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Short Communication

Detection of SARS-CoV-2 RNA in the Danube River in Serbia associated with the discharge of untreated wastewaters

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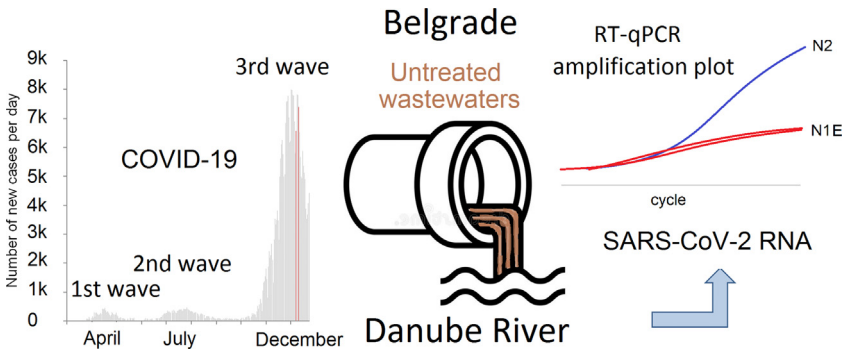
HIGHLIGHTS

- Presence of SARS-CoV-2 RNA was assessed in the Danube River water in Serbia.
- Viral RNA was detected only at the site directly impacted by wastewater discharges.
- N2 primer set (nucleocapsid) gave positive signal in all samples from affected site.
- Concentrations correspond to those in wastewater influents in other countries.
- Epidemiological indicator capacity of the used approach needs further exploration.

GRAPHICAL ABSTRACT



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ABSTRACT

In Serbia less than 13% of collected municipal wastewaters is being treated before their release in the environment. This includes all municipal wastewater discharges from Belgrade (capital city of Serbia; population 1,700,000). Previous research has identified the impacts of raw wastewater discharges from Belgrade on the

Abbreviations: RT-qPCR, Reverse-Transcriptase quantitative-Polymerase Chain Reaction; SARS-CoV-2, Severe Acute Respiratory Syndrome Coronavirus 2; COVID-19, Coronavirus Disease; p.e., Population Equivalent.

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Danube River, and this study investigated if such discharges also provided a pathway for SARS-CoV-2 RNA material. Samples were collected during the most critical circumstances that occurred so far within the COVID-19 pandemics in Serbia. Grab and composite samples were collected in December 2020, during the peak of the third wave (in terms of reported cases) at the site which receives the wastewater loads in Belgrade. Grab samples collected upstream and downstream of Belgrade were also analyzed. RNA was quantified using RT-qPCR with primer sets targeting nucleocapsid (N1 and N2) and envelope (E) protein genes. SARS-CoV-2 RNA (5.97×10^3 to 1.32×10^4 copies/L) was detected only in samples collected at the site strongly impacted by the wastewaters where all three applied primer sets gave positive signals. Determined concentrations correspond to those reported in wastewater influents sampled at treatment plants in other countries indicating an epidemiological indicator function of used approach for rivers with high pollution loads in countries with poor wastewater treatment.

1. Introduction

Since the beginning of the COVID-19 pandemics in 2020, a key research thematic around the globe has been to assess the presence and fate of SARS-CoV-2 in wastewaters and surface waters (as their ultimate recipients) with regard to a possible faecal-oral route of virus transmission (Dada and Gyawali, 2021; Foladori et al., 2020; Gwenzi, 2020). Numerous studies were rapidly undertaken to establish both risks (a source of transmission) and its benefit as wastewater epidemiology tool (and subsequent development of an early warning systems) (Barceló, 2020). The major outcome of these studies was that the virus is detectable in the influents of wastewaters in treatment plants, but rarely in the effluents (Tran et al., 2020). However, a study by Rimoldi et al. (2020) reported the presence of SARS-CoV-2 RNA in rivers receiving treated wastewaters in the province of Milano and Monza e Brianza during the outbreak in April 2020 in Italy, suggesting that treated wastewaters may also be the source. Of particular interest is the situation in low sanitation countries i.e. those lacking wastewater treatment (Adelodun et al., 2020). For example, the study of Guerrero-Latorre et al. (2020) demonstrated that during the peak of the COVID-19 wave in June 2020 in Ecuador, SARS-CoV-2 RNA (up to 3.2×10^6 copies/L) was detectable in natural water bodies receiving untreated wastewaters from the capital Quito.

In Serbia, based on the 2017 data, less than 13% of collected municipal wastewaters were treated before their release to receiving waters (Ministry of Environmental Protection, Environmental Protection Agency, 2019). The impact of untreated wastewaters discharge on the water quality of the Danube River was demonstrated in our previous research (Kirschner et al., 2017). In the river stretch from Novi Sad to its' confluence with the Velika Morava River, all midstream samples were critically polluted based on *Escherichia coli* numbers, and the highest level of faecal pollution was recorded downstream of Belgrade. As ultimate recipients of wastewaters, the Danube River and its largest tributary Sava currently represent the only solution for disposing of wastewaters originating from the Serbian capital's 1,700,000 inhabitants. Knowing that wastewaters from Belgrade significantly deteriorate the microbiological water quality of the Danube, the need to determine the presence of SARS-CoV-2 RNA in surface waters at this highly impacted stretch of the Danube was identified.

National epidemiology data available up to the 25th December 2020 reported a total of 316,344 cases in Serbia with 2882 deaths (https://covid19.rs/). In Serbia, COVID-19 has appeared in three waves among which this third wave was characterized by the highest number of cases and deaths. The major portion of these cases was reported in Belgrade which is expected considering its population size.

The major goal of this study was to investigate if SARS-CoV-2 RNA can be detected in surface waters of the Danube River. The study was carried out in December 2020, at the peak of the third wave (in terms of reported cases) at the site receiving highest wastewater loads from Belgrade. Additionally, samples taken from the site upstream of the urban area and the site 20 km downstream of Belgrade were also analyzed for the presence of the viral RNA.

2. Material and methods

2.1. Sampling sites

Samples were collected from three sites (Fig. 1). SS1 site is located upstream of Belgrade and has been used as control, to eliminate the influence of upstream sources of pollution. SS2 is located in a stretch that is highly influenced by untreated wastewater pollution from Belgrade, while SS3 is located 20 km downstream of Belgrade and has been used to investigate the effect of the potential dilution by the river. All samples were collected approximately 3 m from the shore at 30 cm depth below the surface in clean 500 mL plastic bottles. Samples were immediately stored at 4 °C and transported to laboratory (within 3 h). At SS1 and SS3, a grab sample (2000 mL) was collected on 7th December 2020. At SS2, 1000 mL grab samples were collected each hour over a 12 h period (commencing 07:00) on 10th December 2020. All subsamples were stored at 4 °C until the end of the day. From each SS2 subsample, 100 mL was withdrawn to prepare a 1200 mL composite sample. In each subsample, physical-chemical, chemical and bacterial parameters were measured directly upon sampling. Subsamples used for the composite sample taken at site SS2 that were characterized by the highest and the lowest concentration of faecal indicator bacteria were also processed. All samples used for viral RNA analyses were stored at −20 °C until the analyses (within two weeks). For samples SS1, SS2-composite and SS3, two replicates (identified as "a" and "b") were processed. For the samples SS2-11:00 and SS2-13:00, a single replicate was analyzed.

Higher resolution of the conditions at the site SS2 is given in Supplementary Fig. 1. Belgrade sewer system consists of combined and separate sewers with a total length of over 1500 km, to which 1.2 million inhabitants are connected. Works on upgrading the sewer system and the introduction of wastewater treatment plants is ongoing, but today sewage is still discharged into the rivers Sava and Danube without any treatment through a total of 28 main and numerous smaller sewer outlets. Analysis of the sewer network configuration and the Danube River flow identify three major sewer outlets which can influence river water quality at SS2 (Table 1 and Supplementary Fig. 1).

2.2. Physical-chemical and bacteriological characterization of the samples

In-field measurements (temperature, pH, conductivity and dissolved oxygen levels) were performed using a multi-parameter probe (WTW/Xylem Analytics, Germany). Physical-chemical parameters of water quality were assessed according to the standard methods for the examination of water and wastewater (APHA, 2017). Defined Substrate Technology was used for faecal indicator bacteria (*E. coli* and intestinal enterococci) using a protocol described in Vrzel et al. (2016). Briefly, quantification was performed with the IDEXX Quanti-Tray 2000 system, which provides a Most Probable Number (MPN) result, based on color/fluorescence change in 97 wells. Powdered reagents Colilert-18 and Enterolert-E were used for *E. coli* and enterococci

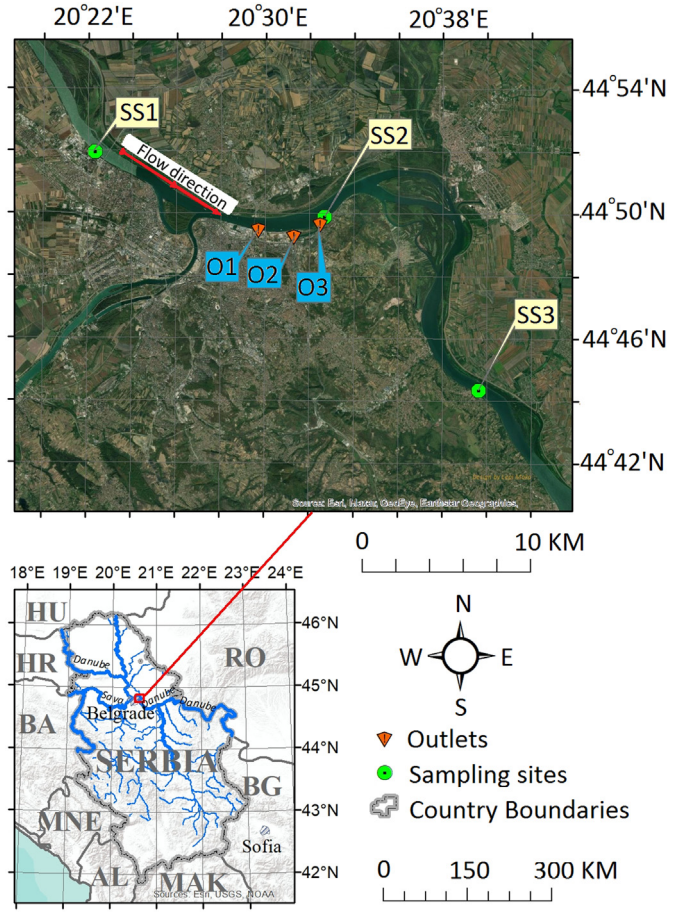

Fig. 1. Study area: SS1 – upstream of the urban area of Belgrade, SS2 – downstream of the WW outlets in Belgrade, SS3 – downstream of Belgrade.

respectively. Trays were incubated at 37 °C for at least 18 h for *E. coli*, and for at least 24 h at 43 °C for enterococci.

2.3. SARS-CoV-2 RNA extraction and RT-qPCR

The protocol developed by KWR Water Research Institute Nieuwegein (Netherlands) described in Medema et al. (2020a) has been applied with modifications. For each sample two parallel preparations (sample concentration and RNA extraction) were performed. Briefly, 50 mL of the samples were centrifuged in duplicates at 4000 ×*g* for 40 min to remove solid suspended matter. Afterwards, 45 mL of the supernatant was removed and concentrated using Amicon Ultra-15 centrifugal filters (Ultracel-100,000 NMWL, Merck Millipore, Carrigtwohill, Ireland), in 3 × 15 mL steps at 4000 ×*g* for 30 min each. The concentrates were collected and the RNA extraction was performed using the NucleoSpin RNA Virus kit (Macharey-Nagel, Düren, Germany), the isolated RNA was eluted with 60 μL of TE buffer.

Table 1
The main wastewater sewer outlets upstream sampling site SS2.

Outlet No	Sewer type	Average sewage flow rate (m^3/day)	Load, p.e.[a] (−)	Distance from SS2 (m)
O1	Combined	46,650	246,000	4450
O2	Separate	7900	37,500	2600
O3	Separate	13,050	61,300	620

[a] p.e. – one population equivalent equals to 60 g of 5-day biochemical oxygen demand per day.

Two targets on the nucleoprotein gene (N1 and N2) and one on the envelope gene (E) were assessed (primer and probe sets shown in Table 2). The N1 and N2 sets published in US CDC (2019) were used to target different regions of the nucleocapsid gene, while the E set published in Corman et al. (2020) was used for the envelope protein gene. The PCR reaction was carried out in 20 μL final volume: 5 μL TaqMan Fast Virus 1-Step Master Mix (ThermoFischer Scientific, Vilnus, Lithuania) supplemented with 0.4 μL BSA (Bovine Serum Albumin 20 mg/mL, ThermoFischer Scientific, USA), primers and probes (Merck, Darmstadt, Germany) at final concentration as in Table 2, and 5 μL of the isolated RNA. The presence of potential inhibitory substances in the isolated RNA was assessed by testing and evaluating a two-fold dilution of the isolated RNA. The RT-PCR was carried out with a QuantStudio 5 real-time PCR device (ThermoFischer Scientific, USA) with 5 min at 50 °C, followed by 45 cycles of 10 s at 95 °C and 30 s at 60 °C. Reactions were considered positive if the cycle threshold was below 40 cycles (as in Randazzo et al. (2020) and Medema et al. (2020a)). Each RNA was analyzed in technical duplicate and each assay included negative and positive template controls. Standard curve was derived from the ATCC Heat Inactivated 2019 Novel Coronavirus VR-1986HK, (ATCC, Manassas, USA) by isolating RNA with the above described method and preparing ten-fold dilutions. Verified limit of detection for N2 region was 3.47×10^2 genomic copies/L. A process control (sample spiked with in-house SARS-CoV-2 material) was processed with the each batch.

The recovery efficiency was assessed by spiking the 45 mL of pre-centrifuged river water sample with ATCC Heat Inactivated 2019 Novel Coronavirus VR-1986HK in triplicate to a final concentration of 10^6 genome copies/L. The recovery efficiency was calculated based on the copies quantified by RT-qPCR as follows: recovery efficiency (%) = (virus recovered / virus seeded) × 100.

2.4. Additional verification of N2 PCR products by sequencing

Sequencing of the PCR products from the diagnostic reaction involved an initial clean-up step by using MN NucleoSpin Gel and PCR Clean-up kit, and a subsequent sequencing protocol using ThermoFisher BigDye™ Terminator v3.1 Cycle Sequencing Kit and N2-F and N2-R primers, the sequencing reaction clean-up by ethanol precipitation and the final capillary electrophoresis step on a ThermoFisher ABI3130xl device. Due to short length of amplicon (PCR product size: 66 bp), sequences were analyzed manually. The obtained sequences were aligned against the submitted sequence (accession number: MN985325) of the SARS-CoV-2/human/USA/WA-CDC-WA1/2020 viral strain. This strain was used as reference material in quantification and sequencing.

2.5. Epidemiological data

Data were retrieved from https://www.worldometers.info/coronavirus/country/serbia/, official national data (https://covid19.rs/), the European Centre for Disease Prevention and Control (ECDC) and public news reports in Serbia.

3. Results

3.1. Epidemiological data

Sampling was performed in the 50th week of 2020 when the 14-day incidence rate in Serbia was 1396 per 100,000 citizens according to ECDC. In Belgrade, the 14-day incidence rate of new cases was slightly higher - 1548 per 100,000 citizens. On the date of sampling of sites SS1 and SS3, the number of new cases in Serbia was 6557, while at the date of sampling of the site SS2, 7393 cases were reported (Fig. 2). In Belgrade, 1583 new cases were reported at 7th December, and 1777 at 10th December 2020.

Table 2
Primer-probe sets used for RT-PCR assays.

Target gene	Primer/probe	Sequence[a]	Final concentration	Ref
Nucleo-capsid (N1)	2019-nCoV_N1-F	5′-GACCCCAAAATCAGCGAAAT-3′	200 nM	US CDC (2019)
	2019-nCoV_N1-R	5′-TCTGGTTACTGCCAGTTGAATCTG-3′	200 nM	
	2019-nCoV_N1-P	5′-FAM-ACCCCGCATTACGTTTGGTGGACC-BHQ1-3′	200 nM	
Nucleo-capsid (N2)	2019-nCoV_N2-F	5′-TTACAAACATTGGCCGCAAA-3′	200 nM	
	2019-nCoV_N2-R	5′-GCGCGACATTCCGAAGAA-3′	200 nM	
	2019-nCoV_N2-P	5′-FAM-ACAATTTGCCCCCAGCGCTTCAG-BHQ1–3′	200 nM	
Envelope (E)	E_Sarbeco_F	5′-ACAGGTACGTTAATAGTTAATAGCGT-3′	400 nM	Corman et al. (2020)
	E_Sarbeco_R	5′-ATATTGCAGCAGTACGCACACA-3′	400 nM	
	E_Sarbeco_P1	5′-FAM-ACACTAGCCATCCTTACTGCGCTTCG-BHQ1-3′	200 nM	

[a] FAM: 6-carboxyfluorescein; BHQ1: Black Hole Quencher-1.

3.2. Physical-chemical and bacteriological characterization of the samples

The data for the analyzed parameters is summarized in Table 3. The impact of wastewaters on the water quality at SS2 is evident through increases in concentration of ammonium, orthophosphate and total P at this site in comparison to SS1 and SS3, but also by the increase in numbers of faecal indicator bacteria. When using the obtained data for an indication of the ecological status in the light of the national legislation (Official Gazette of RS, 2011), SS2 site would be classified as class V (the poorest water quality). At SS2, the highest concentration of enterococci was detected in a subsample taken at 11:00 a.m. (1.05×10^6 MPN/100 mL), and the lowest concentration in a subsample taken at 13:00 p.m. (3.59×10^4 MPN/100 mL).

3.3. SARS-CoV-2 RNA extraction and RT-qPCR

The N1, N2 and E gene validation reactions using purified RNA from the ATCC VR-1986 virus preparation yielded the following results: N1 and N2 genes were detected in all three technical replicates at a 2 genome copies/reaction level, the E gene reaction had a slightly higher threshold: 14 genome copies/reaction. At the lowest reliable detectable level the relative standard deviation stayed below 4%. Percentage recovery was 27.1% (standard deviation 1.36%).

Inhibition was checked by comparing the average Ct values of two technical replicates from the original and diluted samples, and assessed to determine whether the values displayed a positive shift in average Ct values (Table S1). Due to the low Ct regime, the differences deviated from the theoretical shift of 1 Ct by 40%–80%, although the shift was positive (i.e.: the diluted RNA gave higher Ct values), indicating that inhibitory substances were not present in such quantities so as to compromise the quantification.

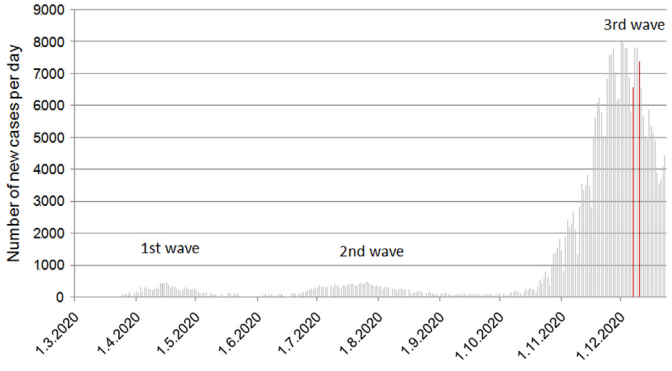

Fig. 2. Number of new COVID-19 cases in Serbia reported per day, sampling dates are marked in red (data retrieved from https://www.worldometers.info/coronavirus/country/serbia/).

In samples taken upstream (SS1) and downstream (SS3) of Belgrade, positive signals have not been detected for any of the primer sets (Table 4). Both replicates of the composite samples taken at SS2 site showed a positive signal for all three primer sets. Additionally, grab samples taken at SS2 with the highest (SS2-11:00) and the lowest (SS2-13:00) concentration of faecal indicator bacteria were also analyzed for the presence of viral RNA. In these samples, only the N2 primer set gave a positive signal. Sequencing of the N2 gene RT-PCR products confirmed the presence of SARS-CoV-2 virus (details provided in Table S2).

4. Discussion

This study was established to target the most critical circumstances that have occurred so far within the COVID-19 pandemics in Serbia. Samples were collected in the period with the highest numbers of reported COVID-19 cases in Serbia to-date at the site SS2, which is by our knowledge the most affected site of the Danube River in Serbia. Among three sewer outlets which were identified to influence Danube water quality at SS2, the biggest outlet is O1, located 4.45 km upstream of the sampling site SS2. Although it discharges high hydraulic and pollution loads into the river, due to significant distance from SS2, its influence on the river water quality at the sampling site is limited (the Danube River has an average discharge of 5600 m^3/s in this section). However, the influence of sewer outlets O2 and O3 on river water quality at the sampling site SS2 is significant: O2 outlet discharges wastewater into a river branch with no natural flow, which is connected at the downstream end to the 60-meter wide Danube river branch, where O3 outlet is located. The flow through this small river branch can be estimated to be much <1% of the total flow of the Danube in this stretch. Pollution loads from O2 and O3 combined amount to almost 100,000 population equivalents (p.e.) and low dilution factors lead to a deterioration of water quality in the river branch and downstream along the right river bank. The evidence of the impact of pollution in this stretch, based on the microbial contamination was already reported in our previous study (Kirschner et al., 2017). Moreover, sewage plumes can be observed on satellite images as well. Considering that the 14-day incidence rate of new cases in Belgrade in the week of sampling was 1548 per 100,000 citizens and that the sampling site is mainly impacted from outlets O2 and O3 (circa 100,000 p.e.) we can roughly estimate that at least 1500 active cases can be attributed to the sewerage system related to the studied site. Still, this should be treated with precaution taking into consideration multiple factors affecting the true estimation of active COVID-19 cases in this area.

To enable comparison of data with other studies we have applied a commonly used method for viral RNA concentration and isolation. Recovery efficiency of RNA extraction was 27.1 ± 1.4% which is the range reported by other authors. Medema et al. (2020b) reported a recovery efficiency of the RNA extraction evaluated with the internal control (RNA fragment with a length of 412 bases) to be 30.4 ± 22.3% while Ahmed et al. (2020b) reported a recovery efficiency of murine hepatitis

Table 3
Physical-chemical and bacteriological parameters measured in samples.

Parameter		SS1 (n=1)	SS2 (mean±SD, n=12)	SS3 (n=1)
Temperature	°C	5.3	6.9±0.3	6.0
Conductivity	µS/cm	433	527±26	440
Dissolved oxygen	%	99.3	86.7±0.7	98.6
	mg/L	12.4	10.4±0.3	11.8
pH		7.7	7.8±0.1	7.7
Ammonium (NH_4^+)	mgN/L	<0.05	3.64±2.12	0.05
Nitrate (NO_3^-)	mgN/L	2.7	1.4±0.2	2.1
Chloride (Cl^-)	mg/L	16.9	22.4±1.5	18.3
Ortophosphate	mgP/L	0.02	0.28±0.23	0.04
Total P	mgP/L	0.2	0.6±0.3	0.1
TOC	mgC/L	6.5	6.9±2.1	2.9
BOD_5	mg/L	NA	5.5±2.5	NA
E. coli	log MPN/100 mL	3.7	5.9±0.4	4.2
Enterococci	log MPN/100 mL	3.3	5.2±0.4	3.4

NA-not assessed, blue - class I, green - class II, yellow - class III, orange - class IV, red - class V, white –not compliant.

virus to be 56.0 ± 32.3%. A potential drawback identified in the applied protocol relates to the pre-centrifugation step which removes solid suspended matter from the sample. There is therefore the potential that a proportion of the SARS-CoV-2 virus particles attached to solid mater may have been removed by the centrifugation step which was also discussed in Medema et al. (2020b) and Ahmed et al. (2020b). In the composite sample from this most affected site, all three assays (N1, N2 and E) gave positive signals, while in subsamples from SS2 only the N2 primer set gave a signal above the threshold (Ct < 40). Discrepancies in sensitivity of the applied primer sets have been also reported by other authors. Medema et al. (2020a) demonstrated the highest sensitivity of the N1 primer set, while in the study of Sherchan et al. (2020) the N2 primer set gave positive signals in most of the samples of wastewater influents in Louisiana. In the study of Philo et al. (2021), it is suggested that a variability of detection among the N1 and N2 could be due the variability in performance among the assays or degradation in the target genetic material. Further, Nalla et al. (2020) found that the N2 and E-gene assays were the most sensitive assays from the 8 assessed protocols, and a study by Lu et al. (2020) demonstrated that the N2 assay was more sensitive than N1 in stool matrix samples spiked with SARS-CoV-2 virus. This study also provided data for a possible explanation why the N2 assay exhibits higher sensitivity in such complex matrices: among the 7158 SARS-CoV-2 genome sequences analyzed, the N1 assay had at least 3 nucleotide positions that exhibited mismatch frequencies of 0.31, 1.46 and 0.54%, whereas the N2 assay had only one nucleotide position with a mismatch frequency of 0.1%. In high diversity matrices like wastewater or river water influenced by raw sewage these mismatch frequencies could affect PCR reaction efficiencies leading to differences in sensitivity.

SARS-CoV-2 copy number in analyzed samples from SS2 site ranged from 5.96×10^3 up to 1.30×10^4/L. The concentrations are similar to those reported in untreated wastewater treatment plant influents. For example, a study of wastewater influents in Louisiana detected concentrations up to 7.5×10^3 genomic copies/L (Sherchan et al., 2020). Data reported in the NORMAN SCORE SARS-CoV-2 in sewage database (an open access datashare platform sharing influent wastewater data from nine countries across Europe and abroad) range from not detected to a maximum of 1.5×10^5 gene copies/L with a median value of 2.5×10^3 gene copies/L in samples where a signal was detected (NORMAN SCORE, 2021). Given that the SS2 sample location is within the receiving water body (providing an opportunity for dilution to occur), it is anticipated that direct outfall samples would be at the upper range of values reported. This indicates that the applied approach could have an epidemiological indicator function not only for wastewaters but also for rivers with high pollution loads in countries with poor wastewater treatment. This present study provides preliminary data on the presence of viral RNA in Danube and further research will be taken to compare the concentrations of RNA in the samples taken directly at outlets and the ones from the Danube. Impacted river sites could be a reasonable sampling location when no obvious sewage outlets occur. Still, the complexity of epidemiological and demographic data, differences in applied sampling, wastewater meta-data and analysis methodologies should be taken into consideration in the standardization of analytical method.

Although SARS-CoV-2 RNA was present in all samples from SS2, positive signals have not been detected for any of the primer sets in samples taken at SS1 and SS3. When looking at the data on the concentrations of faecal indicator bacteria, it can be seen that similar numbers were recorded at the sites SS1 and SS3, which were evidently lower (about two orders of magnitude) in comparison with values from SS2 confirming both Belgrade as a source of pollution and the high dilution potential of the Danube River over the investigated stretch. Further, taking into consideration SARS-CoV-2 RNA concentrations normalized to enterococci concentration of 10^4 MPN/100 mL in SS2 samples, it is expected that in samples from SS1 and SS3 assays will give a negative signal accounting the sample limit of detection (SLOD) of applied methodology (3.47×10^2 copies/L). Still it should be noted that only grab samples were processed for SS1 and SS3, and that preparation of a composite sample at these sites could statistically increase the possibility for detection of viral RNA in this case (Ahmed et al., 2021).

The methodological approach applied in our study does not provide information about the infectious potential of the virus in water samples and thus it is difficult to estimate the human health hazard. Little is known about the potential distribution of the virus in the aquatic environment and the survival of SARS-CoV-2 in water (Ahmed et al., 2020a; Naddeo and Liu, 2020). Knowledge gained so far indicates that detected RNA materials do not occur in the form of an infectious viral particle and thus do not represent a health hazard (Westhaus et al., 2020; Bivins et al., 2020; Rimoldi et al., 2020). While clinical studies of faecal material

Table 4
Results of RT-PCR amplification shown as presence/absence of target gene and SARS-CoV-2 copy number in samples (calculation is based on N2 primer set).

Sample	Gene			SARS-CoV-2 (copy/L)	SARS-CoV-2 (copy/L normalized data[a])
	N1	N2	E		
SS1a	-	-	-	-	-
SS1b	-	-	-	-	-
SS2a	+	+	+	1.31×10^4	5.60×10^2
SS2b	+	+	+	5.97×10^3	2.56×10^2
SS2-11.00	-	+	-	7.22×10^3	6.90×10^1
SS2-13.00	-	+	-	1.32×10^4	3.69×10^3
SS3a	-	-	-	-	-
SS3b	-	-	-	-	-

- not detected.
[a] Normalization to enterococci concentration of 10^4 MPN/100 mL.

from hospitalized patients have isolated the virus in a virulent form (Xiao et al., 2020), environmental studies (i.e. samples collected following dilution with flush water and transportation to the sewer system) have yet to detect viral material in a form that causes infection.

5. Conclusions

To our knowledge, this is the first study that reports the presence of detectable SARS-CoV-2 RNA in surface water of the Danube River, the most international river in the world. Determined concentrations correspond to those reported in wastewater influents sampled at treatment plants in other countries indicating an epidemiological indicator function of the used approach for rivers with high pollution loads in countries with poor wastewater treatment. Results also indicate significant dilution potential of the Danube River at this stretch which sets the RNA copies number below the level of detection 20 km downstream of the affected site.

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CRediT authorship contribution statement

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Declaration of competing interest

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

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