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SARS-CoV-2 circulation in Croatian wastewaters and the absence of SARS-CoV-2 in bivalve molluscan shellfish

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ABSTRACT

The circulation of SARS-CoV-2 in the environment has been confirmed numerous times, whilst research on the bioaccumulation in bivalve molluscan shellfish (BMS) has been rather scarce. The present study aimed to fulfil the knowledge gap on SARS-CoV-2 circulation in wastewaters and surface waters in this region and to extend the current knowledge on potential presence of SARS-CoV-2 contamination in BMS. The study included 13 archive wastewater and surface water samples from the start of epidemic and 17 influents and effluents from nine wastewater treatment plants (WWTP) of different capacity and treatment stage, sampled during the second epidemic wave. From that period are the most of 77 collected BMS samples, represented by mussels, oysters and warty venus clams harvested along the Dalmatian coast. All samples were processed according to EN ISO 15216-1 2017 using Mengovirus as a whole process control. SARS-CoV-2 detection was performed by real-time and conventional RT-PCR assays targeting E, N and nsp14 protein genes complemented with nsp14 partial sequencing. Rotavirus A (RVA) real-time RT-PCR assay was implemented as an additional evaluation criterion of virus concentration techniques. The results revealed the circulation of SARS-CoV-2 in nine influents and two secondary treatment effluents from eight WWTPs, while all samples from the start of epidemic (wastewaters, surface waters) were negative which was influenced by sampling strategy. All tertiary effluents and BMS were SARS-CoV-2 negative. The results of RVA amplification were beneficial in evaluating virus concentration techniques and provided insights into RVA dynamics within the environment and community. In conclusion, the results of the present study confirm SARS-CoV-2 circulation in Croatian wastewaters during the second epidemic wave while extending the knowledge on wastewater treatment potential in SARS-CoV-2 removal. Our findings represent a significant contribution to the current state of knowledge that considers BMS of a very low food safety risk regarding SARS-CoV-2.

1. Introduction

COVID-19 and its causative agent SARS-CoV-2 have reshaped the world we know. The scientists have done a tremendous work in vaccine development (Creech et al., 2021), however the endemicity is expected with many open questions regarding SARS-CoV-2 dynamics (Baker et al., 2021). The circulation of SARS-CoV-2 in the environment has been confirmed numerous times, especially through wastewater surveillance

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approach (Tran et al., 2021). The prerequisite for such surveillance is the knowledge of prolonged excretion of SARS-CoV-2 in faeces of infected individuals (Chen et al., 2020; Wu et al., 2020). These findings and the previous experience in poliovirus environmental surveillance in the Global Polio Eradication Initiative (Asghar et al., 2014), were basis for developing wastewater-based epidemiology (WBE) approach as an early warning system for COVID-19 surveillance (Medema et al., 2020; Nemudryi et al., 2020). It is considered that such investigations allow a detection of rising SARS-CoV-2 incidence in the community 1–3 weeks in advance (Aguiar-Oliveira et al., 2020). Even though promising, the researchers have identified several bottlenecks to overcome in the WBE implementation, highlighting further refinement and validation (Zhu et al., 2021). According to Eurostat, in 2016 and 2018 the proportion of the population connected to at least secondary wastewater treatment plants was at low 36.9% in Croatia compared to more than 80% in 16 other EU member states (Eurostat, 2021). This is important to consider since it is believed that even a conventional wastewater treatment system, including secondary and tertiary treatment, is effective in SARS-CoV-2 RNA reduction and removal (Balboa et al., 2021; Kumar et al., 2021). On the other hand, whether SARS-CoV-2 RNA positive wastewaters could present a source of infection is still a matter of debate. The infectivity of SARS-CoV-2 in water environments has been confirmed in controlled in vitro assays (shorter in wastewater compared to tap water), but it was excluded when SARS-CoV-2 RNA positive samples from wastewater treatment plants (WWTP) were tested (Giacobbo et al., 2021). Nevertheless, the infectivity of wastewaters regarding SARS-CoV-2 cannot be ruled out in all SARS-CoV-2 RNA positive samples since sample pre-treatment and concentration may turn SARS-CoV-2 non-viable, with subsequent impact on infectivity assays (Giacobbo et al., 2021).

Possible implications of the SARS-CoV-2 environmental shedding by wastewaters are the contamination of surface waters and its possible spill over to the wildlife. Even though SARS-CoV-2 RNA was detected in open surface waters (Kolarević et al., 2021; Mahlknecht et al., 2021; Rimoldi et al., 2020), there is no evidence for a transmission of SARS-CoV-2 via water-food-environmental media (Adelodun et al., 2021). To this date SARS-CoV-2 pandemic strain was not detected to circulate in free-ranging wildlife species which may be in contact with surface waters, wastewaters and human waste in general (Colombo et al., 2021; Jemeršić et al., 2021). However, wastewater discharge, primarily those untreated, is the source of contamination for aquatic environments (Bosch et al., 2018). Consequently, bivalve molluscan shellfish (BMS), due to their filter feeding nature, are prone to accumulate different pathogens and cause shellfish-borne disease outbreaks (Bellou et al., 2013). To the best of our knowledge the research on bioaccumulation of SARS-CoV-2 in BMS is rather scarce, with only two papers currently published. Clams (*Ruditapes* sp.) and estuary sediments in Spain were found to be SARS-CoV-2 RNA positive, but no viable viral particles were detected (Polo et al., 2021). On contrary, all tested BMS (oysters, mussels and clams) were SARS-CoV-2 RNA negative in French coastal areas, as well as tested seawater (Desdouits et al., 2021). Nevertheless, the capacity of oysters to accumulate heat-inactivated SARS-CoV-2 strain was confirmed in vitro (Desdouits et al., 2021).

The aim of the present study was to fulfil the knowledge gap on SARS-CoV-2 circulation in wastewaters and surface waters in this region at the start of epidemic and during the second wave in autumn-winter 2020/2021. Therefore the study was oriented towards investigating archive samples of wastewaters and surface waters dating in the time frame of the first documented SARS-CoV-2 case in Croatia and on influents and effluents of nine WWTPs of different capacity and in the range from primary to tertiary treatment stage. This is, to the best of our knowledge the first study of SARS-CoV-2 circulation in wastewaters and surface waters in Croatia. Due to the largely inadequate wastewater treatment infrastructure in Croatian coastal areas, we aimed to investigate the presence of SARS-CoV-2 in BMS, primarily in Mediterranean mussels, followed by European flat oysters and warty venus clams. We believe that this is the first study on potential presence of SARS-CoV-2 contamination in BMS in autumn-winter period (high epidemic burden of enteric viruses) during the second epidemic wave.

2. Materials and methods

2.1. Study area and sampling

Archive samples included in the present study were represented by wastewaters and surface waters for which the sampling was conducted within a research project dealing with rotavirus A (RVA) in Croatian ecosystem (Brnić et al., 2018). These samples (N = 13) were taken between December 2019 and July 2020 and consisted of wastewater effluents (secondary treatment) from Zagreb WWTP (N = 4) and surface waters from Lonjsko polje Nature Park in Sisak-Moslavina County (N = 3), Kopački rit Nature Park in Osijek-Baranja County (N = 3) and from the city lake in Zagreb (City of Zagreb County) (N = 3) (Fig. 1). From late October 2020 until late January 2021, influents (after primary or mechanical treatment) and effluents (after secondary or tertiary treatment) or only influents were sampled from nine WWTP (N = 17) located in the cities of Vinkovci (Vukovar-Srijem County), Slavonski Brod (Brod-Posavina County), Koprivnica (Koprivnica-Križevci County), Zagreb (City of Zagreb County), Karlovac (Karlovac County), Zadar (Zadar County) and Split (Split-Dalmatia County) (Fig. 1). The latter two cities were represented by two WWTPs each (Fig. 1). Three WWTPs were of primary (Zadar-Borik, Split-Stupe and Split-Katalinića Brig), two of secondary (Zagreb and Zadar-Centar) and four of tertiary (Koprivnica, Vinkovci, Karlovac and Slavonski Brod) treatment stage (Fig. 1). WWTPs with secondary or tertiary wastewater treatment implemented activated sludge process (Zagreb, Karlovac, Zadar-Centar, Vinkovci) or sequencing batch reactor (SBR) (Koprivnica and Slavonski Brod) technology. Only influents were sampled in the case of Zadar-Borik, Split-Stupe and Split-Katalinića Brig WWTPs since these three facilities were of the primary treatment stage (only mechanical treatment) and therefore represented an effluent as well. WWTP in Vinkovci discharge the effluents to the Bosut River, WWTPs in Slavonski Brod and Zagreb to the Sava River, WWTP in Koprivnica to the Bistra Stream and WWTP in Karlovac to the Kupa River. These rivers and a stream are part of the Black Sea catchment basin. WWTPs in Zadar and Split discharge the effluents to the Adriatic Sea, 1.3–2.8 km from the coastline at a depth of 34–43 m.

In total, 21 wastewater (10 influents, seven secondary and four tertiary treatment effluents) and nine surface water samples were collected. Data regarding design capacity (PE, population equivalent) and wastewater source are provided in Fig. 1, whereas the wastewater physical and chemical properties (water flow rate, temperature, pH, total suspended solids, total nitrogen and total phosphorous) are provided in Supplementary table 1. It is worth mentioning that a design capacity measured in population equivalent (PE) was provided in Fig. 1 as a reliable constant since data on the actual number of connected residents were not provided by city officials. However, some WWTPs use only around half of design capacity (Koprivnica, Karlovac and Slavonski Brod). None of the tertiary treatment WWTPs uses water disinfection procedures. The volume of sampled effluents was 1 L, whilst the volume of influents and surface waters was 2 L. All water samples were sampled in clean plastic bottles and shipped refrigerated or shipping courier was used which didn't guarantee cold chain. Nevertheless, these samples were shipped with cold blocks in December and January with low outside temperatures.

Bivalve moluscan shellfish (N = 77) were harvested along the Adriatic coast in Dalmatia region at 11 commercial production sites (Fig. 1) in summer 2020 (N = 26) and autumn-winter 2020/2021 (N = 51). Each site was sampled 1–7 times in approximately one month interval. Per sampling 1–2 samples were taken. Among 11 production sites, seven sites were classified as zone A, and four sites were classified as zone A or B (Fig. 1) according to sanitary quality under EU Regulation

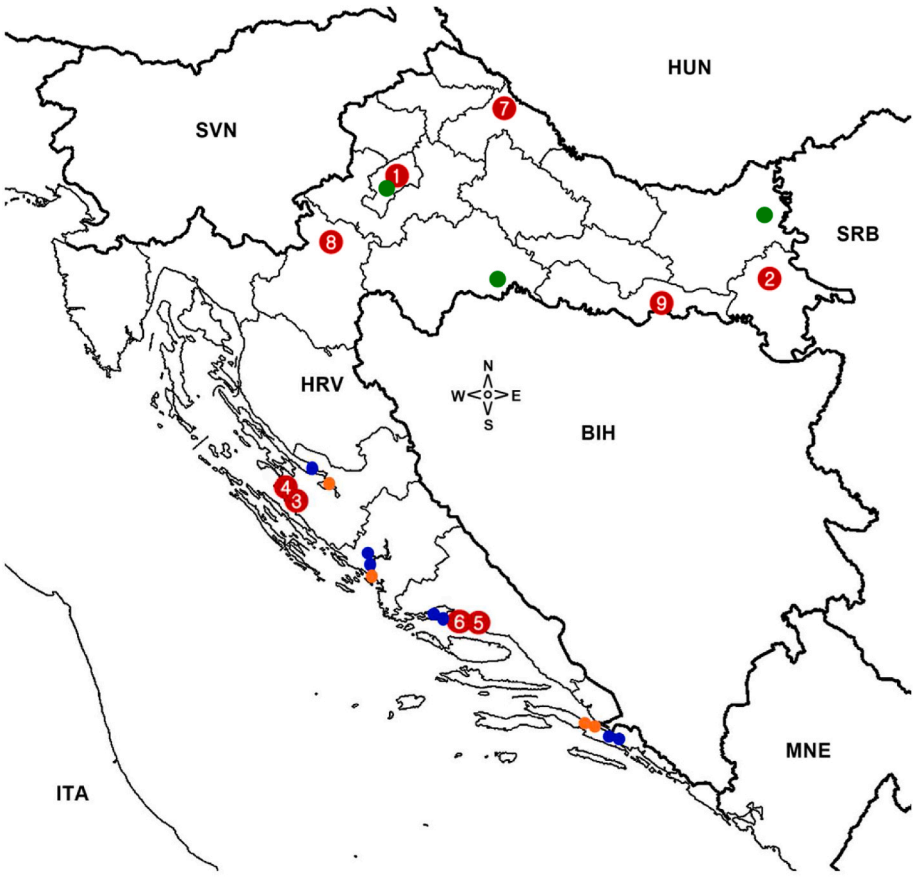

Fig. 1. Geographical distribution of sampling sites included in the present study. Wastewater treatment plants are marked with numbers and their description is provided in the table at the bottom of the figure. Surface water sampling sites are designated with green dots. The easternmost, central and westernmost green dots are located within Kopački rit Nature Park, Lonjsko polje Nature Park and in the city lake in Zagreb, respectively. Eleven bivalve molluscan shellfish production sites are marked with dark blue (zone A) and orange (zone A/B) dots. Attributions: The map source is available at: https://commons.wikimedia.org/wiki/File:Croatia_location_map.svg (Nord-NordWest; CC BY-SA 3.0; https://creativecommons.org/licenses/by-sa/3.0). The central part of the compass icon was created by Tommy Lau from the Noun Project. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Map designation	WWTP	County	Treatment stage	Design Capacity, PE	Wastewater source
1	Zagreb	City of Zagreb	2	1 200 000	Sanitary, Precipitation, Industry, Hospital
2	Vinkovci	Vukovar-Srijem	3	43 000	Sanitary, Precipitation, Industry, Hospital
3	Zadar-Centar	Zadar	2	100 000	Sanitary, Precipitation, Hospital, Industry (one subject)
4	Zadar-Borik	Zadar	1	15 500	Sanitary
5	Split-Stupe	Split-Dalmatia	1	138 000	Sanitary, Precipitation, Industry
6	Split-Kalinića Brig	Split-Dalmatia	1	122 000	Sanitary, Precipitation, Industry, Hospital
7	Koprivnica	Koprivnica-Križevci	3	100 000	Sanitary, Precipitation, Industry, Hospital
8	Karlovac	Karlovac	3	98 500	Sanitary, Precipitation, Industry, Hospital
9	Slavonski Brod	Brod-Posavina	3	80 000	Sanitary, Precipitation, Industry, Hospital

854/2004 on the basis of *Escherichia coli* monitoring. Depending on sampling date A:B zone ratio for samples collected at those four sites was 4:1, 11:3, 5:1 and 10:3. In total there were eight BMS samples with B classification. Regardless of the zone classification, all 11 production sites were not in close proximity to registered wastewater discharge areas. The sampling was organized by the Ministry of Agriculture, Veterinary and Food Safety Directorate for the purpose of monitoring of microbial quality and biotoxins. All BMS samples (2 kg in total) were taken from three levels at each production site in commercial size in order to provide representativeness and shipped to the laboratory on ice. BMS species included in the present study were Mediterranean mussel (*Mytilus galloprovincialis*, N = 52), European flat oyster (*Ostra edulis*, N = 23) and warty venus clams (*Venus verrucosa*, N = 2). Each sample (depending on seashell size) consisted of 10–15 individual oysters and 15–20 individual mussels and warty venus clams since digestive tissues (DT; hepatopancreas) were dissected and pooled to form a 2 g sample.

2.2. Sample processing and nucleic acid extraction

All samples were processed according to the protocol described in EN ISO 15216-1 2017 (Microbiology of the food chain — Horizontal method for determination of hepatitis A virus and norovirus using real-time RT-PCR — Part 1: Method for quantification) with some modifications regarding water processing since the ISO norm was adapted for bottled water.

In order to account for the complexity of wastewaters and in the lesser extent for the surface waters, the protocol was slightly modified. Shortly, each sample was adjusted to pH 7.3–7.5 with 1 M HCl and

mixed with 10 μL of mengovirus MC_0 (MgV) (cell culture supernatant) upon arrival to the laboratory and incubated overnight at 5–7 °C (20–24 h incubation). The added MgV served as a whole process control. Two steps of prefiltration were used combining cellulose filter paper with pore size 12–15 μm (Assistent, Germany) and quantitative fast cellulose filter paper with retention capacity 7–12 μm (Macherey-Nagel, Germany). Influent (0.7 L), effluent or surface water sample (2 L) were membrane filtered using positively charged hydrophilic polyamide membrane filters with pore size of 0.45 μm (Sartorius, Germany) and glass vacuum filtration device with flask combined with Microsart maxi. vac vacuum pump (Sartorius, Germany). Up to four polyamide membrane filters were used per sample depending on clogging frequency. These filters were agitated at 200 rpm in 5 mL of TGBE buffer (pH 9.5 ± 0.2) for 10 min which was adjusted to pH 7.3–7.5 with 1 M HCl upon filter removal. The last step of sample concentration included ultrafiltration step using Amicon® Ultra-4 Centrifugal Filter Units (Merck, Germany) at 3000×*g* until the sample was concentrated at approximately 500 μL. All concentrated samples were further processed immediately or stored at −80 °C. For the nucleic acid extraction of wastewater and surface water samples, we have used complex workflow for MagMAX™ CORE kit (ThermoFisher Scientific, USA) or QIAamp Viral RNA Mini Kit (Qiagen, Germany) by following manufacturer's instructions. The extraction procedure for MagMAX™ CORE kit was automated using KingFisher™ Duo Prime purification system (ThermoFisher Scientific, USA). The extracted nucleic acids were stored at −80 °C or processed immediately in RT-PCR reactions.

Digestive tissues (2 g pools) of BMS were spiked with 10 μL of MgV which served as a whole process control and were further digested with 2 mL of proteinase K (0.1 mg/mL) (Qiagen, Germany) with continuous shaking at 37 °C for 1 h in ThermoMixer® C (Eppendorf, Germany). The enzyme inactivation was performed at 60 °C for 15 min which was followed by centrifugation at 3000×*g* for 5 min. The supernatant was collected (volume was measured) and used as a starting material for nucleic acid extraction. For that purpose, the NucliSENS Lysis Buffer and NucliSENS Magnetic Extraction Reagens (BioMerieux, France) were used according to manufacturer's instructions. The extracted nucleic acids were stored at −80 °C or processed immediately in RT-PCR reactions.

2.3. RT-PCR for MgV, SARS-CoV-2 and RVA detection

The protocol for MgV RNA amplification and virus recovery calculation was described in EN ISO 15216-1 2017. All samples showing recovery (water) or extraction efficiency (BMS) > 1% were considered valid. Primer and probe concentrations were in line with ISO norm while reaction mixture set-up and thermal cycling conditions were as recommended by the manufacturer of VetMAX™-Plus One-Step RT-PCR kit (ThermoFisher Scientific, USA). The runs were performed on a QIAquant 96 5plex (Qiagen, Germany) or on a Mastercycler EP realplex (Eppendorf, Germany). All samples were tested undiluted and diluted 1:10.

Wastewater and surface water samples were tested for the presence of SARS-CoV-2 RNA by targeting E, N and nsp14 protein genes. First line was the application of E protein gene real-time RT-qPCR assay (Corman et al., 2020), followed by N1/N2/RNase P real-time RT-qPCR assay (Lu et al., 2020) and nsp14 real-time (La Rosa et al., 2021) and conventional (La Rosa et al., 2020) RT-PCR assays. BMS samples were tested with E and N1/N2 real-time RT-qPCR assays. Real-time RT-PCR for all target genes was performed on a RotorGene-Q (Qiagen, Germany) or on a QIAquant 96 5plex (Qiagen, Germany) using the qScript XLT One-Step RT-qPCR ToughMix (Quanta Bio, USA). Conventional RT-PCR was performed on the ABI 9700 GeneAmp thermal cycler (Applied Biosystems, USA) using the Superscript III One-Step RT-PCR system with a Platinum Taq DNA Polymerase (Thermo Fisher Scientific, USA). Primer concentrations used in each RT-PCR reaction and the annealing temperature in nsp14 conventional RT-PCR were as recommended by the corresponding article. Other conditions, related to the reaction mixture set-up and thermal cycling, were as recommended by the reagent's manufacturer. All RNA samples were tested in technical duplicate. In each test, a positive human SARS-CoV-2 RNA (kindly provided by Dr Ivan-Christian Kurolt, University Hospital for Infectious Diseases "Dr Fran Mihaljević", Zagreb) was used as a positive control. Possible nucleic acid contamination was monitored using no template control (NTC) or negative control (PCR grade water) and all proved to be negative (no Cq or specific band).

For quantification, triplicate 6-point standard curves were made for E, N1 and N2 assays using synthetic SARS-CoV-2 ssRNA EURM-019 (European Commission Joint Research Center) (kindly provided by Dr Ivan-Christian Kurolt, University Hospital for Infectious Diseases "Dr Fran Mihaljević", Zagreb). The standard curve values were as follows: E assay (R^2 = 0.9996, Slope = −3.47, y-intercept = 37.17, Efficiency = 94%), N1 assay (R^2 = 0.9993, Slope = −3.33, y-intercept = 36.75, Efficiency = 100%) and N2 assay (R^2 = 0.9989, Slope = −3.24, y-intercept = 36.29, Efficiency = 103%). The limit of detection (LOD, ≥ 95% detection in 20 replicates) was 8.63, 6.58 and 4.51 genomic copies (gc)/reaction for E, N1 and N2 assay, respectively. In theory, these three assays provide the LOD of 1.2-2.2 × 10^2 gc/L and 4-7.7 × 10^1 gc/L of SARS-CoV-2 in influent and effluent wastewaters, respectively. The limit of quantification (LOQ, CV ≤ 35% in 20 replicates) was defined to be 29.4, 37 and 24.9 gc/reaction for E, N1 and N2 assay, respectively, which theoretically provides the SARS-CoV-2 LOQ of 6.4-9.4 × 10^2 gc/L in influent and 2.2-3.3 × 10^2 gc/L in effluent wastewaters. For BMS the LOD, in theory, ranged between 5.8 × 10^1 and 1.1 × 10^2 gc/g of DT, whereas LOQ ranged between 3.2 and 4.8 × 10^2 gc/g of DT.

RVA was selected as a highly prevalent enteric pathogen, usually found to circulate in the environment (Ito et al., 2019; Steyer et al., 2011), in order to additionally evaluate the virus concentration techniques applied for the surface water, wastewater and BMS samples. RVA detection was performed on all samples collected for the present study, by the application of real-time RT-PCR that amplifies the fragment of VP2 segment of different RVA genotypes infecting humans and domestic animals (Gutiérrez-Aguirre et al., 2008). Moreover, this protocol was successfully applied on wildlife (Ćolić et al., 2021) and water samples (Steyer et al., 2011). Prior to one-step real-time RT-PCR, the RVA dsRNA was denaturated at 95 °C for 2 min together with primer mix (600 nM) and PCR grade water. The reaction mixture was a combination of a denaturation mix from the previous step, the VP2 probe (200 nM) and reagents of the VetMAX™-Plus One-Step RT-PCR kit (ThermoFisher Scientific, USA). The reaction mixture set-up and thermal cycling conditions were as recommended by the manufacturer. The runs were performed on a RotorGene-Q (Qiagen, Germany) or on a Mastercycler EP realplex (Eppendorf, Germany).

2.4. SARS-CoV-2 nsp14 sequencing and sequence analysis

RT-PCR products were visualized by capillary electrophoresis on QIAxcel Advanced System (Qiagen, Germany) and subsequently purified using ExoSAP-IT™ PCR Product Cleanup Reagent (ThermoFisher Scientific, USA) as previously described (Ćolić et al., 2021). Purified PCR products were Sanger sequenced in both directions on 3730xl DNA Analyzer (ThermoFisher Scientific, USA) by Macrogen Europe (Amsterdam, the Netherlands). Nucleotide sequences generated by this study were deposited to the NCBI GenBank under accession number OK085824. Sequences were aligned in MEGA X Software (Kumar et al., 2018) and compared to SARS-CoV-2 sequences obtained from GISAID (Shu and McCauley, 2017): the reference SARS-CoV-2 sequence (hCoV-19/Wuhan/IVDC-HB-01/2019) and 20 sequences from Croatia originating from different locations and time periods, from the beginning of the pandemic to the present day.

3. Results

3.1. The assessment of MgV recovery and extraction efficiency

In order to test the validity of virus concentration technique employed by the present study, we calculated the recovery (%) for added MgV in wastewater and surface water samples and extraction efficiency in BMS samples. The level of recovery for wastewaters and surface waters was on average 1.08 ± 0.38% (95% confidence level). However, only 40% (12/30) of tested samples had an adequate recovery (>1%) of MgV (Table 1); 22.2% (2/9) of surface waters and 47.6% (10/21) of wastewaters. For those samples, the level of recovery ranged between 1.04% and 3.92%. Among samples with recovery <1% are seven (7/9) surface water samples (from all three locations) and 11 wastewater samples. The latter were represented by influents (8/10) and secondary treatment effluents (3/7). All effluents from WWTPs with tertiary treatment were of adequate MgV recovery. Two influent samples, from Split-Katalinića Brig and Slavonski Brod WWTPs, tested negative (no Cq) for MgV in undiluted and 1:10 diluted sample.

The extraction efficiency for added MgV in BMS was at acceptable levels in 96.1% of tested samples. Only three samples (two mussel and one oyster sample) did not meet the set criterion (extraction efficiency >1%). Among majority of BMS samples that passed the extraction efficiency assessment, the mean value was 2.15 ± 0.21% (95% confidence level) with the range between 1.1% and 5.2%.

3.2. The evidence of SARS-CoV-2 circulation in wastewaters in Croatia

In spite of variable results of MgV recovery, the amplification of SARS-CoV-2 genome was successful in eight out of nine WWTPs in at least influent sample with the application of at least one real-time RT-PCR assay (E, N or nsp14 assay) or conventional nsp14 assay (Table 1). The only WWTP that tested negative for SARS-CoV-2 in influent sample was located in Split (Katalinića Brig) and this sample was also MgV negative. Zagreb WWTP was positive twice, in October 2020 in influent and effluent and in January 2021 in influent (Table 1). In total, nine influents and two effluents after the secondary treatment tested positive on SARS-CoV-2. These two positive effluents were from Zagreb and Zadar-Centar WWTPs during the peak months of the second wave of epidemic in autumn-winter 2020/2021 (HZJZ, 2021a). Zagreb WWTP's SARS-CoV-2 negative effluents were sampled four times before (December 2019 and February, May and July 2020) and once after (end of January 2021) the SARS-CoV-2 positive effluent (sample PV54 from October 2020) when disease was not yet reported (prior to 25th of February 2020) or the incidence in Croatia and Zagreb were significantly lower (HZJZ, 2021a; HZJZ, 2021b). All wastewater effluents after the tertiary treatment (N = 4) were negative for SARS-CoV-2 RNA (Table 1). None of the surface water and wastewater samples were excluded from the analysis due to possible technical difficulties that may impaired the MgV recovery (discussed in detail further in the text). Moreover, among 11 SARS-CoV-2 positive samples (nine influents and two effluents) eight were of inadequate MgV recovery level (<1%) (Table 1).

The performance of different RT-PCR assays, which were applied to our sample set, varied with the evident need for the combination of different SARS-CoV-2 target genes. The best results were obtained for CDC N protein gene real-time RT-qPCR assay (N1/N2) with 10 positive samples (eight influents and two effluents), followed by Sarbeco E protein gene real-time RT-qPCR assay (eight influents, among which one was below LOD) (Table 1). These two assays combined were able to detect SARS-CoV-2 RNA in 10/11 influents sampled during the course of the study (Table 1). The last two assays, nsp14 real-time and conventional RT-PCR, performed worse in our laboratory conditions, having detected only four influents each, respectively (Table 1). The N1/N2 real-time RT-qPCR assay included the assay for human RNase P which was detected in seven samples; three surface waters, two influents and two effluents (Table 1).

3.3. The absence of SARS-CoV-2 in bivalve molluscan shellfish

All 77 BMS samples from 11 production sites along Dalmatian coast tested negative for SARS-CoV-2 with the application of E and N1/N2 real-time RT-qPCR assays, respectively.

3.4. RVA is a highly prevalent pathogen in the environment serving as beneficial additional recovery evaluation criterion

The application of VP2 real-time RT-PCR assay delivered RVA positive results in 22.2% (2/9) of surface water and in 100% (21/21) of wastewater samples (Table 1). Moreover, 17 BMS samples proved to be positive for RVA accumulation in digestive tissue. When three samples (two mussel and one oyster sample) with extraction efficiency less than 1% were excluded, the reported RVA prevalence in BMS was 23% (17/74). The RVA prevalence was higher in BMS sampled in autumn-winter 2020/2021 (30.6%; 15/49) compared to summer 2020 (8%; 2/25). RVA positive BMS samples were detected in higher percentage in A/B production sites (27.8%; 10/36) compared to A production sites (18.4%; 7/38). Among BMS samples with B categorization, 50% (4/8) were RVA positive, whereas 19.7% (13/66) RVA positives were detected among samples with A categorization. All 17 RVA positive samples belonged to mussels (*Mytilus galloprovincialis*) which accounted to the species related prevalence of 34% (17/50).

The applicability of RVA as an additional evaluation criterion for the performance of virus concentration techniques implemented in the present study may be considered beneficial for the wastewater and BMS samples. All 21 wastewater samples were RVA positive, regardless of MgV recovery (Table 1). Even the two MgV negative samples were RVA positive; Cq 34.2 for the influent sample collected at WWTP Split-Katalinića Brig and Cq 24.5 for the influent sample collected at WWTP Slavonski Brod (Table 1). Considering the seasonality of RVA infections in developed countries (Patel et al., 2013), the RVA was detected in all samples from Zagreb WWTP (the only WWTP sampled more than once); even in July (Cq 36.4) which is in the low RVA season (Table 1). Moreover, that effluent sample had a recovery of 0.37% which is below needed 1% for the virus concentration technique to be considered valid. The results on RVA prevalence in BMS confirm the suitability of selected virus concentration technique to provide the ability to detect an autochthonous environmental contamination with a viral pathogen excreted in faeces.

3.5. SARS-CoV-2 nsp14 sequencing results

From the obtained partial nsp14 sequences, 330-nt-long fragment was used for the alignment in MEGA X. Analysed sequences comprised aa positions 142–251 of the nsp14 (nt position 18463-18792 in the total length of Wuhan reference sequence hCoV-19/Wuhan/IVDC-HB-01/2019). Sequences from wastewater were 100% identical to each other and to SARS-CoV-2 reference sequence, and 99.7–100% identical to other Croatian sequences. Previously recognized aa substitution on position 203 (P203S or P203L) was not present in samples from wastewater, but was present in one sample from Croatia (EPI_ISL_1574889) obtained in February 2021. Other previously described substitutions in nsp14 (Eskier et al., 2020; Takada et al., 2020) were not identified due to the short sequence.

4. Discussion

Globally, there are numerous studies on SARS-CoV-2 circulation in wastewaters (Tran et al., 2021) and on the usefulness of WBE as a predictive tool on the state of epidemic within the community (Aguiar-Oliveira et al., 2020). The aim of the present study was to fulfil the knowledge gap on SARS-CoV-2 circulation in wastewaters and surface

Table 1
The results of SARS-CoV-2, MgV, and RVA detection in surface water and wastewater samples.

Sample ID	Sample type	Location/ County[a]	Sampling date	7-day incidence/ 100 000 residents/ County[b]	E (Cq or gc/L)[c]	N (N1/N2 Cq or gc/L)[c]	nsp14 real-time RT-PCR (Cq)	nsp14-conventional RT-PCR	MgV Cq (% recovery)	RVA (Cq)
PV40	Wastewater-effluent	Zagreb/ZG	12.12.2019.	N/A	neg	neg[d]	neg	neg	27.5 (2.4)	pos (30.5)
PV41	Surface water-city lake	Zagreb/ZG	07.01.2020.	N/A	neg	neg	neg	neg	31.7 (0.13)	neg
PV42	Surface water-city lake	Zagreb/ZG	07.01.2020.	N/A	neg	neg	neg	neg	33.9 (0.03)	neg
PV43	Surface water-city lake	Zagreb/ZG	07.01.2020.	N/A	neg	neg[d]	neg	neg	28.1 (1.59)	pos (36.4)
PV44	Wastewater-effluent	Zagreb/ZG	28.02.2020.	N/D	neg	neg	neg	neg	28.7 (1.04)	pos (30.3)
PV45	Surface water-nature park	Lonjsko polje/ SM	04.03.2020.	N/A	neg	neg[d]	neg	neg	29.1 (0.79)	neg
PV46	Surface water-nature park	Lonjsko polje/ SM	04.03.2020.	N/A	neg	neg	neg	neg	31.3 (0.17)	neg
PV47	Surface water-nature park	Lonjsko polje/ SM	04.03.2020.	N/A	neg	neg	neg	neg	32.8 (0.06)	neg
PV48	Surface water-nature park	Kopački rit/ OB	17.03.2020.	N/D	neg	neg	neg	neg	28.1 (1.59)	pos (33.9)
PV49	Surface water-nature park	Kopački rit/ OB	17.03.2020.	N/D	neg	neg	neg	neg	29.5 (0.60)	neg
PV50	Surface water-nature park	Kopački rit/ OB	17.03.2020.	N/D	neg	neg[d]	neg	neg	30.2 (0.37)	neg
PV51	Wastewater-effluent	Zagreb/ZG	15.05.2020.	N/D	neg	neg	neg	neg	28.3 (1.38)	pos (34.7)
PV52	Wastewater-effluent	Zagreb/ZG	06.07.2020.	N/D	neg	neg	neg	neg	30.2 (0.37)	pos (36.4)
PV53	Wastewater-influent	Zagreb/ZG	22.10.2020.	461.9	**pos (33.2)**	**pos ($7.2x10^3$/ 33.4)**[d]	**pos (36.8)**	**pos**	27.1 (3.18)	pos (26.7)
PV54	Wastewater-effluent	Zagreb/ZG	22.10.2020.	461.9	neg	**pos ($6x10^1$/ neg)**[d]	neg	neg	28.4 (1.29)	pos (30.5)
PV55	Wastewater-influent	Vinkovci/VS	24.11.2020.	401	**pos (33.6)**	**pos ($2.1x10^3$/ 33.3)**	**pos (36.4)**	**pos**	30.3 (0.34)	pos (24.8)
PV56	Wastewater-effluent	Vinkovci/VS	24.11.2020.	401	neg	neg	neg	neg	28.4 (1.29)	pos (29.4)
PV57	Wastewater-influent	Zadar-Centar/ ZD	07.12.2020.	558.4	**pos (34.1)**	**pos ($5.4x10^3$/ 35.8)**	neg	**pos**	30.0 (0.42)	pos (26.7)
PV58	Wastewater-effluent	Zadar-Centar/ ZD	07.12.2020.	558.4	neg	**pos ($1.8x10^2$/ 35.0)**	neg	neg	29.1 (0.79)	pos (28.4)
PV59	Wastewater-influent	Zadar-Borik/ ZD	07.12.2020.	558.4	**<LOD**	**pos ($9x10^2$/ 36.3)**	**pos (34.5)**	neg	27.6 (2.25)	pos (30.7)
PV60	Wastewater-influent	Split-Stupe/ SD	14.12.2020.	508.4	**pos (34.7)**	**pos (34.8/ $8.9x10^3$)**	**pos (36.6)**	neg	29.5 (0.60)	pos (27.8)
PV61	Wastewater-influent	Split-Kaplinića Brig/SD	14.12.2020.	508.4	neg	neg	neg	neg	neg	pos (34.2)
PV62	Wastewater-influent	Koprivnica/ KK	16.12.2020.	433.6	neg	**pos ($3.4x10^2$/ 35.6)**	neg	neg	30.8 (0.24)	pos (28.0)
PV63	Wastewater-effluent	Koprivnica/ KK	16.12.2020.	433.6	neg	neg	neg	neg	27.9 (1.82)	pos (32.5)
PV64	Wastewater-influent	Karlovac/KA	19.01.2021.	76.2	**pos (34.0)**	**pos ($1.7x10^3$/ 33.7)**	neg	**pos**	29.3 (0.69)	pos (24.7)
PV65	Wastewater-effluent	Karlovac/KA	19.01.2021.	76.2	neg	neg	neg	neg	26.8 (3.92)	pos (28.0)
PV66	Wastewater-influent	Slavonski Brod/BP	21.01.2021.	89.2	**pos ($8.4x10^2$)**	neg[d]	neg	neg	neg	pos (24.5)
PV67	Wastewater-effluent	Slavonski Brod/BP	21.01.2021.	89.2	neg	neg	neg	neg	27.4 (2.58)	pos (26.5)
PV68	Wastewater-influent	Zagreb/ZG	28.01.2021.	65	**pos ($4x10^2$)**	**pos (neg/ 35.4)**	neg	neg	30.6 (0.28)	pos (26.4)
PV69	Wastewater-effluent	Zagreb/ZG	28.01.2021.	65	neg	neg	neg	neg	33.2 (0.05)	pos (28.3)

SARS-CoV-2 positive samples and corresponding Cq or gc/L values are presented in boldface. Negative samples are referring to "no Cq" detected during respective real-time RT-PCR or to the absence of specific band in the conventional RT-PCR.
N/A (Not Applicable since the first documented human case of COVID-19 in the City of Zagreb County and Sisak-Moslavina County was evidenced on 25th of February 2020 and 13th of March 2020, respectively).
N/D (No Data; available from mid-August 2020 (HZJZ, 2021b)).

[a] ZG (City of Zagreb), SM (Sisak-Moslavina), OB (Osijek-Baranja), VS (Vukovar-Srijem), ZD (Zadar), SD (Split-Dalmatia), KK (Koprivnica-Križevci), KA (Karlovac), BP (Brod-Posavina).

[b] Six days after the sampling date. Data were retrieved from the published weekly reports (HZJZ, 2021b) of the Croatian Institute of Public Health (available from mid-August 2020). These data are county based, and the number of residents connected to WWTPs does not necessarily correspond to the county population.

[c] SARS-CoV-2 quantity in gc/L was provided for the assay which resulted with the highest quantity. Influent samples below LOQ (7.5, 9.4 and 6.4 $\times$ 10^2gc/L for E, N1 and N2 assay, respectively) should be considered as estimated counts. Effluent samples below LOQ (2.6, 3.3 and 2.2 $\times$ 10^2gc/L for E, N1 and N2 assay, respectively) should be considered as estimated counts.

[d] Human RNAse P positive.

waters in this region at the beginning of epidemic and during the second wave in autumn-winter 2020/2021. Wastewaters, especially those untreated in countries with low wastewater treatment infrastructure, may be the source of SARS-CoV-2 environmental shedding (Rimoldi et al., 2020; Westhaus et al., 2021). However current knowledge does not support the evidence of SARS-CoV-2 transmission via water-food-environmental media (Adelodun et al., 2021). Data on the possible SARS-CoV-2 bioaccumulation in BMS is limited to only two studies (Desdouits et al., 2021; Polo et al., 2021). Therefore the second aim of the present study was to extend this knowledge by conducting the study on the possible presence of SARS-CoV-2 contamination in BMS, for the first time in autumn-winter period (high epidemic burden of enteric viruses) during the second epidemic wave.

This is the first report of SARS-CoV-2 circulation in wastewaters of eight WWTPs (out of nine) located in all seven Croatian cities included in the present study (Fig. 1). The only SARS-CoV-2 negative WWTP was located in Split (Kalinića Brig). That sample was probably heavily inhibited (MgV negative, RVA at low Cq 34.2). Moreover, the other WWTP located nearby (Split-Stupe) is SARS-CoV-2 positive on three genome targets and RVA positive at Cq 27.4, even though it does not include hospital wastewaters unlike WWTP Split-Kalinića-Brig (Fig. 1). In total, 11 SARS-CoV-2 positive wastewater samples were detected during the second wave of epidemic among which are nine influents (after primary or mechanical treatment) and two effluents (after secondary treatment). These SARS-CoV-2 positive effluents were detected during the peak months of the second wave in Zagreb and Zadar when the 7-day incidence per 100.000 residents per corresponding county was 461.9 and 558.4, respectively (HZJZ, 2021b) (Table 1). Tertiary treatment effluents from four WWTPs (Vinkovci, Koprivnica, Karlovac and Slavonski Brod), sampled during the second wave (Vinkovci and Koprivnica during the peak months), were all SARS-CoV-2 negative (Table 1). It is important to emphasize that these tertiary treatment effluents were not disinfected like it was reported elsewhere (Mancuso et al., 2021), indicating that even conventional tertiary treatment may be highly effective in SARS-CoV-2 reduction and removal. Possibly, the extended retention of wastewaters in tertiary treatment WWTPs compared to secondary treatment WWTPs has a favourable effect on SARS-CoV-2 removal. Firm conclusions on this effectiveness would arise following a more comprehensive sampling strategy at each WWTP during the extended time frame. Overall, these findings are concurrent with many similar studies performed so far, emphasizing the suitability of wastewaters as an addition to SARS-CoV-2 monitoring within the community (Mancuso et al., 2021; Tran et al., 2021). Higher sampling frequency at each WWTP, reliable number of connected residents reported by city officials and precise city-based epidemiological data for the comparison, would enable the WBE context of the study. Nevertheless, the scientific community has agreed that in spite of its great potential, some difficulties in WBE implementation need to be addressed in future research (Zhu et al., 2021). Apart from focusing on SARS-CoV-2 circulation in wastewaters during the peak months of the second wave of epidemic, the present study investigated the possible circulation at the start of epidemic, which in Croatia began on 25th of February when the first SARS-CoV-2 human case was detected (HZJZ, 2021a). For that purpose we investigated 13 archive samples collected within the on-going RVA research project (Brnić et al., 2018). Among these were four effluents (secondary treatment) from Zagreb WWTP sampled between December 2019 and July 2020 and nine surface water samples collected in March 2020 at two nature parks and one city lake (Fig. 1, Table 1). All proved to be negative on SARS-CoV-2, primarily due to the orientation towards secondary treatment effluents and surface waters. Moreover it is important to emphasize the low SARS-CoV-2 incidence during the first epidemic wave (HZJZ, 2021a). Unfortunately, we were not collecting influents after primary treatment at that time since RVA was readily detected in effluents as well, even in tertiary ones (Table 1). On the contrary, most studies that confirmed the circulation of SARS-CoV-2 in wastewaters prior to the first documented cases within the community were testing untreated wastewaters (Ahmed et al., 2020; La Rosa et al., 2021; Martin et al., 2020). Our results on the application of real-time or conventional RT-PCRs targeting E, N and nsp14 SARS-CoV-2 genes, once more confirmed the necessity to combine different genome targets due to the complex nature of wastewaters (La Rosa et al., 2021; Westhaus et al., 2021). However, the sequencing of the nsp14 protein gene fragment, apart from providing the firm evidence on SARS-CoV-2 circulation, didn't allow us to go further into SARS-CoV-2 variant characterization, for which the NGS implementation provides the needed resolution (Rubio-Acero et al., 2021).

Perhaps the most significant contribution of the present study is related to SARS-CoV-2 investigation in BMS since only two studies, to the best of our knowledge, have been published so far (Desdouits et al., 2021; Polo et al., 2021). All BMS samples included in the present study were SARS-CoV-2 negative by the application of real-time RT-qPCR E and N1/N2 assays. These findings are in compliance with the results from French study (Desdouits et al., 2021); however our results were obtained on BMS collected mostly in autumn-winter season. Winter is known for the high epidemic burden of enteric viruses in the northern hemisphere (Ahmed et al., 2013; Patel et al., 2013). Moreover, the sampling period corresponds with the second, highly abundant, COVID-19 epidemic wave (HZJZ, 2021a). If we take into account a largely inadequate wastewater treatment infrastructure in Croatian coastal areas, these results are even more encouraging. Nevertheless, a dilution factor of wastewaters in the sea and SARS-CoV-2 stability in seawater needs to be taken into account. Previous findings of lower SARS-CoV-2 bioaccumulation efficiency in oysters (compared to non-enveloped virus control) (Desdouits et al., 2021) and accumulated, but non-viable SARS-CoV-2 particles in clams (Polo et al., 2021) talk in favour of low epidemiological risk. Yet, more research on these concerns needs to be done in order to draw firm conclusions.

The confidence of the above mentioned SARS-CoV-2 related results was assessed by monitoring MgV % recovery and extraction efficiency, along with the evaluation of RVA amplification rates which served as an additional control. The virus concentration technique and MgV as selected whole process control produced excellent extraction efficiency results in BMS samples. On contrary, the level of recovery for wastewaters and surface waters were only 40% satisfactory. Nevertheless we are of the opinion that such result was a consequence of extended (20–24 h) incubation of MgV in those samples inducing its attachment to suspended solids (Corpuz et al., 2020) and bacteria which were subsequently removed during membrane filtration procedure. This was reflected in results since influents and surface waters were impacted the most with 80% (8/10) and 77.8% (7/9) of samples having inadequate recovery (<1%), respectively. The recoveries were better in secondary treatment effluents (42.9% (3/7) with inadequate recovery), and completely adequate (>1%) in tertiary treatment effluents (Table 1). We

are aware that MgV might not be the best option for the whole process control since SARS-CoV-2 surrogates were used elsewhere (Desdouits et al., 2021; Mlejnkova et al., 2020), but our intention was to keep MgV which was used in archive samples that were primarily collected to monitor RVA. Even though, MgV was already successfully applied in SARS-CoV-2 related research in wastewaters (Randazzo et al., 2020). In spite of difficulties observed with MgV recovery, SARS-CoV-2 detection in wastewaters was successful, which is evidenced in 11 SARS-CoV-2 positive samples (nine influents and two effluents), even though eight of them had inadequate MgV recovery level (Table 1). The implementation of RVA, a widespread enteric pathogen, as an additional recovery evaluation criterion was beneficial since it was detected in all wastewater samples; even in those which were MgV negative (PV61 and PV66) (Table 1). It is evident that secondary and tertiary treatments at WWTPs reduce RVA concentration (higher Cq values compared to influents) (Table 1), but unlike with SARS-CoV-2, RVA is not completely removed, probably because of the initially higher viral load. In addition, these results reveal that RVA was pretty much present in the community, even though the number of paediatric hospitalizations due to the gastroenteritis sharply decreased during COVID-19 pandemic (personal communication), which was evidenced elsewhere (Maruo et al., 2021). The benefits of RVA implementation were also visible in BMS samples, further confirming adequate MgV extraction efficiency. Moreover, the overall RVA prevalence in BMS was similar as previously reported (Gabrieli et al., 2007), whereas the expected seasonality is visible in higher RVA prevalence in autumn-winter months (30.6% vs 8% in summer). From the public health and food safety perspective, it is important to emphasize that only mussels were RVA positive in the present study, since they are usually consumed cooked. Nevertheless, the RVA positive BMS indicate the presence of anthropogenic influence in the area, even though the production sites were not located nearby the registered wastewater discharge zones. However, we cannot exclude the impact of non-functional cesspits which might be present in those areas and the impact of illegal wastewater discharge from ships passing close by. That further reaffirms the encouraging results of estimated low epidemiological risk that BMS may represent regarding SARS-CoV-2, which was elaborated above.

In conclusion, we can confirm that SARS-CoV-2 was circulating in Croatian wastewaters during the second epidemic wave. Moreover, our results are extending the knowledge of secondary and especially tertiary wastewater treatment contribution to SARS-CoV-2 reduction and removal. The present study confirmed the absence of SARS-CoV-2 in BMS during the autumn-winter season which corresponded to the peak of second epidemic wave. Therefore, our findings represent a significant contribution to the current state of knowledge that considers BMS of a very low food safety risk regarding SARS-CoV-2. Nevertheless, further research is needed in order to establish firm conclusions and public health guidelines.

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Ethical statement

The study on bivalve molluscan shellfish was approved by the Board of Ethics of the Croatian Veterinary Institute (protocol code Z-VI-4-5206/17, approved on 11 December 2017).

Author contributions

D.B. Conceptualization; D.B., L.J. Data curation; D.B., I.L., L.J. Formal analysis; D.B., L.J., B.H. Funding acquisition; D.B., I.L., I.Š. (Ines Škoko), N.K., I.Š. (Ivana Šimić), T.K., M.G., D.Š., B.V., D.K., D.Š. Investigation; D.B., I.L., I.Š. (Ines Škoko), N.K., I.Š. (Ivana Šimić) Methodology; D.B. Project administration; T.K., M.G., D.Š., B.V., D.K., D.Š., B.H. Resources; D.B., I.L. Software; D.B., I.L., L.J. Supervision; D.B., I.L. Validation; D.B., I.L. Visualization; D.B. Roles/Writing - original draft; I. L., N.K., I.Š. (Ivana Šimić), L.J. Writing - review & editing.

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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Appendix A. Supplementary data

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