



Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.



Contents lists available at ScienceDirect

## International Journal of Hygiene and Environmental Health

journal homepage: [www.elsevier.com/locate/ijheh](http://www.elsevier.com/locate/ijheh)

# Useful molecular tools for facing next pandemic events: Effective sample preparation and improved RT-PCR for highly sensitive detection of SARS-CoV-2 in wastewater environment

Magdaléna Rusková<sup>a</sup>, Mária Bučková<sup>a</sup>, Adam Achs<sup>b</sup>, Andrea Puškárová<sup>a</sup>, Jer-Horng Wu<sup>c</sup>, Tomáš Kuchta<sup>d</sup>, Zdeno Šubr<sup>b</sup>, Domenico Pangallo<sup>a,\*</sup>

<sup>a</sup> Institute of Molecular Biology, Slovak Academy of Sciences, Dúbravská cesta 21, 845 51, Bratislava, Slovakia

<sup>b</sup> Biomedical Research Center, Slovak Academy of Sciences, Institute of Virology, Dúbravská cesta 9, 845 05, Bratislava, Slovakia

<sup>c</sup> National Cheng Kung University, Department of Environmental Engineering, University Road 1, East District, 701 01, Tainan City, Taiwan

<sup>d</sup> Department of Microbiology, Molecular Biology and Biotechnology, Food Research Institute, National Agricultural and Food Centre, Priemysel'ná 4, 824 75, Bratislava, Slovakia

## ARTICLE INFO

## Keywords:

Encapsidated S RNA mimic  
Wastewater  
Polyethylene glycol precipitation  
Chaotropic RNA extraction  
Magnetic beads RNA extraction  
Nested quantitative reverse transcription PCR

## ABSTRACT

Viral pandemics can be inevitable in the next future. Considering SARS-CoV-2 pandemics as an example, there seems to be a need to develop a surveillance system able to monitor the presence of potential pathogenic agents. The sewage and wastewater environments demonstrated to be suitable targets for such kind of analysis. In addition, it is important to have reliable molecular diagnostic tools and also to develop a robust detection strategy. In this study, an effective sample preparation procedure was selected from four options and combined with a newly developed improved RT-PCR. First, a model viral system was constructed, containing a fragment of the SARS-CoV-2 gene encoding for the Spike protein. The encapsidated S RNA mimic (ESRM) was based on the plum pox virus (PPV) genome with the inserted targeted gene fragment. ESRM was used for seeding wastewater samples in order to evaluate the viral recovery of four different viral RNA concentration/extraction methods. The efficiency of individual approaches was assessed by the use of a quantitative reverse transcription PCR (qRT-PCR) and by a one-step single-tube nested quantitative reverse transcription PCR (OSN-qRT-PCR). For the detection of viruses in wastewater samples with low viral loads, OSN-qRT-PCR assay produced the most satisfactory results and the highest sensitivity.

## 1. Introduction

The COVID-19 pandemic has shown the importance to have reliable and robust detection methods for attesting the presence of the pathogenic agents and therefore to organize the suitable countermeasures. It became evident that monitoring of anthropized environments such as wastewater can be considered a valuable surveillance tool that consequently can help health operators to follow the pandemic trend and manage it in an effective way (Zhu et al., 2021).

It is necessary to have various molecular tools that can simulate a real situation and can be used to create robust detection methods. Various attempts were already performed by other authors to detect SARS-CoV-2 from wastewater samples and to evaluate several available procedures using model samples. A common characteristic of some

efforts was the use of surrogates for SARS-CoV-2, such as murine hepatitis virus, bovine coronavirus or feline calicivirus (Ahmed et al., 2020; Barril et al., 2021; LaTurner et al., 2021). Another option was the use of naked plasmids harboring SARS-CoV-2 sequences or the use of a commercial reference material (Sapula et al., 2021). As an important step, various approaches to concentration of SARS-CoV-2 from wastewater samples were evaluated, including polyethylene glycol (PEG) precipitation, ultracentrifugation or filtration methods (La Rosa et al., 2020a; LaTurner et al., 2021). Then, the concentrated viral RNA could be extracted by various methods, allowing it to be amplified by a specific real-time reverse transcription PCR assay. Such assays were oriented to viral surrogates or to molecular systems of SARS-CoV-2 (Bivins et al., 2021; Tran et al., 2021). The described strategies were applied to evaluate the recovery rates of viral concentration and RNA extraction

\* Corresponding author.

E-mail address: [domenico.pangallo@savba.sk](mailto:domenico.pangallo@savba.sk) (D. Pangallo).

<https://doi.org/10.1016/j.ijheh.2022.114017>

Received 14 April 2022; Received in revised form 22 June 2022; Accepted 27 July 2022

Available online 3 August 2022

1438-4639/© 2022 Elsevier GmbH. All rights reserved.

methods of wastewater samples based on quantification of viral RNA by quantitative reverse transcription PCR assays (qRT-PCR). Among the potential detection methods, digital RT-PCR (dRT-PCR) or, in particular, droplet-based digital RT-PCR (ddRT-PCR) is suitable for the analysis of samples with low viral load (Yu et al., 2020). However, digital PCR is a low-throughput method with sensitivity comparable to RT-PCR.

Potential SARS-CoV-2 positive control for optimizing diagnostic procedures could involve specifically produced encapsidated RNA mimics and some examples of this strategy have already been published (Chan et al., 2020; Peyret et al., 2022). Plant viruses are non-infectious and non-toxic for humans; thus, they provide a suitable tool for this purpose. Incorporation of partial SARS-CoV-2 sequence in the genome of a plant RNA virus enables its autonomous replication and encapsidation *in vivo*, resulting in the production of RNA mimics with stability comparable to native SARS-CoV-2 particles. Moreover, propagation of viruses in plants is efficient and cheap and their purification is relatively fast and simple, based mainly on ultracentrifugation (Bhat and Rao, 2020).

Produced encapsidated mimics could be used for artificial contamination of various kinds of samples and for testing various detection methods, including qRT-PCR. In order to increase the detection sensitivity of a qRT-PCR assay, the creation and optimization of a nested PCR system could be a useful alternative. Single-tube nested real-time PCR approaches have been frequently used to improve the detection of various pathogen agents and components in food and clinical samples including SARS-CoV-2 (Minarovičová et al., 2011; Costa et al., 2012; Wang et al., 2020; Yip et al., 2020). Wang et al. (2020) in their work demonstrated that clinical samples with low viral load could also be detected and quantified by the one-step single-tube nested quantitative reverse transcription PCR (OSN-qRT-PCR).

The schematic diagram in Fig. 1 describes the two PCR assays. OSN-qRT-PCR is based on the application of two pairs of primers (outer and inner) in one reaction tube, with different annealing temperatures (difference about 10 °C) to control the first and second rounds of PCR. The outer primers have higher melting temperature should anneal during the first 10 cycles of PCR. The inner primers should anneal to the products of the former PCR round at a lower annealing temperature during the next 40 PCR cycles, with the final detection of the fluorescence signal produced by the specific labelled probe.

Contrary to ddRT-PCR, the operation of the OSN-qRT-PCR method is the same as qRT-PCR and there is no need of extra professional training. Besides, the OSN-qRT-PCR assay is feasible in any qPCR-instrument-equipped laboratory. The cost of OSN-qRT-PCR is lower than ddRT-PCR and the turn-around time of OSN-qRT-PCR is shorter (2 h) than that of ddRT-PCR (3–4 h) though a bit longer than that of qRT-PCR (about 1 h and 30 min).

Our aim was to find a reliable and robust qRT-PCR detection method

for SARS-CoV-2 in wastewater by designing a laboratory analysis system, able to mimic real conditions. Such analysis system can provide a model for future development of detection procedures for viral pandemics. For this reason, in this study we compared various strategies for the detection of SARS-CoV-2 in wastewater samples using different molecular tools: i) an encapsidated S RNA mimic (ESRM) based on the plum pox virus (PPV) genome bearing a fragment of SARS-CoV-2 Spike protein-coding sequence, ii) four different viral RNA extraction/concentration methods, iii) the application of two different detection assays: a conventional qRT-PCR and OSN-qRT-PCR.

## 2. Materials and methods

### 2.1. Construction of the encapsidated S RNA mimic (ESRM)

The viral vector pAD-agro consists of a full-length cDNA of the strain PPV-Rec (Predajna et al., 2012) cloned in the commercial plasmid pCambia 1304 (Abcam, Cambridge, UK) with deleted  $\beta$ -glucuronidase gene. The cloning cassette comprising an *EagI/KpnI* linker and sites recognized by viral protease are inserted in the PPV polyprotein-coding region between the replicase (*NiB*) and capsid protein (*CP*) genes (Fig. S1). Biological safety was ensured by mutagenesis of the DAG motif within the CP-coding region, essential for aphid transmission (Blanc et al., 1997; Kamencayová and Šubr, 2012).

The 447 nt long fragment of the SARS-CoV-2 S gene was amplified by PCR from cDNA of the isolate hCoV-19/Slovakia/SK-BMC5/2020 (GISAID.org accession ID EPI\_ISL\_417,879). PCR amplification was performed using EX Taq DNA Polymerase (Takara, Shiga, Japan) under the following conditions: initial denaturation at 94 °C for 3 min followed by 40 cycles of denaturation at 94 °C for 15 s, annealing at 56 °C for 20 s, elongation at 72 °C for 30 s and a final extension at 72 °C for 3 min. Used primers are specified in Table 1. The amplified product was inserted into *KpnI*-digested pAD-agro using In-fusion HD Cloning Kit (Takara) and transformed into *E. coli* JM109. Plasmid DNA was isolated by QIAprep Spin Miniprep kit (Qiagen, Hilden, Germany) and verified by sequencing. The resultant plasmid construct was electroporated into *Agrobacterium tumefaciens* EHA105. *Agrobacterium* from an overnight culture were sedimented by centrifugation (16000 g for 1 min) and resuspended in 10 mM 2-(N-morpholino)ethanesulfonic acid pH 5.6, 10 mM MgCl<sub>2</sub>, 200  $\mu$ M acetosyringone to reach final OD<sub>600</sub> of ~0.1. The suspension was incubated at room temperature for 2 h and subsequently infiltrated into several leaves of 3–4 weeks old *Nicotiana benthamiana* by a needleless syringe (20  $\mu$ l per plant). The plants were cultivated under controlled conditions (temperature 20–22 °C, 12 h light/dark photoperiod). Symptoms of viral infection were evaluated visually, the presence of PPV in plant tissues was confirmed by Western blotting using a specific polyclonal antibody (Šubr and Matisová, 1999), followed by

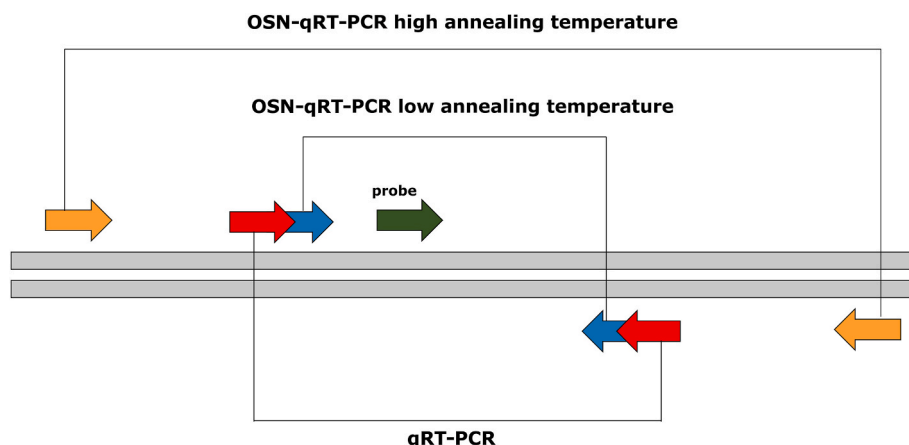


Fig. 1. Schematic diagram of qRT-PCR and OSN-qRT-PCR assays.

**Table 1**  
Oligonucleotides used in this study.

Purpose	Primer/probe name	Sequence (5' – 3')	Amplicon size	Reference
amplification of SARS CoV-2 S gene fragment	IF-CoS For	<u>ATC AGG CCG GCC GGG GTA CCA</u> TTG GCA AAA TTC AAG ACT CAC	447 bp	This work
	IF-CoS Rev	<u>GTG CAC AAC AAC GTT GGT ACC</u> AGG AGC AGT TGT GAA GTT C		
recombinant analysis	NCuniFor NCuniRev	GAG GCA ATT TGT GCT TCA ATG G CGC TTA ACT CCT TCA TAC CAA G	1226 bp	Subr et al. (2010)
qRT-PCR	HOT_Spike_Fw HOT_Spike_Rv	AGT GCA AAT TGA TAG GTT GATC TCT GAT TTC TGC AGC TCT AAT TA	88 bp	This work
OSN-qRT-PCR	LANL_May4.1_Fw LANL_May4.1_Rv	CRC GTC TTG ACA ARG TTG AGG CT TAC ACA CTC TGA CAT TTT AST AGC AGC	155 bp	<a href="https://covid19.edgebioinformatics.org/#/assayValidation">https://covid19.edgebioinformatics.org/#/assayValidation</a>
OSN-qRT-PCR	Inner_Spike_Fw Inner_Spike_Rv	AGT GCA AAT TGA TAG GTT G GAT TTC TGC AGC TCT AAT TA	85 bp	This work
qRT-PCR OSN-qRT-PCR	P-LANL_4.1	FAM-GGC AGA CTT CAA AGT TTG CA-BHQ1	probe	This work

<sup>a</sup> Sequence of the vector adjacent to the cloning site (for hybridisation at in-fusion cloning) is underlined.

further analysis by RT-PCR. Total leaf RNA was isolated using NucleoSpin RNA Plant kit (Macherey-Nagel, Düren, Germany) and cDNA was prepared using random hexamer primers and AMV reverse transcriptase (Promega, Madison, Wisconsin, USA). RT-PCR was performed using primers NCuniFor/NCuniRev (Subr et al., 2010) spanning the cloning cassette of pAD-agro (Fig. S1, Table 1). Electrophoretic analysis and sequencing of amplification products enabled verification of the inserted fragment.

The virus was purified two weeks after infiltration according to Laín et al. (1988) with certain modifications. The protocol included extraction from plant tissues with two volumes (2 mL per gram of tissue) of 18 mM McIlvaine citrate-phosphate buffer pH 7 with 0.2% thioglycolic acid, 10 mM sodium diethyldithiocarbamate, 0.5 M urea, 2 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethylsulfonyl fluoride and 1/3 volume of chloroform, followed by phase separation by centrifugation at 1520g for 30 min and ultracentrifugation of the water phase at 57000 g for 2 h. The sediment was resuspended in 100 mM sodium borate pH 8.2 with 10 mM EDTA and clarified by low-speed centrifugation (1520 g for 15 min). Sucrose was added to the solution to the final concentration of 20% and another round of ultracentrifugation (57000 g for 2 h) was performed. The purified virus serving as ESRM was obtained by resuspension of the sediment in a small volume of 10 mM Tris-HCl pH 8 with 1 mM EDTA and clarification by centrifugation (16000 g for 5 min). The concentration of purified PPV was estimated by UV spectrophotometry, using an extinction coefficient of 2.4 mL/mg/cm (Purcifull, 1966) and concentration of encapsidated RNA calculated as 5% of that value (Hollings and Brunt, 1981) to be  $8.1 \times 10^8$  genomic copies/mL (GC/mL). The final product was stored in aliquots at  $-20^\circ\text{C}$  until used.

## 2.2. Wastewater sample preparation

A sample of untreated wastewater influent was collected from a wastewater treatment plant treating municipal wastewater in Kysucké Nové Mesto (Slovakia). The wastewater sample was kept at  $4^\circ\text{C}$  for 24 h and it had these basic characteristics: Chemical Oxygen Demand (COD) 750 mg/L; N 139 mg/L; Suspended Solids (SS) 312 mg/L; pH 8.14; T  $17^\circ\text{C}$ . The wastewater was negative for SARS-CoV-2 by both qRT-PCR and OSN-qRT-PCR coupled to Method A (section 2.3.1.) with NucleoSpin RNA Virus Kit (Macherey-Nagel). ESRM was added to untreated wastewater at 1% (v/v) to a final concentration of  $8.1 \times 10^6$  GC/mL and subjected to particular virus concentration/extraction protocols. In order to assess the detection limit of the two best concentration/extraction methods, the above samples were serially diluted to concentrations from  $8.1 \times 10^4$  GC/mL to  $8.1 \times 10^2$  GC/mL of ESRM and tested in triplicate.

## 2.3. Virus concentration and RNA extraction methods

The virus particles were concentrated by using four methods (A-D) from the wastewater influent, which was artificially contaminated as shown in Fig. 2.

### 2.3.1. Method A

Method A employed polyethyleneglycol (PEG) precipitation, which is commonly used to concentrate viruses from water matrices (Warish et al., 2020). In this study, we used protocol described by Wu et al. (2020) with some modifications. Artificially contaminated wastewater samples with ESRM (50 ml; wastewater/ESRM) were clarified from particulate biomass by centrifugation for 30 min at 4500 g and  $4^\circ\text{C}$  (Medema et al., 2020). Meanwhile, a 50 mL Falcon tube with 4 g polyethyleneglycol 8000 (PEG 8000; Sigma-Aldrich, Saint Louis, Missouri, USA) and 0.9 g sodium chloride was prepared. After centrifugation, 40 mL of the supernatant was carefully transferred to the Falcon tube with PEG solution. The sample was inverted several times in hand during approximately 15 min at room temperature and permanent agitation and pelleted by centrifugation for 45 min at 12000g and  $4^\circ\text{C}$  without brake. After centrifugation, the supernatant was removed by decantation via the opposing side of the pellet. The tube with the pellet was returned to the centrifuge again and centrifuged at 12,000 g for 5 min and  $4^\circ\text{C}$  with a brake intensity set to 3 (of 9). The supernatant was carefully removed, and the pellet was dissolved in 800  $\mu\text{l}$  of TRI Reagent (solution for RNA isolation; Molecular Research Center, Cincinnati, Ohio, USA) by vortexing for 15 s. After centrifugation at 2000g for 10 s the supernatant was transferred to a clean 1.5 mL microtube and used for RNA extraction.

Two different kits for rapid preparation of highly pure viral nucleic acids from wastewater samples were used: NucleoSpin RNA Virus Kit by Macherey-Nagel and AllPrep PowerViral DNA/RNA Kit by Qiagen.

NucleoSpin RNA Virus Kit was used for the isolation of viral RNA from concentrated wastewater samples according to the manufacturer's instructions.

Alternatively, AllPrep PowerViral DNA/RNA Kit (Qiagen) was used for isolation of viral RNA. The manufacturer's instructions were followed, but the volume of the elution buffer was reduced to 50  $\mu\text{l}$ .

Extracted RNA was used immediately for qRT-PCR and OSN-qRT-PCR or stored at  $-70^\circ\text{C}$ .

### 2.3.2. Method B

Method B was based on ultracentrifugation, which is frequently used to concentrate viruses from wastewater (Fumian et al., 2010). In this study, we used the protocol published by Ahmed et al., (2020) with some modifications. The wastewater/ESRM sample (20 ml) were poured to polycarbonate ultracentrifuge bottles (# 355,618, Beckman Coulter,

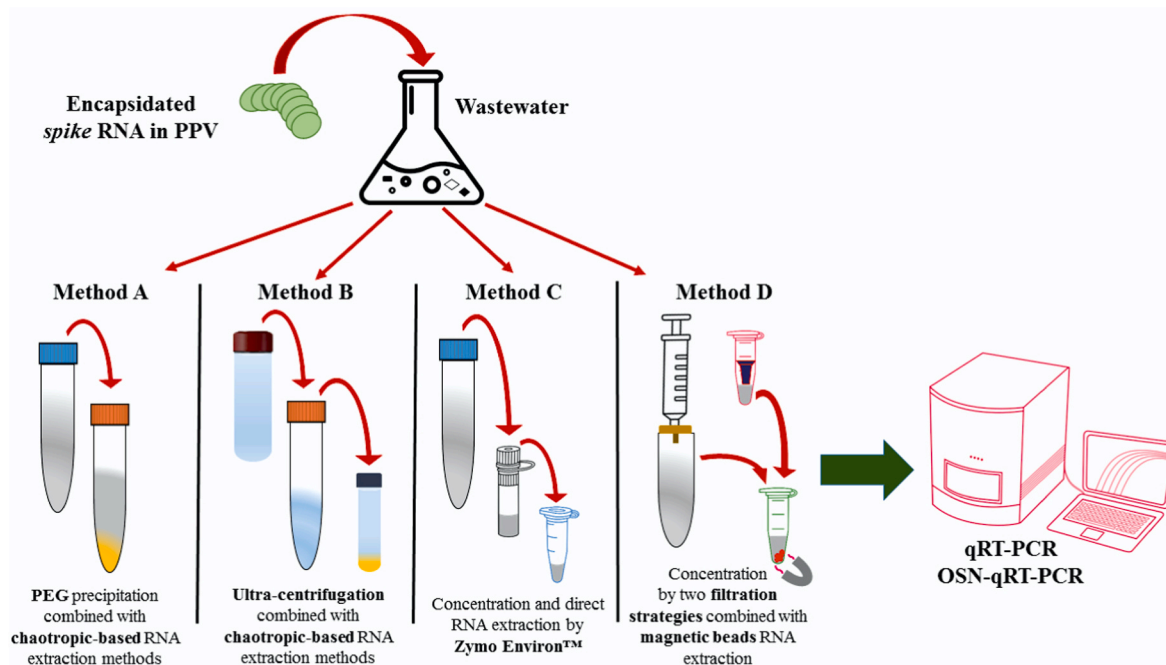


Fig. 2. Illustration of concentration/extraction methods evaluated in this study.

Indianapolis, Indiana, USA) and subjected to ultracentrifugation at 100000g for 1 h at 4 °C (Type 70 Ti rotor, Beckman Coulter, # 337,922) in an Optima XPN-90 Ultracentrifuge (Beckman Coulter, # A94468). The supernatant was removed, and the pellet was resuspended in 3.5 mL of 0.25 N glycine buffer (pH 9.5). Then, the sample was incubated on ice for 30 min and 3 mL of 2x phosphate buffered saline (PBS; pH 7.2) for neutralization was added. The sample was centrifuged at 12000g for 15 min at 4 °C. After that, the virus particles were recovered by ultracentrifugation again in polycarbonate ultracentrifuge bottles (Beckman Coulter, # 355,603) at 100000g for 1 h at 4 °C using a Type 90 Ti rotor (Beckman Coulter, # 355,530) in an Optima XPN-90 Ultracentrifuge. The pellet was resuspended in 400 µl of PBS (pH 7.2) and transferred to a microtube (1.5 mL) for RNA extraction. RNA was extracted using the two kits (NucleoSpin RNA Virus Kit, AllPrep PowerViral DNA/RNA Kit) as described above.

### 2.3.3. Method C

Method C applied commercial Zymo Environ Water RNA kit (Zymo Research, CA, USA). It included viral enrichment (from 4 ml), sample homogenization and RNA purification. We followed the protocol of producer with exclusion that elution volume was increased to 30 µl. RNA was used for qRT-PCR and OSN-qRT-PCR immediately or stored at -70 °C.

### 2.3.4. Method D

NucleoMag DNA/RNA Water Kit (Macherey-Nagel) designed for the isolation of microbial DNA/RNA from water was applied on filter-concentrated samples. The procedure is based on reversible adsorption of nucleic acids to paramagnetic beads under appropriate buffer conditions. The manufacturer's instructions for the extraction of RNA from water samples were followed with slight modifications. The filtration step was performed with two different membrane types. A 0.2 µm pores cellulose ester membrane (Whatman, Little Chalfont, UK) was after filtering wastewater/ESRM samples (10 ml) rolled into a cylinder and inserted into a Bead Tube with lysis buffer.

Alternatively, Vivaclear Mini 0.8 µm polyethersulphone (PES) centrifugal filters by Sartorius (Göttingen, Germany) were after wastewater/ESRM application (500 µl) centrifuged at 2000g for 10 min. The clarified filtrate was transferred to Bead Tube (Qiagen) with lysis buffer.

Samples from both filtration procedures were agitated by Vortex-Genie (Scientific Industries Inc., Bohemia, New York, USA) for 5 min. Then the protocol for NucleoMag DNA/RNA Water kit was followed. RNA was eluted using 50 µl of RNase-free water for each sample. RNA was used for qRT-PCR and OSN-qRT-PCR immediately or stored at -70 °C.

### 2.4. Primer design

Primer sets and probe were designed using the software Primer3Plus (<https://dev.primer3plus.com/index.html>) They were oriented to SARS-CoV-2 Spike sequence between the primers LANL\_MAY-4.1\_Fw/LANL\_MAY-4.1\_Rv of the assay LANL-SARS-CoV-2.May4.1 reported on the webpage: <https://covid19.edgebioinformatics.org/#/assayValidation>. The oligonucleotide sequences and the characteristics of corresponding PCR assays are described in Table 1.

### 2.5. qRT-PCR assay

Quantitative RT-PCR assays were performed in 20 µL of total reaction volume. Each reaction tube comprised 5 µL of RNA, 2x of Luna Universal Probe One-Step qRT-PCR Kit, 20x Luna Warm Start RT Enzyme Mix (New England Biolabs, Ipswich, Massachusetts, USA), 10 µM of primers HOT\_Spike\_Fw/HOT\_Spike\_Rv and 10 µM of probe P\_LANL\_4.1 (Table 1). After vortexing and centrifugation, the reaction tube was transferred to QuantStudio 1 Real-Time PCR System (ThermoFisher, Waltham, Massachusetts, USA). The qRT-PCR amplification consisted of following steps: 55 °C for 10 min, 95 °C for 1 min, 45 cycles of 95 °C for 10 s, 60 °C for 1 min with the collection of fluorescence signal at the end of each cycle. Each run contained positive and negative controls. Data were collected and analysed using the software QuantStudio Design and Analysis Software v1.5.2 (ThermoFisher). Threshold cycle values (Ct) were calculated using the software at the automatic threshold setting. The fluorescence signal showed a typical S-shaped amplification curve and samples with  $C_t \leq 38$  were considered positive.

### 2.6. OSN-qRT-PCR assay

For OSN-qRT-PCR amplification, the mix was identical as for qRT-



PCR with exception of primers. Outer primers LANL\_MAY\_4.1\_Fw/LANL\_MAY\_4.1\_Rv and inner primers Inner\_Spike\_Fw/Inner\_Spike\_Rv were added, each to 10  $\mu$ M (Table 1). The OSN-qRT-PCR amplification contained the following steps: 55 °C for 10 min, 95 °C for 1 min, 10 cycles of 95 °C for 15 s, 64 °C for 30 s, 72 °C for 40 s, followed by 40 cycles of 95 °C for 15 s and 55 °C for 30 s with the collection of fluorescence signal at the end of each cycle. The fluorescence signal showed a typical S-shaped amplification curve and samples with  $C_t \leq 30$  were considered positive. RNA isolated from ESRM by NucleoSpin RNA Virus Kit was used as a standard for the sensitivity analysis of qRT-PCR/OSN-qRT-PCR assay. A standard curve was generated using 10-fold serial dilutions ( $10^{-1}$  to  $10^{-9}$ ) of ESRM RNA, and RNase-free water was used as a negative control. All the amplification reactions were run in triplicates in two independent assays.

## 2.7. Recovery rate

ESRM recovery rate of the four concentration/extractions methods (A-D) were calculated based on the gene copies quantified by qRT-PCR and OSN-qRT-PCR as follows:

Recovery rate (%) = (GC recovered in concentrated wastewater/GC seeded)  $\times$  100. The mean and standard deviation for each concentration method and two quantification assays were calculated.

## 2.8. Statistical analysis

The one-way analysis of variance (ANOVA) was used to determine whether there was a difference in ESRM recovery among the concentration techniques tested. qRT-PCR and OSN-qRT-PCR assays were compared using a Pearson's correlation test.

## 3. Results

### 3.1. Production of encapsidated RNA mimic

The prepared modified PPV was capable of replication and systemic spread in infiltrated plants. Typical disease symptoms (mosaic, vein clearing and leaf distortion) were observed approximately 7 days post infection (dpi) and subsequent Western blot analysis showed the virus accumulation similar to wild-type PPV (Fig. S2). The sequence analysis of RT-PCR products verified the presence of inserted *S* gene fragment, confirming successful production of ESRM *in planta*.

The plants were harvested 14 dpi and used for ESRM purification. In addition to major intact PPV CP, the purified ESRM contained also its partially degraded products, as demonstrated by immunoblotting analysis (Fig. S2). Partial proteolysis commonly occurs in course of potyvirus purification, affecting mainly both CP termini. Similar to mild trypsinolysis, this process has no effect on infectivity or stability of virions in purified samples (Shukla et al., 1988). The yields of ESRM reached 70–80  $\mu$ g per gram of fresh green mass, corresponding approximately to 4  $\mu$ g/g of RNA.

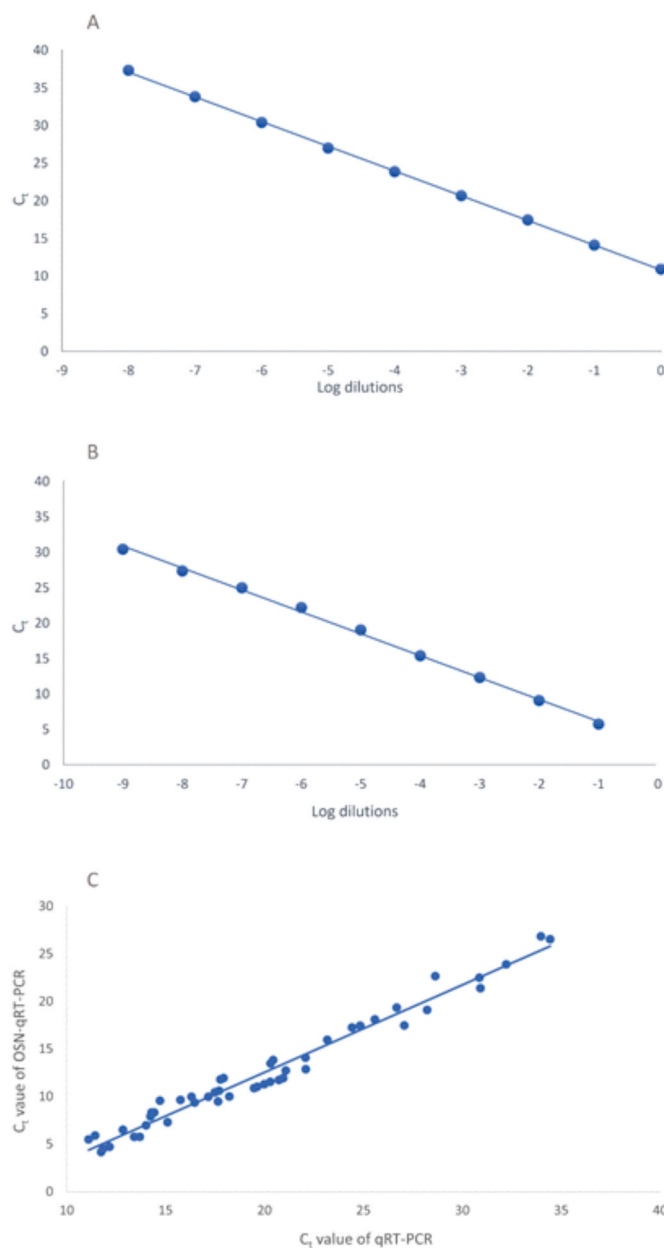
### 3.2. qRT-PCR and OSN-qRT-PCR assays

Two qRT-PCR assays were developed: a conventional qRT-PCR and a one-step single-tube nested qRT-PCR (OSN-qRT-PCR). The  $C_t$  values and standard deviations obtained from dilutions of ESRM's RNA up to  $10^{-9}$  are presented in Table 2. It was observed a  $C_t$  difference of 3.291 and 3.246 between  $\log_{10}$  dilutions for qRT-PCR and OSN-qRT-PCR, respectively. Based on the  $C_t$  values a linear curve (Fig. 3A and B) was created by regression analysis where score  $R^2$  was 1 and 0.999, with efficiency 101.316% and 103.287% for qRT-PCR and OSN-qRT-PCR, respectively.  $C_t$  values of qRT-PCR and OSN-qRT-PCR had a strong correlation ( $R^2$  value 0.985). The linear regression of the two groups had a good goodness-of-fit ( $R^2 = 0.971$ ) with a regression equation  $y = 0.9188x - 5.8258$  (Fig. 3C). The ALOQ (assay limit of quantification, i.e. lowest

**Table 2**

The  $C_t$  values and standard deviations obtained from dilutions of ESRM's RNA.

DILUTION	QRT-PCR		OSN-QRT-PCR	
	$C_t$	SD	$C_t$	SD
UNDILUTED	10.93	0.08	–	–
$10^{-1}$	14.11	0.12	5.75	0.05
$10^{-2}$	17.46	0.02	9.06	0.13
$10^{-3}$	20.68	0.10	12.33	0.09
$10^{-4}$	23.86	0.22	15.40	0.24
$10^{-5}$	27.00	0.16	19.03	0.26
$10^{-6}$	30.40	0.12	22.21	0.12
$10^{-7}$	33.83	0.31	24.98	0.18
$10^{-8}$	37.33	0.27	27.35	0.31
$10^{-9}$	–	–	30.42	0.34
EFFICIENCY (%)	<b>101.316</b>		<b>103.287</b>	
SLOPE	<b>–3.291</b>		<b>–3.246</b>	
$r^2$	<b>1</b>		<b>0.999</b>	



**Fig. 3.** The standard curves of A) qRT-PCR; B) OSN-qRT-PCR; C)  $C_t$  values of qRT-PCR and OSN-qRT-PCR analysed by linear regression and correlation analysis.

copy number detected in 100% of assays) for OSN-qRT-PCR was 1 copy/reaction and for qRT-PCR was 10-fold lower (10 copies/reaction).

### 3.3. Recovery of ESRM

The mean number of gene copies of ESRM recovered and recovery rates by qRT-PCR and OSN-qRT-PCR assay for the four concentration/extraction methods are shown in Table 3. The best performance was demonstrated by Method A using NucleoSpin RNA Virus Kit. The strikingly worst performance was assessed by Method C. Method A and Method B, which were based on different principles, both performed better when combined with NucleoSpin RNA Virus Kit by (Macherey-Nagel) than with AllPrep PowerViral DNA/RNA Kit (Qiagen).

### 3.4. Limit of detection (LOD) for concentration/extraction methods

Concentration methods A and B and RNA extraction by NucleoSpin RNA Virus Kit performed better regarding recovery than the other evaluated methods. To determine LOD of ESRM by these two approaches, three serial dilutions of ESRM were analysed in triplicate. The minimum amount of ESRM detected by qRT-PCR for Method A was equivalent to  $1.03 \times 10^3$  GC/mL and for Method B  $6.75 \times 10^3$  GC/mL (Table 4). The lowest LOD was noted by OSN-qRT-PCR for Method A,  $6.7 \times 10^1$  GC/mL, and for Method B it was  $9.54 \times 10^2$  GC/mL. When the wastewater was seeded with ESRM at a concentration higher than  $8.1 \times 10^2$  GC/mL, PEG precipitation revealed a mean recovery of 8.27% detected by OSN-qRT-PCR.

## 4. Discussion

Wastewater-based epidemiology (WBE) is currently being utilized to monitor the dissemination of SARS-CoV-2 (Sapula et al., 2021). However, due to the high chemical and biological complexity of wastewater, analysis may be skewed by low viral recovery, poorly reproducible results, or both (Shi et al., 2017). In addition, both particulate and dissolved constituents inherently present in wastewater get concentrated along with the target virus and can hinder the detection of viruses thus affecting the viral recovery yield of the concentration method

**Table 3**

Recovery of ESRM achieved by 4 concentration/extraction methods (A-D) from artificially contaminated wastewater.

Concentration/extraction method	Concentration (GC/mL) <sup>a</sup> of recovered ESRM <sup>b</sup>		ESRM recovery rate (%) <sup>a</sup>	
	qRT-PCR	OSN-qRT-PCR	qRT-PCR	OSN-qRT-PCR
<b>Method A</b> + NucleoSpin RNA Virus Kit	$3.13 \times 10^6$ $\pm 6.46 \times 10^5$	$3.92 \times 10^6$ $\pm 5.44 \times 10^5$	$38.61 \pm 7.98$	$48.39 \pm 6.71$
<b>Method A</b> + AllPrep PowerViral DNA/RNA Kit	$2.80 \times 10^6$ $\pm 7.12 \times 10^5$	$3.53 \times 10^6$ $\pm 8.01 \times 10^5$	$34.51 \pm 8.79$	$43.61 \pm 9.89$
<b>Method B</b> + NucleoSpin RNA Virus Kit	$2.17 \times 10^6$ $\pm 6.80 \times 10^5$	$2.89 \times 10^6$ $\pm 9.86 \times 10^5$	$26.81 \pm 8.4$	$35.67 \pm 12.18$
<b>Method B</b> + AllPrep PowerViral DNA/RNA Kit	$1.74 \times 10^6$ $\pm 9.44 \times 10^5$	$2.40 \times 10^6$ $\pm 9.10 \times 10^5$	$21.52 \pm 11.65$	$29.61 \pm 11.23$
<b>Method C</b>	$4.98 \times 10^5$ $\pm 1.09 \times 10^6$	$9.98 \times 10^5$ $\pm 1.43 \times 10^6$	$6.15 \pm 13.5$	$11.09 \pm 17.62$
<b>Method D</b> (first type)	$1.28 \times 10^6$ $\pm 1.39 \times 10^5$	$2.07 \times 10^6$ $\pm 1.36 \times 10^6$	$15.84 \pm 17.11$	$25.52 \pm 16.84$
<b>Method D</b> (second type)	$1.11 \times 10^6$ $\pm 1.18 \times 10^6$	$1.81 \times 10^6$ $\pm 1.22 \times 10^6$	$13.64 \pm 14.6$	$22.32 \pm 15.01$

<sup>a</sup> Values of mean  $\pm$  standard deviation are presented.

<sup>b</sup>  $8.1 \times 10^6$  ESRM seeded (GC/mL).

**Table 4**

Recovery and recovery rate of ESRM achieved by PEG precipitation or ultracentrifugation.

\*Values of mean  $\pm$  standard deviation are presented.

Concentration/extraction method	ESRM seeded (GC/mL)	Concentration (GC/mL) <sup>a</sup> of recovered ESRM		ESRM recovery rate (%) <sup>a</sup>	
		qRT-PCR	OSN-qRT-PCR	qRT-PCR	OSN-qRT-PCR
<b>Method A</b> + NucleoSpin RNA Virus Kit	$8.1 \times 10^4$	$1.52 \times 10^4 \pm 5.76 \times 10^3$	$2.91 \times 10^4 \pm 8.72 \times 10^3$	$18.77 \pm 7.11$	$35.93 \pm 10.77$
	$8.1 \times 10^3$	$1.03 \times 10^3 \pm 8.41 \times 10^2$	$1.57 \times 10^3 \pm 1.1 \times 10^3$	$12.72 \pm 10.38$	$19.38 \pm 13.58$
	$8.1 \times 10^2$	0	$6.7 \times 10^1 \pm 1.15 \times 10^2$	0	$8.27 \pm 14.20$
<b>Method B</b> + NucleoSpin RNA Virus Kit	$8.1 \times 10^4$	$6.75 \times 10^3 \pm 9.82 \times 10^3$	$8.75 \times 10^3 \pm 1.21 \times 10^4$	$8.33 \pm 12.12$	$10.80 \pm 14.94$
	$8.1 \times 10^3$	0	$9.54 \times 10^2 \pm 8.82 \times 10^2$	0	$11.78 \pm 10.89$
	$8.1 \times 10^2$	0	0	0	0

(Michael-Kordatou et al., 2020). Therefore, it is necessary to evaluate various aspects in order to select an efficient method to concentrate, and subsequently detect, SARS-CoV-2 from wastewater, which has been identified as a key research need for WBE (Kitajima et al., 2020; La Rosa et al., 2020b).

In this study, several molecular tools were evaluated in order to be prepared for use in potential future pandemics events. The first step of our evaluation strategy was the construction of ESRM based on the PPV genome bearing the fragment of SARS-CoV-2 Spike protein-coding sequence. In this way RNA is encapsidated which means that is better protected and stabilized. Although, ESRM has a different size comparing to SARS-CoV-2 this should not affect concentration, extraction and detection. ESRM was then used to assess the viral RNA recovery rate of four concentration/extraction methods utilizing two different PCR assays: a conventional qRT-PCR and a newly developed one-step single-tube nested real-time PCR (OSN-qRT-PCR). Since the composition of wastewater lays in a narrow range, the effectiveness of the detection method should not be much different. If the sample of wastewater is very alkaline or very acidic, additional neutralizing step may be necessary.

The different physical and chemical mechanisms involved in the tested concentration methods led to different degrees of recovery of ESRM. All four tested concentration methods led to detection of ESRM RNA at higher concentrations. Since we seeded the wastewater with known concentrations of ESRM, we were able to calculate the full process recovery rate (loss of ESRM through concentration and extraction methods) using two assays. We calculated recovery rate percentage, using qRT-PCR and OSN-qRT-PCR, at  $8.1 \times 10^6$  GC/mL of ESRM seeded in wastewater samples. The other virus particles, present in wastewater, should not influence recovery and detection of ESRM unless they are in a big excess. The detection method should not be affected by any high concentration of competing virus particles (Hong et al., 2021).

The concentration methods used different physical and chemical mechanisms to concentrate SARS-CoV-2. The PEG method concentrates virus particles by precipitation of virus particles upon addition of polyethylene glycol and sodium chloride. Although there is uncertainty in the exact mechanism, virus precipitation is believed to occur similarly to precipitation of proteins by PEG, where water molecules are drawn

from the solution to hydrate PEG molecules, thereby increasing the effective protein concentration, leading to insolubility and precipitation of proteins after reaching saturation (Ingham, 1990; Yamamoto et al., 1970; LaTurner et al., 2021). The combination of PEG precipitation with NucleoSpin RNA Virus Kit displayed the highest recovery rate in our study. A similar recovery rate of  $44.0 \pm 27.7\%$  was reported previously by Ahmed et al. (2020) in a study with enveloped MHV virus. Barril et al. (2021) achieved a higher mean recovery (62.2%) with this method but using it with a non-enveloped feline calicivirus (FCV).

Ultracentrifugation has been used for decades to concentrate viruses from environmental matrices. It has been reported that ultracentrifugation at 100,000 g is required to pellet most macromolecules and viruses (Ammersbach and Bienze, 2011). In this study, good recovery rates were achieved using concentration by ultracentrifugation, RNA extraction by NucleoSpin RNA Virus Kit and detection by qRT-PCR or OSN-qRT-PCR. The determined recovery rates were comparable to the 33.5% reported by Ahmed et al. (2020). Fumian et al. (2010) reported that ultracentrifugation (100,000 g for 1 h at 4 °C) had a mean recovery of 47% (range of 34–60%) of non-enveloped rotavirus A from wastewater samples. This method also involves discarding supernatant a few times, which may have resulted in loss of virus. Nevertheless, the ultracentrifugation method may not be suitable for WBE studies because it requires expensive specialized centrifuges (Ahmed et al., 2020).

When comparing RNA extraction kits after PEG precipitation or ultrafiltration, NucleoSpin RNA Virus Kit performed better than Qiagen All Prep PowerViral DNA/RNA Kit in this study.

Our results are somewhat different from those of O'Brien et al. (2021), who tested 4 commercial kits for RNA extraction from wastewater and reported that pellet-based RNA extraction kits that included inhibitors removal and RNA preservation step yielded the most consistent, timely and accurate results. In that study, the most effective and efficient kit was Zymo Environ Water RNA. However, in our study Method C, which included Zymo Environ Water RNA Kit, had low recovery rates of only  $6.15 \pm 13.5\%$  and  $11.09 \pm 17.62\%$  when combined with qRT-PCR and OSN-qRT-PCR, respectively.

Method D that used NucleoMag DNA/RNA Water Kit with a concentration step by microfiltration or by PES centrifugal filtration was used for the first time in this study to the best of our knowledge. This concentration method was found moderately effective, allowing to recover 13.64–25.52% of ESRM from a small volume of wastewater.

For the two best concentration methods (PEG precipitation and ultracentrifugation), we determined also recovery rate at ESRM seeding levels from  $8.1 \times 10^4$  to  $8.1 \times 10^2$  GC/mL. At  $8.1 \times 10^4$  GC/mL, the greatest recovery rate was determined, which illustrated that the concentration of virus particles in wastewater may influence the performance of the method. Similar results were reported in a recent study where greater recovery was observed, for bacteriophage  $\phi 6$  surrogate, at the highest seeding level (Sangsanont et al., 2022). It was stated that the greater is the SARS-CoV-2 RNA concentration in a wastewater sample, the greater is the recovery and downstream detection probability (Ahmed et al., 2022).

In qRT-PCR, a standard curve is constructed to convert the threshold cycle values ( $C_t$ ) value to virus titers. In a qualitative approach, a negative result is indicated by a lack of the typical amplification curve by a  $C_t$  value higher than a limit usually translated to number of gene copies per volume unit (Zhu et al., 2021). In this study, qRT-PCR and OSN-qRT-PCR assays for the quantification of ESRM were performed using the Luna Universal Probe One-Step qRT-PCR Kit. Selection of this chemical system was based on Sapula et al. (2021) who reported that this master mix had a higher detection efficiency for SARS-CoV-2 compared to TaqPath 1-Step Multiplex Master Mix (No ROX).

When ESRM RNA seeding levels were  $<8.1 \times 10^3$  GC/mL, amplification was observed only by OSN-qRT-PCR assay after concentration by PEG precipitation and extraction by NucleoSpin RNA Virus Kit. PEG precipitation showed LOD of  $8.1 \times 10^2$  for OSN-qRT-PCR and  $8.1 \times 10^3$  for qRT-PCR. These results evidenced that OSN-qRT-PCR had lower LOD

with PEG precipitation compared to the study by Barril et al. (2021), who used just normal qRT-PCR with non-enveloped FCV. LOD with ultracentrifugation was by an order of magnitude higher with each of OSN-qRT-PCR or qRT-PCR. The standard curve at various ESRM levels showed that the OSN-qRT-PCR method is highly sensitive and that OSN-qRT-PCR is able to increase the rate of positive detection of the virus. LOD of OSN-qRT-PCR was at a level of 1 copy/reaction, which is the theoretical optimum, while that of qRT-PCR was 10 copies/reaction. Similar results were published by Wang et al. (2020) with recombinant plasmids. Moreover, Pearson's correlation and linear regression between qRT-PCR and OSN-qRT-PCR results revealed a strong correlation, indicating that the presence of the external amplification step (10 PCR cycles) of the OSN-qRT-PCR assay did not affect the stability of the consequent inner PCR (40 PCR cycles) and that the  $C_t$  value still showed regularity similar to qRT-PCR. In our study, for the first time, OSN-qRT-PCR was evaluated as a reliable detection and quantification method for the analysis of wastewater samples.

## 5. Conclusion

The construction of ESRM and artificial contamination of wastewater with it was useful for optimization of detection of a virus in wastewater. Among the tested concentration/extraction approaches, the PEG precipitation method coupled to chaotropic solid-phase extraction achieved the highest recovery rate of ESRM. OSN-qRT-PCR represented an improvement in detection sensitivity as it had an LOD by an order of magnitude lower than qRT-PCR. Highly efficient concentration/extraction combined with highly sensitive detection can be a valuable molecular tool for detecting viruses in wastewater samples with low viral loads. In the future, the analysis of wastewater by advanced sensitive molecular approaches can play an important role in the surveillance of a range of human pathogenic viruses.

## CRedit authorship contribution statement

**Magdalena Rusková:** Investigation, Methodology, Software, Visualisation, Writing - original draft, Writing - review & Editing. **Mária Bučková:** Formal analysis, Investigation, Methodology, Resources, Software, Validation, Visualisation, Writing - original draft. **Adam Achs:** Investigation, Methodology, Visualisation, Writing - original draft. **Andrea Puškárová:** Investigation, Methodology, Software, Visualisation, Writing - original draft. **Jer-Horng Wu:** Resources, Writing - Review & Editing, Funding acquisition. **Tomáš Kuchta:** Conceptualisation, Writing - Review & Editing, Supervision. **Zdeno Šubr:** Methodology, Validation, Visualisation, Writing- original draft, Writing - Review & Editing, Supervision. **Domenico Pangallo:** Conceptualisation, Resources, Writing- original draft, Writing - Review & Editing, Validation, Visualisation, Supervision, Project administration, Funding acquisition.

## Funding

This study was mainly financed by the bilateral SAS (Slovak Academy of Sciences) – MOST (Taiwan) Joint Research Project (SAS-MOST/JRP/2020/1122/PathogenTracker). We thank also the support of the project VEGA 2/0003/22 of the Scientific Grant Agency of the Ministry of Education, Science, Research and Sports of the Slovak Republic and the Slovak Academy of Sciences and the EIG CONCERT-Japan (EIG CONCERT-Japan/2019/881/SuWaCer).

## 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.



## Acknowledgements

We are thankful to Dr. Nina Kunová for kind assistance with ultracentrifugation runs.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ijheh.2022.114017>.

## References

- Ahmed, W., Bertsch, P.M., Bivins, A., Bibby, K., Farkas, K., Gathercole, A., Haramoto, E., Gyawali, P., Korajkic, A., McMinn, B.R., Mueller, J.F., Simpson, S.L., Smith, W.J.M., Symonds, E.M., Thomas, K.V., Verhagen, R., Kitajima, M., 2020. Comparison of virus concentration methods for the RT-qPCR-based recovery of murine hepatitis virus, a surrogate for SARS-CoV-2 from untreated wastewater. *Sci. Total Environ.* 739 <https://doi.org/10.1016/j.scitotenv.2020.139960>.
- Ahmed, W., Bivins, A., Metcalfe, S., Smith, W.J.M., Verbyla, M.E., Symonds, E.M., Simpson, S.L., 2022. Evaluation of process limit of detection and quantification variation of SARS-CoV-2 RT-qPCR and RT-dPCR assays for wastewater surveillance. *Water Res.* 213, 118132 <https://doi.org/10.1016/j.watres.2022.118132>.
- Ammersbach, M., Bienzle, D., 2011. Methods for assessing feline immunodeficiency virus infection, infectivity and purification. *Vet. Immunol. Immunopathol.* 143, 202–214. <https://doi.org/10.1016/j.vetimm.2011.06.004>.
- Barril, P.A., Pianciola, L.A., Mazzeo, M., Ousset, M.J., Jaureguiberry, M.V., Alessandrello, M., Sánchez, G., Oteiza, J.M., 2021. Evaluation of viral concentration methods for SARS-CoV-2 recovery from wastewaters. *Sci. Total Environ.* 756, 144105 <https://doi.org/10.1016/j.scitotenv.2020.144105>.
- Bhat, A.I., Rao, G.P., 2020. Purification of Plant Viruses. *Characterization of Plant Viruses*. Humana, New York, NY, pp. 133–168. [https://doi.org/10.1007/978-1-0716-0334-5\\_21](https://doi.org/10.1007/978-1-0716-0334-5_21).
- Bivins, A., Kaya, D., Bibby, K., Simpson, S.L., Bustin, S.A., Shanks, O.C., Ahmed, W., 2021. Variability in RT-qPCR assay parameters indicates unreliable SARS-CoV-2 RNA quantification for wastewater surveillance. *Water Res.* 203, 117516 <https://doi.org/10.1016/j.watres.2021.117516>.
- Blanc, S., López-Moya, J.-J., Wang, R., García-Lampasona, S., Thornbury, D.W., Pirone, T.P., 1997. A specific interaction between coat protein and helper component correlates with aphid transmission of a potyvirus. *Virology* 231, 141–147. <https://doi.org/10.1006/viro.1997.8521>.
- Chan, S.K., Du, P., Ignacio, C., Mehta, S., Newton, I.G., Steinmetz, N.F., 2020. Biomimetic virus-like particles as severe acute respiratory syndrome coronavirus 2 diagnostic tools. *ACS Nano* 15, 1259–1272. <https://doi.org/10.1021/acsnano.0c08430>.
- Costa, J., Mafra, I., Kuchta, T., Oliveira, M.B.P.P., 2012. Single-tube nested real-time PCR as a new highly sensitive approach to trace hazelnut. *J. Agric. Food Chem.* 60, 8103–8110. <https://doi.org/10.1021/jf302898z>.
- Fumian, T.M., Leite, J.P.G., Castello, A.A., Gaggero, A., de Caillou, M.S.L., Miagostovich, M.P., 2010. Detection of rotavirus A in sewage samples using multiplex qPCR and an evaluation of the ultracentrifugation and adsorption-elution methods for virus concentration. *J. Virol. Methods* 170, 42–46. <https://doi.org/10.1016/j.jviromet.2010.08.017>.
- Hollings, M., Brunt, A.A., 1981. *Potyriviruses*. In: Kurstak, E. (Ed.), *Handbook of Plant Virus Infections*. Comparative Diagnosis. Elsevier/North-Holland Biomedical Press, Amsterdam-New York-Oxford, pp. 731–808.
- Hong, P.Y., Rachmadi, A.T., Mantilla-Calderson, D., Alkahtani, M., Bashawri, Y.M., Al Qarni, H., O'Reilly, K.M., Zhou, J., 2021. Estimating the minimum number of SARS-CoV-2 infected cases needed to detect viral RNA in wastewater: to what extent of the outbreak can surveillance of wastewater tell us? *Environ. Res.* 195, 110748 <https://doi.org/10.1016/j.envres.2021.110748>.
- Ingham, K.C., 1990. Precipitation of proteins with polyethylene glycol. *Methods Enzymol.* 182, 301–306. [https://doi.org/10.1016/0076-6879\(90\)82025-W](https://doi.org/10.1016/0076-6879(90)82025-W). Academic Press.
- Kamencayová, M., Šubr, Z., 2012. Preparation of vectors based on the genome of Plum pox virus PPV-Rec for heterologous gene expression in plants. *Acta Fytotech. Zootech.* 15, 24–26.
- Kitajima, M., Ahmed, W., Bibby, K., Carducci, A., Gerba, C.P., Hamilton, K.A., Haramoto, E., Rose, J.B., 2020. SARS-CoV-2 in wastewater: state of the knowledge and research needs. *Sci. Total Environ.* <https://doi.org/10.1016/j.scitotenv.2020.139076>.
- La Rosa, G., Bonadonna, L., Lucentini, L., Kenmoe, S., Suffredini, E., 2020a. Coronavirus in water environments: occurrence, persistence and concentration methods-A scoping review. *Water Res.* 179, 115899 <https://doi.org/10.1016/j.watres.2020.115899>.
- La Rosa, G., Iaconelli, M., Mancini, P., Bonanno Ferraro, G., Veneri, C., Bonadonna, L., Lucentini, L., Suffredini, E., 2020b. First detection of SARS-CoV-2 in untreated wastewaters in Italy. *Sci. Total Environ.* 736, 139652 <https://doi.org/10.1016/j.scitotenv.2020.139652>.
- Laín, S., Riechmann, J., Méndez, E., García, J.A., 1988. Nucleotide sequence of the 3' terminal region of plum pox potyvirus RNA. *Virus Res.* 10, 325–341. [https://doi.org/10.1016/0168-1702\(88\)90074-3](https://doi.org/10.1016/0168-1702(88)90074-3).
- LaTurner, Z.W., Zong, D.M., Kalvapalle, P., Gamas, K.R., Terwilliger, A., Crosby, T., Ali, P., Avadhanula, V., Santos, H.H., Weesner, K., Hopkins, L., Piedra, P.A., Maresso, A.W., Stadler, L.B., 2021. Evaluating recovery, cost, and throughput of different concentration methods for SARS-CoV-2 wastewater-based epidemiology. *Water Res.* 197, 117043 <https://doi.org/10.1016/j.watres.2021.117043>.
- Medema, G., Heijnen, L., Elsinga, G., Italiaander, R., Brouwer, A., 2020. Presence of SARS-Coronavirus-2 RNA in sewage and correlation with reported COVID-19 prevalence in the early stage of the epidemic in The Netherlands. *Environ. Sci. Technol. Lett.* 7, 511–516. <https://doi.org/10.1021/acs.estlett.0c00357>.
- Michael-Kordatou, I., Karaolia, P., Fatta-Kassinos, D., 2020. Sewage analysis as a tool for the COVID-19 pandemic response and management: the urgent need for optimised protocols for SARS-CoV-2 detection and quantification. *J. Environ. Chem. Eng.* 8, 104306 <https://doi.org/10.1016/j.jece.2020.104306>.
- Minarovičová, J., Lopašová, J., Valík, E., Kuchta, T., 2011. A method for the detection of *Cryptosporidium parvum* oocysts in milk based on microfiltration and real-time polymerase chain reaction. *Food Anal. Methods* 4, 116–120. <https://doi.org/10.1007/s12161-010-9141-9>.
- O'Brien, M., Rundell, Z.C., Nemeč, M.D., Langan, L.M., Back, J.A., Lugo, J.N., 2021. A comparison of four commercially available RNA extraction kits for wastewater surveillance of SARS-CoV-2 in a college population. *Sci. Total Environ.* 801, 149595 <https://doi.org/10.1016/j.scitotenv.2021.149595>.
- Peyret, H., Gropelli, E., Clark, D., Eckersley, N., Planche, T., Ma, J., Lomonosoff, G.P., 2022. Production and use of encapsidated RNA mimics as positive control reagents for SARS-CoV-2 RT-qPCR diagnostics. *J. Virol. Methods* 300, 114372. <https://doi.org/10.1016/j.jviromet.2021.114372>.
- Predajna, L., Nagyová, A., Glasa, M., Šubr, Z.W., 2012. Cloning of the complete infectious cDNA of the plum pox virus strain PPV-Rec. *Acta Virol.* 56, 129–132. <https://doi.org/10.4149/av.2012.02.129>.
- Purcifull, D.E., 1966. Some properties of tobacco etch virus and its alkaline degradation products. *Virology* 29, 8–14. [https://doi.org/10.1016/0042-6822\(66\)90190-5](https://doi.org/10.1016/0042-6822(66)90190-5).
- Sangsanont, J., Rattanakul, S., Kongprajug, A., Chyerochana, N., Sresung, M., Sriporatana, N., Wanlapakorn, N., Poovorawan, Y., Mongkolsuk, S., Sirikanchana, K., 2022. SARS-CoV-2 RNA surveillance in large to small centralized wastewater treatment plants preceding the third COVID-19 resurgence in Bangkok, Thailand. *Sci. Total Environ.* 809, 151169 <https://doi.org/10.1016/j.scitotenv.2021.151169>.
- Sapula, S.A., Whittall, J.J., Pandopulos, A.J., Gerber, C., Venter, H., 2021. An optimized and robust PEG precipitation method for detection of SARS-CoV-2 in wastewater. *Sci. Total Environ.* 785, 147270 <https://doi.org/10.1016/j.scitotenv.2021.147270>.
- Shi, H., Pasco, E.V., Tarabara, V.V., 2017. Membrane-based methods of virus concentration from water: a review of process parameters and their effects on virus recovery. *Environ. Sci. Water Res. Technol.* 3, 778–792. <https://doi.org/10.1039/C7EW00016B>.
- Šubr, Z.W., Nagyová, A., Glasa, M., 2010. Biolistic transfection of plants by infectious cDNA clones of Plum pox virus. *Julius-Kühn-Archiv* 339.
- Šubr, Z., Matisová, J., 1999. Preparation of diagnostic monoclonal antibodies against two potyviruses. *Acta Virol.* 43, 255–257.
- Tran, H.N., Le, G.T., Nguyen, D.T., Juang, R.-S., Rinklebe, J., Bhatnagar, A., Lima, E.C., Iqbal, H.M.N., Sarmah, A.K., Chao, H.-P., 2021. SARS-CoV-2 coronavirus in water and wastewater: a critical review about presence and concern. *Environ. Res.* 193, 110265 <https://doi.org/10.1016/j.envres.2020.110265>.
- Wang, Ji, Cai, K., Zhang, R., He, X., Shen, X., Liu, J., Xu, J., Qiu, F., Lei, W., Wang, Jinrong, 2020. Novel one-step single-tube nested quantitative real-time PCR assay for highly sensitive detection of SARS-CoV-2. *Anal. Chem.* 92, 9399–9404. <https://doi.org/10.1021/acs.analchem.0c01884>.
- Wu, F., Zhang, J., Xiao, A., Gu, X., Lee, W.L., Armas, F., Kauffman, K., Hanage, W., Matus, M., Ghaeli, N., Endo, N., Duvallet, C., Poyet, M., Moniz, K., Washburne, A.D., Erickson, T.B., Chai, P.R., Thompson, J., Alm, E.J., 2020. SARS-CoV-2 titers in wastewater are higher than expected from clinically confirmed cases. *mSystems* 5, e00614–e00620. <https://doi.org/10.1128/mSystems.00614-20>.
- Yamamoto, K.R., Alberts, B.M., Benzinger, R., Lawhorne, L., Treiber, G., 1970. Rapid bacteriophage sedimentation in the presence of polyethylene glycol and its application to large-scale virus purification. *Virology* 40, 734–744. [https://doi.org/10.1016/0042-6822\(70\)90218-7](https://doi.org/10.1016/0042-6822(70)90218-7).
- Yip, C.C.-Y., Sridhar, S., Leung, K.-H., Ng, A.C.-K., Chan, K.-H., Chan, J.F.-W., Tsang, O. T.-Y., Hung, I.F.-N., Cheng, V.C.-C., Yuen, K.-Y., 2020. Development and evaluation of novel and highly sensitive single-tube nested real-time RT-PCR assays for SARS-CoV-2 detection. *Int. J. Mol. Sci.* 21, 5674. <https://doi.org/10.3390/ijms21165674>.
- Yu, F., Yan, L., Wang, N., Yang, S., Wang, L., Tang, Y., Gao, G., Wang, S., Ma, C., Xie, R., 2020. Quantitative detection and viral load analysis of SARS-CoV-2 in infected patients. *Clin. Infect. Dis.* 71, 793–798. <https://doi.org/10.1093/cid/ciaa345>.
- Zhu, Y., Oishi, W., Maruo, C., Saito, M., Chen, R., Kitajima, M., Sano, D., 2021. Early warning of COVID-19 via wastewater-based epidemiology: potential and bottlenecks. *Sci. Total Environ.* 767, 145124 <https://doi.org/10.1016/j.scitotenv.2021.145124>.