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Viromics and infectivity analysis reveal the release of infective plant viruses from wastewater into the environment

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ABSTRACT

Viruses represent one of the most important threats to agriculture. Several viral families include highly stable pathogens, which remain infective and can be transported long distances in water. The diversity of plant viruses in wastewater remains understudied; however, their potential impact is increasing with the increased irrigation usage of reclaimed wastewater. To determine the abundance, diversity and biological relevance of plant viruses in wastewater influents and effluents we applied an optimized virus concentration method followed by high-throughput sequencing and infectivity assays. We detected representatives of 47 plant virus species, including emerging crop threats. We also demonstrated infectivity for pathogenic and economically relevant plant viruses from the genus *Tobamovirus* (family *Virgaviridae*), which remain infective even after conventional wastewater treatment. These results demonstrate the potential of metagenomics to capture the diversity of plant viruses circulating in the environment and expose the potential risk of the uncontrolled use of reclaimed water for irrigation.

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1. Introduction

Expanding human population, increasing urbanization, predicted global warming and agronomic exploitation of land place enormous pressure on supplies of drinking and irrigation water. Among microbiological water contaminants, viruses are a group of particular concern (Rosario et al., 2009a); although many of them are highly stable pathogens, they remain understudied. Up until now, most of the studies have focused on the presence of bacteriophages and pathogenic human viruses in wastewater. However, as plants make up over 80% of the biomass on Earth (Bar-On et al., 2018), plant viruses, shown to be able to infect plants through the water (Mehle et al., 2014), represent an important, but often overlooked group of pathogens, significantly affecting ecosystems and agricultural production worldwide (Nicaise, 2014). The financial impact of yield losses due to plant viruses in agriculture are globally estimated to cost 30 billion USD annually (Sastry and Zitter, 2014). Studies on water mediated plant virus transmission together with assessment of traditional wastewater treatment efficiency on plant virus inactivation are lacking, even though reclaimed water use for irrigation is being increasingly introduced worldwide (Pedrero et al., 2010; Thebo et al., 2017). Global estimates of the extent to which irrigated croplands are influenced by wastewater, both treated and untreated are 35.9 Mha (~17% of all irrigated croplands), of which the majority are located in countries where less than 75% of wastewater is treated (Thebo et al., 2017).

It was hypothesized that the surface wash out of infected decaying plant residues and the related soil surface layer can bring plant viruses into water (Mehle and Ravnikar, 2012) and they may be washed away by rainwater reaching drainage water and wastewater. Additionally, a number of plant viruses have been found in raw sewage (Cantalupo et al., 2011) and human feces (Shkoporov et al., 2018; Zhang et al., 2006); e.g., pepper mild mottle virus (PMMoV) can survive the transit through the human digestive tract (Zhang et al., 2006) and is released into wastewater after consumption of pepper products (Symonds et al., 2019). PMMoV is globally distributed and present in various water sources in greater abundance than human pathogenic viruses, without substantial seasonal fluctuation (Kitajima et al., 2018), thus, it has been proposed as a water pollution indicator (Kuroda et al., 2015; Rosario et al., 2009b).

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Abbreviations

PMMoV	pepper mild mottle virus
CIM®	convective interaction media
HTS	high-throughput sequencing
WWTP	wastewater treatment plant
QA	quaternary amine
CGMMV	cucumber green mild mottle virus
TEM	transmission electronic microscopy
sRNA	small RNA
SRA	short reads archive
TMGMV	tobacco mild green mosaic virus
ToMV	tomato mosaic virus
ToBRFV	tomato brown rugose fruit virus
CMV	cucumber mosaic virus
PVY	potato virus Y
PepMV	pepino mosaic virus
MNSV	melon necrotic spot virus

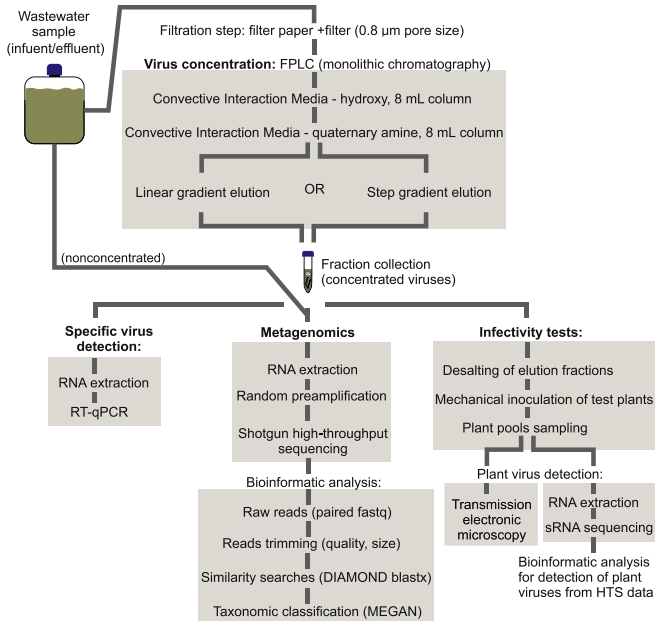

Fig. 1. Schematic representation of methods used for analysis of water virome and infectivity of viruses in water. It represents steps included in virus concentration, specific virus detection using quantitative reverse transcription PCR (RT-qPCR), sample preparation for HTS and consecutive bioinformatics analysis and sample preparation for mechanical inoculation of test plants and subsequent virus detection using transmission electronic microscopy and small RNA sequencing. FPLC – fast performance liquid chromatography.

The low concentration of virions in water samples makes them difficult to detect and requires the use of concentration methods during the sample preparation process. Different concentration approaches and sample preparation modifications can be used to increase the sensitivity of virus detection in wastewater (Haramoto et al., 2018; Hjelmsø et al., 2017). Convective interaction media (CIM®) monoliths have shown to be a fast and efficient tool for concentration of viral particles and free nucleic acids from high volume water samples, pointing to CIM monoliths as a promising tool for sample preparation in water virome studies (Gutiérrez Aguirre et al., 2018). High-throughput sequencing (HTS) based metagenomics studies have recently exposed the composition of viromes from different water environments, e.g., wastewater (Alhamlan et al., 2013; Aw et al., 2014; O'Brien et al., 2017; Tamaki et al., 2012), sewage (Cantalupo et al., 2011; Fernandez-Cassi et al., 2018; Hjelmsø et al., 2017; Ng et al., 2012), reclaimed water (Rosario et al., 2009a) and fresh water (Djikeng et al., 2009; Mohiuddin and Schellhorn, 2015).

Metagenomic approaches reveal an unprecedented spectrum of viral nucleic acids in water samples; however, confirming the infectivity of the viruses discovered through sequencing analysis is essential to assess the relevance of such findings. Although infectious plant viruses have been isolated from various types of water (e.g., rivers, ponds, irrigation and drainage canals) in different locations worldwide (Jeżewska et al., 2018; Koenig, 1986; Mehle et al., 2018), their survival and direct transmission through water is still largely unknown. None of the studies to date have approached infectivity testing for plant viruses detected in wastewater, most probably due to the low viral concentrations in combination with the low sensitivity and time intensive test plant bioassays used for the confirmation of plant virus infectivity.

To identify the potential of reclaimed water as a reservoir and transmission source for infective plant viruses we focus this study on the presence of plant viruses in influent and effluent of a wastewater treatment plant (WWTP), coupling CIM monolithic chromatography concentration, HTS-based shotgun metagenomics and biological infectivity tests (Fig. 1). The combination of fast and efficient virus concentration and metagenomics enables us to study the population of plant viruses in wastewater, highlighting the presence of important plant pathogenic viruses, some of which were not previously detected in the region.

2. Materials and methods

2.1. Samples and description of WWTP

Water samples (5 L) of WWTP influents and effluents were sampled in summer 2016 and 2017. The selected WWTP located in central Slovenia (Central WWTP Domžale-Kamnik, Slovenia) is a conventional two-stage activated sludge plant, upgraded with four sequence batch reactors and additional anaerobic digestion of activated sludge. The capacity of the plant, designed for organic matter removal from wastewater, is 149 000 population equivalents, and an average daily inflow of approximately 20 000 m^3. The facility collects municipal wastewater, rainwater and industrial wastewater from five communities including households, farms, and industry. The recipient of treated water from the plant is the Kamniška Bistrica river.

2.2. Concentration of wastewater samples for viral detection

Before concentration, all samples were filtered through filter paper and cellulose acetate membranes with a pore size of 0.8 µm (Sartorius, Goettingen, Germany). The concentration of each sample was done using CIM quaternary amine (QA) 8 mL monolithic column (BIA separations, Ajdovščina, Slovenia) on a fast protein liquid chromatography system AKTA Purifier 100 (GE Healthcare, Chicago, IL, USA). Influent and effluent samples were loaded at 80 mL/min flow rate onto the CIM QA column. After the column loading and a washing step using 50 mM HEPES buffer, pH 7, we applied either (i) step gradient elution concentration approach (Supplementary Fig. 2a), where an abrupt change in concentration of salt in the elution buffer was used or ii) linear gradient elution concentration approach (Supplementary Fig. 2b), where a gradually increasing concentration of salt in the elution buffer was introduced. The protocol used for concentration of viruses was described

previously (Gutiérrez-Aguirre et al., 2011, 2009) and outlined in detail in Supplementary materials and methods 1.

2.3. Detection of viruses in wastewater samples using RT-qPCR

RT-qPCR was used to assess the performance of chromatographic concentration procedures by determining the presence and viral nucleic acids recoveries of two selected plant viruses (PMMoV and cucumber green mild mottle virus - CGMMV) for step gradient elution (Inf16-Step) and linear gradient elution (Inf17-Lin). Extraction of nucleic acids for RT-qPCR analysis was done following the CIM concentration from a 140 μL aliquot using a QIAamp viral RNA mini kit (QIAGEN, Germantown, MD, USA). RT-qPCR was performed using published assays for PMMoV (Haramoto et al., 2013) and CGMMV (Zhao et al., 2015) using AgPath-ID™ One-Step RT-qPCR Kit (Life Technologies, Carlsbad, CA, USA) on a 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The average Cq obtained for each elution fraction was normalized to the external luciferase control and used to estimate the virus recovery (%) in the fractions relative to the original sample via the standard curve approach as described in previous studies (Balasubramanian et al., 2016; Gutiérrez-Aguirre et al., 2009).

2.4. Metagenomic analysis of wastewater samples

For the metagenomic analysis of wastewater samples, nucleic acids were extracted using TRIzol LS (Life tehnologies, CA, USA), following the manufacturer's protocol with the addition of glycogen (100 μg) to 400 μL of water sample in the beginning of the extraction. Isolated nucleic acid concentration was quantified using Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and Qubit 4 flourometer (Thermo Fisher Scientific, MA, USA). Samples with concentration of RNA lower than 1 ng/μL after extraction were randomly preamplified (see Table 1) as described previously (Fernandez-Cassi et al., 2018; Wang et al., 2003). Briefly, RNA templates were reverse transcribed using SuperScript III (Life Technologies, CA, USA) and Primer A (5′-GTTTCCCAGTCACGATANNNN NNNNN′- 3), which contains a specific sequence followed by 9 random nucleotides for random priming. A second cDNA strand was constructed using Sequenase 2.0 (Thermo Fisher Scientific, MA, USA). To obtain sufficient DNA for library preparation, a PCR amplification step using Primer B (5′-GTTTCCCAGTCACGATA′-3) and AmpliTaqGold (Life Technologies, CA, USA) was performed. PCR products were cleaned and concentrated using Agencourt AMPure XP (Beckman Coulter, IN, USA).

Step gradient elution of samples from 2016 (influent and effluent) and 2017 (effluent) resulted in two elution fractions (Step-E1 and Step-E2), that were separately extracted and sequenced. For influent and effluent samples (year 2017), nucleic acids from the linear gradient elution fractions selected in three different parts of the gradient were separately extracted. Before sequencing, nucleic acids from the elution fractions were pooled together to three fraction groups (Lin^a, Lin^b, Lin^c). For the effluent sample Lin^a (F6–F8), Lin^b (F16–F18) and Lin^c (F35–F37) were sequenced. For the influent sample Lin^a (F6–F8), Lin^b (F16–F20) and Lin^c (F35–F36) were sequenced (shown in Supplementary Fig. 1). The negative control of isolation was spiked with luciferase RNA (Promega, WI, USA) and was also sent for sequencing as a control for contamination in the isolation process and sample cross-talk during sequencing.

We sent isolated RNA (or preamplification products) for reverse transcription, library preparation and sequencing to SeqMatic LLC (Fremont, CA, USA). Nextera XT DNA Library Prep Kit (Illumina, San Diego, CA, USA) was used to construct the sequencing libraries, which were shotgun-sequenced using an Illumina MiSeq (Illumina, San Diego, CA, USA); resulting in 1 million to 3.4 million 2 × 250 bp reads per sample. The remains of sequencing adaptors and primer sequences in the case of preamplified samples were trimmed and resulting reads were further filtered by using quality filter (Limit = 0.01; no ambiguous nucleotide allowed) and by size (reads shorter than 25 bp were discarded) in CLC Genomics Workbench 11 (Qiagen Bioinformatics, Redwood City, CA, USA). In order to normalize samples according to the number of reads, random subsampling was done and all the samples were normalized to

Table 1
Metadata of wastewater samples analyzed by HTS with summary of reads' taxonomic classifications; overview of classified reads (%), reads classified as viral (%), viral reads classified as plant viruses (%), plant virus species richness and corresponding SRA acession numbers.

Sample name	Year of sampling	Sample type	Concentration approach	Random preampli-fication	Classified reads (%)	Reads classified as viral (%)	Viral reads classified as plant viruses (%)	Plant virus species richness	**Accession number (SRA*)**
Inf16-None	2016	influent	none	no	53.12	0.13	48.80	10	SRR9317870
Inf16-Step			step	no	44.88	5.61	1.73	17	SRR9317869 SRR9317872
Ef16-None	2016	effluent	none	no	38.26	0.53	1.22	8	SRR9317871
Ef16-Step			step	no	34.54	2.40	2.81	19	SRR9317874 SRR9317873
Inf17-Lin^a	2017	influent	linear	yes	38.92	6.88	0.57	12	SRR9317876
Inf17-Lin^b			linear	yes	29.77	7.65	11.47	22	SRR9317875
Inf17-Lin^c			linear	no	49.00	3.47	3.96	16	SRR9317868
Ef17-None	2017	effluent	none	yes	79.85	0.72	36.60	8	SRR9317867
Ef17-Step			step	no	20.26	1.88	16.96	12	SRR9317880 SRR9317879
Ef17-Lin^a			linear	yes	14.71	2.63	0.82	3	SRR9317878
Ef17-Lin^b			linear	yes	23.48	6.30	44.53	11	SRR9317877
Ef17-Lin^c			linear	yes	51.81	0.84	26.70	7	SRR9317882

*short reads archive (SRA).

926 418 reads, which was the minimum number of reads obtained among the samples. Trimmed, size and quality filtered normalized reads subsets were exported from CLC Genomics Workbench and compared for similarity with a complete NCBI nr database (June 2018), using DIAMOND (Buchfink et al., 2015) with default parameters. The results of the DIAMOND classifications were used as an input for the taxonomic classification of the reads using MEGAN (Metagenome Analyzer, version 6.12.0) (Huson et al., 2016) with the following parameters for the Lowest Common Ancestor algorithm: min score = 50.0, max expected = 0.01, top percent = 10.0, min support percent = 0, min support = 1, and LCA percent = 100.0. The obtained MEGAN outputs were used to present an overview of taxonomic classification of the sequencing reads. To compare viromes of differently concentrated samples, single-stranded (ss) RNA viral reads were exported as a BIOM file. Differential heat trees of pairwise comparisons between samples were constructed by Metacoder R package (Foster et al., 2017) where the relationships between viral taxons presented in the trees are inferred from NCBI taxonomy hierarchy. All plant infecting viruses whose genomes were detected in metagenomes of wastewater samples were listed in Fig. 3 and the corresponding viral species richness (number of observed viral species) was presented in Table 1. The reads classified as plant viruses were manually inspected and some reads were manually reassigned or left unclassified on the species level to ensure the correct classification of plant virus species. For this, we used an approach that combined BlastN (NCBI nt database, September 2018) and BlastX (NCBI nr database, September 2018) determination of sequence identity to the most similar sequences in the respective databases with detailed review of each identified taxon; reads with lower identity than defined by ICTV species demarcation criteria were classified only on the genus level.

2.5. Infectivity tests and detection of infective plant viruses

Nicotiana occidentalis and *Nicotiana benthamiana* plants, that are susceptible to infection by a wide range of viruses including most of the detected tobamoviruses (Wylie et al., 2015), were selected to test the infectivity of plant viruses after the concentration of wastewater samples using CIM monolithic chromatography. Before inoculation of test plants, selected elution fractions were desalted using PD-10 desalting columns (GE Healthcare, IL, USA) following the manufacturer's instructions and using inoculation buffer (20 mM sodium phosphate buffer, pH 7.6 with 2% PVP (M 10000) (Sigma, Tokyo, Japan)) as an equilibration buffer. Inoculation of test plants with influent sample included eight separate linear gradient elution fractions (F16−F23) and a negative control of inoculation, where plants were inoculated only with inoculation buffer. Inoculation of test plants with effluent sample included a step gradient elution fraction (Ef17-Step-E1) and a negative control of inoculation (Supplementary Fig. 1). All fractions that were used for inoculation of test plants originated from the same concentration experiment as the fractions that were sequenced to ensure the consistency and comparability with metagenomics analysis (Supplementary Fig. 1). Sampling, concentration process, desalting and inoculation of test plants were all done in the same day to avoid freezing of the samples and prevent the possible negative influence of concentrated substances on stability of viral particles.

We inoculated each selected elution fraction on eight plants of each tobacco species (*Nicotiana occidentalis* and *Nicotiana benthamiana*.). We mechanically inoculated two to three leaves per plant using carborundum (0.062 mm, VWR International, Radnor, PA, USA) that was put on each leaf and the elution fraction (approximately 50 μL per leave) was gently rubbed over it. Additional plants inoculated only with inoculation buffer served as negative controls. During the 4-week period following the mechanical inoculation, we observed plants for the appearance of symptomatic changes. We sampled plant material of non-inoculated leaves every week after inoculation for detection of viruses using transmission electronic microscopy (TEM) and small (s) RNA sequencing analysis.

We collected pooled samples of leaf tissue (non-inoculated leaves, approximately 1 cm^2 per leave) separately for different test plants inoculated with different CIM fractions, but exhibiting similar disease symptoms. We also sampled non-inoculated leaves of asymptomatic plants (see Supplementary Fig. 1 for details). Plant material samples were further used to isolate total RNA using TRIzol reagent (Thermo Fisher Scientific, MA, USA). As a control we included a sample of *Phaseolus vulgaris* plant material infected with an endornavirus (kindly provided by Mike Rott, Canadian Food Inspection Agency - Dartmouth, Canada) in the sequencing experiment.

sRNA deep sequencing was used as a generic detection method, which enable detection of all the different types of viruses (different RNA and DNA viruses) infecting plants at the same time (Pecman et al., 2017). Total RNA was sent to SeqMatic LLC (Fremont, CA, USA) for sRNA library preparation (TailorMix miRNA Sample Preparation Kit, SeqMatic LLC, USA) and sequencing. The samples were multiplexed in one run of a NextSeq (Illumina, CA, USA) in 1 × 100 bp mode. Reads were further analyzed using a bioinformatics pipeline for virus detection from HTS data as described in a previous study (Pecman et al., 2017). Shortly, trimmed and size-selected reads were first mapped to the NCBI Viral RefSeq database, containing representatives of all viral genomes with completely sequenced genomes. Results of the mapping were manually inspected. In parallel de novo assembly of trimmed and size-selected reads was performed. Assembled contigs were compared for similarity against all viral sequences deposited in the NCBI GenBank nt database using BLASTn and BLASTx. The presence of suspected viral sequences was confirmed and complete consensus genome sequence was generated by mapping the reads to the complete viral genome sequences of the most similar viral isolates from the NCBI GenBank database.

2.6. TEM analysis of viruses

A sample (25 μL) of the chromatographic fractions was applied to formvar-coated (Agar Scientific, Stansted, UK), carbon-stabilized copper grids that were negatively stained using a 1% (w/v) aqueous solution of uranyl acetate (SPI Supplies, West Chester, PA, USA). Selected elution fractions from year 2 (influent linear gradient fraction 17 and 18) were ultracentrifugated using Airfuge (Beckman Coulter Inc., Brea, CA, USA) onto TEM grids before negative staining as described previously (Hammond et al., 1981). All samples were examined with TEM (Philips CM 100, Netherlands) as described in a previous study (Ruščić et al., 2015).

Plant material of randomly selected plants inoculated with wastewater samples exhibiting distinctive symptoms was also examined using TEM. Homogenates of plant material (20 μl) were applied to Formvar-coated, carbon-stabilized copper grids and negatively stained using a 1% (w/v) aqueous solution of uranyl acetate (SPI Supplies, PA, USA), followed by visualization by TEM.

3. Results

To examine the virome composition of wastewater, we determined the metagenomes of the influent and effluent samples of a WWTP with special emphasis on plant viruses. Viruses were first concentrated using CIM® monolithic chromatography using either: i) step gradient elution concentration approach (Supplementary Fig. 2a) or ii) linear gradient elution concentration approach

(Supplementary Fig. 2b). After linear gradient elution we selected, sequenced and separately analyzed three fraction groups based on the abundance of PMMoV and CGMMV, determined by RT-qPCR (Supplementary Fig. 2b, Supplementary Table 1). Selected fraction groups cover the initial (Lin^{a}), middle (Lin^{b}) and final (Lin^{c}) part of the elution gradient.

3.1. Overview of the highly diverse wastewater virome reveals understudied group of plant viruses

To provide a trustworthy representation of viral species (Rodriguez-Brazzarola et al., 2018) we analyzed HTS data obtained by sequencing wastewater samples (Table 1) using reads-based protein similarity searches. Taxonomic classification showed that a substantial fraction of reads in each metagenome (20–85%) had no matches to known organisms (Table 1, Fig. 2a). This is similar to previous studies, in which the majority of the sequences in wastewater samples had no similarity to known genes or proteins (Aw et al., 2014; Bibby and Peccia, 2013). A large fraction of reads (10–50%) in influent and effluent samples belonged to bacteria (Fig. 2a). A smaller fraction of reads was classified as eukaryotic organisms and archaea (Fig. 2a). Eukaryotic reads were abundantly present in non-concentrated samples (5–13%) and were classified mostly as genomes of plants, aquatic arthropods, rodents and enteric parasites (data not shown). In the same non-concentrated influent and effluent samples the percentage of viral reads was less than 1% (0.1–0.7%), however, it increased to 7.7% in CIM concentrated samples (Table 1, Fig. 2a).

By analyzing metagenomes of the 12 samples (Table 1) we detected members of 56 different viral families, including those with double-stranded (ds)DNA, ssDNA, dsRNA and ssRNA genomes. (Fig. 2b). Examination of wastewater samples by TEM also revealed a diversity of virion morphologies with filamentous viral particles and phage-like virions (Supplementary Fig. 3). As previously shown, the viromes of WWTP influents and effluents contain high abundance of dsDNA bacteriophages. CrAssphage sequences, originally identified from metagenomic analysis of human feces (Dutilh et al., 2014), were abundantly detected in influent samples (Supplementary Table 3). Gut-associated Bacteroides phage B124-14, that can be used to distinguish human gut contaminated metagenomes (Ogilvie et al., 2018) and few reads of circular (Rep)-encoding single-stranded DNA (CRESS DNA) viruses, recently reported in wastewater effluents (Rosario et al., 2019) were also detected in a majority of the samples (Supplementary Table 3).

Important human pathogens such as norovirus, sapovirus, enterovirus, rotavirus A, hepatitis E virus and JC polyomavirus A were also detected in wastewater viromes (Supplementary Table 3). Pathogenic viruses of other hosts such as sequences of members of *Picornavirales* order belonging to *Dicistroviridae* family were detected mainly in influent samples. We also detected bee viruses belonging to *Iflaviridae* family and *Picornavirales* order in both influent and effluent samples (Supplementary Table 3). In influent (Inf17-Lin^{b}) and effluent (Ef17-Lin^{b}) linear gradient elution fractions, we detected representatives of more than 20 families of ssRNA viruses.

Plant viral genomes mostly consist of ssRNA (~75% of all plant viruses) (Hull and Bustamante, 1998) and were therefore mostly detected in analyzed wastewater samples as sequences classified as ssRNA viruses, with exception of the dsRNA pepper cryptic virus (Fig. 3). The most commonly found viruses recovered before and after wastewater treatment belonged to the family *Virgaviridae*, with all of the reads corresponding to the *Tobamovirus* genus (Fig. 2c) most abundantly represented by sequences of PMMoV, tobacco mild green mosaic virus (TMGMV), tomato mosaic virus (ToMV) and CGMMV (Fig. 3). We also detected sequences of tomato

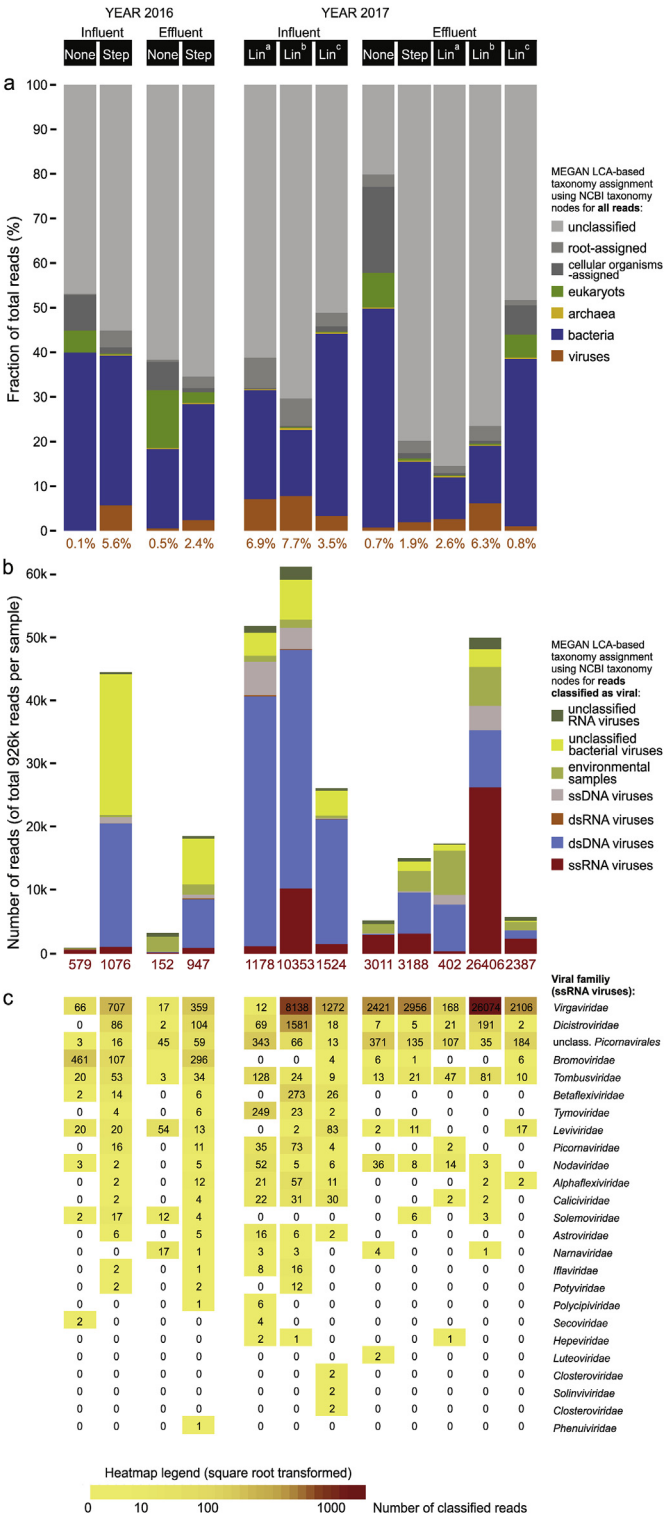

Fig. 2. Taxonomic classification of the sequencing reads from non-concentrated and differently concentrated influent and effluent wastewater samples from two consecutive years (explained in Table 1). (a) Relative amount of the reads classified on the level of domains (the % below the columns represent the fractions of reads classified as viral in corresponding samples). (b) A more detailed overview of the absolute number of viral reads classified on the level of viral groups according to NCBI taxonomy nomenclature (the numbers below the columns represent absolute number of reads classified as ssRNA viruses in corresponding samples). (c) Heatmap for families of positive-sense ssRNA viruses and corresponding number of reads belonging to respective families detected in each sample. The numbers within each cell represent number of reads per normalized sample.

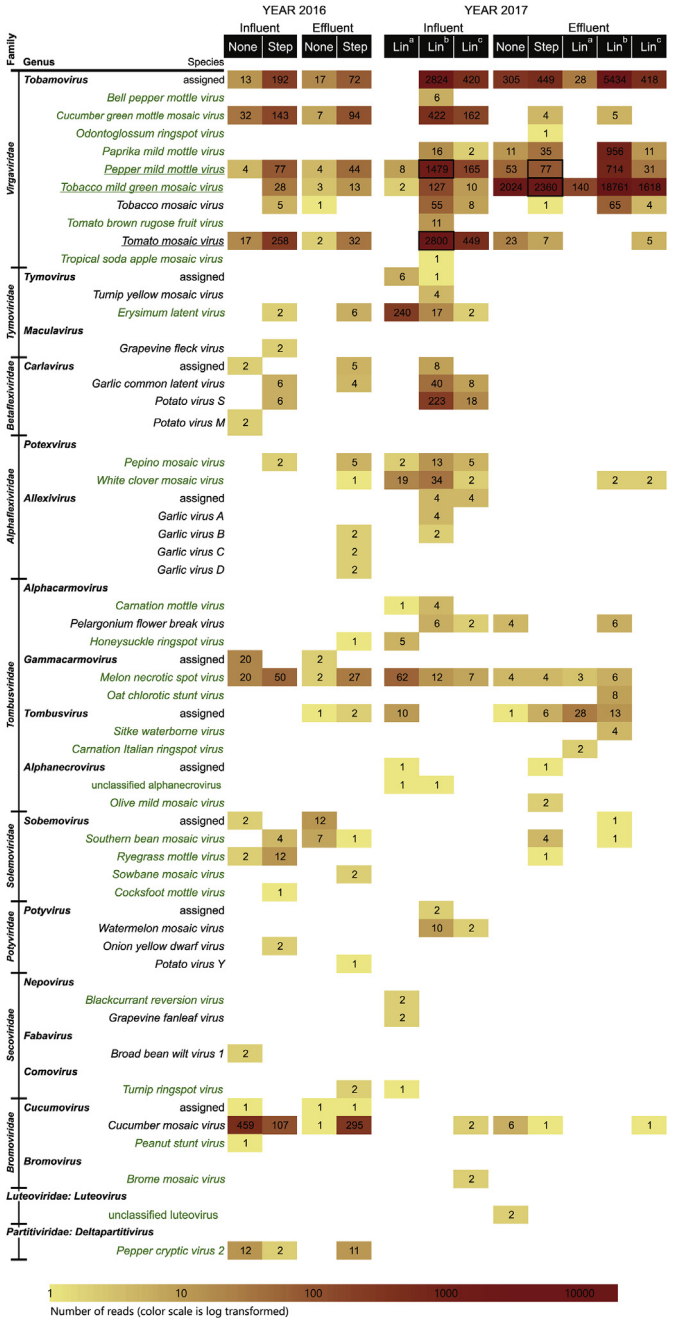

Fig. 3. A heatmap showing the plant viral species and corresponding number of their reads detected in corresponding wastewaters samples. Reads were taxonomically classified using Diamond-MEGAN pipeline and further manually curated. Reads classified on genus level (assigned) represent: (i) sequences of different species from respective genera not classified to the species level with used classification pipeline, (ii) manually curated assignments for sequences with lower species identity than stated in species demarcation criteria (potential novel viral species). Viruses with confirmed infectivity in this study are underlined and viruses that were not reported in Slovenia before are colored green.

brown rugose fruit virus (ToBRFV), which was not found in the country before and is an emerging tobamovirus that has recently brought concern to plant health authorities worldwide. Representatives of less abundant families such as *Potyviridae, Betaflexiviridae* and *Tymoviridae* were mostly present in the influent samples and were rarely detected after the wastewater treatment in the effluent samples (Fig. 2c). Sequences belonging to *Bromoviridae* family were detected abundantly in samples from 2016 and only few reads were detected in samples from 2017 (Fig. 2c). Whilst tobamoviruses were numerically dominant plant viruses in the samples (Fig. 2c), the list (Fig. 3) of detected potentially pathogenic plant viruses includes 47 species from 19 genera including the economically important viruses, such as cucumber mosaic virus (CMV), potato virus Y (PVY) and pepino mosaic virus (PepMV). With in-depth analysis of reads assigned to plant viral families, sequences with low similarity to known plant viruses were also detected, that could only be classified at the genus level and probably represent previously undescribed viral species (Fig. 3).

3.2. Optimized viral concentration enables more detailed insight into diversity of plant viruses

While HTS is a powerful tool for uncovering viruses in the environment, sample and molecular processing steps can alter the distribution of viral groups in the concentrated samples. This is why we have compared non-concentrated and differently concentrated wastewater samples. We analyzed fractions obtained by either step gradient elution or linear gradient elution with RT-qPCR for presence and relative concentrations of two tobamoviruses (PMMoV, CGMMV), calculated their recoveries (Supplementary Fig. 2) and compared the virome composition of differently concentrated samples. We were able to efficiently concentrate PMMoV and CGMMV for approximately two orders of magnitude according to RT-qPCR results. (Supplementary Fig. 2, Supplementary Table 1, Supplementary Table 2).

We observed a higher fraction of viral reads in both step/linear gradient concentrated influent and effluent samples compared to non-concentrated ones (Table 1, Fig. 2a). The enrichment for viral sequences was most noticeable in Lin^b linear gradient samples (Table 1, Fig. 2a). The fractions at the peak of absorbance (Lin^b) have very high recoveries according to RT-qPCR for selected tobamoviruses (CGMMV and PMMoV) and contain the highest number of reads belonging to ssRNA viruses (Fig. 2b: Inf17- Lin^b, Ef17- Lin^b), mostly due to the high abundance of viral reads classified as tobamoviruses (Fig. 2c).

Next, we aimed to get a detailed insight into the plant virus abundance and diversity differences between different concentrations of the same sample. Thus, we visualized pairwise comparisons of taxa abundances for ssRNA viral reads of differently concentrated effluent sample (from year 2017) as differential abundance heat trees (Fig. 4). Overall enrichment of *Virgaviridae* family in Ef17-Lin^b effluent sample was pronounced (Fig. 4). In summary, we detected higher number of plant viral species in concentrated influent and effluent samples compared to non-concentrated ones, especially in Lin^b fractions (Table 1). Both concentration approaches (step and linear) allowed us to focus on the diversity of ssRNA viruses, with the linear gradient having the potential to study specific viral groups, namely tobamoviruses, in more detail.

3.3. Plant viruses in wastewater are infective and survive wastewater treatment

To determine if the plant viruses detected by HTS have a potential to be transmitted to plants by wastewater, we performed biological infectivity tests. Inoculation with non-concentrated wastewater in a preliminary experiment resulted in no detectable infection (results not shown). Therefore, in subsequent experiments we mechanically inoculated test plants using fractions concentrated by either linear (influent) or step (effluent) gradient elution and monitored the plants for symptom development. Using

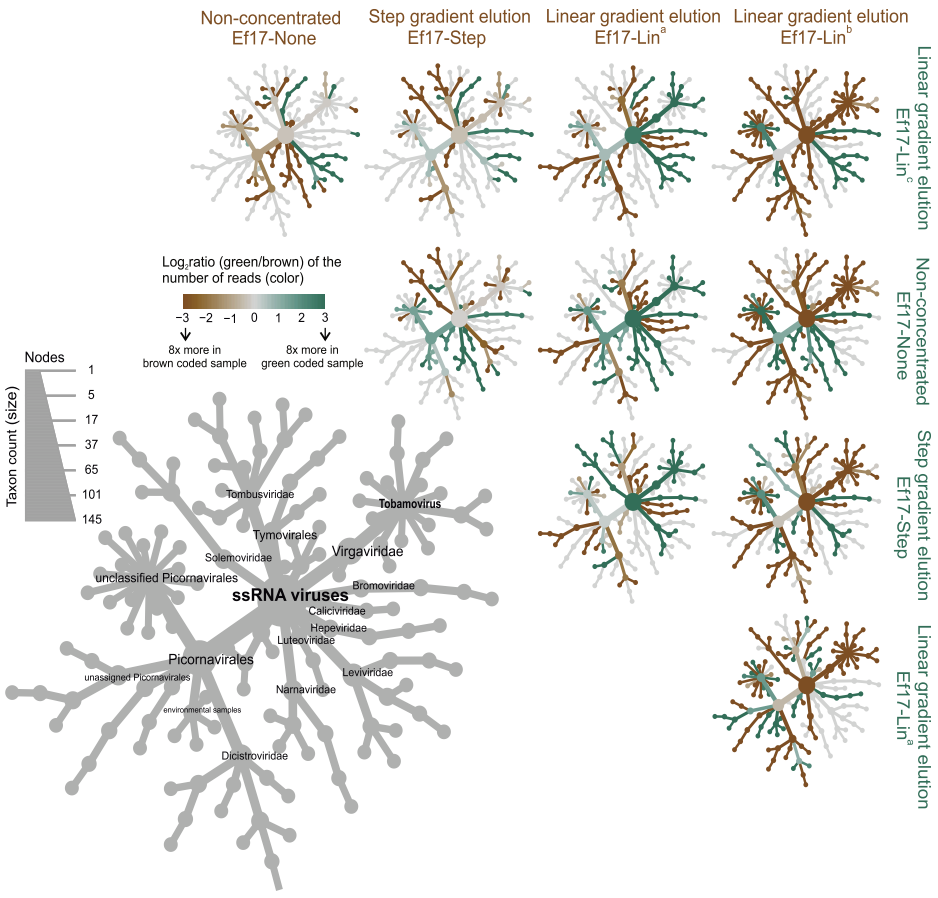

Fig. 4. Pairwise comparisons between ssRNA viromes of differently concentrated wastewater effluent samples from year 2017 highlight the enrichment for tobamoviral reads in linear gradient fraction group b (Lin[b]). Enlarged grey tree in the lower left corner shows the taxonomic groups represented in the ssRNA viromes of all the normalized samples, where node sizes correspond to the number of taxa based on all samples together. Comparisons between ssRNA viromes of differently concentrated samples are shown for all the pairs of samples as small heat trees above the diagonal. The differences in abundance of the taxa for the two compared samples are shown in color: brown color designates higher abundance of taxa in brown coded sample (listed in the horizontal axis) and green color designates higher abundance in green coded sample (listed in the vertical axis). The color of each taxon represents the log-2 ratio of reads abundance of the two different samples compared.

sRNA HTS (a non-targeted plant virus detection approach) we detected high number of reads assigned to tobamoviruses in pools of symptomatic and also asymptomatic plants (Table 2). Complete genome sequences of detected viruses were covered by sRNA reads and consensus viral genomes of infecting viruses have been reconstructed.

Four weeks after inoculation with influent linear gradient elution fractions from year 2017 (see Supplementary Fig. 1), some *N. benthamiana* plants showed reduction in size, curling and mottling of the leaves (Fig. 5a). sRNA sequencing of the pooled plant samples with similar symptoms (Table 2) identified PMMoV in the pool. *N. occidentalis* plants inoculated with the same fractions showed two different types of symptomatic changes. Again, we confirmed PMMoV infection in pool of *N. occidentalis* samples with the symptoms of curling and mottling (Fig. 5b, Table 2). Moreover, in the pool of *N. occidentalis* plants exhibiting curling and necrotic spots (Fig. 5c, Table 2) we detected mixed infection with PMMoV and ToMV. Finally, also in the pool of *N. occidentalis* plants not showing any distinct disease symptoms at the time of sampling we detected PMMoV (Table 2).

To assess the effect of the wastewater treatment on plant virus infectivity, we also inoculated test plants with the effluent of the WWTP from year 2017, which is released directly into the river. For mechanical inoculation we used step gradient elution fraction E1 (for details, see Supplementary Fig. 1). *N. benthamiana* plants again showed disease symptoms with reduction of the size of the plant and curling, which were linked with PMMoV infection (Fig. 5d, Table 2) using sRNA sequencing of pooled symptomatic plant material. Additionally, a pool of *N. occidentalis* plants with mosaic symptoms (Fig. 5e) as well as pool of asymptomatic *N. benthamiana* plants were shown to be infected with TMGMV (Table 2). All of the influent and effluent fractions that successfully infected test plants contained high number of reads of the detected infective viruses (Fig. 3). TEM examination of symptomatic plant material samples revealed the presence of filamentous virus particles of about 300 nm in length, typical for tobamoviruses (Fig. 5f and g).

4. Discussion

In this study, we explore the diversity of viruses in wastewater, focusing on highly economically relevant ssRNA plant viruses. We described the presence of such viruses in influent and effluent samples of WWTP and confirmed the infectivity of some plant viruses, thus proving their biological significance in wastewater. These results implicate the flux of infective pathogenic plant viruses from anthropogenic environments into environmental waters and open important questions about the role of wastewater in the spread of viral diseases of plants.

In this study we detected members of 11 families of plant pathogenic ssRNA viruses (Fig. 3). Tobamoviruses, which were the

Table 2
Viruses detected using sRNA sequencing in plant tissue pools after inoculation of test plants with wastewaters CIM concentrates from year 2017 and summary of mapping reads to corresponding consensus viral genome sequences.

Plant sample pools	Test plants	Symptoms	Viruses detected	Reads mapped to corresponding viral genome sequence	Reads mapped to corresponding viral genome sequence (%)	Average depth of coverage for corresponding viral species	GenBank accession number (complete genome)	SRA accession number (raw data)
INFLUENT								
No_INF-1	N. occidentalis	curling, mottling	PMMoV	1374 679	16.86	4630.64	MN267898	SRR9319298
No_INF-2	*N. occidentalis*	curling, necrotic	ToMV	1270 247	13.90	4320.37	MN267904	SRR9319297
		curling, necrotic	PMMoV	122 901	1.56	414.2	MN267899	
No_INF-3	*N. occidentalis*	asymptomatic	PMMoV	285 047	5.98	958.18	MN267900	SRR9319300
Nb_INF-4	*N. benthamiana*	curling, mottling	PMMoV	1057 897	13.95	3574.51	MN267901	SRR9319299
Nb_INF-5	*N. benthamiana*	asymptomatic	/	/	/	/		SRR9319304
EFFLUENT								
No_EFF-1	*N. occidentalis*	mosaic	TMGMV	2214 61	28.55	7460.34	MN267903	SRR9319303
No_EFF-2	*N. occidentalis*	asymptomatic	TMGMV	1208 726	15.80	4063.91	MN267902	SRR9319296
Nb_EFF-3	*N. benthamiana*	curling	PMMoV	1 804 063	23.81	6076.79	MN267897	SRR9319305
Nb_EFF-4	*N. benthamiana*	asymptomatic	/	/	/	/		SRR9319302

most abundant plant viruses detected in analyzed samples, are considered a major risk to a range of agriculturally important plant species belonging to the *Solanaceae*, *Cucurbitaceae* and other plant families. Their sequences were previously detected in different environmental waters including ballast water (Kim et al., 2015), irrigation systems (Boben et al., 2007), drinking water (Haramoto et al., 2013) and raw and urban sewage (Cantalupo et al., 2011; Fernandez-Cassi et al., 2018). Viruses from this group are known to have extremely stable virions that can resist high temperatures (Bawden and Pirie, 1959; Mutombo et al., 1992). Tobacco, tomato, and pepper are known hosts for ToMV, TMGMV and PMMoV (Smith, 2017), all three found to be present in wastewater as infective particles in this study. We detected reads belonging to CGMMV, which is the most economically important cucurbit-infecting tobamovirus and currently considered a significant threat to the production of cucumber, melon, watermelon, gherkin, and pumpkin (Smith, 2017). We also detected sequences of ToBRFV, first identified in tomatoes in Jordan in 2015 (Mansour and Falk, 2016). Outbreaks of this emerging virus are causing major concerns for growers of tomato and pepper in different countries worldwide (EPPO, 2019a). The virus is easily transmitted, which together with the lack of ToBRFV resistant tomato varieties generates a significant threat for tomato production worldwide (Luria et al., 2017). The detection of ToBRFV sequences in wastewater in Slovenia, a country where it has not been reported yet, thus raises questions about its origin, possibility of unnoticed presence and risks of its water-mediated transmission.

From the *Bromoviridae* family, CMV sequences were detected in the majority of the samples. CMV is known to infect over 1200 plant species including important vegetable crops and ornamentals (Zitter and Murphey, 2009). In the past, it was isolated from river water (Mehle and Ravnikar, 2012; Piazzolla, 1986). We detected sequences of melon necrotic spot virus (MNSV) from *Tombusviridae* family in all analyzed wastewater samples. MNSV, affecting species of the *Cucurbitaceae* family, is transmitted mainly by attaching itself to the outer layer of the aquatic zoospores of the fungus *Olpidium bornovanus* (Gosalvez et al., 2003). Sequences of potato viruses such as carlaviruses (potato virus S and potato virus M) and potyvirus (PVY), which are on the list of quarantine pests in some countries (EPPO, 2019b) were also detected. PVY is the most important viral pathogen in potato worldwide, with different strains of PVY causing diseases in tobacco, potato, pepper and tomato (Tsedaley, 2015). PepMV (*Potexvirus*, *Alphaflexiviridae*) sequences were found in some influent and effluent samples. PepMV causes great concern in the greenhouse tomato industry and is considered as a pest recommended for regulation as quarantine pest (EPPO, 2019b). Experiments using a hydroponic system showed that PepMV and PVY can be released from plant roots into the nutrient solution and can infect healthy plants through their roots (Mehle et al., 2014).

Recent studies demonstrated the usage of wastewater metagenomics for detection of viral outbreaks in human populations (Fernandez-Cassi et al., 2018; Xagoraraki and Brien, 2019). In the present study we confirmed previous observations, that wastewaters metagenomics can also provide a comprehensive view into the presence and abundance of plant viruses, possibly reflecting the diversity of local plants as well as the plants consumed by local residents and animals (Ng et al., 2012). We detected nucleic acids of many viruses, which were never reported in Slovenia before (Fig. 3) suggesting that wastewater might provide a pathway for the dissemination and gradual globalization of plant viruses, however the number of sequences detected for specific viruses varied from very low to high. While metagenomics studies of plant viruses in wastewater collected from different sources in the region provide an insight into the diversity of plant viruses circulating in a given environment, including emerging ones, it needs to be followed by systematic tests for specific viruses, especially in terms of their infectivity.

The availability of an effective concentration method is crucial for metagenomic detection of viral pathogens for aquatic surveillance purposes (Fernandez-Cassi et al., 2018). The CIM monolithic gradient elution enables both concentration and fractionation of the wastewater virome, resulting in tobamovirus reads being most abundant in specific fraction groups (Inf17- Lin^b, Ef17- Lin^b) (Figs. 3c and 4). The ability of the CIM chromatographic sample preparation step to increase viral concentration and at the same time to remove impurities present in the sample (Rupar et al., 2013) likely contributed to the success of infectivity tests, confirming biological significance of viral sequences detected in HTS based surveillance of wastewater.

In the past, infectious plant viruses have been isolated from various types of environmental water in different locations around the world (Koenig, 1986; Mehle et al., 2018; Mehle and Ravnikar, 2012), including irrigation and drainage canals (Jeżewska et al., 2018). However, infectivity of plant viruses was never studied in

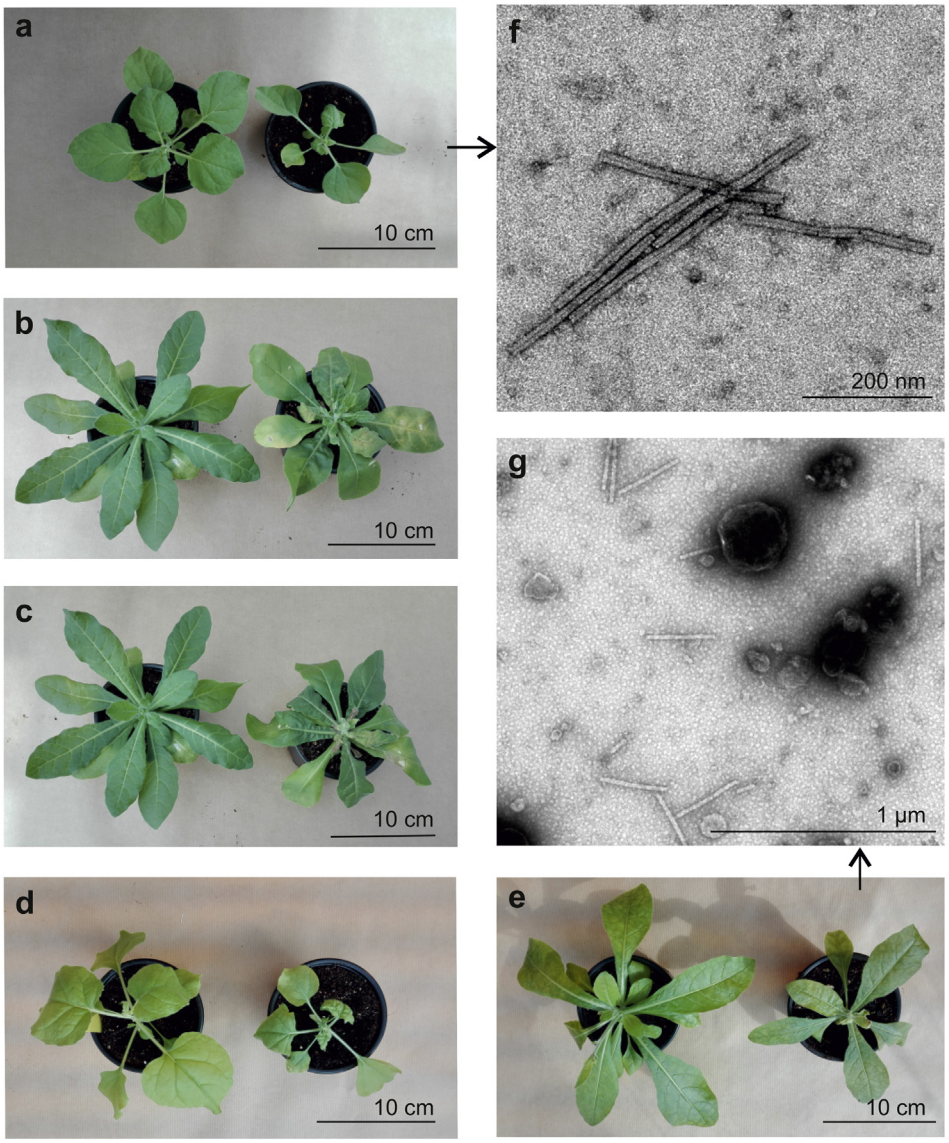

Fig. 5. Disease symptoms on inoculated test plants (right plant) after mechanical inoculation together with corresponding negative controls of inoculation (left plant) and viral particles in symptomatic plant material visualized by TEM. (a) PMMoV infected *N. benthamiana* inoculated with influent sample – fraction 20 (comprised in Inf17- Lin^{b}), (b) PMMoV infected *N. occidentalis* inoculated with influent sample – fraction 18 (comprised in Inf17- Lin^{b}), (c) ToMV infected *N. occidentalis* inoculated with influent sample – fraction 16 (comprised in Inf17- Lin^{b}), (d) PMMoV infected *N. benthamiana* inoculated with effluent sample – fraction E1 (comprised in Ef17-Step), (e) TMGMV infected *N. occidentalis* inoculated with effluent sample fraction E1 (comprised in Ef17-Step). (f) TEM micrograph of PMMoV infected *N. benthamiana* inoculated with influent sample, (g) TEM micrograph of TMGMV infected *N. occidentalis* inoculated with effluent sample.

influents and effluents of WWTP, even though using reclaimed wastewater for irrigation purposes is becoming a widespread practice (Pedrero et al., 2010). The combination of CIM concentration of wastewater samples and mechanical inoculation of selected test plants used here enabled us to observe symptomatic changes in selected test plants and confirm the infectivity of viruses detected by the metagenomic analysis of both treated and untreated wastewater. Using sRNA sequencing that enables generic detection of RNA snd DNA viruses and is widely used in plant virus discovery (Pecman et al., 2017; Roossinck et al., 2015), we were able to detect three infective tobamoviruses: PMMoV, ToMV and TMGMV (Table 2).

High diversity of plant viral sequences and confirmed infectivity of some important pathogenic plant viruses in both treated and untreated wastewater call for the broad consideration of the use of reclaimed water for irrigation purposes, since it poses a risk of disease spread to irrigated plants and plants grown in hydroponic systems. This consideration is especially relevant for highly stable and mechanically transmissible viruses, such as the ones detected in this study. Although virus removal in water was studied and can be achieved by careful selection of treatment processes, such as coagulation-sedimentation, ozonation, microfiltration (Canh et al., 2019; Kato et al., 2018) there are still needs for the development of new cost effective and environmentally friendly technologies for efficient virus inactivation.

This study suggests that plant viruses, especially tobamoviruses, can potentially circulate between urban environment and croplands: from infected plants, through human and animal consumption and excreta, to wastewater, from where they could be transmitted back to plants through, e.g., irrigation with reclaimed wastewater. The release of infective plant viruses into environment through wastewater might have consequences in rapid

transmission of pathogenic viruses to new areas, which cannot be reached by other vectors. Hypothetically, through such process the global incidence of diseases and their impact on the ecosystem can increase more rapidly for a broad range of crops (Mehle et al., 2018). Wastewater-based epidemiology has the potential to predict critical locations and critical moments for viral disease onset (Xagoraraki and Brien, 2019), however it has not yet been applied to predict and prevent plant viral disease outbreaks. The results and methodology used in this study provides a baseline for monitoring reclaimed water for the presence of pathogenic viruses and to monitor and improve wastewater treatment processes for efficient removal or inactivation of viruses.

5. Conclusions

- High-throughput sequencing analysis of wastewater metagenomes confirmed a high diversity and abundance of plant viruses in wastewater treatment plant influents and effluents. Highly stable tobamoviruses were the dominant representatives of plant viruses in wastewater.
- Nucleic acids of plant pathogenic viruses not previously detected in the region were present in both untreated and treated wastewater, including emerging and quarantine viruses. This indicates the applicability of wastewater-based epidemiology for early detection and monitoring of economically important plant viruses, however, detection of nucleic acids needs to be coupled with specific follow-up studies, especially concerning virus infectivity.
- Wastewater contained infective plant viruses, which remained infective after conventional wastewater treatment. This indicates the flux of plant viruses from the urban environment to the ecosystem. The presence of infective plant viruses in wastewater can also have consequences in rapid transmission of pathogenic viruses to new areas, especially when wastewater or reclaimed water is used for irrigation.

Data availability

All sequencing data that support the findings of this study are linked under the accession number PRJNA549409 in the NCBI BioProject database (https://www.ncbi.nlm.nih.gov/bioproject/).

Complete consensus viral genome sequences generated in this study have been deposited in GenBank under the accession numbers MN267897-MN267904.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.watres.2020.115628.

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