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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

Stoimir Kolarević a,⁎, Adrienn Micsinaib, Réka Szántó-Egész c, Alena Lukács c, Margareta Kračun-Kolarević a, Lian Lundyd,e, Alexander K.T. Kirschnerf,g,h,1, Andreas H. Farnleitnerg,h,i,1, Aleksandar Djukiċ j, Jasna Čolić k, Tanja Neninl, Karolina Sunjogl, Momir Paunović l

a University of Belgrade, Institute for Biological Research “Sinisa Stankovic”, National Institute of Republic of Serbia, Department of Hydroecology and Water Protection, Bulevar despot Stefan 142, 11000 Belgrade, Serbia
b WESSLING Hungary Ltd., H-1045 Budapest, Anonymus str 6., Hungary
c Biomi Ltd., H-2100 Gödöllő, Szent-Györgyi Albert str 4., Hungary
d DRIZZE Centre of Excellence, Luleå University of Technology, VA-Teknik, 971 87 Luleå, Sweden
e Middlesex University, The Burroughs, London NW4 4BT, UK
f Medical University Vienna, Institute for Hygiene and Applied Immunology - Water Microbiology, Kinderspitalgasse 15, Vienna, Austria
g Interuniversity Cooperation Center Water and Health (ICC), Austria
h Karl Landsteiner University of Health Sciences, Division Water Quality & Health, Dr.-Karl-Dorrek-Straße 30, A-3500 Krems, Austria
i Technische Universität Wien, Institute of Chemical, Environmental and Bioscience Engineering, Research Group for Environmental Microbiology and Molecular Diagnostics, Gumpendorferstraße 1a, A-1060 Vienna, Austria
j Faculty of Civil Engineering, University of Belgrade, Bulevar kralja Aleksandra 73, 11000 Belgrade, Serbia
k Jaroslav Černi Water Institute, Jaroslava Černog 80, 11226 Belgrade, Serbia
l University of Belgrade, Institute for Multidisciplinary Research, kneza Vlêeslava 1, 11000 Belgrade, Serbia

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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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.
⁎ Corresponding author at: Bulevar despot Stefan 142, 11000 Belgrade, Serbia.
E-mail address: stoimir.kolarevic@ibiss.bg.ac.rs (S. Kolarevic).
1 www.waterandhealth.at.
2 Authors equally contributed to the study.

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1. Introduction

Since the beginning of the COVID-19 pandemics in 2020, a key research theme 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 possible faecal-oral route of virus transmission (Dada and Gyawali, 2021; Foladori et al., 2020; Gwenzé, 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 (Kirscher 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.
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 (Machera–Nagel, Düren, Germany), the isolated RNA was eluted with 60 μL of TE buffer.

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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.

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 (ThermoFisher Scientific, Vilnus, Lithuania) supplemented with 0.4 μL BSA (Bovine Serum Albumin 20 mg/mL, ThermoFisher 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 (ThermoFisher 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. Verification limit of detection for N2 region was 3.47 × 10^5 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.

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3. Results

Table 2

| Outlet No | Average sewage flow rate (m³/day) | Load, p.e.a | Distance from SS2 (m) |
|-----------|---------------------------------|------------|----------------------|
| O1        | 46,650                          | 246,000    | 4450                 |
| O2        | 7900                            | 37,500     | 2600                 |
| O3        | 13,050                          | 61,300     | 620                  |

**p.e.** – one population equivalent equals to 60 g of 5-day biochemical oxygen demand per day.
Table 2
Primer-probe sets used for RT-PCR assays.

| Target gene          | Primer/probe       | Sequencea                | Final concentration | Ref                |
|----------------------|--------------------|--------------------------|---------------------|--------------------|
| Nucleo-capsid (N1)   | 2019-nCoV_N1-F     | 5′-GCACCGAAATCAGCGAAAT-3′ | 200 nM              | US CDC (2019)      |
|                      | 2019-nCoV_N1-R     | 5′-TGTCCCTCAGTGGTCAATG-3′ | 200 nM              |                    |
|                      | 2019-nCoV_N1-P     | 5′-FAM-ACCGGATTTGCTGAGGAC-BHQ1-3′ | 200 nM |                    |
| Nucleo-capsid (N2)   | 2019-nCoV_N2-F     | 5′-TTCAAAACATTGGCCCCAA-3′ | 200 nM              |                    |
|                      | 2019-nCoV_N2-R     | 5′-GGCCGACCTTCCAGAAGA-3′ | 200 nM              |                    |
|                      | 2019-nCoV_N2-P     | 5′-FAM-ACATTTGCCCCAGCGGCTCAG-BHQ1-3′ | 200 nM | Corman et al. (2020) |
| Envelope (E)         | E_Sarbeco_F        | 5′-ACAGTGATCTAATTACACCTG-3′ | 400 nM              |                    |
|                      | E_Sarbeco_R        | 5′-ATATGGCAGGCTACACACAAC-3′ | 400 nM              |                    |
|                      | E_Sarbeco_P1       | 5′-FAM-ACACTGGCATCTTACTGGCTTCG-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. (the poorest water quality). At SS2, the highest concentration of enterococci was detected in a subsample taken at 11:00 a.m. (the poorest water quality).

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 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 m³/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

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/).
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 matter 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 × 10^5/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 × 10^5 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 influential wastewater data from nine countries across Europe and abroad) range from not detected to a maximum of 1.5 × 10^6 gene copies/L with a median value of 2.5 × 10^5 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 10^8 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 3

| 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      |
| pH                        |           |                     |           |           |
| Ammonium (NH₄⁺)            | mg/L      | <0.05               | 3.64±2.12 | 0.05      |
| Nitrates (NO₃⁻)           | mg/L      | 2.7                 | 1.4±0.2   |           |
| Chloride (Cl⁻)            | mg/L      | 16.9                | 22.4±1.5  | 18.3      |
| Orthophosphate            | mg/P/L    | 0.02                | 0.28±0.23 | 0.04      |
| Total P                   | mg/P/L    | 0.2                 | 0.6±0.3   | 0.1       |
| TOC                       | mg/C/L    | 6.5                 | 6.9±2.1   | 2.9       |
| BOD₅                      | 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.

Table 4

| Sample | Gene | SARS-CoV-2 (copy/L) | SARS-CoV-2 (copy/L normalized data) |
|--------|------|---------------------|-------------------------------------|
|        | N1   | N2                  | E                                   |
| SS1a   | -    | -                   | -                                   |
| SS1b   | -    | -                   | -                                   |
| SS2a   | +    | +                   | +                                  |
| SS2b   | +    | +                   | +                                  |
| SS2-11.00 | -   | +                   | -                                  |
| SS2-13.00 | - | +                   | -                                  |
| SS3a   | -    | -                   | -                                   |
| SS3b   | -    | -                   | -                                   |

- not detected.

a Normalization to enterococci concentration of 10^8 MPN/100 mL.

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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 infections.

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

Stoimir Kolarević: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft. Adrienn Micsinai: Conceptualization, Methodology, Formal analysis, Investigation, Resources, Writing – original draft. Réka Szántó-Egész: Methodology, Investigation, Formal analysis. Alena Lukács: Methodology, Investigation, Formal analysis. Margareta Kračun-Kolarević: Conceptualization, Methodology, Investigation, Resources, Visualization. Lian Lundy: Methodology, Validation, Writing – review & editing. Alexander K.T. Kirschner: Methodology, Validation, Resources. Andreas H. Farnleitner: Validation, Writing – review & editing. Aleksandar Djukić: Conceptualization, Formal analysis, Writing – original draft. Jasna Ćolić: Methodology, Investigation. Tanja Nenin: Methodology, Investigation. Karolina Sunjog: Methodology, Resources. Momir Paunović: Conceptualization, Resources, Writing – original draft, Supervision.

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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