [a], Věra Očenášková [c], Eva Juranová [c], Milena Bušová [d], Milan Tuček [d], Vladimír Bencko [d], Hana Zvěřinová Mlejnková [c]

[a] T. G. Masaryk Water Research Institute p.r.i., Brno Branch, Mojmírovo náměstí 16, 612 00 Brno, Czech Republic
[b] Masaryk University, Faculty of Science, Kotlářská 267/2, 611 37 Brno, Czech Republic
[c] T. G. Masaryk Water Research Institute, Podbabská 30, 160 00 Prague, Czech Republic
[d] Charles University, First Faculty of Medicine, Institute of Hygiene and Epidemiology, Studničkova 7, 128 00 Prague, Czech Republic

A B S T R A C T

This study presents the results of systematic wastewater monitoring of SARS-CoV-2 RNA and basic wastewater parameters from four different wastewater treatment plants (WWTPs) in the Czech Republic over the 2020–2022 epidemic. Two-step reverse-transcription quantitative PCR targeting genes encoding the N and Nsp12 proteins was employed to detect SARS-CoV-2 RNA loading in 420 wastewater samples. The results obtained were used to evaluate the potential of wastewater analysis for describing the epidemiological situation in cities of different sizes and determining temporal differences based on the prevailing SARS-CoV-2 variant. Strong correlations between the number of active and hospitalised COVID-19 cases in each WWTP catchment area and the concentration of SARS-CoV-2 RNA detected in the wastewater clearly demonstrated the suitability of this wastewater-based epidemiological approach for WWTPs of different sizes and characteristics, despite differences in SARS-CoV-2 variant waves, with some WWTPs showing high predictive potential. This study demonstrated on the data from the Czech Republic that targeted systematic monitoring of wastewater provides sufficiently robust data for surveillance of viral loads in sample populations, and thus contributes to preventing the spread of infection and subsequent introduction of appropriate measures.

Introduction

COVID-19 is a respiratory infection caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). First identified in the general population in December 2019, it soon reached pandemic levels, eventually resulting in millions of hospital cases and deaths (WHO, 2023).

Multiple methods have been developed for SARS-CoV-2 surveillance and assessment of virus circulation in the population (Alhamid et al., 2022). SARS-CoV-2 monitoring has mainly been carried out by clinical testing of symptomatic patients; however, a significant proportion of patients proved to be either asymptomatic, pre-symptomatic or had mild, non-specific, symptoms and were therefore underreported, leading to significant underestimation of infection (Girum et al., 2020; Oran and Topol, 2021). Moreover, clinical data may also be influenced by testing

capacity and availability and by factors associated with human behaviour.

While transmission of SARS-CoV-2 is mainly through respiratory secretion, Xiao et al. (2020) suggested that it could also be excreted in faeces and urine, which is supported by our previous unpublished results. O'Keeffe (2021) showed that SARS-CoV-2 RNA could even be detected in the faeces of COVID-19 patients with mild or no symptoms, often after their respiratory samples showed as negative.

The successful use of wastewater-based epidemiology (WBE) to monitor drug consumption, pharmaceuticals use, water pollution and antibiotic resistance suggested that the same concept could be transferred to the surveillance of viruses, which could provide epidemiological information on the prevalence of viruses in the population and the emergence of new viral strains (Polo et al., 2020). Since then, the WBE approach has been applied as part of the global polio eradication

programme, as well as for measuring the prevalence of hepatitis A virus, hepatitis E virus, enterovirus and norovirus (O'Keeffe, 2021) and its use has been suggested even for SARS-CoV-2 surveillance, as both a suitable and objective complement to clinical testing.

Since 2020, the WBE approach has been widely used for SARS-CoV-2 surveillance (Medema et al., 2020; López-Peñalver et al., 2023; Rusiñol et al., 2021), with wastewater monitoring applied in >55 countries (O'Keeffe, 2021). The results of such monitoring programmes have confirmed that the concentration of SARS-CoV-2 RNA in sewage is dependent on the number of infected people in the community and on the rate of spread of infection (Medema et al., 2020). In some studies, SARS-CoV-2 RNA was even detected in wastewater before COVID-19 cases had been reported or before any increase in COVID-19 cases had been observed in clinical data, suggesting that WBE could act as a potential early warning system, preventing disease outbreaks (Medema et al., 2020; López-Peñalver et al., 2023). The WBE approach overcomes some of the limitations of clinical testing by providing integrated information for the whole community, while the monitoring of several WWTPs can provide a similar degree of population-level information as huge numbers of clinical tests. As such, WBE can provide important complementary information on the prevalence of SARS-CoV-2 in investigated areas and help identify potential hot-spots at an early stage in the infection process (Rusiñol et al., 2021).

The first cases of COVID-19 in the Czech Republic were reported on March 1, 2020 (Komenda et al., 2020), since when there have been several epidemic waves, characterised by six peaks occurring in spring and autumn 2020; winter, spring and autumn 2021; and winter 2022. The first and second epidemic waves were caused by the SARS-CoV-2 Wild type variant, followed in 2021 by the Alpha and Delta variants, and the Omicron variant wave in 2022. Over this period, several recommendations and restrictions were adopted at different levels of decision-making, including social distancing, the compulsory wearing of masks and respirators, and lockdowns.

Here, we present the results of long-term WBE at four WWTPs in the Czech Republic over the 2020–2022 epidemic, which was conducted as the only one in the Czech Republic covering all waves of the SARS-CoV-2 variant. This study is used to assess the relationship between SARS-CoV-2 RNA concentrations and the number of clinical cases in the catchment area of the investigated WWTPs, to describe differences between SARS-CoV-2 waves and the influence of wastewater parameters. Based on these results, we then assess the potential of using the WBE approach for predicting future epidemic trends.

Results

SARS-CoV-2 RNA concentrations in wastewater samples

In this study, 420 wastewater samples from four WWTPs in the Czech Republic were analysed (WWTP A and C from Bohemia, C and D from Moravia, Table 1). The overall positive rate of SARS-CoV-2 RNA detection was 71 %, rising from 42 % during the Wild type wave to 100 % during the Omicron variant wave (Supplementary Table S1). Highest positive rate was observed for the largest WWTP A. During Wild type wave, Moravian WWTPs reached low positive rates (27 and 31 %) compared to Bohemian WWTPs (83 and 60 %). Detection sensitivity in

our study was within 0.01 % for WWTP B, C and D and 0.04 % for WWTP A.

Since the beginning of epidemic, the viral load in wastewater had increased by approximately two orders of magnitude from 10^3 SARS-CoV-2 GE/day/person during the Wild type and Alpha variant waves to 10^5 SARS-CoV-2 GE/day/person during the Omicron variant wave. The overall trend in SARS-CoV-2 RNA concentrations during the epidemic is visualized in Fig. 1. Differences are clearly observed between Moravian (WWTP B, D) and Bohemian (WWTP A, C) WWTPs and are also evident in SARS-CoV-2 RNA concentrations, with significantly higher SARS-CoV-2 RNA concentrations found at Bohemian WWTPs before 2022, and significantly higher concentrations detected at Moravian WWTPs in 2022 (Fig. 1).

Relationship between SARS-CoV-2 RNA concentration and wastewater parameters

Relationship of SARS-CoV-2 RNA concentration and wastewater parameters was assessed in this study. Our results show only weak correlations between SARS-CoV-2 RNA concentration and BOD_5, COD, P_{total}, TSS, FC, EC and ENT (Table 2). Differences in wastewater characteristics between investigated WWTPs are demonstrated in the Supplementary Fig. S1.

Relationship between SARS-CoV-2 RNA concentration and clinical data

The number of active and hospitalised cases of COVID-19 in each catchment area of the WWTP showed similar trends (Fig. 2). A higher prevalence of COVID-19 was observed during Delta and Omicron variant waves. There were no significant differences in numbers of hospitalised COVID-19 cases (per 100,000 population) between SARS-CoV-2 variant waves, despite differences in COVID-19 prevalence during the epidemic (Supplementary Fig. S2). Number of reported active and hospitalised COVID-19 cases is summarized in Supplementary Table S2. The highest number of active COVID-19 cases was found during Omicron variant wave, while the highest number of hospitalised COVID-19 cases was observed during Alpha and Delta variant waves (Supplementary Table S2).

Overall SARS-CoV-2 RNA concentrations obtained during the epidemic correlated well with clinical data (0.48–0.68). The only exception was the insignificant correlation with the number of active COVID-19 cases during Omicron variant wave (Fig. 3). Correlation between SARS-CoV-2 RNA concentrations and number of active COVID-19 cases and between SARS-CoV-2 RNA concentrations and number of hospitalised COVID-19 cases were similar. Spearman analysis revealed that correlations differed across the four SARS-CoV-2 waves. Spearman's rank coefficients ranged from 0.18 to 0.98 for active COVID-19 cases and 0.21 to 0.93 for hospitalised COVID-19 cases. The strongest correlations between SARS-CoV-2 RNA concentrations and both types of clinical data for each WWTP were found during Alpha and Delta variant waves (Fig. 3). Three out of four WWTPs showed strong correlations during Alpha and Delta variant waves, while WWTP D showed mostly insignificant correlations. In contrast, for Wild type variant wave mostly insignificant correlations were observed. Interestingly, during Omicron variant wave SARS-CoV-2 RNA concentrations correlated better to the number of hospitalised COVID-19 cases.

Predictive potential of SARS-CoV-2 RNA wastewater monitoring

In this study, we examined cross-correlation between SARS-CoV-2 RNA concentrations and active and hospitalised COVID-19 cases, finding that highest correlations were mostly detected for active COVID-19 cases at lag = 0 (i.e. no lag), regardless of variant wave (Fig. 4). However, a degree of variability was observed between WWTPs and variant waves. A stronger correlation was recorded at lag=−1 (4–9 days in advance) for larger cities during the Delta variant wave. For
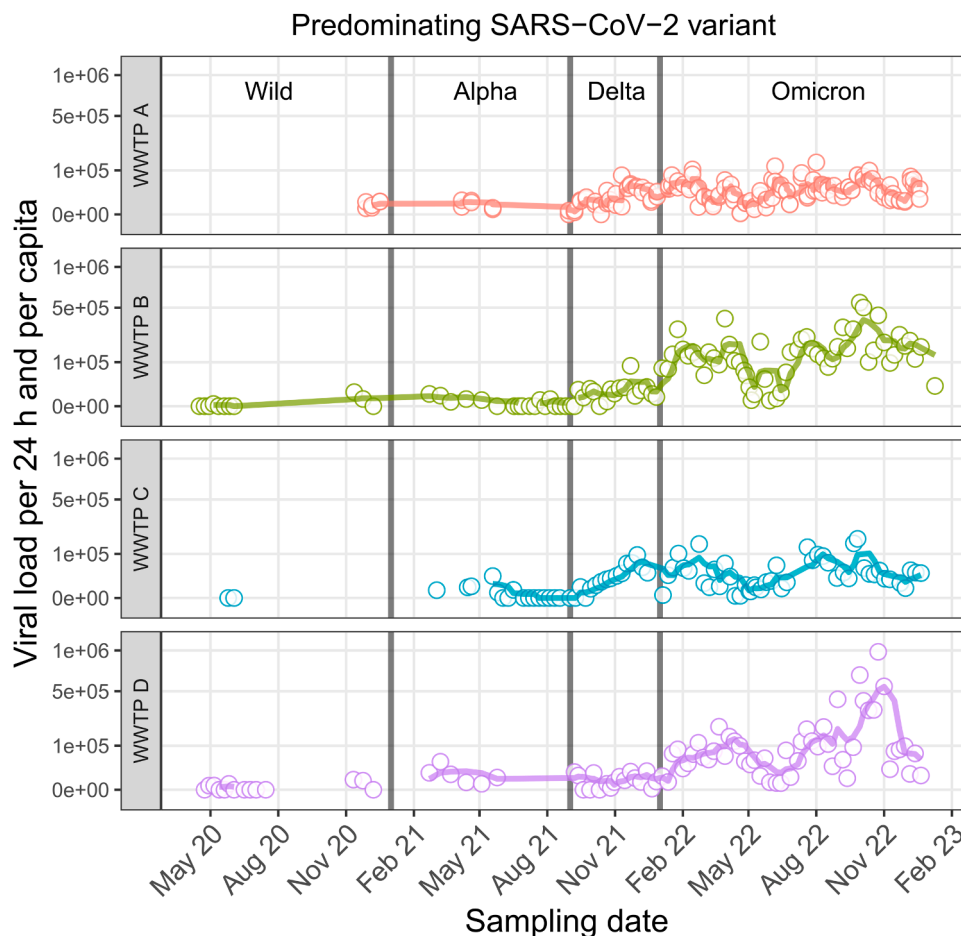
Table 1
Basic data for the four wastewater treatment plants (WWTPs) monitored.

WWTP	Region	Population served	Length of sewage network (km)	Average flow (m^3/day)	Percentage industrial wastewater (%)
A	Bohemia	1,300,000	3,707	354,239	31
B	Moravia	430,000	1,146	137,000	30
C	Bohemia	80,000	182	18,251	20
D	Moravia	24,300	65	7,659	30

Fig. 1. SARS-CoV-2 RNA concentrations at four Czech WWTPs over the 2020–2022 epidemic (rolling mean from 4 values).

Table 2

Correlations between SARS-CoV-2 RNA concentration and wastewater parameters during all epidemic waves (2020–2022).

Parameter	rho	p
BOD5	**0.13**	**0.019**
COD	**0.16**	**0.002**
N_NH4	0.19	0.070
Ptotal	**0.13**	**0.014**
TSS	**0.17**	**0.002**
Ntotal	0.05	0.382
pH	−0.09	0.110
El_conductivity	0.16	0.115
FC	**0.18**	**0.004**
EC	**0.18**	**0.002**
ENT	**0.21**	**0.000**

pH, El. conductivity - electrical conductivity, BOD$_5$ - five-day biochemical oxygen demand, COD - chemical oxygen demand, N_NH$_4$ - ammonium nitrogen, N$_{total}$ - total nitrogen, P$_{total}$ - total phosphorus, TSS - total suspended solids, FC - total faecal coliform bacteria, EC - *Escherichia coli*, ENT – enterococci. Significant correlations in bold.

hospitalised COVID-19 cases, cross-correlations showed highest correlations for the Delta and Omicron variant waves in real-time for WWTP A and WWTP D, and at lag=−1 or =−2 for WWTPs C and B (Fig. 4).

Discussion

SARS-CoV-2 WBE has proven to be an effective tool for understanding the COVID-19 pandemic. It enables to estimate COVID-19 burden and epidemic trends, it is an indicator of infection cases,

hospitalisations and intensive care unit admissions (Acosta et al., 2023; Medema et al., 2020; Polo et al., 2020; Pang et al., 2022). This study covers COVID-19 epidemic in the Czech Republic from 2020 to 2022, in which 420 samples were analysed and relationship between SARS-CoV-2 RNA concentrations and clinical data were sought. Several studies covering different waves of the COVID-19 epidemic have been conducted and have provided strong evidence that SARS-CoV-2 RNA concentrations reflect the COVID-19 burden in the population (Mohapatra et al., 2023; Pang et al., 2022; Rusiñol et al., 2021).

Number of positive samples increased towards Omicron variant wave. During Wild type wave, only 42 % of samples were positive for SARS-CoV-2 RNA, reflecting well the course of the epidemic. First epidemic wave in spring 2020 was characterized by lower number of clinical cases and regionality in terms of the incidence of SARS-CoV-2 infection. Especially in Moravia, the positivity rate was only 27–31 %. During the Alpha and Delta variant waves, SARS-COV-2 infection became widespread, but with fluctuations in the number of clinical cases, as reflected in the positivity rates (60 % and 80 %, respectively). During the Omicron variant wave, 100 % of the samples were positive because the number of infected people was high enough all the time.

There are many factors, which may have impact on positive rate and detection sensitivity (Ahmed et al., 2022; Bertels et al., 2022; Li et al., 2021). One key factor is the design of sampling, sample processing and RT-qPCR (Hart and Halden, 2020; Medema et al., 2020; Li et al., 2023). Other key factors are based on epidemiological variables, including uncertainty in shedding rate, testing capacities, measures taken and reporting of epidemiological data (Li et al., 2023; Xiao et al., 2020). Sewage system characteristics and wastewater properties are also key factors in variability of results and can lead to degradation and dilution

Fig. 2. Trends of SARS-CoV-2 RNA concentration (in red) and 1) numbers of active COVID-19 cases (in blue), and 2) numbers of hospitalised COVID-19 cases (in green) in the catchment area of investigated wastewater treatment plant (WWTP)
Y-axis (left) - number of clinical cases per 100,000 population, secondary Y-axis (right) - viral load per 24 h per capita.

Fig. 3. Spearman's rank correlation between SARS-CoV-2 RNA concentration and active COVID-19 cases (left) and number of hospitalised COVID-19 cases (right) at four Czech WWTPs during all epidemic waves
Significant correlations in bold, highest Spearman's correlation coefficients are represented in dark colour and the lowest in light colour. NA – not available.

of virus particles and inhibition of PCR analyses (Ahmed et al., 2022; Amoah et al., 2022; Bertels et al., 2022; Paul et al., 2021). In this study, we observed detection sensitivity within 0.01 % for WWTP B, C and D and 0.04 % for WWTP A, which is in good agreement with other studies reporting sensitivity of SARS-CoV-2 RNA detection in wastewater ranging from < 1 case per 100,000 to 50 cases (Hart and Halden, 2020; Hewitt et al., 2022; Li et al., 2023). However, reason for lower detectability at WWTP A in this study was mainly caused by lower number of samples collected during periods of low COVID-19 prevalence. The influence of wastewater characteristics was found to be low, with only weak correlations observed between SARS-CoV-2 RNA concentrations and BOD_5, COD, P_{total}, TSS, and microbiological indicators. Differences from published data may be due to different wastewater characteristics

and the amount and type of industrial wastewater (Amoah et al., 2022).

The viral load in wastewater had increased from 10^3 SARS-CoV-2 GE/day/person during the Wild type and Alpha variant waves to 10^5 SARS-CoV-2 GE/day/person during the Omicron variant wave, which is within the range of values reported in other studies (Medema et al., 2020; Rusiñol et al., 2021). The number of active COVID-19 cases raised from 709 per 100,000 population during Wild type wave to 6318 during Omicron variant wave. A higher prevalence of COVID-19 in Delta and Omicron variant waves was likely due to the higher infectivity of these variants. Despite differences in the prevalence of COVID-19 during the epidemic, no significant differences in the number of hospitalised COVID-19 cases between waves of the SARS-CoV-2 variant were observed. Only during Omicron variant wave number of hospitalisations
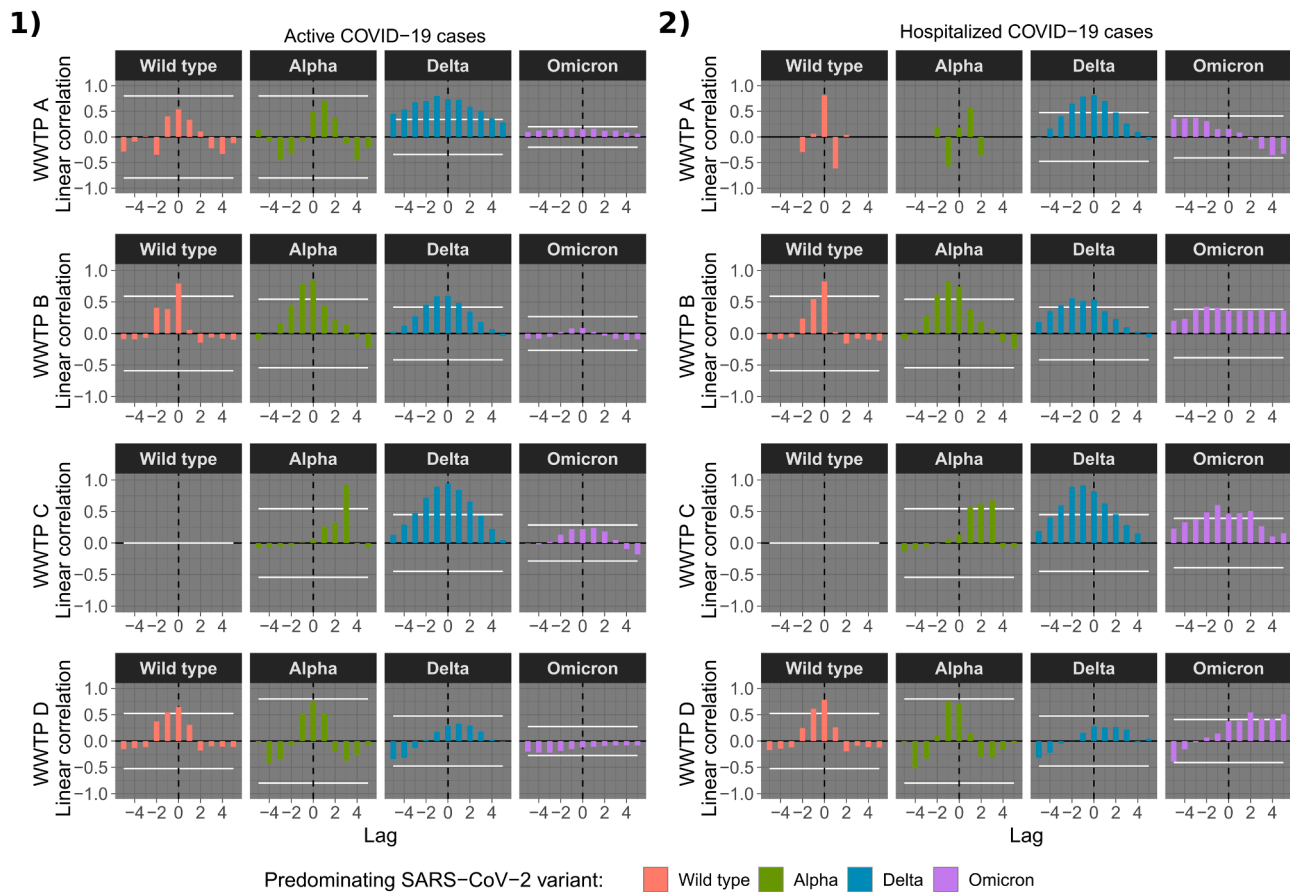
Fig. 4. Cross-correlation between SARS-CoV-2 RNA concentration and 1) active COVID-19 cases, and 2) number of hospitalised COVID-19 cases at four Czech wastewater treatment plants (WWTP) during all epidemic waves (2020–2022).
Lag represents the number of time intervals lapsed to perform the correlation; 95 % confidence intervals for the correlations are indicated as horizontal white lines, the breakpoint at lag = 0 (no lag) being indicated as a vertical dashed line.

was slightly lower. There are two main explanations for similar level of hospitalisations. Firstly, immunisation through vaccination, which began in early 2021, and natural immunisation due to previous infection. Second, the reduced severity of clinical symptoms, especially of Omicron variant infection.

SARS-CoV-2 RNA concentration in wastewater exhibited good relationship with reported active and hospitalised COVID-19 cases and showed good agreement with previous studies (Acosta et al., 2022, 2023; Mohapatra et al., 2023; Wartell et al., 2022). However, there was variability in the strength of the correlation between SARS-CoV-2 RNA concentrations and clinical data throughout the four waves. The highest correlations for the WWTPS examined were observed in the Alpha and Delta variant waves, similarly to the results of Pang et al. (2022). Both waves were characterised by the antiepidemic measures taken, such as social distancing, mask wearing, lockdowns and mandatory testing at schools and works. Due to mandatory testing, the reported data on clinical cases were complex and showed the true status of infection burden in the population. Wild type variant wave showed mostly insignificant correlations, which may have been due to start of clinical testing and data reporting, and also insufficient number of collected samples. The weak correlations for the Omicron variant wave reflected the lower severity of this variant, the optional testing and the subsequent reduction in the number of tests. However, there have been cases where some people did not get tested for SARS-CoV-2 and finally ended up in hospital with COVID-19. This can explain why SARS-CoV-2 RNA concentrations correlated better to the number of hospitalised COVID-19 cases during the Omicron variant wave. In general, however, higher correlations were observed between SARS-CoV-2 RNA concentrations

and active COVID-19 cases, indicating a shift in hospital admissions (López-Peñalver et al., 2023). Our results confirm even local differences as published e.g. Rusiñol et al. (2021) or Pang et al. (2022). In addition to the low number of samples, the stay of infected persons in a different location from where they were registered, the transfer of patients between hospitals from different communities or commuting may have contributed to weak or insignificant correlations at WWTPs A (located in the capital city) and D (located in the border city).

Many authors have confirmed that monitoring SARS-CoV-2 RNA in wastewater could serve as a predictive tool for public health surveillance given the documented time lag between the occurrence of SARS-CoV-2 RNA in wastewater and clinical indicators (e.g. Barrios et al., 2021; Medema et al., 2020; López-Peñalver et al., 2023; Rusiñol et al., 2021; Zhan et al., 2022). Our results showed strong correlations during Delta and Omicron variant waves in larger cities, likely due to the comprehensive clinical data obtained through mandatory testing. Our results are consistent with mentioned studies and suggest that monitoring conducted in larger cities may have higher predictive potential. However, in order to obtain reliable results and use WBE for prediction, attention must be paid to representative and sufficiently frequent sampling, sample processing, virus quantification in wastewater, and normalization (Polo et al., 2020). A key limitation for a reliable early warning system is infrequent wastewater sampling, as incubating or asymptomatic cases may be missed between sampling events (Polo et al., 2020).

Our results confirmed that the WBE method is an effective tool for monitoring the burden of COVID-19 in the population, especially as a supplement of clinical testing. The potential of WBE is also being

exploited to monitor other microbiological (e.g. poliovirus, hepatitis A virus, hepatitis E virus, enterovirus, norovirus, antimicrobial resistance) and chemical agents (e.g. illicit drugs, mycotoxins) and is being implemented in international legislation. The ongoing implementation of the requirements for the determination of SARS-CoV-2 and other pathogenic agents in wastewater in the Urban Waste Water Treatment Directive 91/271/EHS is a significant step in an effective approach to public health protection.

Conclusions

In this study, we used WBE to describe in detail the course of the COVID-19 epidemic in the Czech Republic with respect to different SARS-CoV-2 variants during the epidemic (2020–2022). We recorded significant correlations between population status in the areas monitored, determined from the clinical data (active and hospitalised COVID-19 cases) and SARS-CoV-2 RNA concentration in wastewater.

There was a strong correlation between number of active and hospitalised COVID-19 cases in each WWTP catchment area and concentration of SARS-CoV-2 RNA detected in the WWTP inflow; however, the results varied temporally and spatially and reflected COVID-19 burden in the local community.

On the other hand, there was little or no relationship observed between physicochemical, chemical or microbiological wastewater parameters and the concentration of SARS-CoV-2 RNA found in wastewater samples.

Our data from the early, targeted and long-term monitoring in the Czech Republic demonstrated that the WBE approach can be used for predicting epidemic trends. Furthermore, the study showed that targeted systematic monitoring of wastewater provides sufficiently robust data for such predictions, and that these can be used to monitor trends in the spread of epidemic agents in the population, thereby contributing to the prevention of infection spread and to the introduction of appropriate subsequent measures.

Materials and methods

Wastewater sampling

Between April 2020 and December 2022, a total of 420 samples were obtained from five sampling sites at four mechanical-biological WWTPs of different sizes, between them serving 17.5 % of the Czech population (Table 1, Supplementary Fig. S3; samples from WWTP A collected from two treatment lines, the results presented as the average of the two). The samples (0.5 L raw wastewater) were collected as time or flow-dependent composite samples (24-hr) weekly covering all epidemic waves in the Czech Republic. Immediately after sampling, the wastewater samples were cooled until transport to the analytical laboratory and then processed within 24 h or frozen at −70 ± 4 °C.

Wastewater parameters

All samples were characterised by recording pH, electrical conductivity (El. conductivity), five-day biochemical oxygen demand (BOD_5), chemical oxygen demand (COD), ammonium nitrogen (N—NH_4), total nitrogen (N_{total}), total phosphorus (P_{total}), total suspended solids (TSS), total faecal coliform bacteria (FC), *Escherichia coli* (EC) and enterococci (ENT), according to ISO standards.

Sample processing

Sample concentration and RNA isolation

In spring 2020, wastewater treatment and RNA isolation were performed as described in detail in Mlejnková et al. (2020). Owing to the difficulty of the method, however, a new, more user-friendly procedure was developed and validated (see Vašíčková et al., 2023), and this

method was used from autumn 2020. Briefly, the 0.5 L wastewater sample is precipitated with 40 g of polyethylene glycol 6000 (PEG; Sigma Aldrich, UK) and 8.8 g NaCl (Penta, Czech Republic), shaken until the chemicals dissolve and then stored overnight (12–16 h) at 5 ± 3 °C. Subsequently, the sample is centrifuged at 10 000 × g for 30 min at 4 °C. The pellet produced is then dissolved in 2 ml of 1 × phosphate-buffered saline (PBS) and centrifuged at 3 000 × g for 15 min at 4 °C. Next, 140 µl of supernatant is subjected to RNA isolation using the QIAamp Viral RNA Mini Kit (QIAGEN, Germany), according to the manufacturer's instructions, the extracted RNA being eluted at a volume of 100 µl.

To verify the sample treatment procedure and determine the overall efficiency of the method, transmissible gastroenteritis coronavirus (TGEV; strain M42, Collection of zoopathogenic organisms, Veterinary Research Institute p.r.i., Czech Republic) was used as a control for SARS-CoV-2 RNA detection in wastewater samples, each sample being artificially contaminated with 5 µl TGEV (10^6 genome equivalents [GE]/µl) prior to sample treatment.

Reverse transcription and qPCR analysis

SARS-CoV-2 RNA presence in wastewater samples collected in spring 2020 was assessed using the technique described in Mlejnková et al. (2020). Since then, detection of SARS-CoV-2 RNA has been undertaken using two-step RT-qPCR targeting the N and Nsp12 protein genes (Vašíčková et al., 2023).

Briefly, 5 µl of a mixture containing 10 nmol of dNTP mix (Serva, Germany), 2 pmol of reverse primers (SARS-CoV-2 Ndeg, N—HM, Nsp12 and IAC), 2.2 µl RNase-free water and 1 fg of internal amplification control (IAC) RNA diluted in a carrier RNA solution (50 ng/l; Applied Biosystems, USA) was prepared. This was followed by addition of 5 µl of isolated RNA mixture denaturation at 65 °C for 5 min and immediate cooling on ice. The remainder of the reaction mixture was then added to a final volume of 20 µl: 5 × PrimeScript Buffer (TaKaRa, USA), 100 U of PrimeScript Reverse Transcriptase (TaKaRa, USA), 20 U of Ribonuclease Inhibitor (New England BioLabs, UK), and RNase-free water. Reverse transcription was performed at 50 °C for 60 min, followed by inactivation at 75 °C for 15 min. qPCR amplification was conducted in 20 µl reactions containing 10 µl of LightCycler 480 Probes Master 2 × (Roche, Germany), 7 pmol of SARS-CoV-2 primers, 3 pmol of IAC primers, 2 pmol of each probe, 0.2 U of Uracil-DNA-glycosylase (Roche, Germany), 5 µl of cDNA, and nuclease free water. Amplification was carried out under the following conditions: 95 °C for 10 min, 40 cycles of 95 °C for 10 s, 55 °C for 30 s, 72 °C for 10 s and final cooling at 40 °C for 10 s (sequences for primers and probes listed in Supplementary Table S3). TGEV specific RNA was detected using primers and probes adopted from Vemulapalli et al. (2009), under the same conditions as for SARS-CoV-2 RNA. Both reverse transcription and PCR were validated and performed on LightCycler 480 (Roche, Germany), AriaMX (Agilent, USA) and qTOWER3 G (Analytic Jena, Germany) real-time thermal cyclers. Each sample of isolated RNA was tested in duplicate. The quantification of both SARS-CoV-2 and TGEV GE was performed on the basis of RNA standards prepared *in vitro*. Negative and positive controls were used to avoid false positive or false negative results (Vasickova et al., 2012; Vemulapalli et al., 2009).

While two slightly different analytical methods were used for samples from spring 2020 and samples post 2020 (Mlejnková et al., 2020; Vašíčková et al., 2023), these both showed very similar recovery efficiency (35.5 % and 42.6 %, respectively); thus, the results obtained were considered comparable. Detection limit of both methods of RT-qPCR was 2.5 GE/ml.

Data analysis

Numbers of active (i.e. persons with a positive COVID-19 test) and hospitalised COVID-19 cases were acquired from the National Institute of Public Health and the National Health Information System. The number of active COVID-19 cases on the day of sampling was

represented by a 14-day interval (14-day cumulative incidence) that includes the number of all COVID-19 positive persons 10 days before the date of their positive test by the third day after their positive test. Information on the prevailing SARS-CoV-2 variant in the population was obtained from the National Institute of Public Health. According to this data, the Wild type wave lasted from 04/2020 to 01/2021, the Alpha variant wave then dominated until 09/2021, then the Delta variant wave until 01/2022, after which the Omicron variant became dominant.

Normalisation for copy numbers was performed by flow correction (multiplying gene copies per L with a 24-hour flow rate (m^3) and then dividing the results by the population size in the catchment area of each WWTP). The reduction in yield produced by freezing the sample was counteracted using an empirically obtained factor; thus, the results for frozen samples were multiplied by five to be comparable to results obtained for fresh samples. This factor was calculated based on our degradation studies, which confirmed an average RNA degradation of 20 % when samples were stored at -70 $\pm$ 4 °C (unpublished data).

All analyses were performed in R v.4.1.1 (www.rproject.org) using the *ggpubr* package (Kassambara, 2023) for Kruskall-Wallis and Dunn tests. The Kruskall-Wallis test (Kruskal and Wallis, 1952) and post-hoc Dunn test (Dunn, 1964) were used to compare wastewater parameter levels between WWTPs. Relationships between concentration of SARS-CoV-2 RNA in wastewater, number of active COVID-19 cases, number of hospitalised COVID-19 cases and wastewater parameters were assessed using the Spearman correlation. To assess the potential for prediction, the numbers of active and hospitalised COVID-19 cases were correlated against the concentration of SARS-CoV-2 RNA from the previous sampling date (i.e. a 4–9-day lag). The 14-day cumulative incidence rate for active COVID-19 cases and hospitalised COVID-19 cases was used for all statistical analyses. Normalization by flow correction was used to express SARS-CoV-2 RNA concentration. A moving average of 4 SARS-CoV-2 RNA concentration values was used to visualize epidemic trends. Data associated with each categorized statistical analysis are summarized in Supplementary Table S4.

CRediT authorship contribution statement

Kateřina Sovová: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis. **Petra Vašíčková:** Writing – review & editing, Validation, Resources, Methodology, Investigation. **Vojtěch Valášek:** Investigation. **David Výravský:** . **Věra Očenášková:** Writing – review & editing, Resources, Investigation, Funding acquisition, Conceptualization. **Eva Juranová:** Writing – review & editing, Project administration, Methodology, Investigation. **Milena Bušová:** Writing – review & editing, Visualization, Investigation. **Milan Tuček:** Writing – review & editing, Visualization. **Vladimír Bencko:** Visualization. **Hana Zvěřinová Mlejnková:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could appear to influence the work reported in this paper.

Data availability

Data will be made available on request.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.wroa.2024.100220.

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