DEVELOPMENT AND EMPLOYMENT OF UNTARGETED AND TARGETED TOOLS FOR VIRUS DETECTION IN THE FRAME OF WATER-BASED EPIDEMIOLOGY

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Doctoral Dissertation
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Doctoral Dissertation

RAZVOJ IN UPORABA NETARČNIH IN TARČNIH ORODIJ ZA ZAZNAVANJE VIRUSOV V OKVIRU EPIDEMIOLOGIJE VODA

Doktorska disertacija

Supervisor: Ion Gutierrez Aguirre, PhD

Co-Supervisor: Denis Kutnjak, PhD

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To you, the reader, for taking the time to embark on this journey with me,

remember, as Robert Heinlein said, everything is theoretically impossible, until it is done.

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Abstract

Traditional methods of studying viruses are limited in their ability to detect novel pathogens, but recent advances in high-throughput sequencing (HTS) are changing that. HTS allows researchers to probe deeply into the virome of various hosts and environments, identifying known and unknown viral species. However, a targeted approach, like quantitative PCR, remains the golden standard for large-scale quantitative studies. The increasing popularity of water-based epidemiology (WBE) to monitor wastewater for pathogens has become particularly widespread with surveillance of SARS-CoV-2 during the COVID-19 pandemic. Optimizing this procedure demands an evaluation of the stability of the virus in wastewater and a suitable concentration method for improving detection sensitivity. Real-time quantitative reverse transcription PCR (RT-qPCR) is the primary method used for detecting SARS-CoV-2, and validation of assays using appropriate reference materials and matrices is crucial. Plant viruses present similar challenges, with tomato brown rugose fruit virus (ToBRFV) being highly stable and potentially infectious for extended periods in water. Detection of ToBRFV in Slovenian wastewater before detection in plant samples raises concerns about the virus' s origin, undetected presence, waterborne transmission risks, and using such water sources for agricultural irrigation during water scarcity. Simple and effective methods for ToBRFV concentration and detection from water samples are essential to analyze potential release from infected plants into water sources. Metagenomics analysis of environmental water used in irrigation can provide insight into the types of viruses to which plants are exposed, shedding light on virus transmission dynamics in agricultural settings and aiding in developing effective management strategies. It is a valuable tool for detecting known and unknown viral species in the ecosystem under investigation, providing an early warning system to prevent potential outbreaks. By applying knowledge and techniques from wastewater monitoring for human pathogens, it may be possible to detect plant viruses in irrigation water. In conclusion, advances in HTS-based metagenomics research and WBE can revolutionize virus detection and surveillance in human and plant systems.

Povzetek

Tradicionalne metode preučevanja virusov so omejene pri odkrivanju novih patogenov, vendar nedavni napredek na področju visoko zmogljivega sekvenciranja (HTS) to spreminja. Uporaba HTS omogoča poglobljeno analiyo viromov različnih gostiteljev in okolij ter odkrivanje znanih in neznanih vrst virusov. Kljub temu tarčni pristopi, kot je kvantitativna PCR, ostajajo zlati standard za obsežnejše študije. Uporabnost študij epidemiologije na osnovi vode (water-based epidemiology, WBE) za spremljanje patogenov v odpadnih vodah se je globalno razširila, med pandemijo COVID-19, ko se je spremljalo širenje SARS-CoV-2. Optimizacija takšnega postopka zahteva oceno stabilnosti virusa v odpadni vodi in ustrezno metodo koncentracije za izboljšanje občutljivosti detekcije. Kvantitativni PCR v realnem času z reverzno transkripcijo (real-time quantitative reverse transcription PCR) (RT-qPCR) je osnovna metoda, ki se uporablja za odkrivanje SARS-CoV-2, pri čemer je ključnega pomena validacija testov z uporabo ustreznih referenčnih materialov in matric. Rastlinski virusi predstavljajo podobne izzive, zlasti virus rjave grbančavosti plodov paradižnika (ToBRFV), ki je v vodi zelo stabilen in potencialno kužen dlje časa. Zaradi odkritja virusa ToBRFV v odpadni vodi nekaj let pred njegovim odkritjem v rastlinskih vzorcih, se zastavljajo vprašanja o izvoru virusa, njegovi nezaznavni prisotnosti, tveganju prenosa z vodo in uporabi takšnih vodnih virov za namakanje v. Enostavne in učinkovite metode za koncentracijo in odkrivanje virusa ToBRFV v vzorcih vode so ključne za odkrivanje morebitnega sproščanja virusov iz okuženih rastlin v vodne vire. Analiza metagenoma okoljske vode, ki se uporablja za namakanje, omogoča vpogled v vrste virusov, ki so jim rastline izpostavljene. To prispeva k razumevanju dinamike prenosa virusov v kmetijskem okolju in pomaga pri razvoju učinkovitih strategij zaščite rastlin. Metoda omogoča vzpostavitev sistema zgodnjega opozarjanja in s tem preprečevanje morebitnih izbruhov, in je pomembno orodje za pravočasno odkrivanje znanih in neznanih vrst virusov v preiskovanem ekosistemu. Uporaba znanja in tehnik nadzora humanih patogenov v odpadnih vodah omogoča tudi odkrivanje rastlinskih virusov v namakalni vodi.

Za zaključek lahko povzamemo, da napredek pri raziskavah metagenoma s HTS in WBE prinaša revolucionarne izboljšave pri odkrivanju in nadzoru virusov pomembnih za človeka in rastline.

Contents

Abbreviations

HTS	...	High-throughput sequencing
MSCA-INT	...	Marie Skłodowska-Curie Actions – Innovative Training network
ARRS	...	Slovenian research agency
RNA	...	Ribonucleic acid
WBE	...	Water-based epidemiology
RT-qPCR	...	Real-time quantitative polymerase chain reaction
CDC	...	Center for Disease Control
PCR	...	Polymerase chain reaction
KWR	...	Water research institute, The Netherlands
MIQE	...	Minimum Information for Publication of Quantitative Real-Time PCR Experiments
PEG	...	Polyethylene glycol
qPCR	...	Quantitative PCR
USD	...	United States Dollar
TNA	...	Total nucleic acids
WHO	...	World Health Organization
MWCO	...	Molecular weight cut-off
ToBRFV	...	Tomato brown rugose fruit virus
PMMoV	...	Pepper mild mottle virus
(+)ssRNA	...	Positively charged single stranded RNA
(-)ssRNA	...	Negatively charged single stranded RNA

Chapter 1

Introduction

Viruses infect all known cellular organisms and represent the planet' s most abundant source of genetic material [1]. However, only a minute fraction has been characterized despite their pervasiveness, with less than 1% studied so far [2]. A major challenge in studying viruses remains that conventional investigative methods often allow the identification of only the targeted pathogen, resulting in frequent misses of the novel ones. Fortunately, advances in high-throughput sequencing (HTS)-based metagenomic research are beginning to change this, allowing researchers to probe deeply into the virome of various hosts and environments [1]. One such under-explored environment is water. Significant strides have been made in exploring marine virome [3] and wastewater virome [4]. However, fresh water, especially one dedicated to irrigation, is lagging. Progress in sample preparation techniques, sequencing technologies, and analytics solutions has significantly contributed to these developments in general. Nevertheless, investigations continue to be challenging due to differences in the properties of various matrices and the vast number of unknown viruses.

1.1 Wastewater-Based Epidemiology as a Tool for Monitoring SARS-CoV-2 Pandemic Trends

Despite the potential challenges of conventional methods in detecting novel viruses, they have been indispensable in large-scale studies in cases where the target has been previously sufficiently characterized. The idea of monitoring wastewater for the presence of pathogens has been used in the past [5]. Water-based epidemiology (WBE) was deployed in large cities worldwide early in the COVID-19 epidemic [6]. On the other hand, the vast number of research organizations working on protocol development and implementation extensively amplified the number of protocols and variations, adding to the lack of clarity [7]. Almost all analytical processes in published SARS-CoV-2 WBE experiments differed, from sample type and storage, concentration and extraction, to final detection and quantification [8], complicating the comparison of the results. Time, temperature, and viral structural shape (free RNA, intact particles, and partially degraded particles) influence SARS-CoV-2 RNA stability in wastewater during storage [9]. Before attempting to optimize the procedure for SARS-CoV-2 detection, it is essential to evaluate the stability of SARS-CoV-2 in wastewater. As viruses occur in low environmental concentrations, including wastewater, choosing a suitable concentration method is critical to improving detection sensitivity. Adsorption/extraction/elution, filtration, polyethylene glycol-based precipitation (PEG), and ultracentrifugation are the most common methods for concentrating viruses in water [10], with Centricon ultrafiltration units being the first to be used to concentrate SARS-CoV-2 on wastewater [6]. The global expansion of SARS-CoV-2 monitoring in wastewater

limited the supply of Centricon filtration units, prompting several companies to build wastewater-specific solutions [11]. Most previous protocols, however, have been created for non-enveloped viruses [12], so assessing concentration methods under local laboratory circumstances and unique restrictions should be considered when selecting a method. During the early stages of the pandemic, real-time quantitative reverse transcription PCR (RT-qPCR) was predominately developed to detect SARS-CoV-2, with two or more assays targeting different genes or parts of the gene simultaneously, published by the CDC, USA. Targeting the N1 and N2 genes was among the most commonly used in clinical samples [13]. The number of RT-qPCR assays has steadily risen to include divergent variants of concern [8]. Meta-analysis studies have revealed considerable variability between assays, emphasizing the importance of validating qPCR assays using appropriate reference materials and final target matrices while adhering to stringent checkpoints such as MIQE criteria [8]. The complexity of wastewater dictates performance evaluations on actual samples or samples as similar to real matrices as possible before WBE can be adopted on a broader scale. WHO has addressed these concerns in a preliminary advice document [14] that identifies considerations for employing WBE, emphasizing the importance of evaluating and adapting to the local situation.

1.2 Improving Tools for ToBRFV Outbreak Prediction Using Water Analysis in Closed Irrigation Systems

Plant viruses represent a relevant crop production risk and significant associated economic losses [15]. Their presence in water of various origins has also been known for decades [16]. However, the research regarding plant viruses and the role of water in their transmission has only recently been gaining traction, with several studies showing their prolonged infectivity [4], [17]. One of the better-researched genera is the *Tobamovirus* genus in the family *Virgaviridae*. Tobamoviruses have been detected in diverse environmental samples, including soils [18], clouds [19], and water sources such as drinking water [20], ballast water [21], irrigation systems [22], and raw and municipal wastewater [4]. In 2014, a new tobamovirus was detected in the fields in Israel [23] and named tomato brown rugose fruit virus (ToBRFV). Plants infected with the virus displayed characteristic mosaic signs, such as leaf constriction and brown rugose patches on fruits, resulting in significant yield losses [23]. Since its initial discovery, ToBRFV has rapidly spread worldwide [24] and is now a major pathogen in tomato cultivation. Studies have also confirmed that it also infects peppers [25]. Estimations of disease incidence in affected crops range between 50% and 100% [26], with yield reduction going up to 55% in infected plants [27]. The primary modes of transmission for ToBRFV are through infected seeds, the most common route of entry into greenhouses [28], and mechanical transmission, potentially leading to rapid dissemination within a greenhouse [29]. Like other tobamoviruses, ToBRFV has highly stable virions [30], which confer persistence in soil and water, as well as the potential to remain infectious for extended periods [18], [24]. Reports of infectious tobamoviruses in Slovenia' s influent and effluent wastewater samples have also been documented [4]. Notably, RNA of ToBRFV was detected in wastewater as early as 2017, prompting the inclusion in routine monitoring performed by the Ministry of Agriculture [31]. However, it was not detected in plant samples until 2021, raising concerns about potentially undetected presence, risks of waterborne transmission, the virus' origin, and utilization of such water sources for agricultural irrigation, particularly during water scarcity.

The global hydroponic system market is projected to reach USD 16.03 billion by 2028, and Europe represents the largest market for this industry, accounting for 41% of its share [32]. Tomatoes are one of the crops often grown in hydroponic systems, raising concerns that water-mediated transmission is especially relevant since the viruses from infected plants could potentially spread into the circulating growth medium. Consequently, there is a compelling need for simple and effective methods for ToBRFV water surveillance to detect potential releases from infected plants into water sources and identify the presence of ToBRFV and diseased plants in hydroponic settings. A correctly designed method would provide the cultivation facility with an appropriate early warning system. Such methods would greatly aid in understanding the dynamics of ToBRFV transmission through water and its potential impacts on agriculture and contribute to developing effective management strategies for this emerging plant virus.

1.3 Viromic Analysis of Environmental Irrigation Water

Despite the gradual progress in studying plant viruses in environmental waters, there is still only a limited number of studies focusing on this topic, especially regarding irrigation water and surface water near farms [33], [34]. Researchers in China have confirmed the presence and infectivity of several tobamoviruses, showcasing the possible risks associated with using such water to irrigate susceptible crop plants [35]. Agriculture and especially irrigation already amount to 70% of freshwater withdrawals, with the numbers expected to increase [36]. One of the proposed modalities to reduce the water use burden is introducing treated wastewater for irrigation, potentially increasing the risk of spreading plant viruses even further [4]. In recent years, HTS-based virome studies have enabled comprehensive investigations of virus diversity in environmental water samples [37]. A vast majority of studies are focused on marine viruses (like bacteriophages), including some in rather extreme conditions [3], [38]. Far fewer studies have looked at freshwater from the perspective of viruses [33], although the impact of irrigation of crop plants with potentially infected water cannot be understated [22], [39]. Baseline virome studies are crucial to assess virus presence and understand the potential risks associated with plant viruses in irrigation waters.

Furthermore, HTS-based analysis of environmental water samples provides large sequence datasets that offer more than just presence/absence data for different viruses, as exemplified during the COVID-19 pandemic, where wastewater monitoring helped track virus variants in populations across countries [40]. Expanding this framework to other water types can provide extensive information about the epidemiology of various viruses. Environmental monitoring of water used in, and around agricultural sites can help detect and anticipate the entry and spread of plant viruses in a specific area and enhance our understanding of their epidemiology. Enabling early detection and uncovering plant viruses' presence, diversity, and distribution of plant viruses in irrigation water enhances our understanding of their potential impacts on agricultural systems and facilities management strategies. Assessing the suitability of water analysis for detecting new and emergent viruses can serve as an effective early warning tool of great significance to the containment and control of an epidemic.

1.4 Aims of This Dissertation and Their Scientific Relevance

This dissertation has three main aims:

(1) Evaluation of different steps in the detection of SARS-CoV-2 in wastewater aimed at the implementation of a monitoring scheme,

(2) Investigation of suitable approaches for the detection of ToBRFV in water,

(3) Describe the virome of irrigation waters from Slovenia and describe newly discovered viruses

1.4.1 Evaluation of different steps in the detection of SARS-CoV-2 in wastewater aimed at the implementation of a monitoring scheme

Wastewater treatment plant influents can provide valuable information on the presence of pathogens, including SARS-CoV-2, in the sewer contributing population. Selecting the most suitable protocol will dictate a series of evaluations and comparisons of different methods and techniques used in the process. Investigated parameters will include the stability of SARS-CoV-2 in water/wastewater under different storage conditions and comparing various virus concentration methods to select the most efficient and reliable for high sample throughput. Different RT-qPCR assays for detecting SARS-CoV-2 will also be assessed to formulate a complete and robust method for wide-scale monitoring studies. Addressing these objectives aims to develop a reliable method for monitoring SARS-CoV-2 concentrations in wastewater, which can have important implications for public health surveillance and early detection and warning systems. The findings of this research will provide an overview of the most effective approaches for detecting SARS-CoV-2 in wastewater and will contribute to the advancement of wastewater-based monitoring as a valuable tool in the fight against infectious diseases.

1.4.2 Optimisation of suitable approaches for the detection of ToBRFV in water

ToBRFV has quickly spread around the globe and has caused significant economic losses, especially in hydroponic systems. The second aim of this dissertation is to investigate the various approaches for analyzing ToBRFV in water samples, including testing water directly with RT-qPCR or combined with concentration and nucleic acid extraction steps, to improve sensitivity. Optimizing the protocol will enable a better understanding of the dynamics of ToBRFV detection in hydroponic nutrient solutions and may pave the way for water testing to become the primary screening method for ToBRFV in hydroponic setups. By improving the ToBRFV detection in water, this study will contribute with tools to study water as a transmission route of this virus in hydroponic-like systems. In addition, it may help to identify more effective control measures and preventive strategies to manage the spread of ToBRFV, a devastating tomato pathogen, in hydroponic production systems.

1.4.3 Describe and evaluate the virome of irrigation waters from Slovenia and describe newly discovered viruses

The quality of irrigation water from the perspective of plant viruses remains understudied. The third aim of this dissertation is to provide an overview of the plant virus composition of various types of irrigation water, which can aid in identifying potential co-occurrences of viruses in water samples and plant crops. Additionally, we anticipate the discovery of new viruses in the examined samples, which will contribute to the current understanding of plant viral occurrence and variety in the analyzed regions. By detecting and characterizing viruses in irrigation water, we can monitor their occurrence and spread in the region, allowing for early detection before a severe outbreak occurs. With the ability to monitor a vast area, we can gain valuable insights into the types of viruses to which plants are exposed and develop strategies to mitigate their spread.

1.5 Hypothesis

- SARS-CoV-2 free RNA shows varying stability in wastewater depending on storage conditions (time/temperature), while viral particles are more stable than free SARS-CoV-2 RNA.
- Ultrafiltration (Centricon) and direct TNA extraction (Promega) are relatively simple concentration methods that efficiently concentrate SARS-CoV-2 from wastewater influent with sufficient throughput.
- N1 and N2 (CDC USA) qPCR assays in combination with FAST Virus mastermix will perform best for detection and quantification of SARS-CoV-2.
- ToBRFV can be detected in the water of a hydroponic growth setup early after infection using only RTq-PCR.
- Concentration with Centricon and extraction of nucleic acids improves the sensitivity of the detection, and viral genomes can be detected for a prolonged period of time.
- Diversity and abundance of plant viruses in environmental irrigation waters are influenced by the source of sampled water.
- Metagenomics analysis of water can be used for detection of novel plant viruses.

1.6 Publications Included and Candidate's Contributions

In the paper listed in Section 2.1 (Evaluation of methods and processes for robust monitoring of SARS-CoV-2 in wastewater), we evaluated the challenges and considerations of using the WBE approach for monitoring SARS-CoV-2 in wastewater, focusing on sample storage, concentration and target detection. In order to optimize concentration protocols and compare several RT-qPCR assays, we assessed virus stability in wastewater. The findings highlighted the importance of carefully evaluating and adapting each step in the WBE approach and provided valuable insights for other laboratories implementing similar methods. The candidate is the first author of the publication. She participated in the study design and performed most laboratory work and data analysis, including manuscript preparation.

The paper listed in Section 2.2 (Tomato brown rugose fruit virus in aqueous environments – survival and significance of water-mediated transmission) is a collaborative work in which we examined the detection of ToBRFV in water. The initial detection was confirmed in irrigation water samples from various locations in Slovenia. Subsequently, we

investigated different approaches for detecting the virus in water and under laboratory conditions. Our findings suggest positive detection can be obtained after nine days post-infection of tomato plants, in greenhouse experiments simulating a hydroponic growing system. The candidate is the co-author of this paper. She has designed and performed the experiments related to the section of the article described above, applying different concentration methods for environmental irrigation water samples and for water samples from a hydroponics-like setup, in addition to subsequent extractions and RT-qPCR analysis as well as overall data analysis. She finally drafted the corresponding part of the manuscript.

Lastly, the paper listed in Section 2.3 (Virome analysis of irrigation water sources provides extensive insights into the diversity and distribution of plant viruses in agroecosystems) deals with investigating the presence of plant viruses in environmental water bodies using qPCR and HTS. The results showed a high diversity of plant viruses across the samples, indicating that the water source plays a role in the virome composition. Additionally, we identified seven new plant viruses showing the utility of environmental water testing for plant virus ecology and new species discovery. We compared the virome of water and plants sampled at the exact locations. The co-occurrence of plant viruses in both datasets underscores the importance of water testing for early detection and surveillance of plant pathogens. The candidate is the first author of the publication. She was involved in experimental design, carried out field sampling, and laboratory work (i.e., sample preparation, concentration, RNA isolation and preparation for sequencing), analyzed the data and wrote the manuscript draft.

Chapter 2

Scientific Publications

2.1 Evaluation of Methods and Processes for Robust Monitoring of SARS-CoV-2 in Wastewater

Olivera Maksimović Carvalho Ferreira, Živa Lengar, Zala Kogej, Katarina Bačnik, Irena Bajde, Mojca Milavec, Anže Županič, Nataša Mehle, Denis Kutnjak, Maja Ravnikar, Ion Gutierrez Aguirre

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This paper focuses on the challenges and considerations of implementing a WBE approach for monitoring SARS-CoV-2 in wastewater. Ensuring reliable results demands a thorough evaluation of each critical step in the analysis. Although institutions such as the WHO, CDC (USA) provide guidelines for implementing WBE for SARS-CoV-2 monitoring, each laboratory needs to adapt them to their local conditions. These steps are especially relevant in the early stages when such guidelines are unavailable, as was the case during the first several months of the COVID-19 pandemic. Our study focused on three critical aspects of the procedure: sample storage, sample concentration, and target detection. Sample storage and processing time are critical factors that can impact the integrity of the target due to possible degradation. We conducted experiments to assess the stability of RNA extracted from positive clinical samples versus the stability of thermally inactivated SARS-CoV-2 virus in wastewater at different temperatures and time frames. Our results showed naked RNA degraded faster in wastewater than inactivated virus particles.

Furthermore, storage in the freezer (-20°C or -80°C) increased Cq values in the first 24 hours, indicating that freeze-thawing cycles during sample analysis could significantly impact RNA integrity more than storage time. Next, we compared several approaches to the concentration of wastewater and performance. Based on efficiency, we selected the protocol using Centricon Plus-70 Centrifugal Filters coupled with a commercial RNA extraction kit. The efficacy of the concentration protocol was confirmed based on the reduction of the Cq value after the concentration step. We also compared this system to a semi-automated kit from Promega and found that they were comparable in efficiency, with the Promega kit being superior in throughput. Lastly, we compared several RT-qPCR mastermixes based on their limit and reproducibility of detection. The fine-tuning of the procedure provided the protocol for the national Slovenian monitoring of SARS-CoV-2 in wastewater. Our findings highlight the importance of careful evaluation and adaptation of each step before implementing the WBE approach, providing valuable insights for other laboratories looking to implement similar methods in their local conditions, thus contributing to the advancement of WBE as a reliable tool for epidemiological surveillance of infectious diseases.

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ORIGINAL PAPER

Evaluation of Methods and Processes for Robust Monitoring of SARS-CoV-2 in Wastewater

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Abstract
The SARS-CoV-2 pandemic has accelerated the development of virus concentration and molecular-based virus detection methods, monitoring systems and overall approach to epidemiology. Early into the pandemic, wastewater-based epidemiology started to be employed as a tool for tracking the virus transmission dynamics in a given area. The complexity of wastewater coupled with a lack of standardized methods led us to evaluate each step of the analysis individually and see which approach gave the most robust results for SARS-CoV-2 monitoring in wastewater. In this article, we present a step-by-step, retrospective view on the method development and implementation for the case of a pilot monitoring performed in Slovenia. We specifically address points regarding the thermal stability of the samples during storage, screening for the appropriate sample concentration and RNA extraction procedures and real-time PCR assay selection. Here, we show that the temperature and duration of the storage of the wastewater sample can have a varying impact on the detection depending on the structural form in which the SARS-CoV-2 target is present. We found that concentration and RNA extraction using Centricon filtration units coupled with Qiagen RNA extraction kit or direct RNA capture and extraction using semi-automated kit from Promega give the most optimal results out of the seven methods tested. Lastly, we confirm the use of N1 and N2 assays developed by the CDC (USA) as the best performing assays among four tested in combination with Fast Virus 1-mastermix. Data show a realistic overall process for method implementation as well as provide valuable information in regards to how different approaches in the analysis compare to one another under the specific conditions present in Slovenia during a pilot monitoring running from the beginning of the pandemic.

Keywords SARS-CoV-2 · Method development · Detection · Wastewater · Monitoring

Introduction

The COVID-19 pandemic, caused by SARS-CoV-2, started in December 2019 in Wuhan, China. There are several modes of transmission including respiratory droplets, aerosols and direct contact with surfaces (Santarpia et al., 2020). Additionally, the virus is also shed through faeces and urine (Zhang et al., 2020), although, to date, faecal–oral route of transmission has not been confirmed (Sobsey et al., 2021). A meta-analysis showed that shedding of SARS-CoV-2 through faeces is present in 32–52% of symptomatic cases, and 15–44% of patients continue to shed the virus in the stool for additional 7 days after the loss of detectable viral RNA in their upper respiratory tract (Zhang et al., 2021a, 2021b). Reports indicate that asymptomatic individuals also shed the virus via the gastrointestinal tract (WHO, 2019, 2020). In addition to faeces, similar levels of shedding are also present in the urine of patients (Jones et al., 2020). In urban environments with well-developed communal infrastructure, most of the faeces and urine eventually enter the local sewage system and end up in wastewater treatment plants (WWTP). Monitoring of wastewater for the presence

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and the concentration of SARS-CoV-2 RNA has been shown to provide information about the scale of the epidemic in the population covered by specific WWTP. Wastewater-based epidemiology (WBE) has been successfully deployed in the past, e.g. for poliovirus outbreaks in Borno State in Nigeria (Deshpande et al., 2003). The first successful detections of SARS-CoV-2 in wastewater motivated a quick rollout of WBE in large cities (Medema et al., 2020). In parallel with the increase in the number of research groups working on WBE implementation, a variety of methods for collecting and processing the samples became available (Pecson et al., 2021). Currently, SARS-CoV-2 WBE studies vary with respect to nearly all steps of analysis, from sample type and storage through concentration and extraction method to final detection and quantification (Ahmed et al., 2022, Bivins et al., 2021). For sample storage, temperature and time are probably the most important factors that determine the stability of SARS-CoV-2 RNA in wastewater. Negative effects of ambient temperatures on the stability of the virus were reported relatively early (Ahmed et al., 2020a, 2020b), but the potential impact of prolonged cold storage and freeze/thaw cycles on the stability of SARS-CoV-2 in water samples also needs to be considered. Recent evidence in the literature suggests that freezing and thawing, as well as prolonged storage of the sample at − 20 °C, leads to a reduction in the measured RNA concentration of SAR-CoV-2 or surrogates (Alygizakis et al., 2021; Kaya et al., 2022; Steele et al., 2021). As viruses are generally present at low concentrations in environmental samples, such as wastewater, the selection of an appropriate concentration method is an important step to increase the sensitivity of detection. The main approaches typically used for concentration of viruses in water include: adsorption–extraction/elution, ultra-centrifugal filter devices, polyethylene glycol (PEG)-based precipitation and ultracentrifugation (Pulicharla et al., 2021). Nearly all of these methods were attempted or implemented in the WBE of SARS-CoV-2 (Jafferali et al., 2021; Kocamemi et al., 2020; Medema et al., 2020; Westhaus et al., 2021). For the nucleic acid extraction step, either automated magnetic methods (Kocamemi et al., 2020) or silica membrane spin columns approaches (Ahmed et al., 2020a, 2020b) are the most widely used approaches to purify the viral RNA from the concentrated wastewater samples. As most of these protocols have been developed for non-enveloped viruses, such as enteroviruses (Rusiñol et al., 2020), the protocols should be selected based on an evaluation in local laboratory conditions, as this will accommodate specific limitations introduced by the local wastewater and working conditions. The most widely used method for detection of SARS-CoV-2 in wastewater is real-time quantitative, reverse transcription PCR (RT-qPCR), with two or more assays employed simultaneously, targeting different parts of the same gene or different genes, to minimize the possibility of a false-negative or false-positive result (Zhang et al., 2021a, 2021b). To date an increasing number of different RT-qPCR assays targeting various regions of the virus, have been made available, including assays specifically designed to detect different variants of concern (Alygizakis et al., 2021; Bivins et al., 2021; Yaniv et al., 2021). In the literature there are increasing comparison studies and meta-analyses of all available RT-qPCR assays for detection of SARS-CoV-2 in wastewater, that have exposed the high variability between assays used for wastewater surveillance and call for harmonization efforts and adoption of quality checkpoints as, i.e. adoption of MIQE guidelines (Bivins et al., 2021; Zhu et al., 2021). The complexity of wastewater and its likely impact on the results indicate that any performance comparisons, before deployment of a WBE approach targeting SARS-CoV-2, should be done on real wastewater samples before making the final method choice. This and other important issues have been recently reviewed (Ahmed et al., 2022). On the global level, WHO has addressed these issues in the Interim Guidance document (WHO, 2022), which details points of consideration throughout the process of establishing WBE at a given location. Although it does not provide a recommended protocol, it does emphasize the need to evaluate and adjust to local conditions, which is conveniently described in several real case studies.

Our aim in this study was to describe the optimization steps that led up to the onset of a robust SARS-CoV-2 monitoring in wastewater in Slovenia. It includes an assessment of the influence of wastewater storage conditions in the SARS-CoV-2 detection by RT-qPCR; the applicability of different concentration protocols on real wastewater samples and a more detailed comparison of the best-performing ones; and finally the performance of different RT-qPCR assays in conjunction with different commercial mastermixes.

Materials and Methods

Thermal Stability Evaluation

To evaluate the impact of sample storage temperature and freeze–thaw cycles on the detection of SARS-CoV-2 RNA, we tested the stability of two different SARS-CoV-2 materials spiked in wastewater. Used materials included RNA from positive controls provided by European Virus Archive Global (EVA-GLOBAL) (concentration not calculated) and lyophilized, thermally inactivated SARS-CoV-2 virus propagated in cell culture provided by the Institut für Qualitätssicherung in der Virusdiagnostik, Berlin, Germany as part of INSTAND External Quality Assessment schemes (reconstituted following provider instructions to a concentration range of $4–11 \times 10^9$ copies/mL, as reported by provider). These two materials were evaluated separately under

the same experimental conditions by spiking either RNA from positive controls (EVA-GLOBAL) or the thermally inactivated virus into SARS-CoV-2 free composite wastewater collected in 2018 when SARS-CoV-2 was not present in Slovenia. Replicates with dilutions (1:10) of the spike materials in wastewater were stored at one of the selected temperatures/time sets that included + 4 °C, − 20 °C and − 80 °C, over a time span of 0 h to 7 days (see Supplementary Information; Table S1). For each temperature/time and spike material, the evaluation was done in three biological replicates. All extractions were done using QIAmp Viral RNA Mini Kit (Qiagen, USA, 52,906), following the manufacturer's instructions with adjustments. Adjustments included double elution in 2 × 40 µL of nuclease-free water (Sigma-Aldrich, 3098) heated to 65 ℃ Each extraction batch was accompanied with at least one negative control of extraction (NCE) consisting of nuclease-free water added instead of the sample. Two ng of Luciferase Control RNA (Promega, USA, L4561) was spiked into each sample and NCE prior to extraction, to confirm the success of the extraction and to account for any inhibitory effects during the PCR reaction (data not shown). Viral RNA in the samples was determined by RT-qPCR with N1 and N2 assays as described in Sect. Evaluation of RT-qPCR Assay/Mastermix Combination. Cq values were plotted using the ggplot2 package in RStudio (v.1.2.1106) and visually inspected for noticeable trends.

Sample Concentration Method Assessment

Concentration with Centricon 70-Plus Centrifugal Filters

Prior to processing any real wastewater samples, we performed a small evaluation trial on Centricon Plus-70 Centrifugal Filters with 10 kDa molecular weight cut-off (MWCO) (Millipore, Germany, UFC701008), the first method reported for detection of SARS-CoV-2 in wastewater (Medema et al., 2020). Two different spike materials (RNA from positive controls (EVA-GLOBAL) and thermally inactivated virus, described in Sect. Thermal Stability Evaluation) were used to confirm the performance of the filters. Each spike material was prepared as a 1:10 dilution in SARS-CoV-2 free composite wastewater influent sample and tap water. Concentration protocol was based on Medema et al., 2020, with some modifications. One hundred millilitres of the sample were centrifuged in two 50 mL Falcon tubes (Corning, USA 352,070) on 3200 × g for 50 min without break, using a swing-out rotor bucket (Eppendorf S-4–72) at room temperature. The supernatant was then filtered through the Centricon Plus-70 Centrifugal Filter unit in two consecutive rounds (50 mL + 50 mL) on 3200 × g with break and acceleration on ambient temperature, for 15 min or until the complete sample volume had passed through the filter (most often an additional 15 min cycle is enough). Collection of the concentrate was done by upside down centrifugation of the filter units on 1000 × g for 2 min, on ambient temperature. RNA from concentrated and non-concentrated fractions was extracted immediately using QIAmp Viral RNA Mini Kit (Qiagen, USA, 52,906) as described in Sect. Thermal Stability Evaluation and stored at − 80 °C. Both the fraction collected before and the fraction collected after concentration, for each spike/matrix combination, were tested with RT-qPCR in triplicate using E assay (BHQ probe) as described in Sect. Evaluation of RT-qPCR Assay/Mastermix Combination. Additionally, they were tested with RT-qPCR assays for Luciferase Control RNA (Toplak et al., 2004), used as an RNA extraction and inhibition control (data not shown) and, for samples with wastewater as the matrix, also with pepper mild mottle virus (PMMoV) assay (Haramoto et al., 2013; Rački et al., 2014), to assess if this known faecal indicator, despite being so different structurally, is concentrated in the used setup with similar efficiency as the SARS-CoV-2, and therefore can be used as concentration efficiency control of the concentration procedure in SARS-CoV-2 surveys in wastewater. All assays were done in accordance with the description in Sect. General Technical Description for RT-qPCR, except the PMMoV assay which was performed in 2 technical replicates instead of 3. Concentration efficiency was evaluated based on the reduction in average Cq value before and after the concentration step.

To confirm the reproducibility of concentration with Centricon Plus-70 Centrifugal Filters 3 replicates from concentration to detection step of wastewater from WWTP Domžale-Kamnik were tested, using the same concentration and extraction protocols as described in the previous paragraph. Samples were then tested with RT-qPCR with N1 and N2 assays (Sect. Evaluation of RT-qPCR Assay/Mastermix Combination), Luciferase Control RNA (Toplak et al., 2004), (data not shown) and PMMoV assay (Haramoto et al., 2013; Rački et al., 2014). Standard deviation within both technical and all replicates was calculated with Excel 2010 built-in functions.

Evaluation of Additional Concentration Methods

Since the pandemic negatively affected the reliability of supply chains, in order to select a backup concentration procedure, we decided to evaluate other methods used for concentration of viruses from different types of water samples. A total of 13 samples from 7 different WWTP were included in the screening of concentration methods (Supplementary Information; Table S2 and Table S4). All WWTP samples were collected as a 24 h-flow-dependent composite sample of influent wastewater, volumes ranging from 200 mL to 2 L depending on the method (see Table 3). Samples were transported from the sampling point to the laboratory in cooling

boxes. Samples were collected in November and December of 2020 and from January to April 2021. All samples were stored at 4 °C for a maximum of 48 h before processing. See Table 3 and Supplementary Information Table 4 for details on the processing of samples included in this experiment. Individual protocols included in the experiment are listed below and a schematic summary of each protocol is available in Supplementary Information Figure S1.

Centricon Plus-70 Centrifugal Filters with 10 kDa molecular weight cut-off (MWCO) (Millipore, Germany, UFC701008), were used to concentrate wastewater samples following the protocol described in 2.2.1. RNA from fractions collected before and fractions collected after concentration were extracted immediately using QIAmp Viral RNA Mini Kit (Qiagen, USA, 52,906) as described in Sect. Thermal Stability Evaluation and stored at − 80 °C.

Vivacell 100, 30,000 MWCO PES (Sartorius, Germany, VC1022) were used in an adapted version of the protocol used for Centricon Plus-70 Centrifugal Filters. 100 mL of the sample was centrifuged in two 50 mL Falcon tubes (Corning, USA, 352,070) on 3200 × g for 50 min without break, using a swing rotor bucket, on ambient temperature. The supernatant was pooled and then filtered through the Vivacell 100 unit on 3200 × g for 20 min, with break and acceleration on ambient temperature, or longer until the complete sample volume had passed through the filter. Fractions collected before and fractions collected after concentration were extracted immediately using QIAmp Viral RNA Mini Kit (Qiagen, USA, 52,906) as described in Sect. Thermal Stability Evaluation and stored at − 80 °C.

CIMmultus™ monolithic columns (BIA Separations, Slovenia) of various sizes (1 mL, 8 mL) and chemistries were evaluated using adapted protocols (Bačnik et al., 2020; Gutiérrez-Aguirre et al., 2011) (see Supplementary Information Table S3 for details listed in this paragraph). In each case, a different volume of wastewater sample (from 600 to 2000 mL) was pre-filtered through filter paper and cellulose acetate filter membrane with a pore size of 0.8 µm (Sartorius, Germany, 11,104–142) and loaded onto the corresponding preconditioned column (following manufacturer's recommendations) using fast protein liquid chromatography system AKTA Purifier 100 (GE Healthcare, USA). The flow rate was adjusted to keep the backpressure stable at a fixed limit. After a wash step using 20 × column volumes, an elution step was performed with different volumes (8 mL to 20 mL) of a high ionic strength elution buffer. The elution peak was monitored by measuring UV absorption at 280 nm and conductivity. In between samples, columns were sanitized with 1 M NaOH for 120 min. Collected fractions included: sample before filtration—raw (R), sample after filtration—load (L), flow-through of the sample through the column (FT), wash step (W) and elution (E), all of which were extracted immediately using QIAmp Viral RNA Mini Kit (Qiagen, USA, 52,906) as described Sect. Thermal Stability Evaluation and stored at − 80 °C.

Concentration using polyethylene glycol (PEG) was performed using a protocol made publicly available by IDEXX Laboratories (IDEXX, 2020) without a pasteurization step. Fractions collected before and fractions collected after concentration were extracted immediately using QIAmp Viral RNA Mini Kit (Qiagen USA, 52,906) as described in Sect. Thermal Stability Evaluation and stored at − 80 ℃.

Concentration using skimmed milk flocculation was based on the protocol described in Calgua et al., 2008, with adaptations. Sample (200 mL) was left to stir at room temperature for 6 h and then centrifuged at 3200 × g for 30 min on ambient temperature. The supernatant was carefully discarded and the pellet suspended in 800 µL of phosphate buffer. Fractions collected before and fractions collected after concentration were extracted immediately using QIAmp Viral RNA Mini Kit (Qiagen, USA, 52,906), as described in Sect. Thermal Stability Evaluation and stored at − 80 °C.

For direct RNA capture, Wizard Enviro TNA Kit (PROMEGA, USA, A2991) and Maxwell RSC Enviro TNA Kit (PROMEGA, USA, AS1831) were used according to manufacturer's instructions. Both kits already include an RNA extraction step (Wizard Enviro TNA Kit relies on silica spin columns and Maxwell RSC Enviro TNA Kit on semi-automated magnetic beads system);

Each extraction batch was accompanied by at least one negative control of extraction (NCE) consisting of nuclease-free water added instead of the sample. Also, in order to evaluate extraction efficacy and potential inhibitory effects, each sample and the NCE were spiked with 2 ng of Luciferase Control RNA (Promega, USA, L4561) at the beginning of extraction. Extracted RNA was stored on −80 °C until further analysis.

RNA extracts from concentrated and non-concentrated fractions, for each described protocol, were tested with RT-qPCR (N1 and N2 assays). Additionally, they were tested with RT-qPCR assays for PMMoV (Haramoto et al., 2013; Rački et al., 2014), which served as an additional indicator of each method's concentration efficiency, and Luciferase Control RNA (Toplak et al., 2004), used as an RNA extraction and inhibition control. Similar Cq values obtained for luciferase in all analysed samples excluded the presence of inhibition. RT-qPCR reactions were performed as described in Sects. Evaluation of RT-qPCR Assay/Mastermix Combination and General Technical Description for RT-qPCR. Concentration efficiencies for different approaches were evaluated based on the reduction in average Cq value before and after the concentration step.

Both in the screening for concentration methods as well as in the comparison among Centricon Plus-70 and Wizard enviro TNA kit, we report Cq value reductions after

concentration by each method of the same given water sample. The starting and concentrated volumes are shown in Table 3. Recoveries were not calculated, and they could differ due to different start and end volumes for each method. However, our aim was to select the method resulting in the lowest Cq value after the concentration of the same sample, regardless of the recovery, as such method would result in the highest sensitivity of the RT-qPCR quantification.

Evaluation of RT-qPCR Assay/Mastermix Combination

In order to select an assay, which would allow sensitive detection and accurate quantification of SARS-CoV-2 RNA in wastewater, we tested 4 primer/probe sets (assays) available in the early stages of the pandemic for detection of SARS-CoV-2 genome: RdRp (Corman et al., 2020), E (Corman et al., 2020) with two different quenchers, N1 (CDC, USA, 2020) and N2 assays (CDC, USA, 2020). Details regarding primer and probe sequences with quencher information are available in Supplementary Information, Table S5.

Evaluation of the performance of all RT-qPCR assays was done on dilution series of thermally inactivated SARS-CoV-2 virus (described in Sect. Thermal Stability Evaluation). The reconstituted virus was used as a starting point for serial dilutions (scheme in Supplementary Information; Table S6). Dilutions were done in a 24h composite influent wastewater sample that had previously tested negative for the presence of SARS-CoV-2. RNA was extracted from each dilution using QIAmp Viral RNA Mini Kit as described in Sect. Thermal Stability Evaluation RT-qPCR analysis of serial dilutions was done in triplicates for all 5 SARS-CoV-2 target assays using TaqMan Fast Virus 1-Step Master Mix (Thermo Fisher Scientific, USA, 4,444,432). Assays were compared to one another based on the standard deviation between the three replicates in the lowest detected dilution (data not shown).

The second phase of the performance evaluation included the assessment of three commercial mastermixes. As reagent shortages were expected it was important to understand how the change of mastermix could impact the results. Dilutions of thermally inactivated SARS-CoV-2 virus in wastewater, described in the previous section, were tested using N1 and N2 RT-qPCR assays using the following mastermix kits, all used according to manufacturer's recommendations: TaqMan Fast Virus 1-Step Master Mix (Thermo Fisher Scientific, USA, 4,444,432), RNA UltraSense™ One-Step Quantitative RT-PCR System (Thermo Fisher Scientific, USA, 11,732,927) and AgPath-ID™ One-Step RT-PCR Reagents (Applied Biosystems, USA, AM1005). The assessment was done in two independent serial dilutions with different dilution steps (Supplementary Information; Tables S6 and S7). For each assay/mastermix the limit of quantification (LoQ) was defined as the concentration of the highest dilution at which the variance coefficient of measured Cq values between three technical replicates was below 0.5, and the limit of detection (LoD) was set to be the concentration of the dilution, at which at least one out of three technical replicates were positive. When a replicate measurement was clearly different from the other two replicates within the triplicate, it was not considered in the calculations. The selection of the mastermix was based on the sensitivity and accuracy of detection on the highest dilution.

General Technical Description for RT-qPCR

Each RT-qPCR reaction was performed with 2 µL of extracted RNA per reaction in a total reaction volume of 10 µL. RT-qPCR analysis was done using Applied Biosystems™ 7900 HT Fast PCR Instrument (Thermo Fisher Scientific, USA, 4,329,001) with cycling parameters as recommended by the individual mastermix manufacturer. Data were analysed using SDS 2.4.2 Standalone software with automatic setting of the baseline and threshold for RdRp and E assays and manual threshold set up for N1 (0.11) and N2 (0.12) assays. Each amplification plot was checked manually and the result was considered positive if it produced an exponential amplification curve distinguishable from negative controls, and in such cases, Cq values were calculated. Every RT-qPCR plate included positive controls and a no template control. Positive controls were synthetic single-stranded RNA (ssRNA) fragments of SARS-CoV-2 (EC-JRC, EURM-019), 1.000x (expected Cq 21) and 100.000x (expected Cq 28) diluted in nuclease-free water. Results from positive controls were monitored within a control chart, which indicates if the value was within a predefined range (± 3Cqs, data not shown).

Results

Thermal Stability Evaluation

The RT-qPCR detection (Cq values obtained with both N1 and N2 assays) of thermally inactivated virus spiked in wastewater remained stable at each temperature/time (Fig. 1b, d and f). For spiked SARS-CoV-2 RNA from positive controls (EVA-GLOBAL) we observed rapid degradation in wastewater, especially at 4 °C, where the Cq values increased over 6 Cq values already after 24 h (Fig. 1a), but also at − 20 °C and − 80 °C, where we observed an increase of 2 to 3 Cq values. The observed

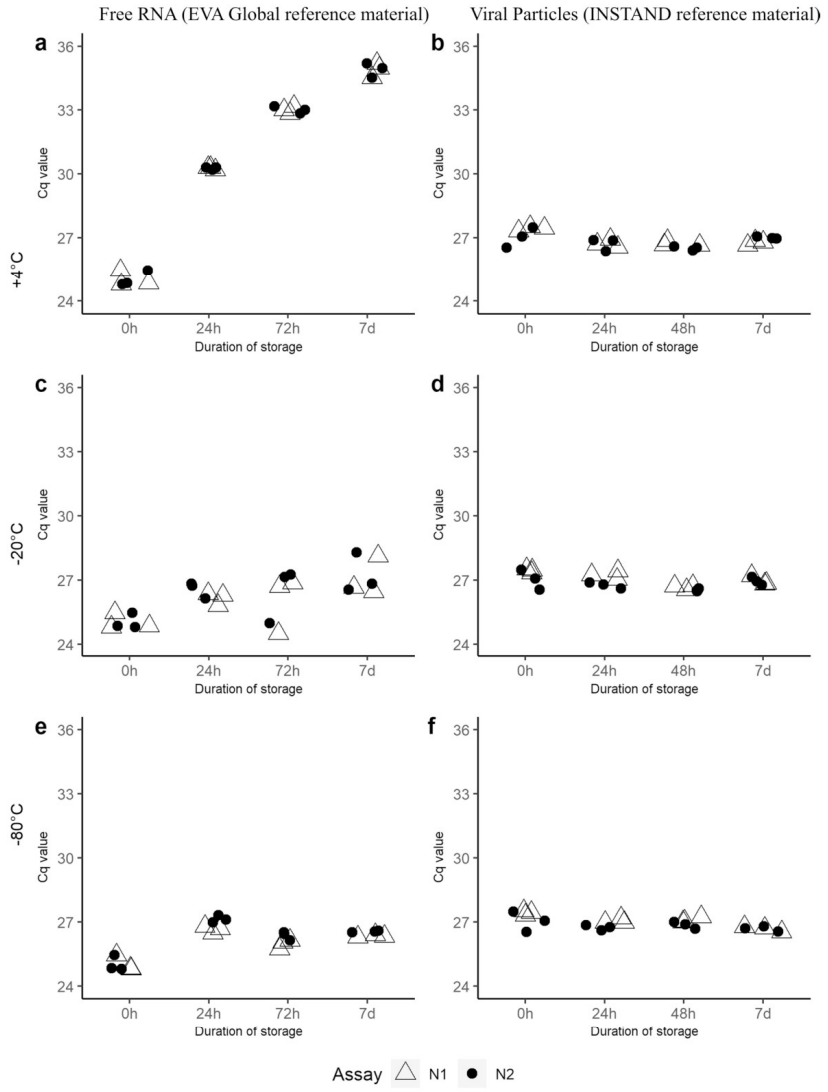

Fig. 1 Overview of stability evaluation for wastewater spiked with RNA from positive controls (EVA-GLOBAL) stored for different time periods on + 4 °C (**a**); − 20 °C (**c**) and − 80 °C (**e**) and wastewater spiked with the thermally inactivated virus for different time periods on same temperatures of + 4 °C (**b**); − 20 °C (**d**) and − 80 °C (**f**). Each incubation was repeated 3 times in parallel and the points show average Cq values from RT-qPCR ($n = 3$) for both N1 and N2 assays. Figure created in RStudio v.1.2.1106

increase in Cq values remained on average similar in all tested storage times (Fig. 1c, e).

Sample Concentration Method Assessment

Centricon 70-Plus Centrifugal Filters

In the first experiment with Centricon Plus-70 Centrifugal Filters both synthetic RNA and thermally inactivated virus were concentrated similarly from 100 mL of spiked tap water to 1 mL, with an average Cq reduction of 5.1 (synthetic RNA) and 5.6 (thermally inactivated virus) (Table 1). In ideal conditions (100% efficiency of the concentration method) the viral concentration should have increased by 100-fold, meaning a Cq decrease of 6.68 (at 100% amplification efficiency of RT-qPCR). The observed Cq reductions thus confirm that Centricon 70 Plus is a suitable tool for concentration of both naked RNA and packed viral particles. Synthetic RNA spike showed different behaviour in tap water and wastewater. Looking at the Cq values before the concentration step, we can see an increase in values in the wastewater, likely as a consequence of faster degradation in comparison to tap water, which seemed to happen at a larger extent with RNA from positive controls (EVA-GLOBAL) (previous section). This is confirmed by the results with synthetic RNA after concentration in wastewater, by that time we already observed a complete loss of a detection signal, which was not due to the inhibition of PCR reaction as confirmed by the lack of inhibition in the same sample spiked with viral particles (Table 1) and also from Cq values obtained for Luciferase Control RNA (internal amplification control) assay, which were comparable in all samples (data not shown). Both, the spiked thermally inactivated virus and the naturally occurring PMMoV, which has been proposed to be used as a measure of the faecal contribution in WBE analysis (Kitajima et al., 2018), were concentrated similarly in wastewater samples with Cq reductions of 4.9 (synthetic RNA) and 5.2 (thermally inactivated virus) (Table 1).

The procedure (from concentration step to detection step) showed good reproducibility in concentrating SARS-CoV-2 and PMMoV from a real wastewater influent sample based on the low intra- and inter- replicate standard deviation of Cq values obtained in three concentration rounds (Table 2).

Evaluation of Alternative Concentration Methods

When looking at the range of tested concentration protocols, with most of them we were able to concentrate naturally occurring PMMoV (Cq reduction ranging from 2.5 to 6.0), except for the positively charged CIMmultus™ monolithic columns (BIA Separations) with SO3 chemistry (Table 3). In most tested protocols we were concentrating SARS-CoV-2 less efficiently than PMMoV. Skimmed milk-based protocol and Vivacell 100, 30,000 MWCO PES (Sartorius, Germany, VC1022) resulted in almost no Cq reductions for N1 and N2 assays (Table 3). Along with Centricon Plus-70 Centrifugal Filters, the Wizard Enviro TNA Kit (PROMEGA, USA, A2991) outperformed all other methods for simultaneous concentration of both targets, with Cq reductions ranging from 4.9 to 6.9 for SARS-CoV-2 and Cq reduction of 4.3 to 6.0 for PMMoV (Table 3) on starting volumes of 40–100 mL. In terms of price per sample, all methods except ones relaying on CIMmultus™ monolithic columns (BIA Separations) have acceptable price points below 300€ per sample. Similarly, the time required to process a batch is within the timeframe of one working day. However looking at the number of samples that can be processed simultaneously within one batch Wizard Enviro TNA Kit (PROMEGA, USA, A2991) provides the best platform for high

Table 1 Cq values and reduction of average Cq values obtained by concentration with Centricon Plus-70 Centrifugal Filters of tap water and wastewater spiked with either synthetic RNA or thermally inactivated virus

Water type	Tap water		Wastewater		Wastewater	
Target	SARS-CoV-2; E-gene		SARS-CoV-2; E-gene		PMMoV	
Spiked with	Synthetic ssRNA	Thermally inactivated virus	Synthetic ssRNA	Thermally inactivated virus	Synthetic ssRNA	Thermally inactivated virus
Before concentration (Cq)	22.4	27.5	27.1	28.0	26.0	26.4
	22.7	27.5	27.2	28.0	25.9	26.1
	22.6	27.8	27.6	27.9	–	–
After concentration (Cq)	17.7	22.1	Undetected	23.1	20.1	21.1
	17.7	21.9	Undetected	23.0	20.2	21.0
	16.8	21.9	Undetected	23.1	–	–
Average Cq reduction	5.1	5.6	N/A	4.9	5.8	5.2

Cq values presented for E assay (BHQ quencher) and PMMoV

Table 2 Cq values (shown in triplicate for N1, N2 and PMMoV assays) obtained for each of the three independent replicate concentration/detection rounds done in the same wastewater influent sample

Replicate/Target	Cq (SARS-CoV-2; N1)	Intra-replicate standard deviation	Cq (SARS-CoV-2; N2)	Intra-replicate standard deviation	Cq (PMMoV)	Intra-replicate standard deviation
Replicate 1	31.3		31.7		21.9	
	32.3	0.4	31.3	0.2	21.4	0.4
	31.8		31.5		21.1	
Replicate 2	30.9		31.3		21.3	
	31.1	0.1	31.7	0.2	20.8	0.3
	31.2		31.3		20.6	
Replicate 3	31.7		31.5		20.8	
	31.4	0.4	31.2	0.1	20.8	0.0
	30.7		31.4		20.8	
Inter-replicate standard deviation among all replicates	0.5		0.2		0.4	

Intra- and inter-replicate standard deviations are also shown

Table 3 Concentrations achieved by different methods from the same wastewater sample expressed as a reduction in average Cq value, from RT-qPCR replicates (marked as ΔCq; $n=3$) before and after concentration for each performed assay (N1, N2, PMMoV)

Concentration method	Start volume (mL)	End volume (mL)	ΔCq N1	ΔCq N2	ΔCq PMMoV	Samples per batch	Price per sample	Time per batch
Centricon Plus-70 Centrifugal Filter *Experiment 1*	100	0.5	5.1	5.9	5.7	8	245 €	7 h
Centricon Plus-70 Centrifugal Filter *Experiment 2*	100	0.5	6.2	5.7	4.3			
Centricon Plus-70 Centrifugal Filter *Experiment 3*	100	0.5	4.9	5.0	6.0			
CIMmultus™-QA (8 mL) *Experiment 1*	2000	20	2.9	3.2	6.0	1	1400 €	10 h
CIMmultus™-QA (1 mL) *Experiment 2*	600	8	3.4	3.1	5.1	1	1400 €	8 h
CIMmultus™-SO3 (1 mL) *Experiment 1*	900	8	1.1	2.3	0.5	1	1400 €	8 h
PEG-based concentration *Experiment 1*	35	0.4	2.6	3.4	4.9	5	255 €	5 h
Skimmed Milk-based concentration *Experiment 1*	200	0.8	− 1.7	− 0.4	4.3	8	206 €	8 h
Vivacell 100. 30.000 MWCO PES *Experiment 1*	150	0.5	− 1.0	0.5	2.5	8	248 €	7 h
Wizard Enviro TNA Kit; *Experiment 3*	40	1	6.7	6.9	5.4	14	175 €	7 h

Associated sample volume for load (start) and elution (end), number of samples and time used per batch and price per sample are also shown

throughput analysis as it can accommodate the highest number of samples per batch.

All three independent experiments, involving three different wastewater samples, Centricon Plus-70 and Wizard Enviro TNA kit, resulted in the lowest Cq values for SARS-CoV-2 assays in the concentrated samples, which would result in the most sensitive RT-qPCR detection of the virus in wastewater. Based on our results these two approaches were further compared using real wastewater samples containing SARS-CoV-2. For this comparison, Wizard Enviro TNA Kit (PROMEGA, USA, A2991) was substituted with Maxwell RSC Enviro TNA Kit (PROMEGA, USA, AS1831) which uses magnetic-based purification of RNA in the second step of the procedure and enables higher throughput. Maxwell RSC Enviro TNA Kit (PROMEGA, USA, AS1831) and Centricon Plus-70 Centrifugation Filters coupled with

QIAmp Viral RNA Mini Kit (Qiagen, USA, 52,906) RNA extraction were compared using 12 different SARS-CoV-2 positive wastewater samples. The concentration efficiency for PMMoV was higher using Maxwell RSC Enviro TNA Kit (PROMEGA, USA, AS1831) for all 12 compared samples: Cq reduction of 7 or above, whereas with Centricon-based method this reduction is in the range of 4–6 Cqs (Fig. 2). On the other hand, for concentration efficiency for SARS-CoV-2 (measured by N1 and N2 assays) there is no clear conclusion as to which method performs better based on the observed Cq reductions.

Screening for the Optimal RT-qPCR Assay/Master Mix Combination

Analysis of the serial dilutions of the thermally inactivated virus showed that N1 and N2 assays with Zen/Iowa Black probes were 10 × more sensitive than E assay (with either BHQ and BBQ650 quenchers) and 100 × more sensitive than RdRp assay (with BBQ650 quencher) assays, achieving also lower variability between the technical replicates (Table 4). Based on these results subsequent evaluations were done only using N1 and N2 assays.

In a comparison of three different RT-qPCR commercial kits using dilutions of thermally inactivated virus in wastewater with N1 and N2 assays, all 6 assay/kit combinations performed similarly, based on the Cq values obtained for dilutions (Fig. 3). TaqMan Fast Virus 1-Step Master Mix showed lower overall variability between technical replicates, especially at higher dilutions, resulting in larger dynamic range in both dilution series done (Fig. 3, Supplementary Information, Table S8 and Table S9). The slope and intercepts of regression curves, indicative of the amplification efficiency of the RT-qPCR, were most comparable among N1 and N2 assay when using Fast Virus 1-mastermix (Fig. 3). Based on these results we determined for the TaqMan Fast Virus 1-Step Master Mix a practical LoQ for quantification of SARS-CoV-2 in wastewater of 0.69 copies/µL for N1 and 1.37 copies/µL for N2 assay and an LoD for the detection in wastewater of 0.09 copies/µL) for both assays. Other evaluation parameters and regression equations are available in Supplementary Information Table S10. Besides the performance of the mastermix, TaqMan Fast Virus 1-Step Master Mix was the easiest to implement and modify if needed as it is only one reagent that contains the whole mix, whereas the other two are comprised of 2 or more reagents that have to be mixed just before us.

Discussion

Adopting a WBE approach for monitoring SARS-CoV-2 in wastewater requires an extensive evaluation of each critical step of the process. Nearly three years into the pandemic several institutions, such as the WHO, CDC, USA, and KWR, Netherlands have publicized guides to implementation that list available options for each step in the analysis (WHO., 2022, CDC, USA, 2022; KWR, Netherlands, 2022), Initially, the rapid onset and progression of COVID-19 pandemic forced environmental virologists globally to come up with optimized protocols and method evaluations in reduced time and with diverse limitations such as reagent shortages. Critical phases in the analysis process include: sample collection, sample admission and storage, concentration and nucleic acid extraction, target detection/quantification and data analysis. Here we focused on the core three parts of the procedure from sample admission to virus detection, demonstrating a method set up in a rapid-evolving environment as it was experienced in our laboratory. The outcome of these evaluations was the establishment of a complete analysis workflow that is being currently used for the official national wastewater monitoring in Slovenia.

Sample storage and processing time could have an impact on the final result, due to the possible degradation of the target. This degradation can be driven by various factors, such as time, temperature and the complex composition of wastewater (Markt et al., 2021). Having this in mind, we conducted an experiment that looked into the stability of both extracted RNA from positive controls (EVA-GLOBAL) and thermally inactivated SARS-CoV-2 virus in wastewater at different temperatures for different time frames. We selected the two different spike materials to simulate two extreme forms in which the virus could be present in the sample. Neither of the two reference materials used is likely to entirely resemble the shape in which the virus exists in wastewater and its actual storage stability; however, they are close approximations of two extreme structural viral forms, namely, packed particles versus naked genomic RNA. Based on the obtained results we could see that extracted RNA from positive controls (EVA-GLOBAL) degrades faster in wastewater in comparison to inactivated virus particles. As visible in Fig. 1a, extracted RNA gradually degraded in wastewater over the course of 7 days at + 4 °C, with the biggest change occurring in the first 24 h of storage. When RNA-spiked wastewater was stored frozen at either − 20 °C or − 80 °C, we observed an increase of cca. 2 RT-qPCR Cq values already at 24-h storage. Longer storage times did not translate into higher Cq increase, suggesting that the freeze-thawing cycle required when analysing samples stored at such temperatures could have a larger influence than the storage time itself on the observed increase by affecting

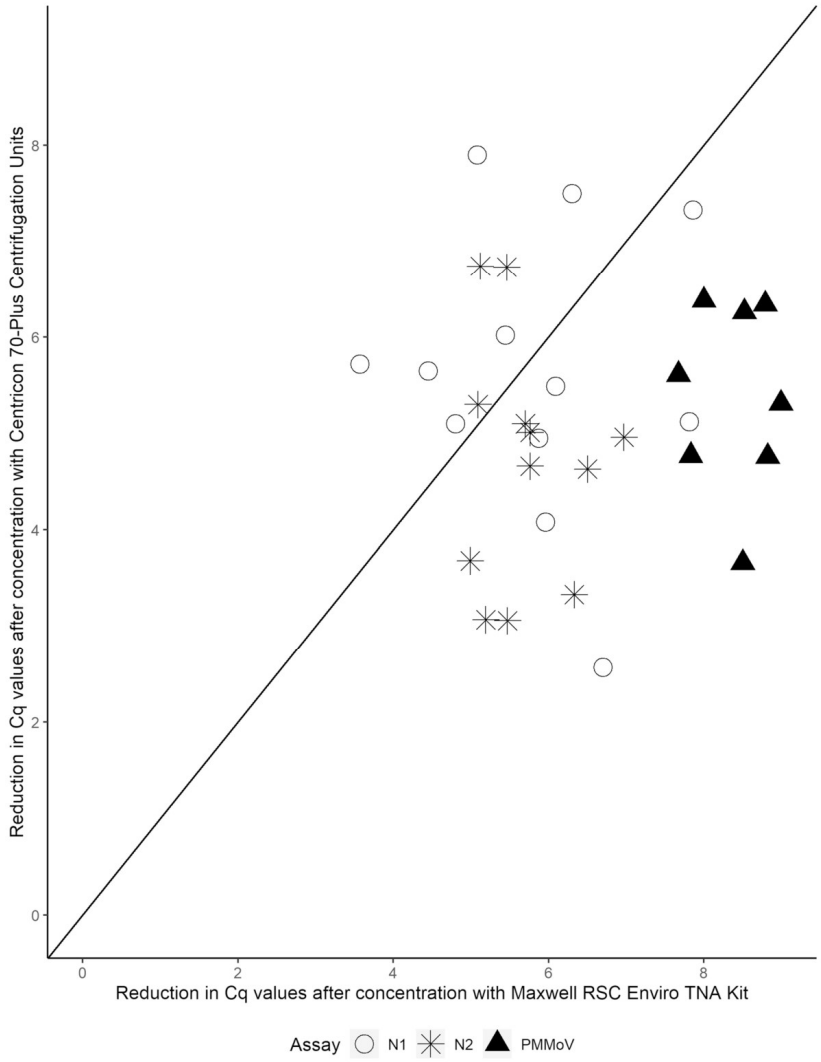

Fig. 2 Reduction in average Cq values ($n=3$) for N1, N2 and PMMoV assays achieved after concentration of real wastewater samples depending on the concentration methods used (Maxwell RSC Enviro TNA Kit or Centricon Plus-70 Centrifugal Filters). Each point represents the relation between the Cq reductions obtained for each target when using the two concentration methods on a given wastewater sample. Points below the line show sample and assay combinations that were concentrated with greater efficiency using Maxwell RSC Enviro TNA Kit and points above the line represent the ones concentrated more efficiently with Centricon Filtration Units and Qiagen extraction step. Figure created in RStudio v.1.2.1106

Table 4 Cq values obtained from the dilution series of thermally inactivated viruses in wastewater, tested with different assays and different probe quencher modifications (detailed primer/probe sets and their sequences are presented in Supplementary Information; Table S5)

Dilution Factor*	E (FAM/BHQ)	Variance coefficient	E (FAM/BBQ650)	Variance coefficient	RdRp (FAM/BBQ650)	Variance coefficient	N1 (FAM/ZEN/IOWA)	Variance coefficient	N2 (FAM/ZEN/IOWA)	Variance coefficient
0	25.2		24.0		25.9		23.8		24.7	
	24.6	0.09	24.0	0.00	25.7	0.01	23.6	0.02	24.4	0.03
	24.8		23.9		25.7		23.5		24.4	
10	27.5		27.9		28.8		26.9		27.9	
	27.6	0.01	27.5	0.07	28.9	n/a	26.8	0.00	27.8	0.02
	27.7		27.4		Undetected		26.9		27.6	
10^2	31.1		39.9		Undetected		30.3		31.2	
	Undetected	n/a	33.8	n/a	Undetected	n/a	30.0	0.16	30.7	0.08
	32.1		31.6		Undetected		29.5		30.7	
10^3	Undetected		Undetected		Undetected		33.0		34.1	
	Undetected	n/a	Undetected	n/a	Undetected	n/a	33.8	0.16	33.5	0.09
	41.3		Undetected		Undetected		33.3		33.8	
5×10^3	Undetected		Undetected		Undetected		Undetected		36.4	
	Undetected	n/a	Undetected	n/a	Undetected	n/a	Undetected	n/a	Undetected	n/a
	Undetected		Undetected		Undetected		34.4		Undetected	
2.5×10^4	Undetected		Undetected		Undetected		35.6		Undetected	
	Undetected	n/a	Undetected	n/a	Undetected	n/a	Undetected	n/a	Undetected	n/a
	Undetected		Undetected		Undetected		Undetected		36.5	
**1.25×10^5	Undetected	n/a	Undetected	n/a	Undetected	n/a	Undetected	n/a	Undetected	n/a
**6.25×10^5	Undetected	n/a	Undetected	n/a	Undetected	n/a	Undetected	n/a	Undetected	n/a

*Concentration range of undiluted thermally inactivated virus as reported by the manufacturer $4–11\times109$ copies/mL

**all 3 technical replicates were undetected

n/a: not applicable

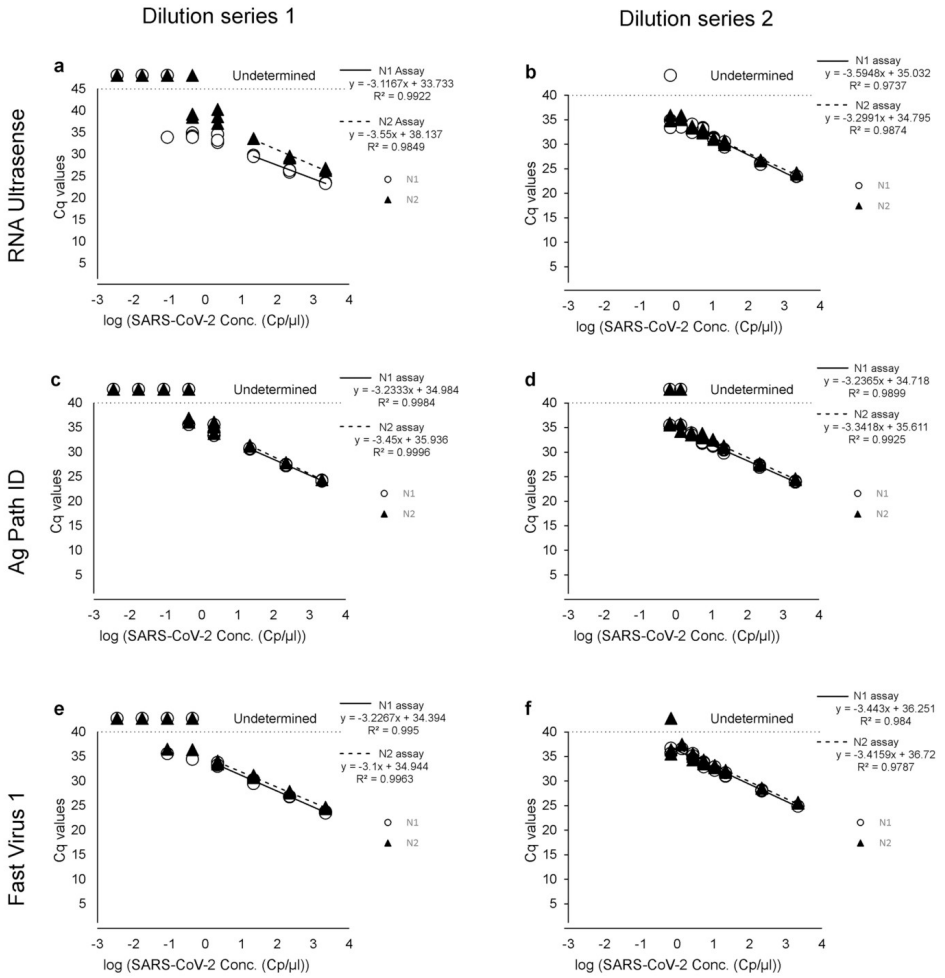

Fig. 3 Evaluation of the performance of RT-qPCR (N1 and N2 assays) on serial dilutions 1 (**a**, **c** and **e**) and 2 (**b**, **d** and **f**) of thermally inactivated SARS-CoV-2 in wastewater influent, using three different commercial mastermixes: RNA Ultrasense (**a** and **b**), Ag-Path (**c** and **d**) and Fast Virus (**e** and **f**). Cq values obtained in triplicate measurements for each dilution are plotted against the log of the virus concentration (as calculated from the concentration indicated by the reference material provider). Regression lines were calculated considering only the points that meet the criteria for being included within the quantification range (see Sect. Evaluation of RT-qPCR Assay/Mastermix Combination, and Supplementary Information Table S8 and S9), and obtained regression equation and square error are shown in each graph. The points that gave no signal in the RT-qPCR (undetermined) are shown above the graph for a clearer picture. The Y axis has been moved to the left, as not to overlap with measurements. More detailed information on dilution series, Cq values and other parameters are shown in Supplementary Information Tables S7–10. Figure created in Excel 2016

the RNA integrity (Fig. 1c and e). Comparable results were also found in wastewater samples with naturally occurring SARS-CoV-2 indicating cca. 3 RT-qPCR Cq values increase in samples that were first frozen on −20 °C compared to ones stored at 4 °C (Qiu et al., 2022). In the case of the inactivated virus spiked in wastewater (Fig. 1b, d and f), we did not observe any increase in the Cq values at none of the temperatures tested, suggesting that the RNA, when protected by the capsid and the lipid envelope is more resilient to environmental degradation than in free RNA form. There is no conclusive information in the literature on the structural form in which SARS-CoV-2 exists in wastewater on the route from households to the WWTP, although suggestions have been made that the virus is present in the form of fully intact enveloped particles (Robinson et al., 2022). However, it can be expected that unlike enteric viruses, which are highly stable in the environment (Sanchez et al., 2016), SARS-CoV-2 enveloped particles will degrade faster in such a milieu, exposing their RNA over time making it susceptible to degradation, as confirmed by our results with RNA from positive controls (EVA-GLOBAL) spiked in wastewater. Since routinely collected samples represent a 24 h-flow-dependent composite sample, the viruses are exposed for an additional 24 h to ambient temperatures during the sampling and in this time, degradation of exposed SARS-CoV-2 RNA can continue.

Based on the available literature early in the pandemic, the first concentration protocol we set up was Centricon Plus-70 Centrifugal Filters coupled with QIAmp RNA Mini Kit for RNA extraction. We checked if the protocol was fit for purpose with two different spike materials in both tap and wastewater and the initial results confirmed the applicability of the protocol, based on the concentration efficacy derived from the reduction of Cq value after the concentration step (Table 1). Additionally, we saw that reference material consisting of synthetic RNA is degraded fast in wastewater, faster and to a higher extent than the RNA from positive controls (EVA-GLOBAL), and thus does not represent a good spike-in for testing the SARS-CoV-2 degradation in real wastewater samples. The rapid fast degradation of both synthetic RNA and RNA from positive controls (EVA-GLOBAL) in wastewater is something to expect considering the complex composition of wastewater influent, which very likely contains substances that cause degradation of RNA. Our results also showed that Centricon Plus-70 concentrated both SARS-CoV-2 and PMMoV similarly. This confirmed the usability of naturally occurring PMMoV as a tool for normalizing the SARS-CoV-2 measurements for changes in faecal load in the wastewater and as concentration efficiency control when using this protocol. The analysis done on 3 replicates from concentration to detection step of a SARS-CoV-2-positive wastewater sample resulted in high repeatability of the concentration using the Centricon protocol, further confirming that this protocol was fit for its purpose (Table 2).

Due to the supply shortages that were experienced globally during the pandemic we decided to test other protocols that could be used instead or alongside Centricon Units. The main parameter to evaluate the concentration step was the measured reduction in Cq values for both SARS-CoV-2 and PMMoV. For all method comparisons we used real wastewater samples. Based on the results we immediately excluded protocols using skimmed milk, Vivacell 100, and CIM-SO3, as they resulted in none or suboptimal concentration (Cq reduction) of SARS-CoV-2. Possible explanations as to why these methods did not work are mainly related to the charge, form and size at which the SARS-CoV-2 virus is present in wastewater. The Vivacell 100 used here, had a molecular cut-off point of 30,000 kDa which is significantly higher than 10,000 kDa in Centricon units. It is also possible that the different material of which the filters are made (polyether sulfone in Vivacell vs cellulose acetate in Centricons) plays a role in the losses due to non-specific binding of viral particles or viral RNA to the filter. Monolithic column CIM-SO3 is negatively charged so its failure likely means that SARS-CoV-2. Skimmed milk flocculation and, especially PEG precipitation are methods that have been widely used in environmental virology for decades (Hamza et al., 2009). Skimmed milk did not concentrate SARS-CoV-2 at all; however, it concentrated PMMoV optimally. PMMoV is known to be present in wastewater as intact infective rod-shaped particles (Bačnik et al., 2020) protected by a protein capsid and thus, can flocculate optimally with the skimmed milk protocol suggesting that SARS-CoV-2 might be present in the wastewater samples in a form difficult to flocculate, likely different to that of intact enveloped particles. It could also explain why another method based on virus particle precipitation, PEG, also concentrated PMMoV better than SARS-CoV-2. It is also worth noting that here we tested just a single variation of both the skimmed milk and PEG protocols forced by the time constraints and requirements of the ongoing SARS-CoV-2 monitoring. Additional optimizations or choosing other variations of the protocols could result in a better performance also for SARS-CoV-2, for example, in the case of PEG precipitation, the initial step of removing solids used here may have resulted in about 30% reduction in recovery (Kaya et al., 2022) and extension of the incubation period to overnight, not used here, could have helped to improve the yield (Farkas et al., 2017). CIM-QA column gave acceptable results, with Cq reductions of approximately 3 for SARS-CoV-2 and 5 for PMMoV, but we excluded it mainly due to the length and complexity of the protocol and difficulties for adaptation to high throughput scenarios such as routine high-scale monitoring, which was the final goal of the method

adaptation. Apart from the concentration efficiency of the selected method we also took into account the price per sample, batch size and time to process the batch, as these factors also impact the practicality of wide-scale monitoring. The majority of the methods displayed acceptable prices per sample (apart from column-based methods) and were able to support scaling up. The outlier was the Wizard Enviro TNA Kit from Promega (parameters for Maxwell RSC Enviro TNA Kit are similar but not displayed here) which provided both the lowest price per sample and the highest number of samples per batch. Ultimately, the only two methods that efficiently concentrated both SARS-CoV-2 and PMMoV were Centricon units coupled with Qiagen extraction and either of two tested kit versions from Promega (Wizard Enviro TNA Kit and Maxwell RSC Enviro TNA Kit). These conclusions are in accordance with previous similar studies (Mondal et al., 2021; Pecson et al., 2021), where they also point out that the Promega kits offered higher throughput in comparison to Centricon Filters. The two methods perform similarly for the detection of SARS-CoV-2, but there is a preferentially higher concentration efficiency of PMMoV with the Promega kit (Fig. 2). These findings indicate that although usable, the two methods should they be interchanged, this should be done with caution in cases where PMMoV is used for data normalization. In such cases running both methods in parallel during a transition period will help asses any major changes induced in the data trend by the method exchange.

Two aspects of the RT-qPCR quantification of SARS-CoV-2 were evaluated individually; assay and mastermix selection. As expected, the selected assay has an impact on the sensitivity. RdRp assay showed significantly lower sensitivity compared to E assay and N1 and N2 assays by 10 and 100-fold, likely due to a mismatch in the reverse primer annealing region of the RdRp assay (Nalla et al., 2020). When comparing E assay to N1 and N2 assays, we noticed around a tenfold reduction in sensitivity for E assay. The N1 and N2 assays had double quenched probes, as opposed to the single quenched probes used with E assay. The increased sensitivity of N assays after the addition of a second quencher in the probe has been reported elsewhere (Hirotsu et al., 2020). It is likely that RdRp and E assays would have also benefited from using double quenched probes; however, we did not test for this and our final decision was to use the N1 and N2 assays. Using two assays to confirm the presence of the genetic material of the virus in the sample will aid in decision-making in regards to samples with low virus concentration, which could have been more inconclusive if only one assay was employed. It is important to continuously evaluate the sequences of both assays annealing regions in the new emerging variants that might bear mutations in said regions that could affect their efficiency.

Second tested parameter was the choice of the mastermix used for RT-qPCR. Having several mastermix options seemed like a good contingency plan in response to potential reagent shortages. All observed parameters (regression factor, deviation between technical replicates and sensitivity) were comparable (Supplementary Information Table S10); however, TaqMan Fast Virus 1-step mastermix showed a better performance at low virus concentrations and better

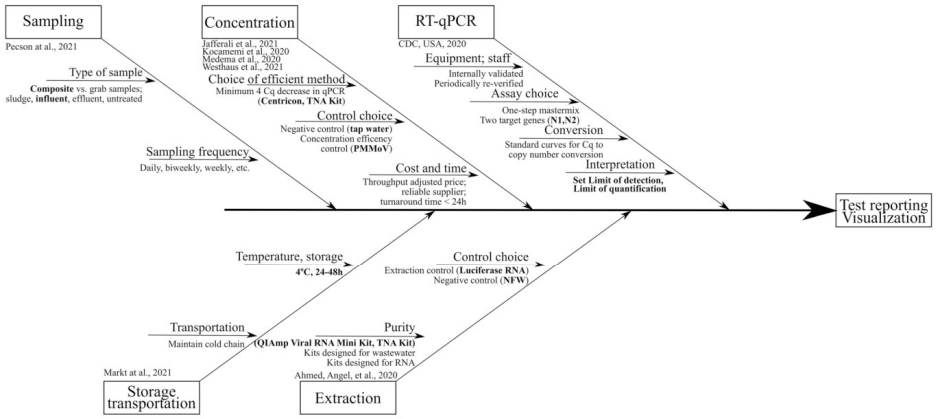

Fig. 4 Scheme depicting different factors influencing the outcome of the analysis aimed at detecting SARS-CoV-2 in wastewater. Steps not discussed in this manuscript have appropriate references cited. The steps that were evaluated in the manuscript have the determined choices in bold. The rest of the steps were decided based on the experience of the authors and/or available resources

correlation between N1 and N2 assays (Fig. 3) and was selected as the main mastermix option.

The outcome of the overall method evaluation described here was the implementation of a complete testing procedure in the work frame of nationwide wastewater monitoring. Taking the time to individually evaluate each step of the procedure allowed us to select the most fit-for-purpose methods. Having properly evaluated methods also enables optimal tech transfer and more accurate inter-laboratory comparisons in the frame of quality assurance environments. Training of additional personnel is also straightforward and time efficient. Thus, we strongly encourage laboratories to implement the WBE approach targeting SARS-CoV-2 or any other viral pathogen, to properly verify each step of the overall procedure. A simplified diagram depicting important factors to be considered in each analysis step is presented in Fig. 4.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s12560-022-09533-0.

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Data Availability The datasets generated during and/or analysed during the current study are available from the corresponding author.

Declarations

Conflict of interest The authors have no competing interests to declare that are relevant to the content of this article.

Ethical approval All authors contributed to the study conception and design and have read and approved the final manuscript.

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2.2 Tomato Brown Rugose Fruit Virus in Aqueous Environments – Survival and Significance of Water-Mediated Transmission

Nataša Mehle, Katarina Bačnik, Irena Bajde, Jakob Brodarič, Adrian Fox, Ion Gutiérrez-Aguirre, Miha Kitek, Denis Kutnjak, Yue Lin Loh, Olivera Maksimović Carvalho Ferreira, Maja Ravnikar, Elise Vogel, Christine Vos, Ana Vučurović

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Our study delved into various aspects of detecting ToBRFV in water. Direct contribution includes experiments regarding the detection of ToBRFV in environmental waters and evaluation of approaches to detect ToBRFV in a hydroponic-like setup.

ToBRFV RNA was detected in environmental irrigation water samples from Slovenia in 2019 and 2020, after its first detection in Slovenia in a wastewater sample (Bacnik et al., 2020), but before its first detection in plants in the frame of routine testing done by Slovenian plant protection officials (2021). We did not determine whether the detected ToBRFV in the waters was present as infectious particles, non-infectious particles, or only as RNA. The source of water contamination with ToBRFV remains unknown, and the available data did not allow us to draw any definitive conclusions.

Setting up the greenhouse experiment, we simulated hydroponic tomato farm conditions. Several trays containing tomato plants were kept in water-based growth media. The growth media was static in the tray and manually topped up regularly to maintain constant volume per tray. Within this experiment, we inoculated tomato plants and tested the growth media for the presence of ToBRFV. Our research revealed that the ToBRFV RNA signal could be reliably detected from the growth media approximately nine days after plant infection. We compared three different strategies (i. direct RT-qPCR analysis, ii. RNA extraction followed by RT-qPCR analysis, iii. concentration with Centricon Filtration Units, 10 kDa MWCO, followed by RNA extraction and RT qPCR analysis) and evaluated their performance. As expected, a combination of concentration and extraction provided the lowest Cq signal. However, even direct use of the growth media around the infected plants gave an RT-qPCR signal reliably distinguishable from the control. The results of this experiment served as the base for additional experiments to assess ToBRFV' s release and survival in water, further described in this publication.

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Tomato brown rugose fruit virus in aqueous environments – survival and significance of water-mediated transmission

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Tomato brown rugose fruit virus (ToBRFV) has recently emerged as a major disease of tomatoes and peppers. ToBRFV is a seed- and contact-transmitted virus. In Slovenia, ToBRFV RNA was detected in samples of wastewater, river, and water used to irrigate plants. Even though the source of detected RNA could not be clearly established, this raised the question of the significance of the detection of ToBRFV in water samples and experimental studies were performed to address this question. The data presented here confirm that the release of virus particles from the roots of infected plants is a source of infectious ToBRFV particles in water and that the virus can remain infective up to four weeks in water stored at room temperature, while its RNA can be detected for much longer. These data also indicate that irrigation with ToBRFV-contaminated water can lead to plant infection. In addition, it has been shown that ToBRFV circulated in drain water in commercial tomato greenhouses from other European countries and that an outbreak of ToBRFV can be detected by regular monitoring of drain water. A simple method for concentrating ToBRFV from water samples and a comparison of the sensitivity of different methods, including the determination of the highest ToBRFV dilution still capable of infecting test plants, were also investigated. The results of our studies fill the knowledge gaps in the epidemiology and diagnosis of ToBRFV, by studying the role of water-mediated transmission, and provide a reliable risk assessment to identify critical points for monitoring and control.

KEYWORDS

tomato brown rugose fruit virus, water-linked epidemiology, survival, tomato, hydroponics

1 Introduction

Tomato brown rugose fruit virus (ToBRFV, genus *Tobamovirus*, family *Virgaviridae*) was first detected in field-grown tomatoes, which showed typical mosaic symptoms as well as leaf narrowing and yellow or brown rugose spots on the fruits (Salem et al., 2016; Luria et al., 2017). This resulted in huge yield losses in Israel in 2014 (Luria et al., 2017), and in Jordan in 2015 (Salem et al., 2016). After its initial discovery in Israel and Jordan, it was reported in more than 50 countries (EPPO GD, 2022). Therefore, ToBRFV is considered a pathogen that is changing global tomato production (Caruso et al., 2022), which takes place on more than five billion hectares and tomato is considered one of the most important vegetables (FAOSTAT, 2020). Economic losses due to ToBRFV infection have also been reported for pepper plants (OEPP/EPPO, 2020). The disease incidence in affected crops was estimated from 50 to 100% (Salem et al., 2016; Alkowni et al., 2019), while observed yield reduction was 10-55% (Avni et al., 2019). Infected plants exhibit mild to severe mosaic, and deformation of leaves, while the fruits may develop brown rugose (rough) patches, marbling, and growth deformation. The rapid spread of ToBRFV to different countries across multiple continents in less than a decade after its emergence was most likely due to the transfer of infested seeds from its place of origin (Caruso et al., 2022). Seed transmission rates are low, however, infection can have a major impact on intensive greenhouse production (Oladokun et al., 2019; Salem et al., 2022). Once established in the production facility, the virus can spread rapidly through plant-to-plant contact (Panno et al., 2020) and, during the course of common cultivation practices, through wounds on leaves or on the roots of seedlings (e.g., after transplanting) (Salem et al., 2016). Like other tobamoviruses, ToBRFV has extremely stable virions (Zhang et al., 2022). Due to their virion stability, tobamoviruses exhibit high persistence in soil, as well as irrigation and drainage water, and remain infectious over long periods of time (Li et al., 2016; Caruso et al., 2022). In previous studies, tobamoviruses have been detected in different environmental samples, including soil (Fillhart et al., 1998), clouds (Castello et al., 1995), and water (Kuroda et al., 2015). Additionally, sequences of tobamoviruses have been detected in different environmental waters, including drinking water (Haramoto et al., 2013), ballast water (Kim et al., 2015), irrigation systems (Boben et al., 2007), and raw and urban sewage (Cantalupo et al., 2011; Fernandez-Cassi et al., 2018). A high diversity of tobamoviral species was reported for reclaimed water (Rosario et al., 2009) and wastewater influents and effluents from a central Slovenian wastewater treatment plant, where some tobamoviruses were confirmed to be infective (Bačnik et al., 2020). Sequences of ToBRFV were detected in wastewater influent sample, although the virus at that time had not yet been reported as infecting plants in Slovenia (Bačnik et al., 2020). This raises questions about its origin, the possibility of its unnoticed occurrence, and the risks of its transmission through water, as well as about the possible use of such water sources in agriculture for irrigation during water shortages.

Modern agriculture requires the use of irrigation in crop production. According to reports, water use in agricultural production accounts for >80% of global water consumption (Xinchun et al., 2017). Moreover, global food security may be threatened by severe water scarcity due to climate change (Murtaza et al., 2019). Agricultural producers use freshwater, wastewater, groundwater, and surface water for irrigation. The use of wastewater in agriculture is considered one of the ways to overcome water scarcity; however, water from alternative sources such as wastewater is sometimes of low quality and requires continuous management and monitoring (Murtaza et al., 2019).

Water-mediated transmission may accelerate global disease emergence and ecosystem impacts for a wide range of crops (Mehle et al., 2018), including tomato and pepper. This is especially true, when hydroponic systems are widely used for the production of some crop species because they require significantly less water, and can contribute to solving the global problem of water scarcity (Sambo et al., 2019). Crop production in soilless cultures using open or closed hydroponic systems has been increasing worldwide. Soilless culture is an alternative for plant growers facing soil-related problems such as nematodes and pathogens, as well as nutrient imbalances (Stanghellini and Rasmussen, 1994; Schnitzler, 2004). In contrast, the use of circulating nutrient solutions in hydroponic systems has the potential for the rapid and effective spread of water-transmissible plant pathogens throughout the crop (whether the pathogens are contained in the original water source or enter the water *via* the distribution pathway), increasing the likelihood of disease outbreaks if the system is not intensively managed (Stewart-Wade, 2011). Therefore, the risk of spreading and the required management of plant viruses, which often lead to crop losses, needs to be assessed before recirculating used water (Bandte et al., 2009).

Previous studies have shown that various tobamoviruses can be transmitted from contaminated soil to the upper parts of plants through the roots (Broadbent, 1965; Fletcher, 1969; Allen, 1981; Koenig, 1986; Pares et al., 1992; Antignus et al., 2005; Li et al., 2016; Avni et al., 2019). In addition, tomato mosaic virus has been shown to persist in root debris in the soil for almost two years and to be infectious for even slightly longer in dry soils (Fletcher, 1969; Broadbent, 1976).

To date, there have been few comprehensive studies on water-mediated transmission of plant viruses, and none have been conducted on emerging tobamoviruses. In a previous study, it has been shown that pepino mosaic virus (PepMV, genus *Potexvirus*), potato virus Y (PVY, genus *Potyvirus*), and potato spindle tuber viroid (PSTVd, genus *Pospiviroid*) can be released from plant roots into the nutrient solution, where they remain infectious, and are able to infect healthy plants through the roots and eventually spread to the green parts, where they can be detected after several months (Mehle et al., 2014). It should be noted, however that the virus/viroid was not detected in the green parts of all plants examined in the study by Mehle et al. (2014), suggesting that water may not be the main route of transmission of PepMV, PVY, and PSTVd between plants. However, it may allow the infection of individual plants, whereupon both viruses and viroids can spread rapidly and effectively to neighboring plants, either mechanically, by vectors or by other means. As is the case for PepMV, PVY, PSTVd, tobamoviruses are also very stable and easily transmissible, which must be taken into account when investigating the potential of

water as a transmission route. Furthermore, when interpreting the results, it should be noted that the infection of plants with stable and easily transmissible viruses can occur not only through the roots, but also through the upper parts of the plant when contaminated water is used for irrigation (e.g., through small wounds that may appear on the leaf surfaces during irrigation, by wind action, or by the application of common agricultural practices) (Mehle and Ravnikar, 2012).

The main objective of this study was to explore the water-linked epidemiology of ToBRFV. Experiments were designed to reveal the possible role of water contaminated with ToBRFV in hydroponic systems as well as in conventional production systems. The main concern was to answer the following questions: (i) can ToBRFV be released from the roots of infected plants into irrigation water, (ii) how long does ToBRFV remain infectious in water, and (iii) can ToBRFV infect plants through the roots when plants are irrigated with contaminated water? In addition, the utility of water monitoring for ToBRFV in commercial tomato greenhouses was evaluated, and some improvements were made to the diagnostic procedure for water analysis.

2 Materials and methods

2.1 Detection of ToBRFV in different environmental water samples

Fourteen samples (5 liters) of wastewater, river water, and irrigation water from different locations in Slovenia from 2017 to 2022 (Supplementary Table 1) were filtered through filter paper and cellulose acetate membranes with a pore size of 0.8 µm (Sartorius, Germany) to remove larger particles. Concentration of each sample was performed using an 8-ml convective interaction media quaternary amine (CIM QA) monolith column (BIA Separations, Slovenia) on an AKTA Purifier 100 FPLC system (GE Healthcare, USA) as previously described (Bačnik et al., 2020). RNA was extracted from the concentrated and non-concentrated water samples using QIAamp Viral RNA mini kit (Qiagen, USA), stored at -20°C, and then tested for the presence of ToBRFV by one step real-time quantitative reverse transcription PCR (RT-qPCR) using primers and probes from Menzel and Winter (2021) and ISHI-Veg (2019) as described in EPPO standard PM 7/146(1) (EPPO, 2021) (hereafter: M&W RT-qPCR and ISF-ISHI-Veg RT-qPCR, respectively). As a control of the RNA extraction procedure and to account for potential inhibition of the qPCR reaction, luciferase control RNA (Promega) was added to each sample and to a negative buffer control (2 ng per sample) immediately prior to the RNA extraction and then tested with RT-qPCR using luciferase RNA-specific primers and probe (Toplak et al., 2004). Analysis of luciferase control RNA showed that the extractions were successful and the inhibition was not present. Negative controls were included in all concentration steps, RNA extractions, and RT-qPCR runs to monitor possible contamination during the procedures. Analysis of these negative controls did not reveal any contamination during the process. In addition, for confirmation, RNA from the 2021 concentrated water sample was further analyzed by conventional RT-PCR with ToBRFV-specific primers (Panno et al., 2019) and by nested RT-PCR with generic tobamovirus primers (Dovas et al., 2004), and the obtained amplicons were analyzed by Sanger sequencing.

2.2 Handling of test plants and water samples from greenhouse experiments

Experiments were performed in a quarantine greenhouse with temperatures of 22 ± 2°C during the light period (16 h) and 19 ± 2 °C during the dark period (8 h). ToBRFV isolate (DSMZ PV-1236) was propagated on tomato (*Solanum lycopersicum*) cv. Moneymaker. Briefly, two to three fully developed lower leaves of tomato were dusted with Carborundum powder (400 mesh, VWR Chemicals, the Netherlands) and then inoculated with the extract of leaf inoculum prepared in a 0.02 M phosphate buffer (pH 7.4) containing 2% polyvinylpyrrolidone 10,000. Mechanical inoculation of test plants with water or nutrient solution samples was performed in such a way that a few drops (approximately 300-500 µl) of these samples were used as inoculum. Five to ten minutes after inoculation, the test plants were rinsed with tap water to remove the abrasive and kept in a quarantine greenhouse. Symptom development on test plants was monitored weekly, and infection was confirmed on newly developed leaves using a rapid one-step assay based on lateral flow immunochromatography (ImmunoStrip®, Agdia, USA) (hereafter: LFD) according to the manufacturer's instructions or by testing extracted RNA using M&W RT-qPCR.

Total RNA was extracted from leaf material (approximately 200 mg) using RNeasy Plant mini kit (Qiagen, USA), following the manufacturer recommendations, with minor modifications: specifically, without using 2-mercaptoethanol, and performing the final RNA elution with two consecutive washes with 50 µl (total of 100 µl) of RNase-free water pre-warmed to 65°C. To assess the quality of the RNA in the extractions, RNA samples were tested with RT-qPCR using *nad5*-specific primers and a probe (Menzel et al., 2002), and only RNA samples with a Cq value for *nad5* of less than 33 were analyzed further. RNA from water and nutrient solution samples was extracted using QIAamp Viral RNA mini kit, and its quality was measured as described above.

Periodic testing with M&W RT-qPCR of control plants grown in the same chamber of the quarantine greenhouse as the experimental plants were used to check the adventitious spread of ToBRFV during greenhouse manipulations. Due to limited space in the quarantine greenhouses, the tops of tomato plants were occasionally pruned to keep them at a maximum height up to one and half meter.

2.3 Comparison of different approaches for the detection of ToBRFV RNA in water

Three trays of three tomato plants each were placed in a greenhouse chamber: one with all three plants inoculated with

ToBRFV, one with just one plant out of three inoculated with ToBRFV, and one with three healthy tomato plants. Plants were grown in Grodan rockwool cubes (100 mm cubes, Grodan, Netherlands) floating on nutrient solution (Johnson et al., 1994). The nutrient solution was sampled three times per week (each time 3 × 50 ml per tray) while the level of nutrient solution was kept constant. Nutrient solution samples were stored at 4°C for six months, and then M&W RT-qPCR was performed for each nutrient solution sample. The nutrient solution was analyzed using RT-qPCR in three different ways: (i) directly from non-concentrated water sample without RNA extraction; (ii) RNA extracted with QIAamp Viral RNA mini kit from non-concentrated sample; (iii) RNA extracted from the concentrated sample from water sample concentrated using Centricon Plus-70 Centrifugal Filter Units, 10 kDa (Millipore, Germany, UFC701008). For concentration, samples were first centrifuged at 3,200 g for 10 min, and the supernatant (2 × 40 ml) was applied to Centricon units. The Centricon units were then centrifuged at 3,200 g for 10–15 min, or until the entire volume had passed through the units. The Centricon units were then inverted, and the eluate was collected by centrifugation at 1,000 g for 1 min. A total of 350–1000 µl of eluate was collected per sample, from which RNA was extracted using QIAamp Viral RNA mini kit. In all RNA extractions, luciferase control RNA was added and tested as describe above. Two concentrated samples with the lowest Cq value were also analyzed under electron microscope using negative staining as described previously (Bačnik et al., 2020) and one by mechanical inoculation of four tomato plants cv. Moneymaker. Four weeks after mechanical inoculation, the infection of tomato plants on newly developed leaves was checked using M&W RT-qPCR.

2.4 Determination of the highest dilution of ToBRFV infected plant material still able to infect test plants

Tomato leaves (1 g) infected with ToBRFV were macerated in tap water (10 ml) using extraction bags with synthetic intermediate layer for filtration (Universal Extraction bags 12 × 15 cm, Bioreba, Switzerland) and tenfold dilutions were prepared using tap water as diluent. These dilutions were tested by LFD. RNA was extracted from the dilutions with QIAamp Viral RNA mini kit and tested using M&W RT-qPCR (Cq values for the obtained dilution series are provided in Table 1). Approximately 300-500 µl of each dilution of ToBRFV infected plant material were used for mechanical inoculation of four test plants of tomato. Two control plants were inoculated with non-infested tap water (confirming absence of ToBRFV with RT-qPCR). Symptom development on the test plants was monitored for up to nine weeks. Infection with ToBRFV on newly developed leaves was checked two weeks after

TABLE 1 Results of testing dilutions series of ToBRFV infested sap.

Dilution	ToBRFV infested water		Test plants[f]			
	LFD	RT-qPCR (Cq)[a]	Symptoms[b]	LFD[c]	RT-qPCR (Cq)[a,d]	RT-qPCR (Cq)[a,e]
10^{-1}	+	4	+	+	NT	NT
10^{-2}	+	7	+	+	NT	NT
10^{-3}	+	10	+	+	NT	NT
10^{-4}	+	13	+	+	NT	NT
10^{-5}	+	17	+	+	NT	NT
10^{-6}	+	20	+	+	NT	NT
10^{-7}	–	22	–	–	NT	undet
10^{-8}	–	25	–	+	13	NT
10^{-9}	NT	25	–	–	NT	undet
10^{-10}	NT	26	–	–	30	NT
10^{-11}	NT	29	–	–	37	38
10^{-12}	NT	33	–	–	NT	undet
10^{-13}	NT	34	–	–	38	37
10^{-14}	NT	35	NT	NT	NT	NT
NC	–	undet	–	–	37	undet

[a]The presence of ToBRFV RNA in water samples investigated by M&W RT-qPCR. The average Cq values of three replicates are given. Variation among technical replicates was ±0.5 from the mean Cq for Cq values <32 and ±0.7 from the mean Cq for Cq values ≥33.
[b]Symptoms observed were leaf curling, shoestring, bubbling and mosaic.
[c]Test plants were tested 2 weeks after mechanical inoculation with LFD.
[d]Test plants were tested 4 weeks after mechanical inoculation.
[e]Test plants were tested 9 weeks after mechanical inoculation.
[f]Each dilution was inoculated on 4 tomato plants, except NC, which was inoculated on 2 tomato plants.
+, Positive; -, Negative; Undet, No signal obtained with RT-qPCR; NT, not tested; NC, negative control.

inoculation with LFD and four and nine weeks after inoculation with M&W RT-qPCR (analysis was performed on RNA extracted from a pool of all four test plants together).

2.5 Survival of the ToBRFV in an aqueous environment

With the aim of investigating how long ToBRFV can remain infectious in aqueous environments, the following studies were performed. The ToBRFV spike source was obtained as described in previous section and was then used to spike 1 L of tap water. Three dilutions of ToBRFV infected plant material in water were prepared: 10^{-2}, 10^{-4}, and 10^{-6}. These dilutions of spiked infested water were stored in a quarantine greenhouse and sampled weekly. RNA from these water samples was extracted using QIAamp Viral RNA mini kit followed by M&W RT-qPCR analysis. ToBRFV infectivity in collected samples was determined *via* the mechanical inoculation of an aqueous solution onto four or five test plants of tomato (Figure 1A). Symptom development was monitored up to four weeks post inoculation and results were confirmed by M&W RT-qPCR or LFD analysis (analysis was performed on a pool of all four/five test plants together). Two control plants were included in the study each week, inoculated

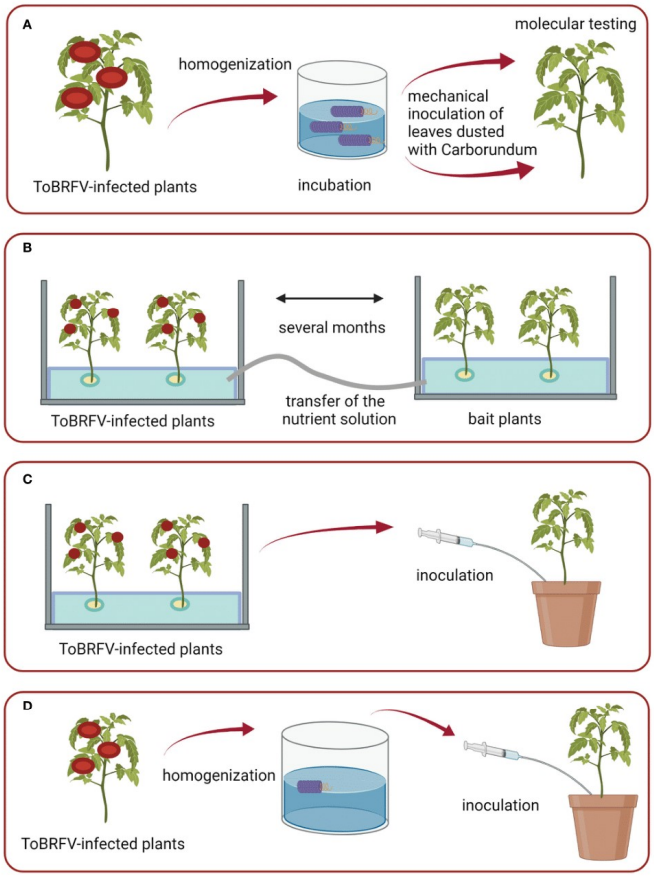

FIGURE 1
Schematic of conducted experiments: **(A)** Set-up of the study on the survival of ToBRFV in aqueous environment; **(B)** Study of water-mediated transmission in an experimental hydroponic system; **(C, D)** Studies on the transmission of ToBRFV to plants growing in substrate irrigated with infested water. Figures were created with BioRender.com.

with non-infested tap water (confirming absence of ToBRFV with RT-qPCR).

2.6 Water-mediated transmission in experimental hydroponic systems

Three separate experimental hydroponic systems were designed to study the possibility of water-mediated transmission of ToBRFV. In each, six ToBRFV-infected tomato plants cv. Moneymaker were placed in a glass tank (dimensions: 0.6 × 0.4 × 0.4 m) filled with nutrient solution (Johnson et al., 1994). Few weeks later, healthy (bait) plants of tomato cv. Moneymaker were placed into separate tanks as two-week-old seedlings (approximately 5-10 cm high). In each of these tanks, six bait plants were placed. Before the plants were placed into the tanks, the substrate was washed away from the roots with water. In each tank, the plants were grown in 10 cm-diameter plastic pots filled with stone-wool substrate (Grotop Master Dry, Grodan, the Netherlands). The bait plants were irrigated with the nutrient solution from the tank with the inoculated plants. Two separate tanks of bait plants were used for the first and third experiments, while only one tank of bait plants was used for the second experiment.

Special care was taken to prevent any contact between the mechanically inoculated plants and the bait plants, and between the nutrient solution from the inoculated plants and the upper green parts of the bait plants. Styrofoam (thickness, 3 cm; positioned in the tanks approximately 5 cm above the bottom) was used keep the upper green parts separate from the root parts and the nutrient solution. During the first experimental period, infected plants and bait plants were grown in the same chamber of the greenhouse, and nutrient solution was pumped directly from the tanks containing inoculated plants to the root zones of the tanks containing bait plants using pumps and plastic tubes (Statuary fountain pump PondoCompact 300, Pontec, Germany) (Figure 1B). During the second and third experimental periods, infected plants and bait plants were grown in separate chambers of the greenhouse, and nutrient solutions were transferred from the tanks containing inoculated plants to the root zones of the tanks containing bait plants using a bottle and glass funnel. Occasionally, the roots of inoculated plants and bait plants of first and second experiments were gently stirred by hand to imitate the real conditions in a hydroponic system, where injury to root systems is expected due to the presence of macrobiota and growth of roots through glass wool. The lower parts of the tanks were wrapped with aluminum foil, to prevent algal growth in the nutrient solution.

Both nutrient solution and leaves and root tissue of inoculated and bait plants were sampled at regular intervals. RNA was extracted from both nutrient solution samples using QIAamp Viral RNA minikit and from plant tissue samples (pools from six plants) using RNeasy Plant mini kit. All extracted RNA was analyzed using M&W RT-qPCR. In addition, the infectivity of ToBRFV in the nutrient solution was checked with mechanical inoculation of tomato plants as described above. Several control plants of tomato grown in substrate at the same time in the same greenhouse, and fresh nutrient solutions were also tested.

2.7 Transmission of ToBRFV through injection of infested water into the substrate

Ten-day-old seedlings (about 5–10 cm tall) of tomato plants cv. Moneymaker were each planted in 18 cm diameter plastic pots with the substrate (Fruhstorfer Erde Aussaat und Stecklingserde, Hawita, Germany). For the first three weeks, 50 ml of infested water/nutrient solution was added to the substrate in each pot once a week using a syringe, and then 100–150 ml of infested water/nutrient solution was added per pot two to three times a week depending on the growth stage of plants. Precautionary measures were taken to prevent contact of green parts of the bait plants with the exterior of syringe and infested water/nutrient solution. The nutrient solution from the tank of inoculated plants from the second experiment described above was injected into the substrate of six pots (Figure 1C), while freshly prepared ToBRFV-infested tap water was injected into the substrate of the other four pots (Figure 1D). This ToBRFV-infested water was prepared each week by macerating infected tomato leaves in tap water in extraction bags (Universal Extraction Bags, Bioreba, Switzerland). Control plants watered with the same amount of non-infested nutrient solution/tap water were also included. Each week, RNA extracted from a pool of upper leaves of all six/four plants per experiment and from a pool of control plants were analyzed by M&W RT-qPCR.

2.8 Analyses of the water from commercial tomato greenhouses

Tomato commercial greenhouses from different countries from north-west Europe were used to monitor the presence of ToBRFV. Greenhouses with (Greenhouse D) or without (Greenhouse A, B, C) previous ToBRFV outbreaks were chosen and drain water samples were gathered from a collection point before disinfection in the greenhouse water circulation system. RNA was extracted from the samples using the RNeasy Plus kit (Qiagen, USA) and the samples were tested using ISF-ISHI-Veg RT-qPCR.

To monitor the correlation of ToBRFV building up in water with the development of infection in plants, leaf samples from the young leaves at the top of the plant and from the sepals were also taken. RNA from collected samples was extracted and analyzed using the same procedure as for water samples.

The infectivity of ToBRFV in drain water was assessed using bio-assay in a growth chamber. Five tomato plants of cv. Climbo of 10 days old were planted in styrofoam pots (480 ml) containing a soil mixture suitable for young plants (DCM potting mix for sowing and cutting). For the duration of the experiment, the plants were watered three times a week with 50 ml of ToBRFV-infested drain water collected from a grower with an active ToBRFV outbreak. Irrigation with ToBRFV-infested water was carried out for four weeks, and in the meantime the water was stored at 4°C. Before the treatment, RNA extracted from the infested water sample was tested with ISF-ISHI-Veg RT-qPCR. Additionally, five tomato plants of cv.

Climbo of 10 days old were first dusted with Carborundum and then inoculated on the cotyledons with the same ToBRFV-infested drain water sample. Symptom development was monitored for up to four weeks after the start of the treatment and confirmed by ISF-ISHI-veg RT-qPCR analysis of extracted RNA from upper newly developed leaves. As a positive control, five plants were mechanically inoculated with ToBRFV-infected leaf material. The inoculum was prepared by homogenizing 1 g of frozen ToBRFV-infected leaf material (-20°C) in 3 ml phosphate buffer (pH 7.4) using metallic beads. As a negative control, five plants were inoculated with the phosphate buffer. The experiment was performed in a growth chamber under controlled conditions (25 ± 2°C during the light period (14 h) and 19 ± 2°C during the dark period (10 h)).

In addition, genomic and coat protein degradation in drain water over time was assessed. A drain water sample of 2 L was collected from a grower with an active ToBRFV outbreak. Subsequently, 1 L of the sample was stored at 4°C and 1 L of the sample was stored at room temperature to monitor the integrity of the ToBRFV particles in drain water over time. Both samples were sub-sampled every two weeks. After RNA extraction using an RNeasy Plus kit, the water samples were tested with ISF-ISHI-Veg RT-qPCR and a near-full genome PCR (hereafter NFG-PCR) to determine the integrity of the RNA genome. The cDNA for the PCR was created using the qPCRBIO cDNA synthesis kit (PCR biosystems) with viral RNA as a template. The RT reaction was performed using the F-22 and F-6392 primers published by Eldan et al. (2022). Following synthesis, the viral cDNA was then amplified using the Herculase II fusion DNA polymerase (Agilent) using the same primers as for the RT reaction (Eldan et al., 2022). Additionally, the water samples were tested with DAS-ELISA (Agdia, Reagent set for ToBRFV) to determine the presence of the ToBRFV capsid protein.

3 Results

3.1 Detection of ToBRFV in Slovenian environmental water samples

The first detection of ToBRFV RNA in Slovenia was done in a wastewater sample from 2017 using high-throughput sequencing (Bačnik et al., 2020). Later, its RNA was detected by RT-qPCR in samples from a river in central Slovenia (one ToBRFV-positive sample from 2019 and one from 2020; both collected at the same location) and in samples from rivers and a pond used for crop irrigation from south-western Slovenia (one sample in 2019 and two samples in 2020; all collected at different locations, once in a pond and twice in a river), from south-eastern Slovenia (one sample from 2020; source: river) and from north-eastern Slovenia (one sample from 2021; source: unknown) (Supplementary Table 1). The concentration of ToBRFV RNA in these eight water samples is estimated to be low because a concentration step of the water samples was required for reliable detection; even after the concentration step, relatively high Cq values were obtained with RT-qPCRs: between 28 and 30; in the case of a concentrated water sample from 2021, the Cq value of the M&W RT-qPCR was 32. The presence of ToBRFV RNA in this particular sample was additionally confirmed by sequencing the PCR and nested-PCR products (data not shown). M&W RT-qPCR or ISF-ISHI-Veg RT-qPCR for other water samples from 2019–2022 revealed either no signals or Cq values above 34.

3.2 Comparison of different approaches for the detection of ToBRFV RNA in water

Centricons were found to be efficient for concentrating ToBRFV from infested nutrient solution samples stored at 4°C for six months, as analysis of RNA from concentrated nutrient solution samples by RT-qPCR yielded values up to 8 Cq lower than analysis of RNA from nonconcentrated nutrient solution samples (Table 2). The concentration step proved unnecessary for samples in which high virus concentrations were expected. In such cases, late signals (high Cq values) were obtained even when samples were analyzed directly, i.e., without RNA extraction. In addition, it was found that the extracted RNA from concentrated and non-concentrated samples of the nutrient solution from the tray containing healthy plants also gave some late signals in RT-qPCR, most likely due to contamination from growing these plants in the same chamber as the ToBRFV-infected plants.

The concentrated samples of nutrient solution with the estimated highest virus concentration (two samples with Cq 15 from the tray with three infected plants) were further examined by electron microscopy and one of these two also by mechanical inoculation of test plants. No virus particles were observed in these two samples under the electron microscope, and the virus did not replicate in test plants.

3.3 Determination of the highest dilution of ToBRFV infected plant material still able to infect test plants

The tests of dilution of ToBRFV infected plant material in water confirm that a higher viral load is required to detect the virus with LFD and by inoculation of test plants than when using RT-qPCR (Table 1). With test plants assays and LFD, the presence of infectious ToBRFV was confirmed in all samples with Cq ≤ 20 by M&W RT-qPCR. However, in one example, infection of plants was observed at an even higher dilution (10^{-8}, Cq = 25), indicating that sporadic transmission to test plants from water samples with higher Cq is possible, although accidental transmission during the course of the experiment cannot be completely ruled out, although the results of controls were negative.

3.4 Survival of ToBRFV in aqueous environment

Infected leaves of test plants were macerated and incubated in water under quarantine conditions in the greenhouse. ToBRFV

TABLE 2 Results of RT-qPCR analyses of nutrient solution exposed to ToBRFV-infected plants.

No. of days[a]	Three infected plants per tray[b]			One infected plant and two healthy per tray[b]			Three healthy plants per tray[b]		
	D	E	C+E	D	E	C+E	D	E	C+E
2	Undet	35	35	Undet	37	Undet	Undet	36	36
5	Undet	36	30	Undet	40	Undet	Undet	36	Undet
7	Undet	34	28	Undet	Undet	36	Undet	37	37
9	35	30	24	34	28	25	Undet	36	Undet
12	35	23	17	35	24	20	Undet	35	32
14	36	23	17	36	22	15	Undet	34	31
16	33	19	15	33	25	18	Undet	32	34
19	33	22	15	33	23	15	Undet	34	28

[a]Days after exposure of the nutrient solution to the ToBRFV-infected plants.
[b]The nutrient solution was analyzed by M&W RT-qPCR in three different ways: D, directly without RNA extraction; E, RNA extracted; C+E, RNA extracted from the concentrated sample. The average Cq values of three replicates are given. Variation among technical replicates was ±0.7 from the mean Cq. Undet, No signal obtained with RT-qPCR.

remained infectious for up to four weeks in water spiked with ToBRFV at dilutions of 10^{-2} and 10^{-4}, and only one week in water spiked with ToBRFV at a dilution 10^{-6} (Table 3). In all cases, ToBRFV RNA was detected for much longer, at least 15 weeks after preparation of the infested water (Table 3 shows data only up to Week 9).

3.5 Water-mediated transmission of ToBRFV in hydroponic and substrate systems

ToBRFV RNA was detected in the nutrient solution as early as the first week after infected plants were placed in a glass tank (Cq value of M&W RT-qPCR for the nutrient solution was 25, whereas the Cq value of infected leaves was 7; data not shown). In each experiment, six tomato plants were placed in the tanks with bait plants. Experiments 1 and 2 had two replicas. Five, seven, and eight weeks after the start of the experiment, the Cq values of M&W RT-qPCR of this nutrient solution were 19, 15, and 16, respectively, and the ToBRFV in the nutrient solution proved infectious at all these time points *via* the mechanical inoculation of test plants (data not shown). In addition, three separate experiments examined infection *via* the roots of tomato plants grown in other glass tanks in which only the roots were exposed to ToBRFV-infested water (i.e., bait plants; Figure 2). In the first experiment, symptoms such as leaf curling, shoestring, blistering, and mosaic were observed on the leaves of bait plants in one tank eight weeks and in the other tank nine weeks after the start of irrigation with the infested nutrient solution. This correlated with the decrease in RT-qPCR Cq value below 10 for root and leaf samples of the bait plants.

TABLE 3 Detection of ToBRFV in artificially infested water stored in quarantine greenhouse.

Time (weeks)[a]	ToBRFV 10^{-2} dilution		ToBRFV 10^{-4} dilution		ToBRFV 10^{-6} dilution	
	RT-qPCR (Cq)[b]	Test plants[c]	RT-qPCR (Cq)[b]	Test plants[c]	RT-qPCR (Cq)[b]	Test plants[c]
0	7	+	14	+	21	+
1	7	+	14	+	21	+
2	7	+	15	+	22	–
3	7	+	16	–	23	–
4	8	+	15	+	27	–
5	10	–	21	–	26	–
6	10	–	20	–	21	–
7	10	–	21	–	22	–
8	12	–	21	–	22	–
9	11	–	21	–	21	–

[a]Weeks after water inoculum was prepared.
[b]The presence of ToBRFV RNA in water samples investigated by M&W RT-qPCR. The average Cq values of three replicates are given. Variation among technical replicates was ±0.5 from the mean Cq.
[c]Infectivity in water was monitored by observing the development of symptoms on inoculated test plants (for each time point and for each ToBRFV dilution 4 to 5 test plants were used) along with RT-qPCR or LFD analysis. If no symptoms developed on test plants 4 weeks after mechanical inoculation, the absence of ToBRFV was confirmed by RT-qPCR. +, Positive (symptoms and LFD positive or Cq less than 10); -, Negative (no symptoms and ToBRFV presence not confirmed by RT-qPCR). Negative controls were always negative.

Also, in the second and third experiments, the symptoms observed on the bait plants correlate with the drop in RT-qPCR Cq value below 10 for the leaf samples (root samples were not tested). However, in the second experiment, this was observed 13 weeks after beginning irrigation with the infested nutrient solution, and in the third experiment, in which roots were not occasionally stirred by hand as in the first two experiments, this was observed even later (between five and six months after irrigation with the infested nutrient solution began). The Cq values of the leaves of control plants grown in the same chamber of the quarantine greenhouse as the bait plants were above 30, except at Week 8 in the period of Experiment 1, when the Cq value of the control plants was 22. However, this appears to be due to adventitious spread rather than infection, as later tests on the same plants showed a much higher Cq value and, in addition, the control plants did not show typical virus-like symptoms.

Similar to the experiments described above, ToBRFV was also detected in leaves of tomato plants grown in a substrate that had been irrigated with ToBRFV-infested nutrient solution and with ToBRFV-infested tap water (Figure 3). The virus detection signal strength in infested tap water was lower than in the nutrient solution, and plants irrigated with infested tap water became infected with ToBRFV earlier than plants irrigated with infested nutrient solution.

3.6 Results of ToBRFV monitoring in drain water from commercial tomato greenhouses

In Greenhouse A, eight rows were monitored weekly for a possible outbreak of ToBRFV, by randomly collecting pooled leaves of different tomato plants spread randomly across the row. In Week 5, a sudden drop in ToBRFV Cq value was measured in one row (143) (Figure 4). Symptoms were monitored in all plants from greenhouse A, and the first symptomatic plants were observed at week 6. It is notable that in Week 5 (i.e., one week before symptoms were observed), the Cq value for ToBRFV from the drain water dropped below 30. Similarly, in Greenhouses B and C, the decreasing Cq values of the drain water samples indicate a possible propagation of the virus in the tomatoes before the first symptoms were observed (in Greenhouse B, symptoms were observed at Week 43, and in Greenhouse C at Week 50; in both cases, the plants already showed low Cq values at this time). In practice, however, it is possible that a residue (not necessarily an infectious virus) of ToBRFV is detected. This is illustrated by an example from commercial Greenhouse D in the season following a ToBRFV outbreak. In this greenhouse, where no symptoms were observed on new tomato plants and where authorities also did not

FIGURE 2
Evaluation of ToBRFV infection in tomato plants grown in an experimental hydroponic system with ToBRFV-infested nutrient solution in three separate experiments. Y-axes show Cq values from M&W RT-qPCR. Plotted Cq value represent average of three technical replicates. Variation among technical replicates was ±0.5 from the mean Cq for Cq values <32 and ±0.7 from the mean Cq for Cq values ≥33. The X-axes show the weeks after the start of irrigation with infested nutrient solution. The black signs and lines designate the results of the leaf and roots samples of bait plants. The results of the examination of the nutrient solution, the leaves of the control plants and, in the case of Experiment 1, the results of the root samples of the bait plants are shown in grey. The red line shows the Cq value of 30 below which the result was considered positive.

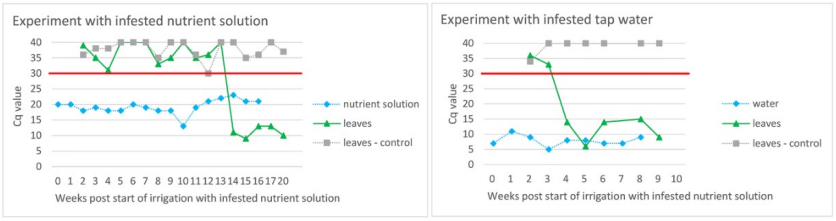

FIGURE 3
Evaluation of ToBRFV infection in tomato plants grown in substrate irrigated with ToBRFV-infested nutrient solution/tap water. The Y-axes show Cq values from M&W RT-qPCR. Plotted Cq value represent average of three technical replicates. Variation among technical replicates was ±0.5 from the mean Cq for Cq values <32 and ±0.7 from the mean Cq for Cq values ≥33. The X-axes show the weeks after the start of irrigation with infested nutrient solution/tap water. The black symbols and lines designate the results of the leaf samples of tomato plants grown in substrate watered with infested nutrient solution/tap water. The results of the examination of the nutrient solution and the leaves of the control plants are shown in grey. The red line shows the Cq value of 30 below which the result was considered positive.

detect the virus during random sampling of plants throughout the greenhouse, the ToBRFV concentration in the drain water gradually decreases (shown as increasing of Cq values of ISF-ISHI-Veg RT-qPCR) over time.

Using drain water from a commercial grower with a ToBRFV outbreak, it was possible to infect plants *via* mechanical inoculation of leaves (three of five mechanically inoculated test plants became infected); however, this did not work *via* roots (none of the plants irrigated with this drain water for four weeks became infected) (Table 4). The drain water sample that was used to inoculate the plants had a Cq-value of 16. Finally, the results which followed the degradation of ToBRFV viral particles in drain water stored at 4°C showed that ToBRFV RNA in drain water can be detected using RT-qPCR and by NFG-PCR for at least fourteen weeks; however, clear detection of the viral coat protein by ELISA was possible for eight weeks, and only a weak signal was obtained at weeks 12 and 14 (Table 5). Similarly, the detection of viral RNA in drain water stored at room temperature was possible for at least fourteen weeks, but the detection of viral coat protein in water stored at room temperature was not possible even at Week 2 (Table 5).

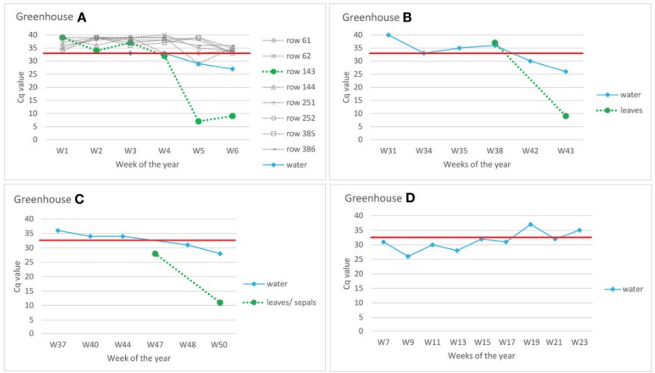

FIGURE 4
Detection of ToBRFV in recirculating drain water from commercial tomato greenhouses. Y-axes show Cq values of CSP1325 primers and probe of ISF-ISHI-Veg RT-qPCR. Similar Cq values were also obtained with CaTa28 primers and probe of ISF-ISHI-Veg RT-qPCR (data not shown). The X-axes show the week number at the time the samples were taken for analysis. The black dots are the results of the water samples, the grey dots are the results of the leaf samples. Greenhouse **(A)** pooled samples of leaves from different rows (row 61, row 62, etc.) and drain water samples were analyzed weekly to monitor for a possible outbreak of ToBRFV. Plants from row 143 were symptomatic at Week 6 (W6). Greenhouses **(B, C)** drain water samples were analyzed at six/five selected time points; leaf samples were analyzed at the time when symptoms first appeared: W43 in the case of Greenhouse **(B)** and W50 in the case of Greenhouse **(C)** In addition, leaf sample of W38 was analyzed in Greenhouse B and sepal sample of W47 in Greenhouse **(C)** Greenhouse **(D)** example of testing drain water in greenhouse in the season following a ToBRFV outbreak; no symptoms appeared in the new crop, and random sampling of plants by authorities did not detect the virus. The red line shows the Cq value of 32.5 below which the result was considered positive.

TABLE 4 Results of analyzing test plants 4 weeks after mechanical inoculation and 4 weeks after the start of irrigation with drain water from the greenhouse with ToBRFV outbreak.

Replicate	Watering with ToBRFV infested water	Mechanical inoculation of leaves		
		ToBRFV infested water	Positive control	Negative control
1	40 [a]	39 [a]	6 [a]	37 [a]
2	37	11	6	Undet
3	36	7	6	40
4	38	9	6	Undet
5	37	32	6	Undet

[a]The Cq values of CSP1325 primers and probe of ISF-ISHI-Veg RT-qPCR are given. Similar Cq values were also obtained with CaTa28 primers and probe of ISF-ISHI-Veg RT-qPCR (data not shown). Undet, No signal obtained with RT-qPCR. Symptoms were observed on all test plants that had a Cq value below 12.

4 Discussion

In Slovenia, ToBRFV RNA was detected in a wastewater sample from 2017 (Bačnik et al., 2020) and then in samples from a river and in samples from rivers and a pond used for crop irrigation in different parts of Slovenia (Supplementary Table 1) before the ToBRFV-infected plants were found (Vučurović et al., 2022). None of the locations where ToBRFV-contaminated water was sampled is close to the location of that finding of ToBRFV-infected plants (Vučurović et al., 2022), which is also the only finding of ToBRFV-infected plants in Slovenia to date. Therefore, the source of water contamination with ToBRFV in Slovenia remains unknown. Many other studies indicated that tobamoviruses, including ToBRFV can be found in human gut and oropharynx (Aguado-García et al., 2020) or in wastewater samples at high relative abundance (Rothman et al., 2021). This opens up the possibility that the origin of the ToBRFV sequences in Slovenian wastewater may be faecal contamination rather than agricultural runoff but based on the available data it is not possible to draw any conclusions. However, the source of ToBRFV contamination of drain water in commercial tomato greenhouses from north-west Europe was clearly associated with the infected tomato plants growing in these greenhouses.

As described in Mehle and Ravnikar (2012), potential sources of plant viruses in environmental waters include roots of infected plants growing in an ecological niche near the water, injured or decaying plant material, and sewage. Some plant viruses present in vegetables or fruits may pass through the digestive tract (Zhang et al., 2006) and be released into wastewater that could find its way into environmental waters, or surface wash-out of locally scattered and infected decaying plant debris and associated soil surface layers, including animal feces, virus-containing seeds, etc., could bring the plant viruses into waters (Mehle et al., 2018). Under experimental conditions, we have shown that infectious particles of ToBRFV can be released from tomato roots of ToBRFV-infected plants into nutrient solution (Figure 2). The release of viruses from roots into water has already been demonstrated for some other plant viruses, such as tobacco necrosis virus (TNV), tobacco mosaic virus (TMV), PepMV and PVY, as well as for PSTVd (Yarwood, 1960; Schwarz et al., 2010; Mehle et al., 2014).

The ToBRFV concentration in Slovenian water samples was below the detection limit of serological testing (LFD) and mechanical inoculation of test plants, so it is not known whether the detected ToBRFV in Slovenian waters was present as infectious particles, as non-infectious particles, or only as RNA. However, the data presented here shows that in case of an active ToBRFV outbreak in commercial greenhouses, a much higher ToBRFV concentration can be reached in the drain water, and by mechanical inoculation of test plants it was confirmed that the ToBRFV particles detected in the water are infectious (Table 4). In

TABLE 5 Results of the analysis of drain water from the greenhouse with ToBRFV outbreak after storage at room temperature and at 4°C.

No. of weeks	Room temperature			4°C		
	RT-qPCR[a]	ELISA	NFG-PCR	RT-qPCR[a]	ELISA	NFG-PCR
0	16	+	+	16	+	+
2	17	-	+	17	+	+
4	16	-	+	14	+	+
8	16	-	+	17	+	+
10	16	–	+	15	–	+
12	14	–	+	14	+[b]	+
14	15	–	+	13	+[b]	+

[a]The Cq values of CSP1325 primers and probe of ISF-ISHI-Veg RT-qPCR are given. Similar Cq values were also obtained with CaTa28 primers and probe of ISF-ISHI-Veg RT-qPCR (data not shown).
[b]Weak positive result. +, Positive; -, Negative. The negative controls included in each run of each method were always negative.

addition, the data presented here demonstrate that under experimental conditions ToBRFV remains infectious in water at room temperature for up to four weeks (Table 3). There are also some data on the survival of other plant viruses in water and nutrient solutions under greenhouse conditions (Mehle et al., 2018). For example, PVY has been shown to remain infectious in aqueous environments for up to one week, PepMV for up to three weeks, and tomato mosaic virus (ToMV) for at least six months (Pares et al., 1992; Mehle et al., 2014). The differences in survival observed between these viruses are likely due to their different structures and to different experimental conditions (Mehle et al., 2018). For example, the survival time of PVY in water has been shown to be much longer (up to 10 weeks) when stored at 4°C, likely due to the higher stability of the coat protein at lower temperatures (Mehle et al., 2014). Correspondingly, the coat protein of ToBRFV was detected in a drain water sample stored at 4°C even after fourteen weeks by ELISA, whereas this was no longer possible after two weeks when the sample was stored at room temperature (Table 5).

The determined survival time in aqueous environments also depends on the virus concentration in the water, and this was demonstrated by including different dilutions of ToBRFV infected plant material in the survival experiments (Table 3). In addition, several other factors may influence these results, such as the susceptibility of the test plants used, the presence of clay particles or organic matter that may protect plant viruses from inactivation in water (Mehle et al., 2018). In the case of the experiment with ToBRFV 10^{-4} dilution, infectivity in water three weeks after water inoculation could not be confirmed, while four weeks after water inoculation, transmission to test plants by mechanical inoculation was successful (Table 3). The reason for this unexpected result could be a combination of the low number of infected virions present and the limited number of test plants per time point and/or the different susceptibility of the test plants (although we used test plants of the same batch, the plants differ slightly in growth stage at the time of inoculation).

The presence of plant viruses in waters may have epidemiological importance if the viruses can enter plants through the roots or through the upper parts of plants when contaminated water is used for irrigation (Mehle et al., 2018). Our experimental data show that ToBRFV from the nutrient solution or irrigation water can infect healthy tomato plants through the roots and eventually spread to the upper parts of the plants, where it can be detected after one to six months (Figure 2). This study demonstrates that the time required for symptom development and reliable detection of ToBRFV in the upper green parts of tomato plants depends on the virus concentration in the water as well as the severity of root damage. Under conditions expected in production systems or in nature, root systems are damaged by the presence of macrobiota and root growth through soil or glass wool. In two of three experiments mimicking the hydroponic system, we damaged the roots by lightly stirring them by hand, and in these two experiments, infection of the plants was observed earlier (similar to the experiment in which the plants were grown in soil) than in the experiment in which the roots were not additionally damaged and in which only stirring with the water flow generated by the work of the pump was used to mix the nutrient solution. Our results show that infection occurred even when tomato roots were not severely damaged but were exposed to moderate concentrations of ToBRFV in the nutrient solution (about 20 Cq), probably due to the high infectivity of ToBRFV.

For a reliable assessment of the role of water as a route of spread for the virus, it is important to conduct long-term experiments because in practice, when recycled water is used for irrigation, tomato plants can be inoculated repeatedly throughout the growing season, which can take 10 months (Mehle et al., 2014). For example, for PSTVd, in the short-term experiment (repeated addition of inoculum to the rooting substrate of tomato for up to 10 consecutive days) by Verhoeven et al. (2010), transmission through the roots was not confirmed, whereas this was confirmed in the long-term experiment (several months) by Mehle et al. (2014). However, due to technical limitations, it was only possible to conduct a short-term study of ToBRFV transmission using drain water from a commercial tomato greenhouse with an active ToBRFV outbreak (Table 4). This was done by repeatedly adding the drain water to the rooting substrate of the tomato over a period of only four weeks, and therefore it is not surprising that the plant did not show ToBRFV transmission in this experiment. In addition, drain water used in this study was not taken fresh but stored at 4°C, which could also affect the final result.

Waterborne transmission of some other plant viruses has also been confirmed (Mehle and Ravnikar, 2012; Mehle et al., 2014). As discussed in Mehle et al. (2018) for other waterborne viruses, this is likely not the most efficient route of virus transmission for ToBRFV either. Considering that ToBRFV can quickly and effectively spread mechanically to neighboring plants, the possibility that it can also be transmitted by water should be considered an important issue. This is especially important to consider in hydroponic systems or other systems in which recycled water is used, and in such cases our data show that it is worth monitoring the water for ToBRFV to predict critical locations and moments for viral disease onset. In addition, the data presented in this study shows that an outbreak of ToBRFV can be detected with regular monitoring of drain water. Using the example of a commercial tomato greenhouse (Greenhouse A in Figure 4), it is clear that monitoring drain water is more efficient than analyzing the large number of plant samples that must be taken in a greenhouse for early detection and screening purposes. Although the RNA of ToBRFV is detectable by PCR-based methods long after the virus has lost its infectivity, long-term monitoring can reveal changes in the amount of virus in water by qPCR, which could be used to monitor ongoing infections.

Viruses are usually present in water at very low concentrations but can still pose a significant health risk, because very low titers are often required for infection (Mehle et al., 2018). Our data suggest that monitoring large bodies of water, where ToBRFV may be highly diluted, requires an appropriate concentration step. Concentration of ToBRFV from water samples using CIM

monolithic chromatography proved to be efficient but requires complex equipment that is not available in many laboratories. For this reason, ultrafiltration with Centricon Plus-70 Centrifugal Filter Units, which can be used in any laboratory with a benchtop centrifuge, was evaluated and found to also be very efficient for concentrating ToBRFV from water samples (Table 2). However, it has yet to be determined whether the use of a lower initial sample volume in Centricon units (up to 100 ml) compared to CIM monoliths (up to 5 l) has an adverse effect on the sensitivity of the final detection when used in large water bodies. For the moment, for analysis of cleaner water sources (river, tap, underground) analyzing larger volumes (CIM monoliths) would be recommended, while for waters with a higher contamination burden, e.g., hydroponics, wastewater, recirculating water, lower volumes (Centricon) may suffice. The other limitation of the experiment comparing different approaches for the detection of ToBRFV in water (direct analysis of water samples with RT-qPCR, analysis of extracted RNA, and analysis of RNA from the concentrated water samples) is that six-month-old water samples were used. This means that although the water samples were stored at 4°C, our tests most likely detected ToBRFV RNA and not virus particles. This is also indicated by the unsuccessful transfer of ToBRFV from a concentrated water sample to test plants and the fact that no virus particles were found by electron microscopy in two concentrated water samples examined. Nevertheless, it confirms once again the resilience of the ToBRFV RNA detection by RT-qPCR which can persist and stay infective for several months on some glasshouse surfaces (Skelton et al., 2021).

The main problem faced in performing all these experiments was the adventitious spread of ToBRFV signal in the greenhouse. Using RT-qPCRs, high Cq values were obtained (between 30 and 40) in many control plants, in water samples, and in swabs from walls that were never in direct contact with contaminated water or with infected plants, but were from the same chamber as heavily infected plants or heavily contaminated water (data not shown). In one case, ToBRFV was detected in collected control plants with a Cq value of 22, but a week later the analysis of the same plants resulted in a much higher Cq value. Since there was no significant drop in Cq values in these samples over periods from two to six months, and no symptoms were seen in the negative control plants, it is assumed that these were environmental contaminations that never led to real infection. However, this is very worrying as such low signals may also mean low virus titer, which theoretically could lead to infection in individual cases. Therefore, this should be investigated in further studies, because the situation found under experimental conditions is also likely to be the situation in commercial greenhouses during and after ToBRFV outbreaks as shown by Loh et al. (2022).

The results of our studies have shown that water can be an important source of ToBRFV inoculum under the experimental conditions. Potentially, such a scenario in intensive production can lead to significant yield losses in tomato production. Therefore, the results of this study are very important for the introduction of new water monitoring systems that can be used for larger scale studies. An improved, more affordable water monitoring system can play an important role in controlling ToBRFV and other tobamoviruses.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Author contributions

NM participated in the design of the study, drafting of the manuscript, and supervised with AV the conduct of the hydroponic transmission, soil transmission, and survival study experiments, conducted by MK, AV, IB, and JB. OMCF, IG-A, DK, JB, AV, and NM conceived and performed the experiments ToBRFV detection in various environmental waters. OMCF, AF, YL, IG-A, DK, KB, and NM planned and performed the experiments for testing the different approaches for ToBRFV RNA detection in water. EV and CV planned and performed the experiments related to commercial greenhouses. All authors contributed to writing and reviewing the manuscript and approved the submitted version.

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Conflict of interest

Authors AF and YL are employed by Fera Science Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2023.1187920/full#supplementary-material

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2.3 Virome Analysis of Irrigation Water Sources Provides Extensive Insight into the Diversity and Distribution of Plant Viruses in Agroecosystems

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In this study, we investigated the presence of plant viruses in environmental water bodies, including surface and underground water sources, using advanced molecular techniques. Our results confirmed the presence of selected tobamoviruses through targeted detection using qPCR. To gain a broader understanding of viral diversity, we employed HTS analysis to study viral abundance and diversity within the samples. Our findings revealed that viral reads accounted for approximately 1% to 7.7% of all reads, consistent with previous reports. Looking at plant viruses specifically, we have seen a significant diversity in family and genus levels across the samples. The virome' s composition seems to depend on the water source, with underground samples showing lower diversity and abundance in species compared to surface water sources. Remarkably, we identified seven new plant viruses from five different genera, expanding the list of known plant viruses and highlighting the utility of environmental water testing as a powerful tool for studying the ecology of plant viruses and discovering new ones. Phylogenetic analysis further revealed close connections between some detected and other known viruses. In addition, our study uncovered evidence for the possible exchange of plant viruses between water and plant samples, as 16 viruses were detected in both types of samples, including four new viruses detected in plant samples for the first time. One of those viruses, Plantago tobamovirus 1, although detected in only one plant sample, was present in water samples across all sampling regions. Phylogenetic analysis showed that individual sequences clustered together depending on the sampling area. The geographical distance was consistent with how the sequences clustered together. Overall, our study provides critical insights into the presence of plant viruses in environmental water sources, demonstrating the potential benefits of water testing as a tool to detect the introduction of known and new plant viruses in an agroecosystem and survey their distribution and diversity.

Virome analysis of irrigation water sources provides extensive insights into the diversity and distribution of plant viruses in agroecosystems

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Abstract

Plant viruses pose a significant threat to agriculture. Several are stable outside their hosts, can enter water bodies and remain infective for prolonged periods of time. Even though the quality of irrigation water is of increasing importance in the context of plant health, the presence of plant viruses in irrigation waters is understudied. In this study, we conducted a large-scale high-throughput sequencing (HTS)-based virome analysis of irrigation and groundwater sources to obtain complete information about the abundance and diversity of plant viruses in such waters. We detected nucleic acids of plant viruses from 20 families, discovered several novel plant viruses from economically important taxa, like *Tobamovirus* and observed the influence of the water source on the present virome. By comparing viromes of water and surrounding plants, we observed presence of plant viruses in both compartments, especially in cases of large-scale outbreaks, such as that of tomato mosaic virus. Moreover, we demonstrated that water virome data can extensively inform us about the distribution and diversity of plant viruses for which only limited information is available from plants. Overall, the results of the study provided extensive insights into the virome of irrigation waters from the perspective of plant health. It also suggested that an HTS-based water virome surveillance system could be used to detect potential plant disease outbreaks and to survey the distribution and diversity of plant viruses in the ecosystems.

Keywords

Irrigation water, plant viruses, virome, environmental water testing, high-throughput sequencing, agroecosystems

Abbreviations

RT-qPCR – reverse transcription quantitative polymerase chain reaction
HTS - high throughput sequencing
PMMoV - pepper mild mottle virus
ToMV - tomato mosaic virus
CGMMV - cucumber green mottle mosaic virus
LUC – Luciferase Control RNA
NCI - negative control of isolation
CLC-GWB – CLC Genomics Workbench
LCA - lowest common ancestor
ICTV – International Committee on Taxonomy of Viruses
ORF - open reading frame
(-) ssRNA - negative sense single stranded RNA
(+) ssRNA - positive sense single stranded RNA

dsRNA - double stranded RNA

PTV1 – plantago tobamovirus 1

RdRp - RNA-dependent RNA polymerase

1. Introduction

Plant viruses are a well-known risk factor in crop production, resulting in at least $30 billion in yield losses annually [1]. The presence of plant viruses in the aqueous environment has been known for nearly four decades [2]. They have been detected in a variety of water bodies including rivers, lakes, ice, and tap water [3], and in wastewaters [4]–[7]. For some of them, the stability in water for prolonged period and the ability to infect plants after this time were demonstrated [8]. Although the research of plant viruses in environmental waters has been gradually developing, there are still relatively few studies focusing on this topic. There are even fewer studies that focused on irrigation water and/or surface water near farms [9]–[11]. In one study, researchers from China have confirmed presence and infectivity of eight selected tobamoviruses [12]. These findings put forward possible risks associated with the use of water containing plant viruses for irrigation of plants, which need to be further investigated. This is especially important since currently, 70% of the freshwater withdrawals are used for irrigation and general agricultural needs and this trend is expected to increase [13]. For example, in the European Union, the main irrigation water resource is on-farm underground water or surface supply networks in more arid areas like Greece [13]. In addition to these two sources, on-farm surface water (e.g., rivers, streams, ponds) is also often used [13].

In recent decade, virome studies, based on high-throughput sequencing (HTS), enabled high resolution generic studies of virus diversities in environmental water samples. Many studies addressed diversity of viruses (e.g., bacteriophages) in oceans [14], [15], however, less attention has been given to freshwater bodies [16]. Virome studies of freshwater bodies, specifically focusing on plant viruses are sparse [9], [17], even though this aspect is important in the view of the possible detrimental effects of plant viruses in agriculture, if such waters are used for irrigation of plants [3], [8]. To better understand the potential risks associated with the presence of plant viruses in irrigation waters, baseline virome studies are first needed to provide information about the presence of viruses in such samples.

Moreover, large sequence datasets obtained by HTS-based analysis of environmental water samples can bring additional information, reaching beyond the presence/absence data for different viruses. This was most evidently demonstrated during the COVID-19 pandemic where wastewater monitoring has been used for tracking virus variants in populations across countries [18]. Extending such framework to other water types could bring extensive information about different aspect of epidemiology of other viruses. For example, environmental monitoring of water used in, and around agricultural sites could be used to detect and anticipate the entry and spread of plant viruses in the given area and to better understand their epidemiology.

In this study, we investigated the virome of diverse types of irrigation water and nearby environmental ground water samples, collected in several agroecosystems (tomato farms) in Slovenia. Focusing on plant viruses, we aimed to (1) provide a baseline virome data for plant virus presence in sampled waters, (2) compare viromes of different irrigation water types,(3) use water virome data to discover novel plant viruses, and (4) obtain information about plant virus diversity and distribution in the wider environment. Moreover, these results were associated and compared with results from a previous study [19] that looked at the virome of tomato and surrounding weed plants at the same tomato farms. This enabled us to obtain unique and unprecedented comparative insight into the virome compositions of two different but connected agroecosystem components: plants and water.

By elucidating the presence, diversity, and distribution of plant viruses in irrigation water, we can better understand their potential impacts on agricultural systems and develop strategies for their early detection and management. The applicability of water analysis for detection of new and/or emergent viruses is also discussed herewith, following its usability as an early warning system for viruses just entering the environment. This research contributes to the growing body of knowledge on plant viruses in environmental waters and highlights the need for further studies in this area that would improve our understanding of the ecological dynamics of plant viruses in agroecosystems.

2. Materials and Methods

2.1. Samples and sampling locations

Water samples (5 L) were collected in the summer of 2019 and 2020 at different locations in Slovenia (Figure 1, Supplementary Information 1, S1). Selected locations were farms with tomato as the main growing crop. The sampled water was either used at the farm to irrigate crops or from the nearby groundwater body not primarily used for irrigation. Water was collected in autoclaved glass bottles and transported back to the laboratory in cooler boxes. Before further processing, samples were stored at 4 ℃ for up to 48 hours. Different types of samples are labelled further in the text as: (T) - tap water from municipal water system; (U) - underground water originating from any underground source; (P) - pond or any standing freshwater body, and (R) - rivers or any type of ground watercourse, including canals and streams. The sample nomenclature shows the type of water, year of sampling, and site number (e.g., U-19-01 represents underground water sampled in 2019, at location 1).

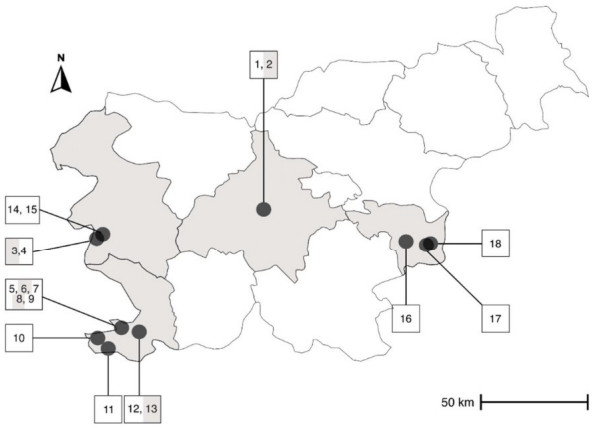

Figure 1 – Sampling locations and their geographical distribution. Dots represent sampling location (if locations were very close together, they are designated with the same dot). Lines separate different statistical regions. Greyed-out regions contain sampling locations. Grey shaded numbers represent locations where ground water not directly used for irrigation was sampled. For exact longitude and latitude information refer to Supplementary Information 1, S1.

2.2. Concentration of water samples and nucleic acids extraction

All samples (5L) were concentrated using convective interaction media (CIM) monolithic chromatography, with a CIM quaternary amine (QA) 8 mL column (Sartorius BIA separations, Slovenia), and with step gradient elution to a final elution volume of 25 mL**.** The collected fractions (one before concentration (raw) and one after concentration (elution) for each sample) were stored at -80 °C until nucleic acids extraction. RNA from elution fraction of each sample was extracted using two different protocols (QIAmp Viral RNA Mini kit (Qiagen, USA) and TRIzol LS (Invitrogen, USA)), and RNA from raw fraction only with QIAmp Viral RNA kit, with each batch accompanied by negative control of isolation (NCI, nuclease-free water used instead of sample). The detailed protocol is available in a previous published study [4]. Before extraction using the QIAmp Viral RNA Mini Kit, samples, including NCI were spiked with 2 ng of Luciferase Control RNA (LUC) (Promega, USA). These extracts were used for real-time quantitative PCR (RT-qPCR) analysis. TRIzol LS extracts were used for sequencing, wherein only the NCI was spiked with LUC RNA. The RNA extracts were stored at -80°C until further analysis.

2.3. RT-qPCR

RNA extracted using QIAamp Viral RNA kit from raw and elution fraction were analysed using RT-qPCR for the targeted detection of three selected tobamoviruses, namely, pepper mild

mottle virus (*Tobamovirus, Virgaviridae,* PMMoV), tomato mosaic virus (*Tobamovirus, Virgaviridae,* ToMV), and cucumber green mild mottle virus (*Tobamovirus, Virgaviridae,* CGMMV). For PMMoV and ToMV, both raw and elution fraction were tested, and for CGMMV, only the elution fraction was tested by RT-qPCR. In addition, an RT-qPCR assay specific for luciferase RNA was used to quantify LUC RNA that was spiked during the nucleic acid extraction step. Published primer/probe sets were used for all targets (PMMoV [20], ToMV [11], CGMMV [21] and LUC [22]). Each RT-qPCR reaction was performed with 2 µL of extracted RNA per reaction in a total reaction volume of 10 µL. RT-qPCR was performed using the AgPath-ID™ One-Step RT-qPCR Kit (Life Technologies, USA) on a 7900HT Fast Real-Time PCR System (Applied Biosystems, USA), using cycling parameters as recommended by the mastermix manufacturer. Samples were tested in duplicates and prepared as undiluted and 10-fold dilutions. A negative template control (nuclease-free water instead of the RNA) and a positive control (with known presence of the corresponding target virus) were included for each assay in each RT-qPCR analysis. Data were analysed in standalone software (SDS v4.0) with automatic setting of the baseline and threshold set up to a value of intersection between amplification curves at the exponential phase of the amplification, namely, 0.065 for PMMoV, 0.02 for ToMV, 0.15 for CGMMV and 0.15 for LUC assays. Each amplification plot was checked manually, and the result was considered positive if it produced an exponential amplification curve distinguishable from negative controls. In such cases, Cq values were calculated. Cq values for LUC RNA were monitored in a control chart and extraction was considered successful if obtained Cq was within ±3 standard deviations from the mean Cq value (data not showed).

2.4. Shotgun high-throughput sequencing (HTS)

RNA isolates of each sample, along with 3 spiked NCIs (spiked with LUC RNA), all obtained by the TRIzol LS extraction protocol, were randomly pre-amplified according to the protocols described previously [4]. The pre-amplification products were sent to SeqMatic LLC (Fremont, USA) for library preparation and sequencing. Nextera XT DNA Library Prep Kit (Illumina, USA) was used to prepare the sequencing libraries, which were shotgun-sequenced using an Illumina MiSeq platform (Illumina, USA) in a 2x250 bp mode.

2.5. Analysis of HTS data

2.5.1. Data preparation

After obtaining the raw sequencing data, sequencing reads were trimmed-off of sequencing adaptors and primer sequences from the pre-amplification step and filtered with a quality filter (Supplementary Information 1, S3) in CLC Genomics Workbench (CLC-GWB) (v. 20-22). Datasets were then normalized by random subsampling of each sample with the subsampling

size equivalent to the lowest number of reads observed among the samples within the same year (Supplementary Information 1, S1).

2.5.2. Virome analysis focusing on plant viruses

A general overview of metagenome and detection of known viruses was done by first exporting normalized reads subsets from CLC-GWB (v. 20-22) and comparing them for similarity to the entire NCBI nr database (v. 237) using DIAMOND (v. 9.34) blastx [23] with default parameters. The results of the DIAMOND similarity searches were used as input for the taxonomic classification of reads using MEGAN (Metagenome Analyzer, v. 6.20.16, May 2020 database) [24] with the LCA algorithm (Supplementary Information 1, S3). The obtained MEGAN outputs in the form of summarized reads were used to provide an overview of the taxonomic classification of the sequencing reads (Supplementary Information 1, S4). Additionally, viral reads binned on the level of order were used to determine the genome organisation (as denoted by International Committee on Taxonomy of Viruses (ICTV) for each order [25]) and viral reads binned on the level of family were used to determine the expected host (as denoted by ICTV for each family [25]), data in Supplementary Information 1, S5, S6. Read classifications on different levels were visualized as bar plots using RStudio (v. 2021.09.0) and edited in Inkscape (v. 0.92).

Information on classification of reads for each plant virus genus was exported from MEGAN as a BLAST table (Export→Matches). The table was further curated using a custom script (Supplementary Information 2) which classified reads that were assigned to a specific species up to the level of genus if they were below the ICTV [25] species demarcation criteria for that genus. Data generated in this manner for each genus is summarized in Supplementary Information 1, S7. This way, we obtained the number of reads assigned per virus species and genera for each sample. Reads classifications for plant virus families, genera and species were visualized as bubble charts in RStudio (v. 2021.09.0) and edited in Inkscape (v. 0.92).

2.5.3. Assembly of genomic sequences for new plant viruses

Performed read classifications showed the possible presence of new plant virus species (e.g., reads classified only at the genus level). Thus, we aimed to assemble genomic sequences of new viruses using two different approaches. In the first approach, all reads, assigned to the genus level for plant virus genera, which had over 100 reads assigned in MEGAN, were *de novo* assembled in CLC-GWB (v. 20-22) (parameters in Supplementary Information 1, S3). After assembly, any contig longer than 500 bp was checked using BLASTx against an entire NCBI nr database (v. 248). Contigs that did not match to a known virus and with percent identity above the species demarcation criterium (as listed by the ICTV [25]) for that genus were kept for further analysis. Such contigs were extended in Geneious Prime (v. 2022.2) by

iterative mapping of reads from the corresponding sample back to the contig until there was no more extension (parameters in Supplementary Information 1, S3). Finally, all relevant contigs were checked for open reading frames (ORFs) in CLC-GWB (v. 20-22) to confirm the genome structure of complete or near-complete genomes of novel viruses.

In the second approach, all reads (after normalisation) per sample were *de novo* assembled using SPAdes (v. 3.14) [26], and compared for similarity to an entire NCBI nr database (v. 237) using DIAMOND (v. 9.34) blastx [23] with default parameters. The results of the DIAMOND alignment were used as an input for the taxonomic classification of reads using MEGAN (v. 6.20.16, May 2020 database) [24]. Contigs were manually inspected, and those matching plant viruses on the level of genus, but not matching a known virus on the level of species (based on the percent identity species demarcation) were extended in Geneious Prime (v. 2022.2) and checked for ORFs as described above.

2.5.4. Pairwise identity comparisons and phylogenetic analyses for putative new plant virus species

For each genus that contained a putative novel virus, complete genome sequences from selected members of the genus from the NCBI GenBank database and the sequences of the potential new viruses from this study were aligned in MEGA X (v. 10.0.5) [27] using ClustalW with default parameters. These alignments were used in SDT (v. 1.2) [28] to calculate pairwise identities using the software's MUSCLE algorithm and were visualized in RStudio. Sequences of potentially novel viruses for which pairwise identities were below the species demarcation criteria for a corresponding genus (ICTV [25]) were further analysed.

Phylogenetic trees were constructed using the amino acid sequence of RNA-dependent RNA polymerase (RdRp) gene for selected known member species of that genus, new virus species discovered in this study, and an outgroup virus. The only exception is for *Sobemovirus* genus, where the complete genome nucleotide sequences were used instead. Outgroup viruses were selected from a different genus of the same virus family, following ICTV [25] recommendations, where possible. The selected sequences were aligned in CLC-GWB (v. 20-22, Supplementary Information 1, S3), and the most conserved region was selected for phylogenetic analysis (Supplementary Information 1, S8). This alignment was further trimmed using the ‘automated1’ method in trimAl (v. 1.3) [29]. Phylogenetic trees were constructed using IQtree (v. 1.6.12) using the maximum-likelihood approach, with the selection of the most appropriate substitution models performed in the same software [30] (Supplementary Information 1, S9). The phylogenetic trees were visualised in iTOL (v. 6.7) [31].

2.5.5. Comparison of water viromes and viromes of surrounding plants

In a parallel study [19] samples from tomatoes and surrounding weed plants, from the same locations as in this study, were analysed for the presence of plant viruses. In order to compare viruses that were found in these plants to the viruses found in water samples, reads from each water sample were mapped using CLC-GWB (v. 20-22, parameters in Supplementary Information 1, S3) to a user-defined database consisting of all viral sequences detected in the analysed plants (a complete list of viruses detected in plants can be found in [19]). Occurrence of viruses detected both in water and plants, and abundance of reads corresponding to selected viruses found in water, were plotted on the map of the sampling locations using RStudio (v. 2021.09.0) and edited in Inkscape (v. 0.92).

2.5.6. Analysis of the genomic diversity of Plantago tobamovirus 1 in water and plant samples

Plantago tobamovirus 1 (*Tobamovirus, Virgaviridae*) (PTV1) was detected in *Plantago major* for the first time in a plant [19] at one of the sampled locations. Subsequent analysis showed its presence in water samples in various other locations covered in the study. To explore the variability of PTV1, contigs (generated using the second assembly approach, Section 2.5.3) from all samples that had > 90% identity with PTV1 in BLASTn analysis against an entire NCBI nr database (v. 248) were aligned in CLC-GWB (V. 20-22, parameters in Supplementary Information 1, S3). Two longer genome parts in which several contigs overlapped, were used for further analysis (Supplementary Information 1, S9). Pairwise identities based on those two alignments were calculated using SDT (v. 1.2) [28]. In the next step, the two selected alignments were additionally aligned using the same algorithm to include ribgrass mosaic virus (*Tobamovirus, Virgaviridae*) as an outgroup. Phylogenetic trees were constructed from those assemblies using neighbour joining method in CLC-GWB (v.20-22, parameters in Supplementary Information S3, S9) and the leaves representing different consensus virus genomes were connected to corresponding locations on the map, using Inkscape (v. 0.92).

3. Results

3.1. Targeted detection confirms the efficiency of concentration approach and shows the presence of nucleic acids of pathogenic plant viruses in analysed water samples

Each water sample was concentrated using the method described to increase the relative abundance of present viruses. The efficiency of concentration was tested by targeted qPCR for two selected viruses expected to be present in many samples. The method performed efficiently as seen from the reduction of Cq values from raw to elution fraction for PMMoV and ToMV (Supplementary Information 3, Figure 9). In the cases where Cq reduction could be

calculated, it ranged from 1.8 to 8.6. In addition, CGMMV was tested only in the elution fraction (Supplementary Information 1, S2). The Cq values obtained for the detected viruses (elution fraction) varied between Cq 36 and 20. ToMV was most abundantly present and detected in 21 out of 22 samples, followed by CGMMV (18/22) and PMMoV (15/21).

3.2. The metagenome overview and the effect of the water source on the amount and diversity of detected plant virus sequences

Taxonomic classification of reads was performed, to obtain a general overview of the metagenome of the concentrated water. A considerable proportion of reads (37.6-80.5%, depending on the sample) did not provide matches in the used database. Bacteria accounted for the second largest proportion (5.9-58.8%), followed by eukaryotes (1.3-44.5%). As for viral reads, they account for 0.01 to 7.7% reads per sample (Figure 2a). Across the samples, we detected reads corresponding to all the genome organization types for viruses, with (-) ssRNA and dsRNA viruses being the least abundant (< 1% of all viral reads for samples in which they were detected). We further focused on ssRNA viruses, since 75% of known plant viruses have this genome organization [32]. Per sample, between 0.6% and 95.6% of reads assigned to viruses were indeed classified as ssRNA viruses. In addition, unclassified RNA viruses (which can have any variation of RNA genome organization) were the second most abundant group, accounting for 0.3-60.4% of viral reads per sample (Figure 2b). High abundance of plant virus sequences was confirmed by looking at the number of viral reads binned by the expected host organism. In this analysis, in almost all samples, majority of viral reads were assigned to viruses infecting bacteria and archaea (when excluding the wide host range/unclear category), followed by viruses infecting plants, fungi and protists. In the case of samples P-19-03, U-19-01 and R-19-13, plant/fungi/protist viruses are the most abundant subset (Figure 2c).

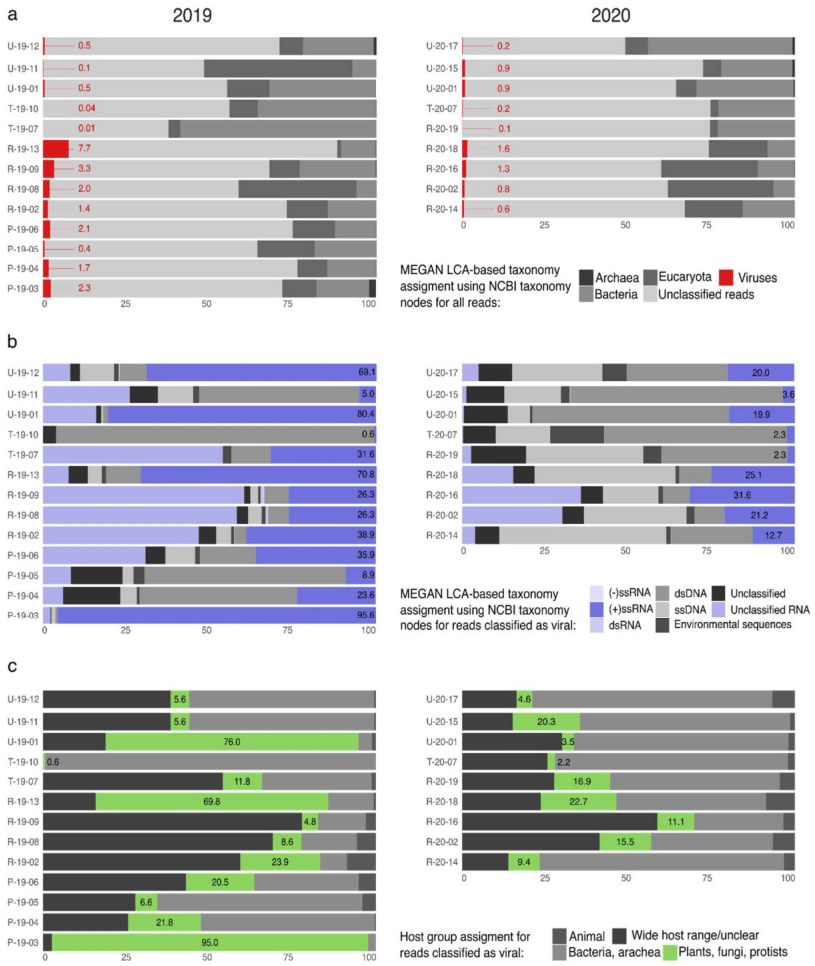

Figure 2 – Classification of sequencing reads for different collected water samples, based on DIAMOND blastx similarity search, followed by MEGAN-LCA binning. (a) “Domain” level classification of all reads – highlighted and noted in red are proportions (%) of reads belonging to viruses. (b) Classification of viral reads according to their genome types based on the "order” level classification associated with available genome type information from ICTV – highlighted in shades of purple are proportions (%) of viral reads belonging to RNA viruses with different genome types. Numbers denote relative proportion (%) of (+)ssRNA viral reads. (c) Classification of viral reads according to predicted host groups based on the "family” level classification associated with available host information from ICTV [25] database – denoted and highlighted in green are proportions (%) of reads belonging to host group “Plants, fungi and protists”. In all panels, samples are separated in two columns by year.

Plant viruses from a total of 20 families were detected across samples (Figure 3). The most abundant viruses belonged to the *Virgaviridae* and *Tombusviridae* families, for which reads were detected in 19 out of 22 analysed samples (Figure 3). We can observe a difference between groundwater and underground water samples subgroups at this taxonomic level. For the groundwater samples, the number of families detected per sample ranged from 2 to 17, with the average number being 10. On the other hand, for the underground samples, the average number of detected families is 5 and in none of the samples we detected more than 9 families (Figure 3).

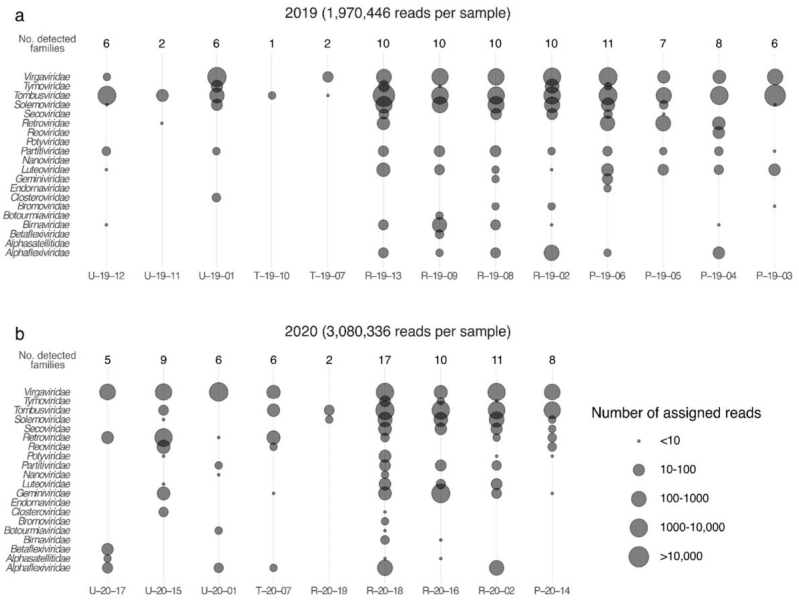

Figure 3 – Classification of plant virus reads for different collected water samples, on the level of families. Bubble charts depict detected families of plant viruses in each sample and their corresponding read counts. The size of the bubble shows the number of assigned reads, based on DIAMOND blastx similarity search, followed by MEGAN-LCA binning. Samples are divided by sampling year: (a) 2019 and (b) 2020, due to the different subsampling size (listed next to the year designation); number of detected plant virus families for each sample is shown at the top of each panel.

Similar trend can also be corroborated by the results of the targeted qPCR tests for three selected tobamoviruses (ToMV, PMMoV, CGMMV). A difference in both the number and signal strength for tested viruses was present between the two main water subtypes (groundwater and underground water). In eight out of 13 groundwater samples, all 3 targets

were detected with the strongest signal recorded for ToMV in sample P-20-14 (Cq 20). On the other hand, we did not detect all 3 viruses in any of the underground water samples and Cq values in these samples were above 25.

3.3. Many known plant viruses were detected in analysed water samples

We have next looked further into classification of the viral reads on the genus and species level to search for presence of known plant viruses in irrigation and other ground water samples. Reads corresponding to plant viruses from 18 different genera were detected. All of the genera were found in the groundwater samples, while only 12 of them were detected in underground water (Figure 4, 5, Supplementary Information 1, S8). Reads assigned to *Tobamovirus, Tombusvirus,* and *Sobemovirus* genera were most abundant across samples.

Overall, reads of 73 different plant virus species were detected (Supplementary Information 1, S8). Thirty-seven percent of detected viruses were present only in individual samples and 10 viruses had more than 100 associated reads in an individual sample (Figure 4, 5, Supplementary Information 1, S8). These viruses belong to the genus *Aureusvirus* (1 species), *Tombusvirus* (3 species) and *Tobamovirus* (6 species). Some of the species, detected in both ground and underground samples, include ToMV (detected in 14 out of 22 samples), Moroccan pepper virus (*Tombusvirus, Tombusviridae*) (11/22), tobacco mosaic virus (*Tobamovirus, Virgaviridae*) (11/10) and pelargonium leaf curl virus (*Tombusvirus, Tombusviridae*) (10/22).

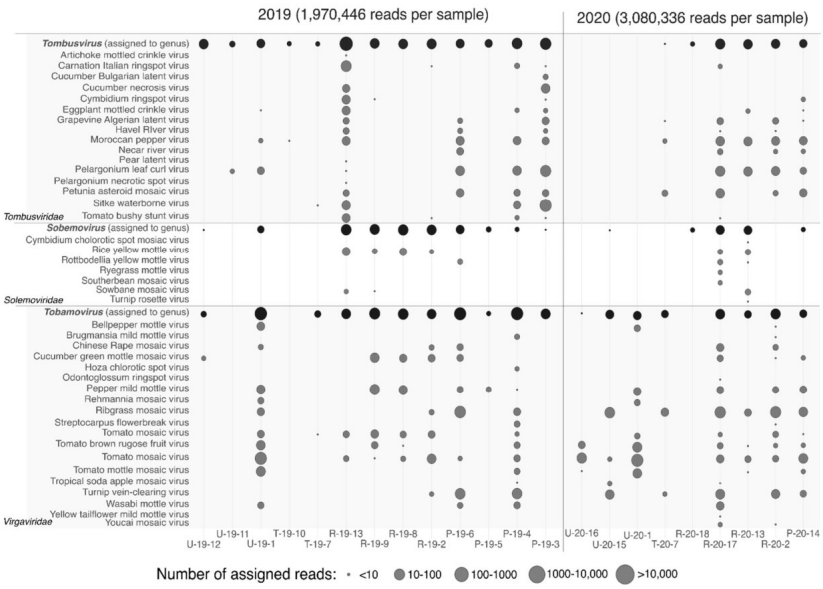

Figure 4 – Classification of plant virus reads for three most abundant plant virus genera for different collected water samples, on the level of species and genera. Bubble charts depict detected species/genera of plant viruses in each sample and their corresponding read counts. The size of the bubble shows the number of assigned reads, based on DIAMOND blastx similarity search, followed by MEGAN-LCA binning and additional curation. Reads that could not be classified to the level of species (because they were too divergent) were classified on the level of genera (black bubbles). Samples are divided by sampling year: (a) 2019 and (b) 2020, due to the different subsampling size (listed next to the year designation).

Although nearly all viruses were present in ground and underground water sources, exceptions exist, such as the case of rice yellow mottle virus (*Sobemovirus, Solemoviridae*) that was detected only in groundwater, and the Rehmannia mosaic virus (*Tobamovirus, Virgaviridae*), detected only in underground water.

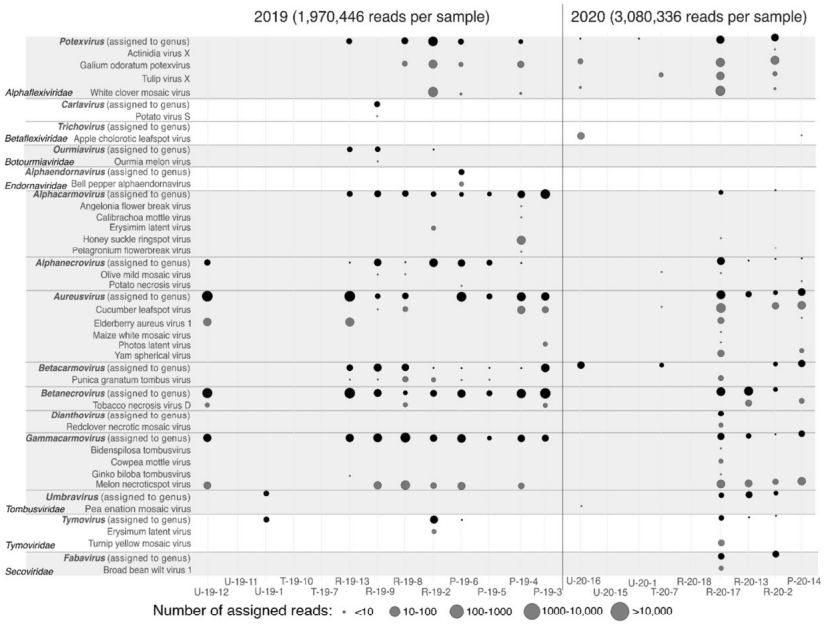

Figure 5 - Classification of plant virus reads for fifteen less abundant plant virus genera for different collected water samples, on the level of species and genera. Bubble charts depict detected species/genera of plant viruses in each sample and their corresponding read counts. The size of the bubble shows the number of assigned reads, based on DIAMOND blastx similarity search, followed by MEGAN-LCA binning and additional curation. Reads that could not be classified to the level of species (because they were too divergent) were classified on the level of genera (black bubbles). Samples are divided by sampling year: (a) 2019 and (b) 2020, due to the different subsampling size (listed next to the year designation).

3.4. Virome analysis of water samples revealed new plant viruses present in the environment

We assembled complete or near-complete genomes of seven new viruses spanning five different genera, obtained from five different water samples (Supplementary Information 1, S11). Complete genome sequences were reconstructed for three new viruses, and partial genomic sequences, lacking one or more ORFs, were reconstructed for four new viruses.

Partial genomes of three novel viruses from three different genera were obtained: Novo mesto aureusvirus 1 (*Aureusvirus, Tombusviridae*), Gorica betanecrovirus 1 (*Betanecrovirus, Tombusviridae*), and Bericevo sobemovirus 1 (*Sobemovirus, Solemoviridae*) (Supplementary Information 3, Figure 1-3). Percent pairwise identities comparisons showed that each virus was below the species demarcation criteria for the corresponding genus (Supplementary

Information 3, Figure 4-6). Based on the phylogenetic analysis, we provided details on how the new viruses clustered within their genus in Supplementary Information 3, Figure 10-12.

Tombusvirus

Partial genome sequence of Gorica tombusvirus 1 (*Tombusvirus, Tombusviridae)* (location 3, year 2019), lacking the movement protein and silencing suppressor ORFs, and complete genome sequence of Krkavce tombusvirus 1 (*Tombusvirus, Tombusviridae)* (location 13, year 2019) were reconstructed (Figure 6). Percent pairwise identities for the amino acid sequence of coat protein (CP) showed that both new viruses have 80% or less identity with other viruses in the genus, which is below the current species demarcation criteria (Figure 6). Phylogenetic analysis including tombusviruses revealed that Gorica tombusvirus 1 is not closely related to other tombusviruses, although its assignment to the genus is well supporter with bootstrap analysis, and that Krkavce tombusvirus 1 is clustering with cucumber necrosis virus (Figure 6).

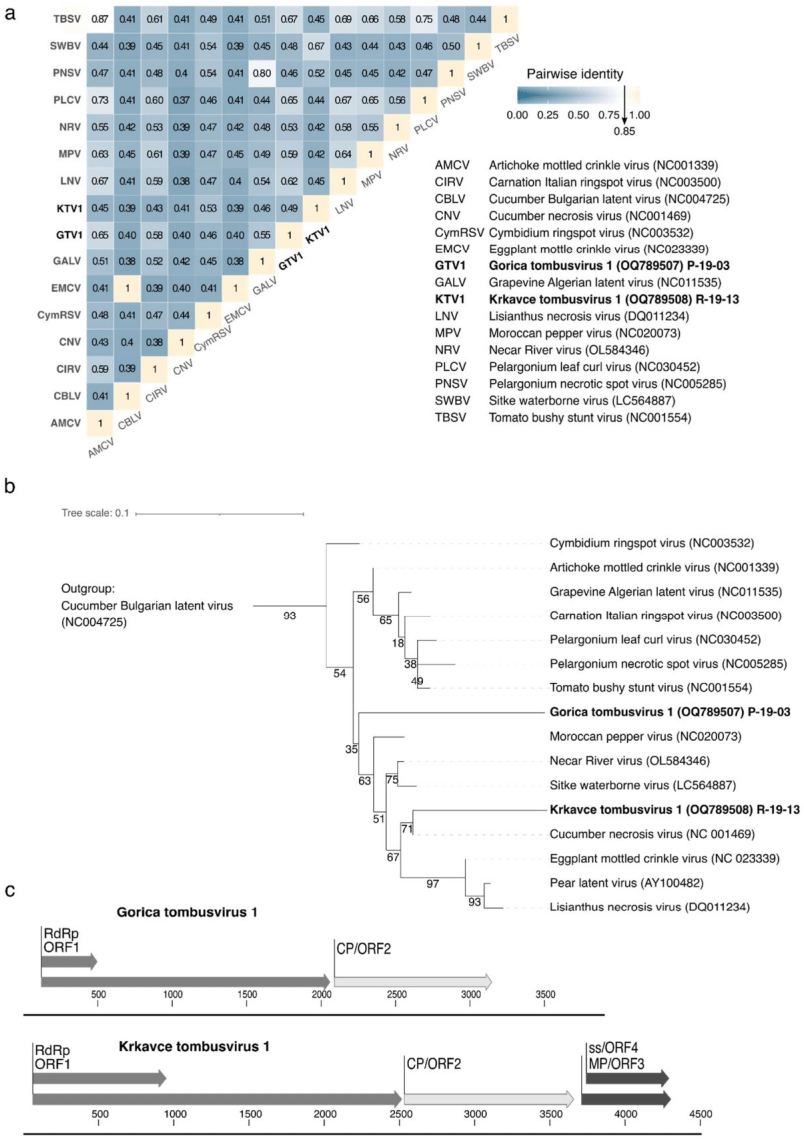

Figure 6 – Genomic characteristics of two new tombusviruses discovered in water samples. (a) Percent pairwise identities for the amino acid sequence of coat protein (CP) for the two new viruses and members of the *Tombusvirus*

genus; arrow on the colour key designate the ICTV proposed species demarcation criterion (b) Maximum likelihood phylogenetic tree based on the alignment of conserved RdRp protein sequences of the two new viruses (bolded) and members of the *Tombusvirus* genus, rooted using the outgroup (maize white line mosaic virus, *Aureusvirus*, *Tombusviridae*); numbers in brackets represent NCBI GenBank accession numbers of corresponding nucleotide sequences; numbers on the branches represent bootstrap support values; the branch length represents average number of amino acid substitutions pre site. (c) Genome structure of the two new tombusviruses with annotated predicted ORFs.

Tobamovirus

Two complete novel tobamovirus genome sequences were reconstructed, Gorica tobamovirus 1 (*Tobamovirus, Virgaviridae*) (location 4, year 2019) and Bertoki tobamovirus 1 (*Tobamovirus, Virgaviridae*) (location 17, year 2020). Both viruses show genome composition and organisation typical of tobamoviruses (Figure 7a). Percentage pairwise identity comparisons based on complete genome nucleotide sequence revealed that both viruses have pairwise percent identities with other tobamoviruses lower than the species demarcation criteria (Supplementary Information 3, Figure 7). Phylogenetic analysis showed that Bertoki mosaic virus 1 is clustered (with high bootstrap support) in the subclade containing ribgrass mosaic virus and related viruses. Consistent with the results of the pairwise identity comparisons, Bertoki tobamovirus 1 is most closely related to PTV1. Gorica tobamovirus 1 represents more divergent branch on the tree, which still clusters with the same bigger subclade within the genus (Figure 7b).

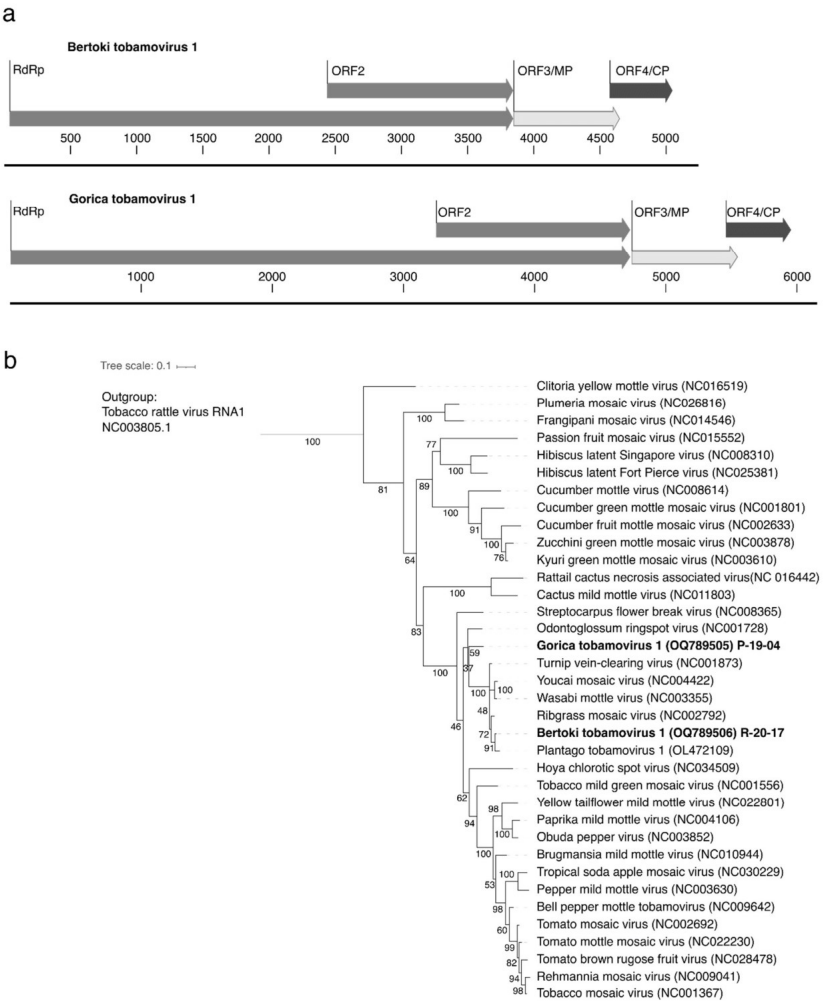

Figure 7 – Genomic characteristics of two new tobamoviruses discovered in water samples. (a) Genome structures with annotated predicted ORFs. (b) Maximum likelihood phylogenetic tree based on the alignment of conserved RdRp protein sequences of the two new viruses (bolded) and members of the *Tobamovirus* genus, rooted using the outgroup (tobacco rattle virus, *Tobravirus*, *Virgaviridae*); numbers in brackets represent NCBI GenBank accession numbers of corresponding nucleotide sequences; numbers on the branches represent bootstrap support values; the branch length represents average number of amino acid substitutions per site.

3.5. Overlaps in detection of viruses in water and plant samples

In a recently published parallel study, we analysed samples of tomato and weed plants from the same locations as the water samples used in this study, to detect plant viruses [19]. Here, we now compared viruses found in plants and in water to see if there were any overlaps in detection (e.g., year, location, region). A total of 14 viruses and 2 satellite viruses were detected in both plant and water samples, disregarding the location and sampling year (Supplementary Information 1, S10). Out of those 16, four are not associated with plants as hosts, another four are new viruses detected for the first time in the previously mentioned plant virome study and the remaining 6 viruses and 2 satellite viruses are previously known to infect plants. Five plant viruses, (olive latent virus 1 (*Alphanecrovirus, Tombusviridae*), white clover mosaic virus (*Potexvirus, Alphaflexiviridae*), ToMV, and tomato bushy stunt virus (*Tombusvirus, Tombusviridae*), with tomato bushy stunt virus satellite RNA B10), were detected in both plant and water samples at the same location, at the same sampling time (Figure 8a). Instances of plant virus detection in both water and plant samples were observed in 3 out of 4 regions. However, the regional distribution of viruses detected in plants that were also detected in water varied, from detection in water in all 4 regions (i.e., ToMV), to detection in only one (i.e., olive latent virus), (Figure 8b). Additionally, also the 4 new plant viruses, which we detected and reconstructed from water for the first time in this study, were not found only in single samples; we were able to detect their nucleic acids in several different samples across the sampled sites (Supplementary Information 1, S11).

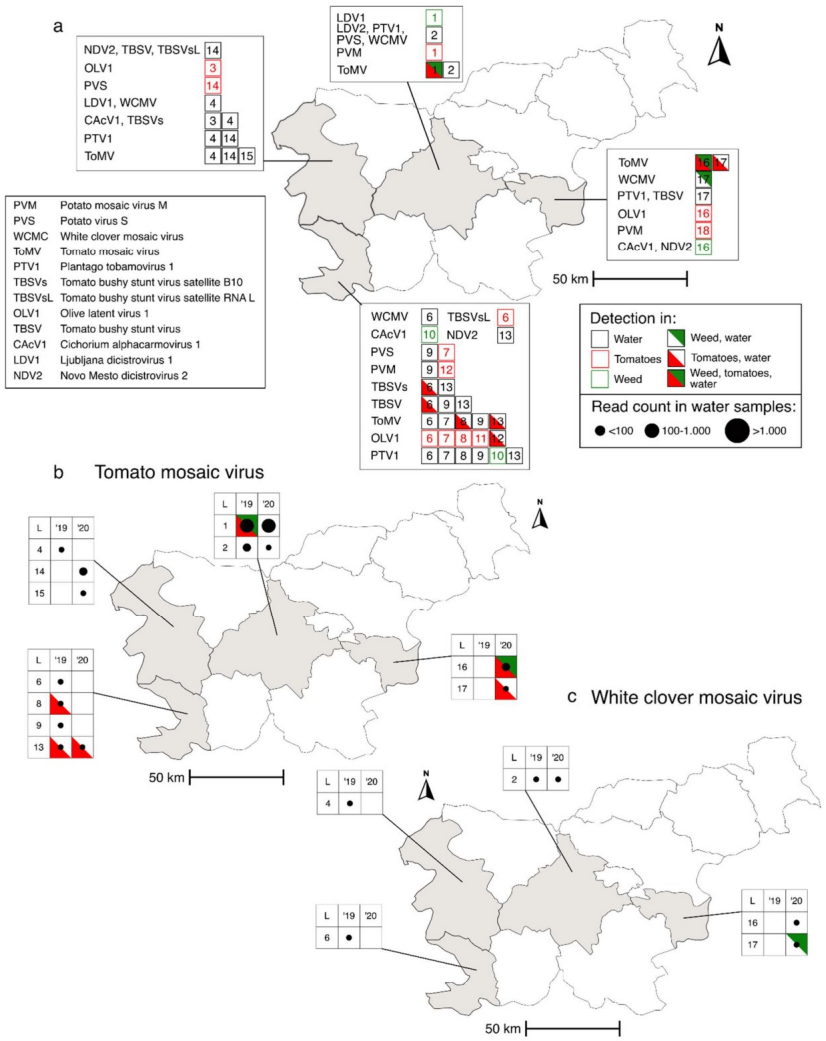

Figure 8 – Geographical distribution of plant viruses detected both in water and plant samples. (a) Map showing the exact locations (next to the virus names' abbreviations) of the detection of all such viruses in tomatoes, weeds, water or combination of these sample types (denoted by the colour coding explained in the legend). Viruses detected both in plants and in water at the same location were always detected in both sample types at the same sampling time. (b) – (c) Detailed results about the abundance of virus reads in water samples for two selected viruses, (b) tomato mosaic virus, (c) white clover mosaic virus. The first column of the matrices represents the

location (L) where the virus was detected (Supplementary Information 1, S1), second two columns represent the years of sampling. The size of the dot in each cell corresponds to the number of virus reads in water samples. The type of the plant sample (tomato, weed) in which the virus was detected is denoted by the colour coding explained in the legend as in (a).

3.6. Virome analysis of water samples informs us about the diversity of a recently discovered virus

To further explore the potential of the obtained water virome data we performed a more in-depth genomic analysis of a selected plant virus, a recently discovered PTV1, which was detected in several analysed water samples. A total of eight partial consensus genomic sequences with highest similarity to this virus from seven different water sampling locations, collected across two years, were reconstructed and compared against each other and with the original virus genomic sequence reported from a plant [19]. Four of them covered the partial genomic sequence including ORF coding for RdRp and six partial genomic sequence including ORF coding for CP. Pairwise identities of sequences ranged from 89-99% (Supplementary Information 3) and phylogenetic analysis revealed clustering of isolates in subgroups (Figure 9) for both alignments. Sequence obtained from the plant clustered together with a sequence obtained from the water in the same region, in both analyses (Figure 9). Phylogenetic analysis for partial RdRp included four water-derived sequences, all from the western region of the country, and revealed two subclusters among them. Phylogenetic analysis for partial CP included six water-derived sequences and revealed several subclusters corresponding with geographic location of samples: sequences from the western part of the country clustered together and sequences from central and eastern part of the country formed a separated divergent cluster.

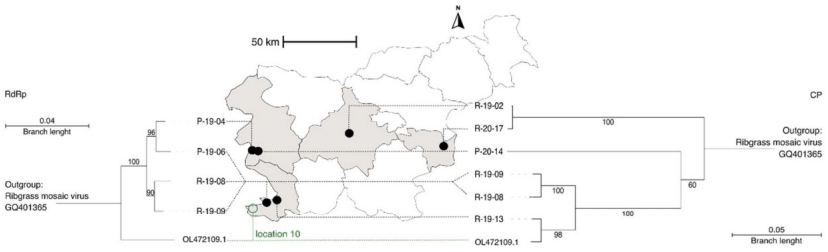

Figure 9 – Genetic diversity of PTV1 in plant and water samples. Neighbour joining phylogenetic trees based on the alignment of partial consensus genomic sequences of PTV1 obtained from water samples and plant derived sequence from a previous study, rooted with the outgroup (ribgrass mosaic virus). Tree on the left is based on the alignment of partial genomic sequence including ORF coding for RdRp and tree on the right is based on the alignment of partial genomic sequence including ORF coding for CP. Numbers next to the branches represent bootstrap support values; the branch length represents average number of nucleotide substitutions per site. Viral genomic sequences on the tree are designated with sample numbers for water-derived consensus genomes and

NCBI GenBank accession number for plant derived sample and are connected to the sampling locations as indicated on the map.

4. Discussion

The quality of irrigation water can play a vital role in the health and productivity of agricultural crops. Presence of plant viruses in water has been known for a long time [2], and interest in their presence in irrigation water is slowly increasing. This study described a comprehensive virome analysis that explored diversity of plant viruses in irrigation water. Shotgun HTS analysis, that was applied to water samples, shed light on the viral abundance and diversity within the samples. Viral reads accounted for around 1% of all reads, comparable to previously reported results [4], [9]. Although the sample preparation used here aimed at concentrating RNA sequences, we observed all possible variations of viral genome types within each sample. With this amount of data, we were able to detect members of 20 viral families infecting plants. There is a notable difference in both abundance and richness of detected viral families between ground and underground water sources, which can implicate diverse water quality for irrigation purposes. Two most abundantly present plant virus families in our samples, *Virgaviridae* and *Tombusviridae*, are frequently detected in aqueous environments [4], [12], [33]. Member species of both of these families have experimentally been shown to retain infectivity in extreme conditions, such as the human gastrointestinal tract [34]. Therefore, their abundant presence in water in comparison to less stable viruses is consistent with previous results. Individual species from these families are considered an economical threat to various agriculturally important plant species, mainly from *Solanaceae* family (e.g., ToMV in tomato). Out of 73 plant viruses detected in this study, ToMV was the most frequently and abundantly detected virus throughout the samples.

Using HTS-based water virome analysis we were able not only to detect wide variety of known plant viruses that circulate in the environment, but also discovered new plant viruses. Few instances of discovery of novel plant viruses in water samples exist prior the use of HTS. For example, Sikte waterborne virus, was first detected by inoculation of test plants with water from Sikte river in Germany [35]. However, it took another 15 years for its detection in plants outside the laboratory and a few more years to obtain its complete genome [35]. By analysing the sequence data obtained from water samples in this study we were able to discover seven new virus species from five different plant virus genera, reconstruct their complete or near-complete genomes, and show their presence in several water samples, often in different regions.

Within this study we were able to leverage previously available information on plant virome from the same sampling locations [19]. This enabled us to conduct a pioneering approach to

compare the presence and abundance of plant viruses both in water and in plants within same agroecosystems. For example, by comparing the detection instances in water with detection in tomatoes [19], we observed a simultaneous detection of ToMV in both sample types for location 1 (Figure 8b). At the time of sampling, a large-scale outbreak of ToMV in tomato was observed in one of the greenhouses at the tomato farm that comprises this location. This brings to light the usefulness of water testing for the surveillance of plant virus outbreaks, since the viruses can be released from the plants into the environment. This concept was to some extent already explored in our previous research, where we showed that tomato brown rugose fruit virus (*Tobamovirus*, *Virgaviridae*), a damaging tomato pathogen, can be detected in water in experimental hydroponics system and in drain water from commercial greenhouses, when plants are infected [8]. As we have seen during the COVID-19 pandemic, wastewater monitoring schemes proved their applicability as an early warning system [18]. It is likely that a well-positioned (irrigation) water monitoring scheme can provide similar benefits in the context of managing plant health.

The detection of ToMV and only few other viruses in both plants and water samples from the same location and year (Figure 8a) might indicate that this pattern can be observed only if there is a high virus infection burden in the planted crops. In total, we detected 16 viruses that were previously found in plants at sampled locations [19], and also in water. However, most often they were not found in water at the same location or at the same sampling time as in plants. In many water samples, where virus reads counts were low and same viruses were not detected in nearby plants, deducing the connection between the plants and water is not possible. Nevertheless, detection of plant viruses in water can have several important implications. For example, the detection of important plant pathogen (e.g., a virus on quarantine list for a region) in water samples can motivate increased survey for the virus in nearby host plants or intensified research on the characteristics of such virus in the environment [8]. Moreover, as we have demonstrated in this study, water virome data can rapidly provide a wealth of information about possible distribution of known and newly discovered viruses in the environment. For example, white clover mosaic virus was detected in plant virome analysis in a single plant sample on studied locations, however, addition of data from water analysis indicated much wider distribution of virus in the environment, since it was detected in nearly all water samples, covering all analyzed regions (Figure 8c). However, this study also highlights the limitations of using water samples as a proxy for distribution of plant viruses, as the lack of detection in water does not guarantee lack of detection in plants, like in the example of olive latent virus 1, which was detected at seven different locations in plants, but at only one in water (Figure 8a). These discrepancies can be expected, since many different factors such as virus titer in plants, abundance and distributions of host plants, and

virus stability in the environment, would affect the possible presence, abundance and spread of different viruses in environment.

HTS-based virome studies result in large sequence datasets that can be further exploited to study not only presence, but also diversity of viruses in a region under study. In this study, we demonstrated that water virome data can bring extensive additional insights into diversity of recently discovered viruses, for which limited information is available. Plantago tobamovirus 1 was recently discovered in a single plant sample [19] at one of our sampling locations. Here, we detected it in multiple water samples, covering all regions under study. We reconstructed partial consensus genome sequences of this virus from several water samples and confirmed notable genetic diversity within the species. Moreover, clustering of isolates derived from water virome data is largely matching the geographic distribution of sampled waters. Thus, having only a single occurrence and genomic information about the virus from its plant host, using water virome data, we were able to discover that virus is likely widespread and genetically diversified in the studied ecosystems. Interestingly, closely related tobamovirus from the ribgrass mosaic virus subgroup have been isolated from river water from Hungary in 1986 [36]. Together with results from this study, this opens interesting questions about the wide presence and survival of these viruses and abundance of their hosts in ecosystems, likely close to the surface water bodies.

Within the irrigation water virome study presented here, we obtained rich information about the presence of plant viruses in the region (also for some that were previously not reported in the country). We demonstrated usability of environmental water virome analysis as a tool for studying the diversity and ecology of plant viruses, as well as for the discovery of novel virus species. In contrast to more traditional plant virus surveillance targeting host plants, water virome analysis allows monitoring of a larger area with a single sample and might provide early warning prior to large outbreaks in agroecosystems. Additional research should be focused on assessing the biological relevance of new viruses found in environmental irrigation waters and to study the directionality of spread between viruses found in water and plant components of the ecosystem. Moreover, HTS-based analysis of irrigation waters can be employed to study within-species diversity of plant viruses and detect potentially emergent virus variants that could represent threat for global plant health.

Conclusions

- HTS-based virome analysis of irrigation and other ground water sources informed us about the presence of a wide range of plant viruses in the regions under study, with environmentally stable viruses, such as tobamoviruses and tombusviruses, being the most abundantly detected in the analysed water sources.

- Notable differences in both abundance and richness of plant virus nucleic acids were observed between different water types, whereas ground water samples in general had higher count of plant viruses present in higher abundance, which might imply different quality of investigated water types.
- The information obtained from water virome analysis allowed us to detect both known viruses previously not found in an area and new virus species, which overall,provided us with a clearer understanding of environmental presence of a given virus and its diversity in the agroecosystem.
- Understanding the presence and diversity of plant viruses in irrigation water might be a contributing factor in ensuring effective management of plant health in future. Water virome analysis was shown here to be a useful tool for the surveillance and discovery of known and new plant viruses in a wider environment.

Data availability

HTS data produced in this study is available via European Nucleotide Archive (ENA) under project code PRJEB60028. Complete or partial genomes of new virus species discovered in this study were submitted to NCBI GenBank, and their accession numbers can be found in Supplementary Information 1, S11. Consensus genomic sequences of PTV1 obtained from water samples are available in Supplementary Information 2. Codes used for additional reads classification curation and generation of figures in R are available in Supplementary Information 2.

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Chapter 3

Discussion

3.1 Setup of a Large-Scale Methodology for Wastewater Monitoring – Lessons Learned

Monitoring viral pathogens by implementing wastewater-based epidemiology (WBE) can be a valuable tool for early detection and assessing the effectiveness of public health interventions. However, implementation of WBE system that ensures accuracy, efficiency, and reproducibility comes hand in hand with several challenging factors/issues.

In the first study, we addressed the significance/implication of the fundamental steps of the WBE approach, including sample storage, sample concentration, and target detection, highlighting the importance of adapting guidelines provided by institutions such as the WHO [14], CDC [41], and KWR, Netherlands, to local laboratory conditions.

Sample storage and processing time are critical factors potentially impacting the target's integrity in wastewater samples[42]. Assessing the stability of RNA (extracted from positive controls, EVA-GLOBAL) and thermally-inactivated whole viruses showed that RNA, compared to virus particles, degrades significantly faster at 4°C, as indicated by the increase in RT-qPCR Cq values, suggesting that the state in which the virus is present in the sample can affect detection of the virus in wastewater [9]. Moreover, storage up to 24h in the freezer (-20°C) increased Cq values by about 2. However, prolonged storage times of up to 7 days did not additionally increase Cq values, indicating that freeze-thawing cycles during sample analysis could have a more relevant impact on RNA integrity than storage time itself [9]. The findings highlight the importance of carefully considering sample storage conditions and processing times while implementing the WBE approach to ensure accurate detection and quantification of viral targets. Accordingly, samples were processed within 24h of admission and kept at +4°C for the analysis duration. A subsequential interlaboratory evaluation confirmed our results [43]. Furthermore, the researchers did not observe any significant differences in final results after up to 3 days of storage at +4°C.

Setup of the WBE protocol should recognize that other aspects of the method (e.g., choice of target gene) can affect the results, and previously mentioned study has established that the storage conditions affect concentration methods targeting whole intact particles more than methods targeting naked RNA [43]. Optimizing sample concentration is another crucial step in the WBE approach necessary to increase detection sensitivity. We described the concentration protocol using Centricon Plus-70 Centrifugal Filters coupled with QIAmp RNA Mini Kit for RNA extraction and its applicability for SARS-CoV-2 concentration in wastewater. The protocol' s performance was estimated by reducing the Cq value after the concentration step [9]. We also compared this system to alternative

protocols (i.e., PEG precipitation, skimmed milk, Vivacell 30 kDa ultrafiltration units, CIM-QA chromatography columns). Time constraints permitted only screening of the alternative protocols, leaving room for additional improvements to the workflow, potentially producing even better results. Nevertheless, our screening highlighted the performance of the semi-automated kit from Promega as the most comparable to Centricon filters, with significantly higher throughput.

Other studies reported comparable results [11], providing valuable insight for laboratories looking to implement similar concentration methods for WBE in their local conditions.

Since Centricon filtration units were the method of choice in the first studies at the beginning of the pandemic [6], they rapidly became the method of choice for most laboratories, subsequently resulting in global shortages, only to generate widespread development of alternatives (like the abovementioned Promega kit) found to be as effective and often more cost-efficient.

Lastly, we evaluated various RT-qPCR assays and associated mastermixes. Selecting a suitable target gene (N1 and N2 in our case) is crucial for accurately detecting and quantifying viral targets in wastewater samples. Contingency plans are advantageous in crises, such as the pandemic, as reagent shortages are expected. The comparison showed that the TaqMan Fast Virus 1-step mastermix performs better at low virus concentrations while maintaining a correlation between the two tested genes [9]. Our findings emphasize the importance of thoroughly evaluating different assays and selecting the most appropriate one for the specific target virus and laboratory conditions to ensure reliable results. The evaluation conditions should be as close as possible to the expected scenario; in this case, accounting for expected throughput and using wastewater as the matrix. The research highlighted the need to carefully select the most appropriate reference material, which can be an additional difficulty in crisis times. The evaluations and comparisons paved the way for establishing a successful methodological procedure implemented in Slovenia's national wastewater monitoring scheme, thus answering this thesis' s first aim.

3.2 Different Methodological Approaches and Impact on Sensitivity in Closed Water Systems

The second publication moves from wastewater monitoring for human pathogens towards detecting economically relevant plant viruses, namely ToBRFV, in environmental water and closed irrigation systems, such as hydroponic growing facilities. Following the detection of the virus in irrigation water used in the fields in Slovenia, we have set up experiments that investigated the presence of ToBRFV in a growing medium surrounding infected tomato plants, mimicking a hydroponic setup inside a research greenhouse facility.

ToBRFV is now a well-known viral pathogen affecting mostly tomatoes and peppers, causing significant economic loss to the crop [24]. The virus is a member of the *Tobamovirus* genus, which are known to be highly environmentally stable [30]. In Slovenia, the ToBRFV's RNA was detected in wastewater samples as far back as 2017 [4] and in samples of water used for crop irrigation in subsequent years [22]. Its presence in plants was only confirmed several years later, following a routine inspection by the Ministry of Agriculture [31]. The source of water contamination with ToBRFV remains unknown, and further research is needed to determine the potential transmission routes [22]. Possibilities include farm run-offs, decaying plant material, or entry via treated wastewater, similar to other tobamoviruses such as PMMoV. Irrigation water could be a potential route for plant infections, which becomes even more relevant in a closed system such as hydroponic

growing facilities. There have been confirmed instances of pathogen infection in green houses where the culprit was the infected irrigation water [44]. The instances cover a variety of pathogens [45], as well as numerous virus species [22], [46]. An appropriate inspector standardly visits large-scale hydroponic facilities and collects leaf samples for further testing. The process can take several days (or weeks), and it is up to the inspector's expertise to select the appropriate sample. Exploring a more cost-efficient and less time-consuming approach to monitoring the presence of the virus in such close systems, we explored various strategies to detect ToBRFV in a growing medium instead of leaves. Simulating hydroponic facility, the test plants were kept in a growing medium instead of soil, with 3 plants per tray. Only the roots were in contact with the medium. Test plants were inoculated with the virus and kept for up to 1 month. The growing medium was sampled approximately every two days and tested for ToBRFV's RNA with 3 different approaches. Attempting to detect the virus with as little sample preparation as possible, we tested the growth medium directly with RT-qPCR. Nine days after inoculation, we saw a reliable positive signal indicating the presence of the target RNA in the growth medium, clearly distinguishable from the control (no signal). Although the experiment confirmed the detection of the reliable signal with little to no sample preparation, it is not possible to determine if the signal truly came exclusively from the virus being released from the roots, as the trays were not covered, nor in which form was the virus present in (whole particle, just RNA, fragments, etc.).

Striving to increase detection sensitivity, we used the same water samples and processed them using the QIAmp Viral RNA Mini kit, directly extracting the RNA from the medium or concentrating the growth medium using Centricon Filtration units. In both cases, we obtained low-level signals as early as day two post-infection and significantly lower Cq values (up to 8 Cqs difference) for later days post infection (up to day 9) compared to non-concentrated, non-extracted samples. Although the concentration-extraction step increases sensitivity of the detection, it also introduces a bias as exposure to environmental contaminations increases, mandating stricter procedures and selecting more rigorous conditions to avoid false positives. Establishing fixed thresholds would not be recommended in this case, as it could easily lead to missing truly low-level positive results due to the aggressive nature of the virus leading to devastating economic losses.

These findings provide a valuable tool for rapid water screening in hydroponic sites to detect circulating ToBRFV early, correlating with similar findings for other plant viruses [17].

These preliminary findings pointed out the risk of transmission and prompted several other experiments, which were conducted as a part of the same publication but are beyond the contributions of this thesis. The complete study's outcome unveiled that infectious particles of ToBRFV could be released from the roots of infected tomato plants into nutrient solutions causing infection in bait plans after several weeks to months [22]. These findings highlight the need for effective management strategies to prevent water contamination with ToBRFV. Appropriate methodology for detection is essential, as it is needed to balance the detection sensitivity, speed, and ease of testing.

3.3 The Untapped Potential of Irrigation Water for Viral Discovery and Monitoring

Broadening the scope of our research beyond closed systems, we applied untargeted HTS analysis to study plant viruses in environmental water bodies, emphasizing irrigation water sources. Our third publication described the plant virome and determined sequences

of novel plant viruses from our dataset, placing the detected viruses in a broader context connected to previous work focusing on crop and weed plants from the same locations [47].

Examining the sequencing data, we observed a relatively low abundance of viral reads (from below 1% to 7.7% of total read count), consistent with previously published data [4], [33]. Notwithstanding the low proportion of viral reads in our samples, we distinguished a wide diversity of genome organizations. The least abundant were sequences of (-)ssRNA viruses, accounting for less than 1% of viral reads, whereas (+)ssRNA, which is the most frequent genome organization of plant viruses [48], accounted for up to 95.6% of viral reads. Classifying reads based on expected host groups showed that viruses that affect plants, fungi and protist account for anywhere between 0.6 and 95% of all viral reads, making them the most abundant group in cases of some samples. In addressing the third aim of this thesis, we found that the virome profiles of surface and underground sources depended on the water source. We detected 20 plant virus families across the sample range, *Virgaviridae* and *Tombusviridae* accounting for most reads.

Both of those families are well known to be commonly present in the water environment [4], [35], [48]. The most abundantly present genus, *Tobamovirus*, has several member species previously shown to be highly environmentally stable, even to the extremes, to retain infectivity after *in vitro* simulations of passing the gastrointestinal tract like pepper mild mottle virus [39]. Therefore, their more abundant presence in irrigation water is unsurprising, especially compared to other less stable genera. Taking into account all samples, we detected 73 individual viral species. The most predominant virus was the tomato mosaic virus (ToMV), also frequently detected in plant samples (tomato and various weed plants) from the same locations [47]. Comparing the detection instances in water and plant sample sets, we observed a connection between datasets, especially if an active outbreak of the particular virus had been confirmed for the location. The intensity of the signal (read number, subsequent RT-qPCR signal) was significantly higher compared to other locations. Although this trend persisted in both sampling years, it is difficult to discern if the virus entered the waterways from infected plants or if the contaminated water contributed to the infection of plants. Applying HTS on environmental samples like water has given a unique opportunity to detect various known and unknown viruses. However, it presents certain limitations. It is possible to detect false positives depending on the complete analysis pipeline, especially the data analysis [49]. In this case, one must be additionally careful when interpreting results that show low-level detection, particularly when the virus in question is on, i.e., a quarantine list for a given area. In such cases, it is necessary to confirm the presence of the virus with additional tests based on a different biological principle (e.g., ELISA) and make additional efforts to locate its presence in plants since detection in water samples does not give sufficient information about the origin of the infection.

Concerning new viruses, the situation is slightly more complicated. HTS analysis can reveal complete genomic sequences of unknown viruses and place them within the taxonomic classification. Our study revealed the presence of seven new plant viruses belonging to 5 different genera. Phylogenetic analysis further disclosed close connections between some newly detected plant viruses and previously characterized ones. Contrarily, isolated identification by HTS does not elucidate the biological relevance of newly found viruses, as long-term surveillance deems essential. Without additional biological characterization, we can only broadly assume the host range based on the genera, but we cannot discern the potential symptoms or impact on the overall plant health. In addition, the detected viruses (depending on their taxonomic classification) could potentially have other organisms as hosts or be integrated into the plant genome [49]. The selected methodology has left unresolved questions, nonetheless, obtained virome of irrigation water provides us with an abundance of useful data we would otherwise miss, such as the newly

detected Plantago tobamovirus 1. The initial analysis of plant samples revealed it only at a single location [47]. However, when testing water samples, many sampling points revealed positive detections, indicating that the virus is more widespread than the initial results for plants alone suggested. Further analyzing this virus' s sequences showed sufficient genetic diversity, with a well-supported clade comprised of sequences within close geographical proximity, whereas sequences from other regions mapped outside this clade.

Our study underscores the importance of employing HTS analysis for a comprehensive assessment of viral diversity in environmental water samples, as traditional detection methods and/or plant tissue analysis alone may not capture the full extent of viral diversity. However, taking a broader view, it should be noted that positive detection in water does not guarantee detection in plants at that site. Nevertheless, it should be a warning, especially for emerging pathogens. Implementing appropriate water treatment protocols, maintaining good agricultural practices, and regularly surveilling water sources are crucial steps in mitigating exposure to waterborne transmission of plant viruses.

3.4 Water-Based Epidemiology and Its Application

The insights gained from our studies collectively contribute to the advancement of water-based epidemiology as a reliable tool for monitoring infectious diseases, including SARS-CoV-2 and plant viruses, in environmental water samples. These studies highlight the challenges and considerations laboratories should expect when implementing WBE approaches. The first study emphasizes the technical aspects of how factors, such as storage duration and temperature, concentration, nucleic acid extraction, and viral target detection/quantification, may influence the accuracy of results and should be assessed discretely. The second study highlighted how water-based analysis could be a good alternative tool for viral detection, especially in the context of early detection in systems such as hydroponic facilities.

In conclusion, we demonstrated that the analysis of environmental water provides an abundance of information essential to studying plant viral ecology, diversity and epidemiology. The results of the last two studies raise concerns about exposure to potential threats associated with waterborne transmission of plant viruses, particularly in agricultural settings and in wastewater reuse irrigation circumstances, underlining the necessity for robust measures to prevent water contamination with plant viruses, such as implementing appropriate water treatment protocols, maintaining good agricultural practices and regularly monitoring water sources.

Additionally, our studies highlight the power of advanced molecular techniques, such as HTS analysis, to study viral diversity and ecology in environmental water samples. Identifying new plant viruses from different (lineages) genera expands the list of known plant viruses and provides insights into their potential evolutionary relationships, transmission routes and ecological dynamics in aquatic environments. HTS analysis allows a more comprehensive assessment of viral diversity compared to traditional detection methods and has become an invaluable tool in viral discovery and characterization in various environmental samples, including wastewater.

Further research in this field will advance our understanding of the dynamics of viruses in environmental waters, their potential threat to human and plant health, and the development of effective viral monitoring, prevention, and control strategies. Integrating advanced molecular techniques such as HTS analysis with other interdisciplinary approaches, such as epidemiology, virology, environmental science, and public health will further enhance our ability to detect, monitor, and control viral pathogens in water resources and minimize human and environmental health exposure.

Chapter 4

Conclusions

Understanding the intricate connections between viral diversity, wastewater-based epidemiology, and environmental water sources is crucial for the comprehensive study and management of viral pathogens in water environments. Water, often overlooked as a source of information, can provide invaluable insights into the communities from which it originates. Monitoring the presence and dynamics of viral pathogens in wastewater has shown great promise in the early detection of viral outbreaks and assessing the effectiveness of public health interventions. The lessons from implementing wastewater-based epidemiology can be extrapolated to other areas, expanding our understanding of viral dynamics in different contexts. A fundamental aspect of studying viruses in water environments is understanding the specifics, including their stability and survival in aqueous environments, forming the basis for assessing their potential threat in the broader environment. Developing specific and rapid detection tools can be of immense value in accurately timing preventive and corrective measures, enabling swift responses to potential viral threats. Furthermore, applying this knowledge and techniques to the wider environment allows us to prudently identify and respond to novel pathogens. Understanding the baseline viral diversity in each area through comprehensive monitoring provides the necessary background information to raise alarms upon detecting new pathogens, offering additional time to react appropriately and implement effective measures. The findings of this thesis have opened up new avenues for research and optimization. Understanding the persistence and potential impact of the diverse array of plant viruses detected in irrigation water is crucial. While we have evidence of long-term infectivity for certain viruses like tobamoviruses, data on most plant viruses is still lacking. To fully comprehend their behavior, it is important to consider real-life conditions and factors influencing degradation dynamics. One intriguing aspect is the presence of the same viruses in plants and water at the same location, but determining the direction of movement remains challenging. While it is plausible to imagine bidirectional movement, unraveling the directionality of virus flux would greatly enhance the utility of water monitoring. Taking inspiration from human virology, where targeted panels have improved cost efficiency and data analysis by focusing on specific virus groups (e.g., respiratory viruses), a similar approach could be adopted to develop panels for plant viruses, enabling a more targeted evaluation of irrigation water. However, the scope does not have to be limited to plant viruses alone.

Water, either environmental or wastewater, offers a comprehensive snapshot of the circulating pathogens in the environment. Designing a well-structured panel could proactively identify and capture potential zoonotic viruses before they trigger the next epidemic. Although this area of research is still in its infancy, it presents numerous opportunities for improvement that can ultimately benefit society on a larger scale.

This thesis recognizes the importance of linking viral diversity, water-based epidemiology, and environmental water sources to the holistic management of viral

pathogens in aquatic environments. Leveraging water as a source of information, understanding specific pathogens, and developing rapid detection tools can inform timely interventions, enabling proactive measures to protect public health and the environment.

Appendix A

Supplementary Material of Included Publications.

A.1 Supplementary Material for Publication 2.1

The supplementary material for this article can be found online at: https://static-content.springer.com/esm/art%3A10.1007%2Fs12560-022-09533-0/MediaObjects/12560_2022_9533_MOESM1_ESM.docx

A.2 Supplementary Material for Publication 2.2

The supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2023.1187920/full#supplementary-material

A.3 Supplementary Material foP publication 2.3

The supplementary material for this article can be found online at: https://www.biorxiv.org/content/10.1101/2023.06.20.545696v1.supplementary-material

Appendix B

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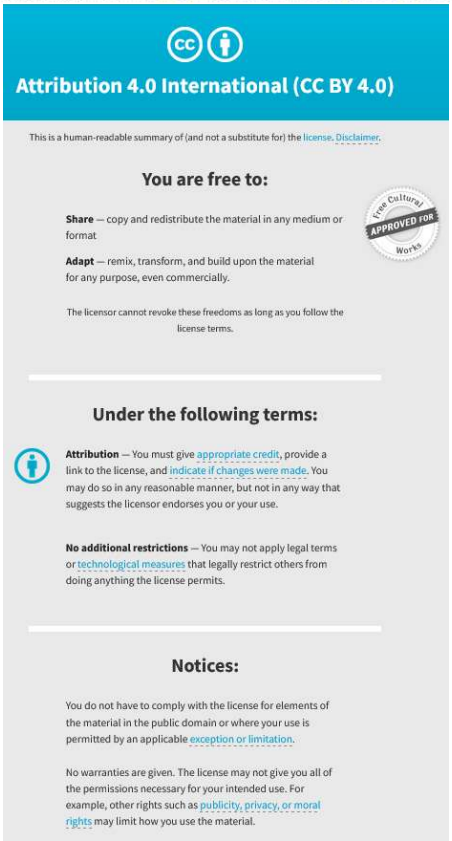

B.2 Permission for Reproduction for Publication 2.2

Keywords: Tomato brown rugose fruit virus, water-linked epidemiology, Survival, Tomato, Hydroponics

Received: 16 Mar 2023; **Accepted:** 16 May 2023.

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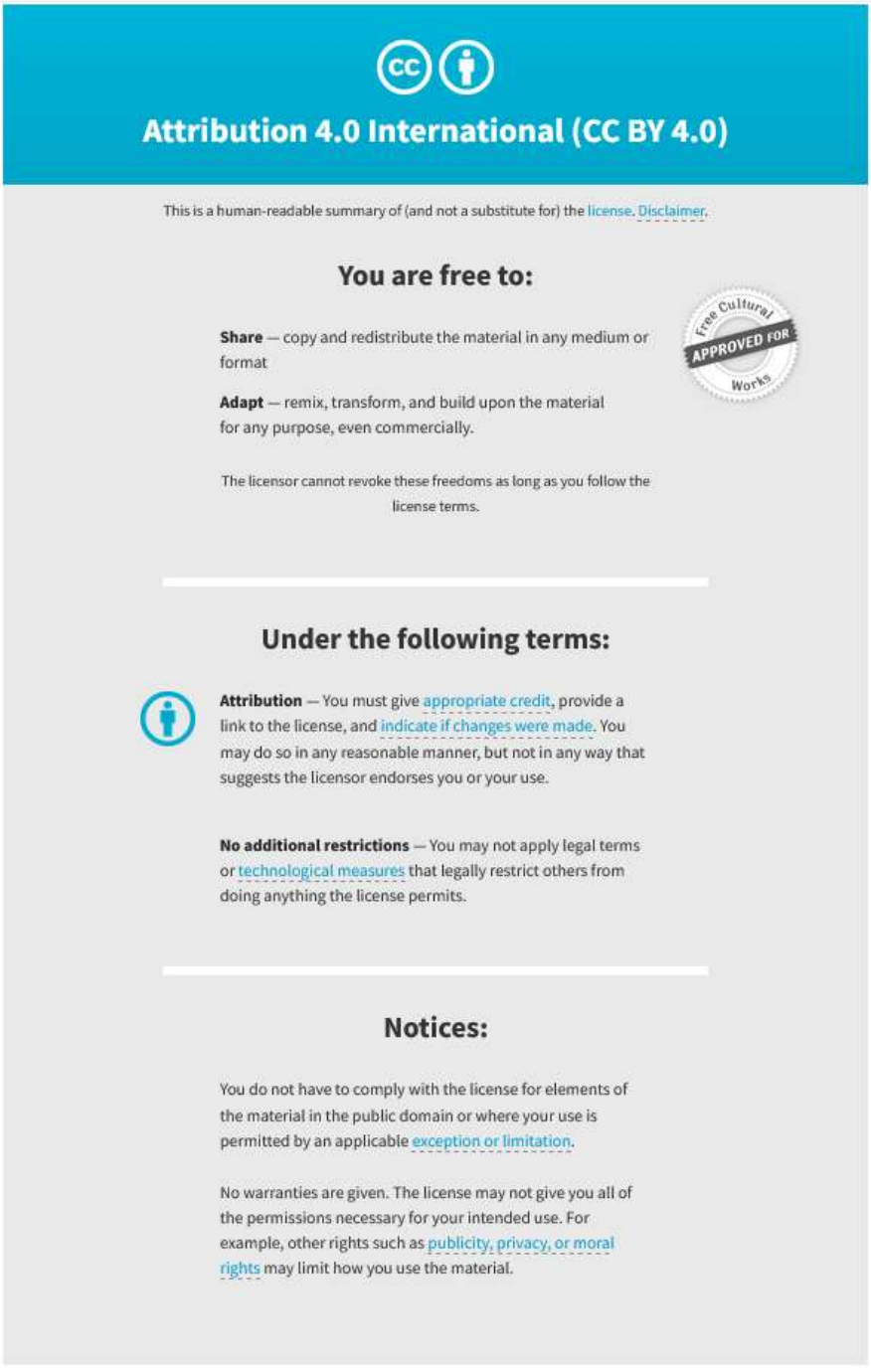

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Bibliography

Publications Related to the Thesis

Journal Articles

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Biography

Olivera Maksimović is a PhD student, employed at the National Institute of Biology (NIB), Ljubljana, Slovenia. She graduated in 2016 from the Faculty of Agriculture, University of Belgrade, Serbia, as a BSc of Food Technology. During her studies, she did several internships in different food manufacturing sites like dairy factory, Mlekoprodukt d.o.o, Zrenjanin, Serbia, in departments related to food safety and quality control. Olivera obtained her MSc degree in Food Science, Technology and Business, within a consortium of KU Leuven, Belgium, Universidade Catolica Portuguesa, Portugal, and Hochschule Anhalt, Germany, in 2018. During this time, she worked at Campden BRI, UK in the microbiology laboratory setting up the foodborne virus subunit. This work included development of molecular-based methods for detection of various foodborne viruses (norovirus, hepatitis E) in food and on surfaces. The method for detection of norovirus in food was also accredited under UKAS, which was the first accreditation of this type in the country. The second part of work included the setting up of cell laboratory for evaluation of infectivity for above-mentioned viruses. She had her training related to cell cultures at Glasgow Caledonian University, UK.

In 2019, she enrolled in the PhD study program of Sensor Technologies at the Jožef Stefan International Postgraduate School (IPS) and started working as a young researcher at NIB, Department of Biotechnology and Systems Biology, under the supervision of Dr. Ion Gutierrez Aguirre and Dr. Denis Kutnjak. She was a member of Student Council (2019-2021) and took active part in the organization of yearly IPS Student Conference. Her research work is focused on detection of viruses in different water samples using different methods for virus concentration, quantitative real time PCR (qPCR) and high-throughput sequencing (HTS) technologies. The PhD is a part of the European Commission MSCA ITN action INEXTVIR coordinated by NIB.

In the first part of her PhD research, she focused initially on the optimization of a workflow for concentration and quantification of SARS-CoV-2 in wastewater. This work resulted in a publication (Section 2.1), and also became the basis for the ongoing national wastewater monitoring scheme. The second part of her PhD research focused on plant viruses in irrigation water. This included analysis of the metagenomics profile of Slovenian irrigation water, described in a publication (Section 2.3). Applicability of the methodology was studied during a short scientific visit to the UPM-CBGP laboratory in Madrid, Spain. Focusing on a specific virus, tomato brown rugose fruit virus, led to a 3-month scientific visit to Fera Ltd. York, UK. Work performed during this visit and later at NIB focused on optimizing concentration and detection methods for studying the release of the virus from infected plants to nutrient water in a setup mimicking hydroponic growth conditions. This work forms part of a larger study (Section 2.2).

During her PhD studies, she was also involved in various other studies using high throughput sequencing methods. Olivera presented her work on many national and international conferences in the form of posters and lectures. At Monolith Summer School (2022, Portorož, Slovenia) she had won second place for best young researchers' presentation.