Доклади на Българската академия на науките
Comptes rendus de l'Académie bulgare des Sciences

Tome 79, No 6, 2026

MEDICINE

Microbiology

SUMMER PEAK OF SARS-CoV-2 VIRUS IN WASTEWATER FROM THE LARGEST BULGARIAN CITIES IN THE 2024 SEASON

Sergei Ivanov[1,2]✉, Zoltan Urshev[3], Stoyan Shishkov[1,4], Anton Hinkov[1,4], Mihaela Kirilova[1,5], Silvia Mileva[1], Kalin Atanasov[1]

Received on February 22, 2026
Presented by B. Petrunov, Member of BAS, on April 28, 2026

Abstract

Wastewater-based epidemiology (WBE) was applied to monitor human-pathogenic respiratory viruses, including SARS-CoV-2, in the four largest Bulgarian cities during the summer and autumn of 2024. Starting from 1 liter wastewater samples, concentration of the virus fraction with PEG 8000/NaCl, extraction and purification of DNA/RNA were applied. Detection of SARS-CoV-2, FluA/B, RSV and HCoV229E was performed with TaqMan qPCR. A total of 42 samples were processed over a 7-month period. Two summer peaks of SARS-CoV-2 were detected in the capital Sofia, in July and September 2024. With some differences, a similar result was observed in Plovdiv and Burgas. The concentration of SARS-CoV-2 in wastewater decreased significantly in November and December. Other respiratory viruses started to be detectable only at the beginning of October. The results of this study show an unusual summer peak of SARS-CoV-2 in wastewater from large cities, followed by a significant decrease of virus concentrations in the fall. The established trend correlates with the statistics of COVID-19 incidence in Bulgaria.

Key words: coronavirus, epidemiology, TaqMan qPCR, wastewater

This study was funded by the European Union-Next Generation EU, through the National Recovery and Resilience Plan of the Republic of Bulgaria, project No BG-RRP-2.004-0008, scientific group 3.2.4.

https://doi.org/10.7546/CRABS.2026.06.10

Introduction. The COVID-19 pandemic had a major impact on public health, the economy and the general public perception of safety. For the first time since the Spanish flu, an infectious disease claimed millions of lives worldwide. In Bulgaria, the pandemic death toll reached 5661/1M population, the highest in Europe and second in the world [1]. The main reason was the poor management of health policy, including non-existent infectious disease monitoring programmes. Now, after the termination of emergency diagnostics in 2024, health authorities have returned to the pre-pandemic pathogen surveillance regime – i.e. no testing for pathogens outside hospitals, except for private patient-paid analyses. Providing an alternative for monitoring of pathogenic viruses, the current study, part of an EU-funded project, selected Wastewater-based epidemiology (WBE) as the preferred methodology. WBE serves as a complementary surveillance tool for monitoring of community-level infectious diseases caused by viruses and other microorganisms. WBE is a scalable and cost-effective methodology, does not require patients' consent and it is independent from the usual medical diagnostic pathways and healthcare administration. Recent experience with SARS-CoV-2, influenza virus, etc., proves that changes in the concentration of these viruses in wastewater correlates with the local disease incidence [2]. Moreover, wastewater surveillance can be used to predict disease outbreaks, because usually peaks in virus concentrations are detected before changes in hospital statistics. Additional advantages of WBE are that it takes into account a population that usually avoids routine clinical surveillance, and it has the potential to integrate into monitoring plans for other health risk indicators such as antibiotic and drug consumption, detection of the spread of antibiotic-resistant genes, etc. [3].

The current WBE project was launched in early 2024. Initially, it included only detection of SARS-CoV-2, with influenza, RSV, and other viruses added to the monitoring programme from the summer of 2024. Using WBE, we followed the dynamics of the concentration of the studied pathogens in four Bulgarian cities. As early as the first few months of monitoring, a summer epidemiological peak in the concentration of SARS-CoV-2 in wastewater was established. This publication presents some unusual results of the monitoring of respiratory viruses in the summer and autumn of 2024.

Materials and methods. The sampling programme includes the wastewater treatment plants of the capital Sofia, as well as the next most populous cities – Plovdiv, Varna, and Burgas. Together, they represent almost 35% of the population of Bulgaria. Over a period of about 7 months (June – December, 2024), 42 samples were collected, including 18 from Sofia, 10 from Plovdiv, and 7 each from Varna and Burgas. One liter of wastewater was collected from each location, transported immediately to the laboratory in a refrigerator, and processed for up to a maximum of 24 h. The wastewater samples were first placed on a shaker at room temperature for 45 min. Then they were filtered through four layers of gauze and 400 ml of the filtrate were centrifuged at 7000g/45 min to

remove bacteria. The supernatant was divided into two halves of 200 ml and each half was saturated to 10% PEG 8000 and 0.5 M NaCl, incubated for 2 h at 6 °C and centrifuged 13 500g/60 min. The precipitate containing the concentrated viral fraction was stored long-term in liquid nitrogen. Total nucleic acids were isolated with the Quick-DNA/RNA™ Viral Kit after resuspension of the pellet in DNA/RNA Shield™ (Zymo). With this protocol the wastewater samples were concentrated 1600 times. The purified DNA/RNA were stored at −20 °C and used up to 72 h for analysis of RNA viruses.

Virus detection was performed with TaqMan qPCR using laboratory-developed protocols on a BioRad CFX Opus 96 instrument. One-step Reliance multiplex supermix™ and SsoAdvanced probes™ (BioRad) were used for the enumeration of RNA- and DNA-viruses, respectively. Typically, the reaction mixture were multiplex between 2 and 4 targets per reaction. A list of the primers used for virus detection is shown in Table 1. Each qPCR run included a negative, positive and internal controls. Quantitative positive controls were prepared by the synthesis of oligonucleotides corresponding to the target sequences in published viral genomes. The results were processed with BioRad CFX Maestro software. Each value in the figures is the average of at least three measurements ± standard error. The detection of crAssphage as well as samples artificially spiked with bacteriophage BsXeu269p/3 were applied as validation controls for viral nucleic acid extraction procedures from wastewater.

Results and discussion. The proportion of positive for SARS-CoV-2 samples was 15/18, 9/10 and 5/7, for Sofia, Plovdiv and Burgas/Varna, respectively. In the capital Sofia (Fig. 1A) in June and early July 2024, the virus was not detected. This was followed by a rapid short-term escalation to 2300 copies/ml at the end of July. A second peak in the concentration of the virus in Sofia wastewater was recorded from the sampling on 25.09, where the value exceeded 6000 copies/ml. This was followed by a gradual decrease, and before Christmas the amount of SARS-CoV-2 was below the detection limit – like in June! In Plovdiv (Fig. 1B) the peak in the concentration of the coronavirus began a little later, in August, and continued until October. The peak values for SARS-CoV-2 in Plovdiv were lower compared to Sofia. In Burgas, the dynamics of SARS-CoV-2 was like in Plovdiv, i.e. from August to October. In Varna, the trend was different – a peak in the concentration of the virus was observed only in October. In general, in all four cities, during the months of November and December, the level of SARS-CoV-2 decreased and became undetectable in wastewater. Table 2 presents summarized data for other respiratory viruses. Unlike SARS-CoV-2, they were not detected in wastewater in the summer months. There were single positive samples for influenza in October and November, but this virus was mainly detected in December. RSV followed the opposite trend – it was detected mainly in October. Another coronavirus, HCoV 229E, causing "common cold" appeared in October and was detectable in wastewater throughout the fall. In summary,

Table 1

Primers, probes and other oligos, used in this study

Virus	Primers, probes and other oligos	Sequence 5′–3′	Reference
SARS-CoV-2	CoV_N1_F	GACCCCAAAATCAGCGAAAT	US CDC
	CoV_N1_R	TCTGGTTACTGCCAGTTGAATCTG	
	CoV_N1_IN	5′ HEX -ACCCCGCATTACGTTTGGTGGACC- 3′ BHQ1	
	EURM-019	universal synthetic ssRNA of 880 nt, quality control material	Eur. Comm, Joint Research Centre
Influenza A	IA_F	CTTCTAACCGAGGTCGAAACGTA	Whiley & Sloots [4]
	IA_R	GGTGACAGGATTGGTCTTGTCTTTA	
	IA_IN	5′ FAM -TCAGGCCCCCTCAAAGCCGAG- 3′ BHQ1	
	IA-control	Synthetic oligonucleotide target sequence from Influenza A virus (A/swine/Ontario/42729A/01(H3N3)) (Acc. No. AY619976)	This study
Influenza B	IB_F	AAATACGGTGGATTAAACAAAAGCAA	Van Elden et al. [5]
	IB_R	CCAGCAATAGCTCCGAAGAAA	
	IB_IN	5′ TexR -CACCCATATTGGGCAATTTCCTATGGC- 3′ BHQ2	
	IB-control	Synthetic oligonucleotide target sequence from Influenza B virus (B/Townsville/2/2005) (Acc. No. CY150499)	This study
RSV A	A21	gctcttagcaaagtcaagttgaatga	Hu et al. [6]
	A102	tgctccgttggatggtgtatt	
	APB48	5′ FAM -acactcaacaaagatcaacttctgtcatccagc- 3′ BHQ2	
	RSV A control	Synthetic oligonucleotide target sequence from Human respiratory syncytial virus (Acc. No. M11486)	This study
RSV B	B17	gatggctcttagcaaagtcaagttaa	Hu et al. [6]
	B120	tgtcaatattatctcctgtactacgttgaa	
	BPB45	5′ TexR -tgatacattaaataaggatcagctgctgtcatcca- 3′ BHQ2	
	RSV B control	Synthetic oligonucleotide target sequence from Human respiratory syncytial virus (Acc. No. D00736)	This study
CoV 229E	229E_F	ggatgacatcatgaaggcag	Boehm et al. [7]
	229E_Rev	tacccgttttcgctgacttt	
	229E_IN_mod	5′ HEX -tttttcctgaggcttgtcaaaacctaaa- 3′ BHQ1	This study
	229E control	Synthetic oligonucleotide target sequence from Human coronavirus 229E clone 4311700484 (Acc. No. MN488627)	This study
Phage BsXeu269p/3	YK-MjCPF1	ccagtacgccgtgttcatca	Shopova et al. [8]
	YK-MjCPR1	gtcgttcatcaggccgtagt	
	YK-MjCPF1/R1	5′ HEX -cggcatcgactgggctgcgcgcc- 3′ BHQ1	
CrAssphage	056F1	cagaagtacaaactcctaaaaaacgtagag	Stachler et al. [9]
	056R1	gatgaccaataaacaagccattagc	
	056P1	5′ FAM AATAACGATTTACGTGATGTAAC 3′ BHQ1	

Table 2

Distribution of positive samples for other respiratory viruses for the last 3 months of 2024. Data points are identified according to the city/week pair. White fields indicate that no measurement was made, and a zero value means that the target virus is below the qPCR detection limit. The +/++/+++ scale for positive results depends on the concentration of viruses in the wastewater

	Influenza A and B										**RSV A and B**										**CoV 229E**									
	October			November				December			October			November				December			October			November				December		
City	40	41	42	44	46	47	48	49	50	51	40	41	42	44	46	47	48	49	50	51	40	41	42	44	46	47	48	49	50	51
Sofia	0	0	0	0	+++	0		0	++	++	+	+	+	0		0	0	0	0	0	+	+		+++	+	++	++	+	0	+
Plovdiv			0	+	0			+		0			+	0			0	+		0			+	0	0		++	++		+
Burgas		0				0			++			+				0			0			0				+			0	
Varna		+				0			+			+				0			0			+				+			0	

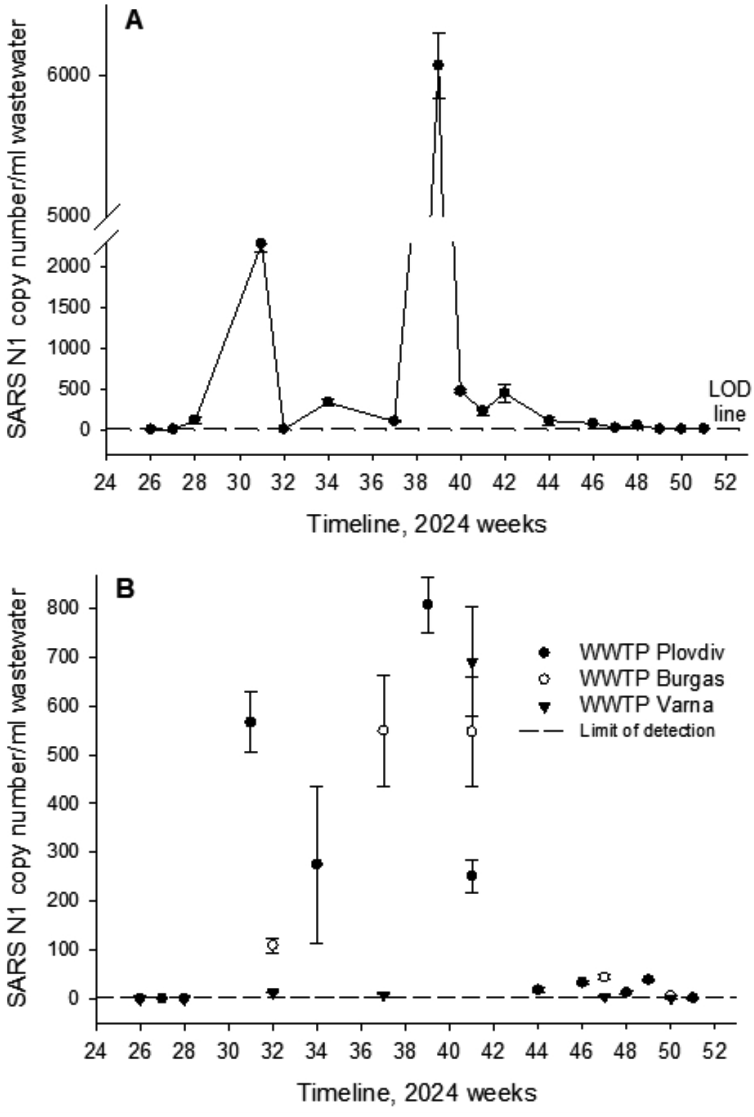


Fig. 1. Monitoring of the SARS-CoV-2 virus in wastewater of the capital Sofia (A), and the cities of Plovdiv/Burgas/Varna (B), for the period from week 26 to week 51 of 2024. The values are presented in copy number/ml wastewater ± standard error. The dotted horizontal line indicates the detection limit for this virus. WWTP, wastewater treatment plant

Flu, RSV and 229E have a "normal" trend for respiratory pathogens, appearing in the autumn season. The opposite was observed with SARS-CoV-2, it circulates during the warm months, and with the cold in October it decreased and later became undetectable.

The observed trend correlates with the official health statistics of Bulgaria [10]. In 2024, a peak in the incidence of COVID-19 was reported starting at the end of

August, i.e. with a latent period of 2–3 weeks after the increase in SARS-CoV-2 concentrations in wastewater. Similarly, at the end of October, after the established decrease in the virus, a decline in the number of reported cases followed. Summer SARS-CoV-2 activity is unusual. Results from similar wastewater monitoring show typical peaks of SARS-CoV-2 in the second half of autumn, winter, and early spring [11–13]. An exception, as reported here, was found in the same summer of 2024 in some cities in Switzerland [14]. In conclusion, questions remain open for discussion and new research. For example, how widespread are such "exceptions", why does respiratory SARS-CoV-2 prefer summer heats, etc. An interesting additional observation in this study was the specific dynamics of SARS-CoV-2 concentrations in individual cities of a small country like Bulgaria – a reason to think about in future planning of monitoring programmes.

Acknowledgments. The authors thank the management teams of Wastewater Treatment Plants in Sofia, Plovdiv, Varna, and Burgas for the fruitful collaboration for the benefit of public health.

REFERENCES

[1] Coronavirus Tracker, representative until 13.04.2024. Available online: https://www.worldometers.info/coronavirus/ (accessed on 03.10.2025).

[2] Grassly N. C., A. G. Shaw, M. Owusu (2025) Global wastewater surveillance for pathogens with pandemic potential: opportunities and challenges, Lancet Microbe, **6**(1), 100939, https://doi.org/10.1016/j.lanmic.2024.07.002.

[3] Robins K. A., F. C. Leonard, K. Farkas et al. (2022) Research needs for optimising wastewater-based epidemiology monitoring for public health protection, J. Water Health, **20**(9), 1284–1313, https://doi.org/10.2166/wh.2022.026.

[4] Whiley D. M., T. P. Sloots (2005) A 5′-nuclease real-time reverse transcriptase–polymerase chain reaction assay for the detection of a broad range of influenza A subtypes, including H5N1, Diagn. Microbiol. Infect. Dis., **53**(4), 335–337, https://doi.org/10.1016/j.diagmicrobio.2005.08.002.

[5] Van Elden L. J., M. Nijhuis, P. Schipper et al. (2001) Simultaneous detection of influenza viruses A and B using real-time quantitative PCR, J. Clin. Microbiol., **39**(1), 196–200, https://doi.org/10.1128/JCM.39.1.196-200.2001.

[6] Hu A., M. Colella, J. S. Tam et al. (2003) Simultaneous detection, subgrouping, and quantitation of respiratory syncytial virus A and B by real-time PCR, J. Clin. Microbiol., **41**(1), 149–154, https://doi.org/10.1128/JCM.41.1.149-154.2003.

[7] Boehm A. B., B. Hughes, D. Duong et al. (2023) Wastewater surveillance of human influenza, metapneumovirus, parainfluenza, respiratory syncytial virus (RSV), rhinovirus, and seasonal corona-viruses during the COVID-19 pandemic, Lancet Microbe, **4**(5), e340-e348, https://doi.org/10.1101/2022.09.22.22280218.

[8] Shopova E., L. Brankova, S. Ivanov et al. (2023) Xanthomonas euvesicatoria-specific bacteriophage BsXeu269p/3 reduces the spread of bacterial spot disease in pepper plants, Plants, **12**(19), 3348, https://doi.org/10.3390/plants12193348.

[9] Stachler E., C. Kelty, M. Sivaganesan et al. (2017) Quantitative CrAssphage PCR assays for human fecal pollution measurement, Environ. Sci. Technol., **51**(16), 9146–9154, https://doi.org/10.1021/acs.est.7b02703.
[10] National Centre of Infectious and Parasitic Diseases, Epidemiological Bulletin. Available online: https://www.ncipd.org/index.php/en/epi-bulletin (accessed on 03.10.2025).
[11] Yuehan A., A. Davis, D. Jones et al. (2021) Wastewater SARS-CoV-2 monitoring as a community-level COVID-19 trend tracker and variants in Ohio, United States, Sci. Total Environ., **801**, 149757, https://doi.org/10.1016/j.scitotenv.2021.149757.
[12] Zafeiriadou A., L. Kaltsis, M. Kostakis et al. (2023) Wastewater surveillance of the most common circulating respiratory viruses in Athens: The impact of COVID-19 on their seasonality, Sci. Total Environ., **900**, 166136, https://doi.org/10.1016/j.scitotenv.2023.166136.
[13] Robotto A., D. Lembo, P. Quaglino et al. (2022) Wastewater-based SARS-CoV-2 environmental monitoring for Piedmont, Italy, Environ. Res., **203**, 111901, https://doi.org/10.1016/j.envres.2021.111901.
[14] Pitton M., R. E. McLeod, L. Caduff et al. (2024) A six-plex digital PCR assay for monitoring respiratory viruses in wastewater, Nat. Water, **3**, 1174–1186, https://doi.org/10.1038/s44221-025-00503-x.

[1]*Research group: Microbiological Risks in the Environment, Sofia University "St. Kliment Ohridski", 8 Dragan Tsankov Blvd, 1164 Sofia, Bulgaria*
e-mails: sivanov714@abv.bg, silviadimitrova@abv.bg, polaris.bg@gmail.com

[2]*CFB Laboratory Ltd., Sofia, Bulgaria*

[3]*RnD Department, LB Bulgaricum Plc., 66 James Bourchier Blvd, 1407 Sofia, Bulgaria*
e-mail: zoltan_urshev@yahoo.com

[4]*Laboratory of Virology, Faculty of Biology, Sofia University "St. Kl. Ohridski", 8 Dragan Tsankov Blvd, 1164 Sofia, Bulgaria*
e-mails: sshishkov@biofac.uni-sofia.bg, ahinkov@biofac.uni-sofia.bg

[5]*Department of General and Applied Hydrobiology, Faculty of Biology, Sofia University "St. Kl. Ohridski", 8 Dragan Tsankov Blvd, 1164 Sofia, Bulgaria*
e-mail: mihaela.kirilova@uni-sofia.bg