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The role of pipe biofilms on dissemination of viral pathogens and virulence factor genes in a full-scale drinking water supply system

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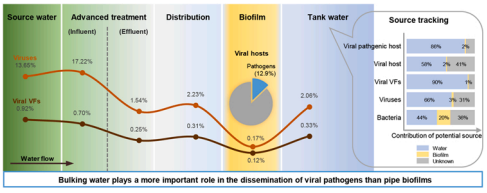
HIGHLIGHTS

- Viral pathogens and VFs can disseminate from source water to tap water.
- Propotion of viral pathogens and VFs in biofilms was far less than that in water.
- Bulking water plays the main role in the dissemination of viral pathogens.
- *Mycobacterium* and TOC were key influencing factors for viral virulence variation.

GRAPHICAL ABSTRACT



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ABSTRACT

Water is an important medium for virus transmission and viral pathogens are increasingly appreciated as a significant water safety issue. However, the effect of pipe biofilms on viral pathogens remains unclear. This research aimed to investigate the dissemination of viruses in a full-scale drinking water supply system (DWSS) and the effect of pipe biofilms on viral pathogens in bulking water. Viral pathogens, pathogenic viral hosts, and viral virulence factors (VFs) were found to disseminate from source water to tap water. The proportion of virus and viral VFs in the biofilm was far less than that in water. The contribution of biofilms in pipe wall to viruses and viral VFs in bulking water was less than 4%, and viruses in the biofilm had no obvious effect on pathogenic viruses in water. Dominant viruses carrying VFs changed from *Cyanobacteria* virus to *Mycobacterium* virus after advanced water treatment. *Mycobacterium* and organics were identified as the key factors influencing composition and abundance of viral VFs, which could explain 41.1% of the variation in viral virulence in the water supply system. Host bacteria and organics may be used as the key targets to control the risk of viruses in DWSSs.

1. Introduction

It is well known that water is an important medium for virus transmission. Water-transmitted viral pathogens, as classified by the World Health Organization (WHO), include adenovirus, astrovirus, and hepatitis viruses (World Health Organization (WHO), 2011). The recent outbreak of coronavirus disease 2019 (COVID-19) has become a public health emergency worldwide (Alexander et al., 2020). There is no current evidence to confirm that human coronaviruses are transmitted through contaminated drinking water (La Rosa et al., 2020).

The biofilm on drinking water supply pipe wall can provide an ideal place for microorganisms to survive, propagate and interact (Zhang et al., 2021). Biofilm in pipe wall showed significant influence on tap water bacteria, and the contribution of biofilm to tap water bacteria was nearly 18% (Liu et al., 2018). Biofilm formation and detachment can also affect the transmission of antibiotic resistant bacteria in drinking

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water supply systems (DWSSs) (Zhang et al., 2018). However, these researches on biofilm in DWSSs always focus on bacteria despite the evidence that other microbial taxa such as viruses are present in biofilms (Douterelo et al., 2018).

The role of biofilms in the dissemination of viruses in aquatic environments is controversial. During epidemic outbreaks, viral pathogens are discharged into wastewater, and some of these viruses will attach to biofilms (Skraber et al., 2009). Some viruses (F-specific bacteriophages) persist longer in biofilms than in wastewater, so wastewater biofilms may contribute to the dispersal of pathogenic viruses during epidemic periods (Skraber et al., 2009). The presence of biofilms increases the retention time of the virus (MS2 as a model virus) on aquifer surfaces in managed aquifer recharge systems (Amirhosein et al., 2020). However, there is also evidence for biofilm control by bacteriophage viruses (Donlan, 2009). Specific bacteriophage viruses can be used to inhibit the development of biofilms on ultrafiltration membranes (Goldman et al., 2009). The combined treatment of bacteriophage viruses and chlorine oxidation is more effective than chlorination for the control of *Pseudomonas aeruginosa* biofilms in wastewater (Zhang and Hu, 2013). The present studies on the relationship between viruses and biofilms often focus on targeted or model microorganisms. F-specific phages or other model viruses could hardly be detected in drinking water biofilms (Skraber et al., 2005; Lehtola et al., 2004). Knowledge of the role of pipe biofilms in the dissemination of viruses is still in its infancy, especially in DWSSs.

Second-generation sequencing has facilitated leaps in our knowledge of viruses in the aquatic environment. It was reported that bacteriophages are the most dominant viruses in aquatic environments (Kavagutti et al., 2019). One of the most striking features of bacteriophage genomes is their apparent mosaic structure, so bacterial virulence factor genes may be incorporated into the viral genome (Hatfull, 2008). The present studies on viral pathogens were often performed by using real-time PCR, which shows high accuracy but has the limitation of fewer targets and makes the analysis far from comprehensive (Emelko et al., 2019; Morrison et al., 2020; O'Brien et al., 2017). Furthermore, the survival mode of the virus depends on the metabolism of its host (Brum et al., 2016). Thus, clarifying the relationship between the virus and the host in DWSSs can help to not only identify viral pathogens accurately but also predict the potential influence of the host on viral pathogens. However, how viral pathogens and pathogenic viral hosts disseminate and the key influencing factors for the dissemination of viral pathogens remain largely unknown.

To obtain viral information in DWSS, we collected water samples covering source rivers, reservoirs, waterworks, pipe networks, communities, and biofilm samples at the end of the pipeline. Subsequently, the water quality was measured, and shotgun metagenomic sequencing was performed. The aims of this research were (1) to investigate the dissemination of viruses in a full-scale chloraminated DWSS, (2) to study the effect of pipe biofilms on the occurrence of viral pathogens in water, and (3) to explore the key influencing factors for viral virulence variation.

2. Materials and methods

2.1. The drinking water supply system and sampling

Water samples were collected from source water (SW) (SW1: standing in one of the outlets of Taihu Lake; SW2: the influent of Jinze Reservoir, which is situated in the Taipu River; SW3: the effluent of Jinze Reservoir), a drinking water treatment system (DWTS) (TW1 and TW2: the influent and effluent of the waterworks), a primary drinking water distribution system (PrimDWDS) (DW1: water in the start of pipelines; and DW2: water at the end of pipelines), and two secondary drinking water distribution systems (SecDWDSs) (SeW1: water sample from the underground tank; SeW2: water from the top tank; and SeW3: water sample from the underground tank) (Fig. 1). Water samples were collected according to the standard examination methods for drinking water (GB/T 5750.2–2006). Sterilized bottles were used to collect water samples from each site. All the water samples were collected in August 2018, and transported on ice to the laboratory and processed within 12 h. Locations and years of sampling sites are shown in detail in Tab. S1.

The Jinze Ecological Reservoir is the main source of drinking water for southwest Shanghai. The water comes from the Taipu River, the largest tributary of Taihu Lake. The total storage capacity of the Jinze Reservoir is approximately 9×10^7 m^3. Its water pretreatment process includes sedimentation, aeration, and absorption by aquatic plants. The DWTS is 46.1 km away from the Jinze Reservoir. The drinking water production rate is 3×10^5 m^3/day. The water treatment process in the DWTS includes coagulation, sedimentation, sand filtration, ozonation, biological activated carbon (BAC) filtration, and chloramine disinfection. The pipe material in the PrimDWDS is ductile iron, and the pipe diameter ranges from 300 mm to 1600 mm. The stainless steel water tanks were selected in the SecDWDSs.

Four biofilm samples were obtained by scraping the inner wall of actual pipelines which have been running stably before sampling with sterilized cotton (Fig. 1). The pipes included ductile iron pipes with diameters of 600 mm (BF1) and 100 mm (BF2) respectively, polypropylene-random pipe (BF3, 20 mm in diameter), and stainless steel pipe (BF4, 20 mm in diameter). BF1 was obtained in the terminal PrimDWDS. BF2, BF3 and BF4 were obtained from the SecDWDSs.

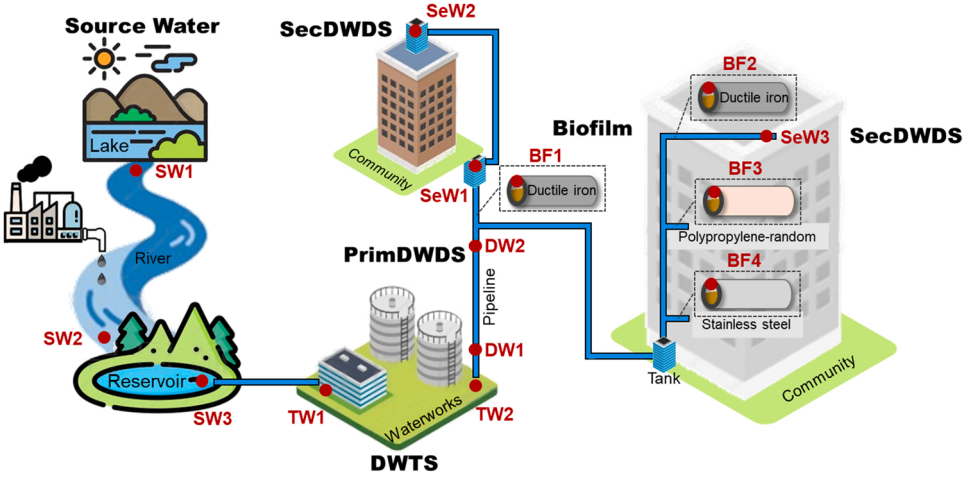

Fig. 1. Flowchart of the drinking water supply system. DWTS: drinking water treatment system, PrimDWDS: primary drinking water distribution system, and SecDWDS: secondary drinking water distribution system.

Sterilized tubes (50 mL) were used to collect the biofilm sample, and transported on ice to the laboratory and processed within 12 h.

2.2. Water quality analysis

The measurements of total residual chlorine and temperature were performed in situ. The conductivity, pH, resistivity, turbidity, alkalinity, hardness, total dissolved solids, total organic carbon (TOC), chlorides, sulfates, nitrite nitrogen, bicarbonate, total iron, Ryznar saturation index, Larson ratio, standard plate-count bacteria and heterotrophic plate count measurements were performed in the laboratory. The detection methods for the above mentioned water quality parameters are shown in Tab. S2. Assimilable organic carbon (AOC) was determined with a batch growth assay as described previously (Lautenschlager et al., 2013). The enumeration of total and intact cells was performed by using SYTO 9 stain and propidium iodide with a CytoFLEX flow cytometer (Beckman Coulter, Inc.USA). (Lehtinen et al., 2004).

2.3. DNA extraction and quantification

The genomic DNA of biofilms was extracted by using the FastDNA SPIN Kit for Soil (Mpbio, USA). The enrichment of biological cells of water was performed using the membrane filtration method. The volume of water used for enrichment of biological cells is shown in Tab. S3. Water was filtered through a sterile 0.22 μm mixed cellulose membrane (Bandao, China) with a magnetic filter funnel (Pall Life Science, USA) and a vacuum pressure pump (Pall Life Science, USA). Genomic DNA was extracted from the membrane by using an optimized phenol: chloroform-based method (Miao and Bai, 2021). The concentration of DNA was detected by ultraviolet spectrophotometry (OneDrop OD-1000, China). DNA quality was checked by 2% agarose gel electrophoresis. Moreover, qualified DNA was adjusted to 50 ng/μL and stored at − 80 ℃.

2.4. Shotgun metagenomic sequencing and host annotation

Shotgun metagenomic sequencing was performed by using an Illumina HiSeq 2000. Libraries were prepared with a fragment length of approximately 400 bp using a Covaris M220 and a NEXTFLEX™ Rapid DNA-Seq Kit. Raw reads were quality controlled using FASTQ (Chen et al., 2018). CD-HIT (http://www.bioinformatics.org/cd-hit/) and SOAPaligner were applied for abundance calculation of the nonredundant gene set (Li et al., 2009). The host annotation of the virus was performed by using NCBI (http://www.ncbi.nlm.nih.gov/). The annotation of viral virulence factors was performed by comparing the target amino acid sequences with the Virulence Factors Database (VFDB) by using BLASTP (Liu et al., 2019). To reduce gene misestimation, transcripts per million reads (TPMs) were used to express the gene abundance of each sample, which was calculated for each sample as follows:

$$\mathrm{TPM_i} = \frac{(R_i/L_i) \times 10^6}{\sum_1^n (R_j/L_j)}$$

where R_i tracks the read number, L_i tracks the gene length, and $\sum_1^n (R_j/L_j)$ tracks the total number of normalized read numbers by gene length.

2.5. Statistical analysis

All data analyzed in this study were considered statistically significant at $p < 0.05$ unless indicated otherwise. All environmental variables were evaluated using one-way univariate analysis of variance (ANOVA) to select reasonable measurements. Mothur 1.30.1 was used to estimate the Shannon diversity index (Schloss et al., 2009). The comparison of microbial community structures was displayed by using two axes of a nonmetric multidimensional scaling (NMDS) analysis based on the Bray-Curtis distance at the taxonomic genus level. Student's t test was used to analyze the dissimilarities of environmental variables in different groups. Procrustes analysis was used to explore the relationship between the viral and host communities (Zhao et al., 2019). Fast expectation-maximization microbial source tracking (FEAST) was used to identify the microbial percentage contribution of each potential source to the bulking water (Shenhav et al., 2019). The Mantel test was used to find the correlation between viral virulence and environmental factors. Variance partitioning canonical correspondence analysis (VPA) was used to quantify the explanatory degree of the influence of different water quality parameters on the functional change of the microbial community (Rui et al., 2015).

3. Results and discussion

3.1. Occurrence and dissemination of viruses in the water supply system

We obtained 247.9 gigabases of raw reads from the fourteen metagenomic samples. A total of 2501 species were annotated as viruses via megablast against the NCBI nr/nt database online, including 6400,615 genes with a total length of 3307,693,308 bp. The proportion of viral TPMs in water was reduced from 17.2% to 1.5% after the water treatment process and remained at approximately 2.2% in the water distribution system (Fig. 2a, Tab. S3). The proportion of viral TPMs in biofilms was 0.1–0.2%, which was far less than that in the water samples (Fig. 2a, Tab. S3).

A total of 1343 species of viruses were identified in the lake water, double-stranded DNA (dsDNA) virus is the most dominant, accounting for 98.0%. The high proportion of dsDNA viruses in DWSS was consistent with findings from sea and freshwater lake samples (Du et al., 2020; Skvortsov et al., 2016). The variation of the viral TPMs (Fig. S1, Tab. S4) and the number of viral species (Fig. 2b) were similar. From the lake to the influent of the reservoir entrance, the number of viral species decreased to 1310, and the viral TPMs decreased by 44.6%. Some industrial and medical wastewater flows into the Taipu River, which may decrease the stability of the viral community. For example, the concentration of arsenic, which can inhibit the growth of viruses, increased sharply from the lake (0.0018 mg/L) to the reservoir entrance (0.0026 mg/L) (Hwang et al., 2004; Miteva et al., 2005). After pretreatment of the reservoir, the viral TPMs decreased by 9.0%. After source water transportation from the reservoir to waterworks, the number of viral species increased to 1343, and the viral TPMs increased by 52.4%. This may be due to sufficient nutrient substances in the source water and the long reaction time (35.8 h) can provide good conditions for aerobic bacterial metabolism, which may be viral hosts (Liu et al., 2017). The TPMs was dramatically reduced by 90.3% after the DWTS treatment, and only 856 species were detected in effluent water. After entering the PrimDWDS, the number of species increased to 1121. The number of virus species and the TPMs in the SecDWDSs were similar to those in the PrimDWDS.

There were only 234–402 viral species in biofilms, which was far less than the number of species in water (ranged from 856 to 1343) (Fig. 2b, Tab. S3). Additionally, the viral proportion in the biofilm was only 0.5–27.9% of that in water (Tab. S5). The proportion of virus in the biofilm of pipe wall was very small, whatever the pipe materials were. The relative abundance of viruses in the biofilm in polypropylene-random pipe was the highest, followed by viruses in stainless steel pipe biofilm, and the relative abundance of viruses in the biofilm in ductile iron pipe was the lowest. Accordingly, there were abundant bacteria but only a few viruses in the pipe biofilm. On the one hand, this may be because the viral attachment sites on the host surface were blocked. In detail, the extracellular polymeric substances (EPS) produced by the bacteria attached to the surface of the viral host bacteria and blocked the attachment site of the corresponding viruses, so the adsorption capacity of the viruses was decreased (Amirhosein et al., 2020). On the other hand, it was reported that increasingly spatially correlated clusters of viruses and clusters of hosts resulted in sufficient

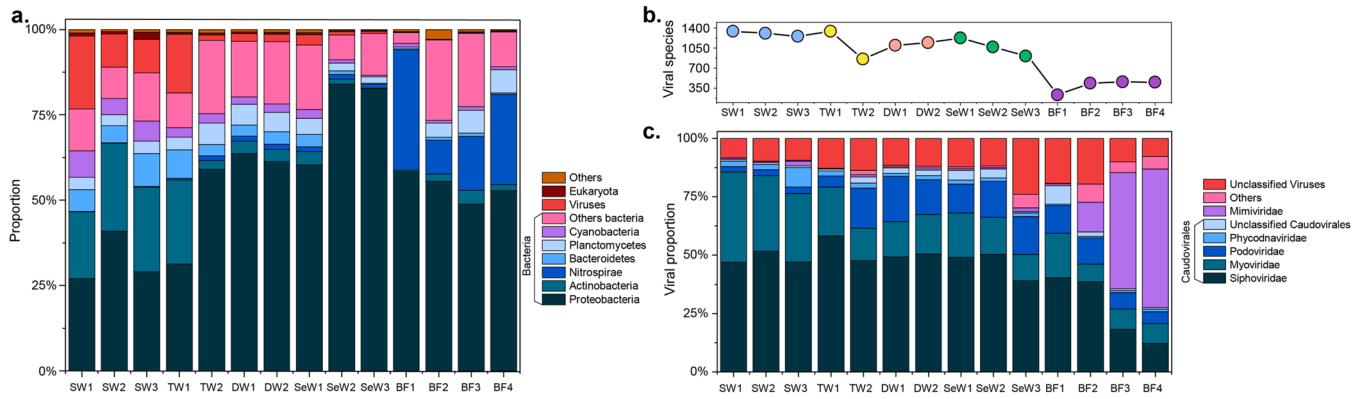

Fig. 2. . Composition and diversity of the microbial communities. a, Proportion of the microbial communities. b, Number of viral species. c, Proportion of viruses.

increases in viral infectivity; therefore, the poor mobility of biofilms caused low viral infectivity and led to fewer viruses in the biofilm than that in the water (Taylor et al., 2017).

The most abundant dsDNA virus in the source river was *Caudovirales,* with a relative abundance of 88.7% (Fig. 2c, Tab. S6). The dominant families of *Caudovirales* included *Siphoviridae* (49.1%), *Myoviridae* (22.2%) and *Podoviridae* (10.4%), which are also widespread in sewage and freshwater lakes (Sharma et al., 2017; Skvortsov et al., 2016). After pretreatment of the reservoir, the TPMs of *Siphoviridae* and *Myoviridae* decreased by 17.2% and 17.4%, respectively (Tab. S7). After source water transmission, the TPMs of *Siphoviridae* and *Podoviridae* increased by 93.9% and 162.1%, respectively. The TPMs of *Siphoviridae* and *Myoviridae*, as the most abundant families in the DWSS, decreased by more than 99.4% after the water treatment. Subsequently, the increases in the TPMs of *Siphoviridae* and *Myoviridae* were high after entering the PrimDWDS (an average increased by 132.2%) and SecDWDSs (an average increased by 12.6%). The dominant viruses in the pipe biofilm included *Siphoviridae*, *Myoviridae*, *Podoviridae*, and *Mimiviridae*. In addition, the dominance of *Mimiviridae* in the biofilm in polypropylene-random and stainless steel pipe was higher than ductile iron pipe.

The NMDS analysis revealed a significant difference in the viral community structure before and after the water treatment (Fig. S2, ANOSIM, R=0.69, $p < 0.05$). The viral Shannon index of water after the water treatment was significantly higher (Student's t, $p < 0.05$) than that of water before the treatment (Tab. S8). Therefore, there were great changes in viral diversity and composition after treatment with DWTS. Furthermore, the viral community structure of biofilms was significantly

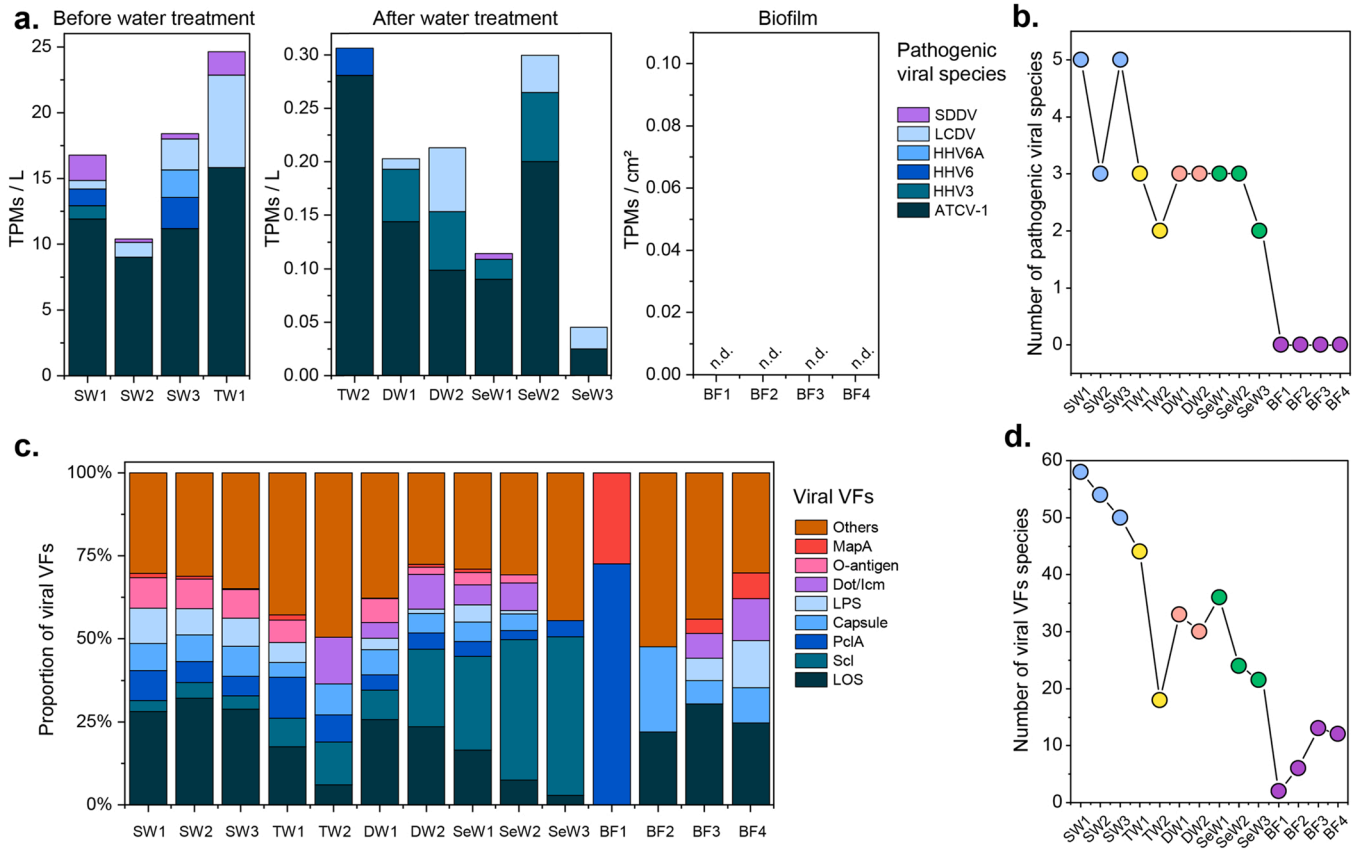

Fig. 3. . Composition of viral pathogens and virulence factors (VFs) in the drinking water supply system. a, Composition of human viral pathogens. n.d.: no detected. b, Number of human pathogenic viral species. c, Proportion of viral VFs. d, Number of viral VFs.

different from that in water by using NMDS (Fig. S2, ANOSIM, R=0.82, $p < 0.05$). Both the bacterial and viral Shannon indices in biofilms were less than those in water (Tab. S8). Accordingly, the few viruses in pipe biofilms may also be due to the low diversity of bacterial hosts.

3.2. Viral pathogens and viral virulence factors in water and biofilms

There were 0.0258% of viral pathogens in the source river, including viruses that infected humans (0.0133%), mammals (0.0085%), insects (0.0038%), and aquatic life (0.0002%) (Tab. S9). CoVs and other viral pathogens classified by the WHO were not detected in our DWSS (World Health Organization (WHO) (WHO), 2011). Waterworks can remove 81.3% of human viral pathogenic species (Fig. 3a) which included 0.095% *Acanthocystis turfacea Chlorella virus 1* (ATCV-1), 0.0018% *human herpesvirus* (HHV), 0.0015% *scale drop disease virus* (SDDV), and 0.0005% *Lymphocystis disease virus* (LCDV). The most abundant human viral pathogen, ATCV-1, is associated with changes in cognitive functions, and human beings may be infected by swallowing water containing it (Yolken et al., 2014). The content of ATCV-1 was observed to decrease during water treatment, but it was present in the DWDS. HHV, which is continuously detected in the DWDS, may cause varicella or herpes zoster and has strong infectivity (Loparev et al., 2004). LCDV and SDDV disappeared after the water treatment and soon reappeared. No viral pathogenic species were observed in biofilms (Fig. 3b). Viral pathogens cannot be removed completely by the water treatment process; thus, there is a potential risk of viral contamination in tap water.

In addition to the viral pathogens, there were also 84 kinds of VFs in the virus (Fig. 3c, Tab. S10), accounting for 0.04% of the total microbial gene TPMs (Tab. S11). A total of 58 kinds of VFs were observed in the source water (Fig. 3d). After the water treatment, the VFs decreased from 44 to 18. Only 2–13 kinds of VFs, were detected in the biofilms. The proportions of viral VFs in biofilms to that in water only ranged from 0.09% to 35.5% (Tab. S12). The contribution of *Siphoviridae* and *Myoviridae* to dominant viral VFs was the largest, accounting for 76.1% ± 18.7% (Fig. S3). Most (75.1%) of the viral VFs in water were offensive VFs, which are related to regulating the adhesion of pathogens, and the dominant (63.4%) microbial VF subtype was adhesion (Fig. S4). Liang's research showed that toxins were the most abundant (25%–60%) VF subtypes in the aquatic ecosystem, which was inconsistent with our DWSS; therefore, the composition of VFs was affected by the environment (Liang et al., 2020). As the most abundant VFs in DWSS, lipo-oligosaccharide (LOS) can assist the adhesion and invasion of pathogenic infection. Moreover, LOS is crucial in the induction of anti-ganglioside antibodies and may cause cross-immunoreaction (Heikema et al., 2010). Except for LOS, the dominant VFs in the DWSS, lipopolysaccharide (LPS) and streptococcal collagen-like protein (Scl), were also associated with adhesion (Caswell et al., 2010; Schaeffer et al., 2004).

The human health risk of viruses in DWSS should be a concern. It was reported that factors for doses were commonly the most influential determinant of risk (Owens et al., 2020). Quantitative microbial risk assessment (QMRA) has become a widely used technique for assessing population health risks posed by waterborne pathogens (Buseyne et al., 2018). However, the estimated infection risk of viral pathogens in our system has not been investigated. Accordingly, quantitative viral risk assessment tools in DWSS remain largely unknown, and suitable viral reference pathogens and dose-response models of viruses need rigorous evaluation (clinically relevant animal models and clinical trials) given the diverse viral VFs in DWSSs.

3.3. Composition of viral hosts and their correlation with viruses

The major hosts of viruses in the DWSS included algae, bacteria, and

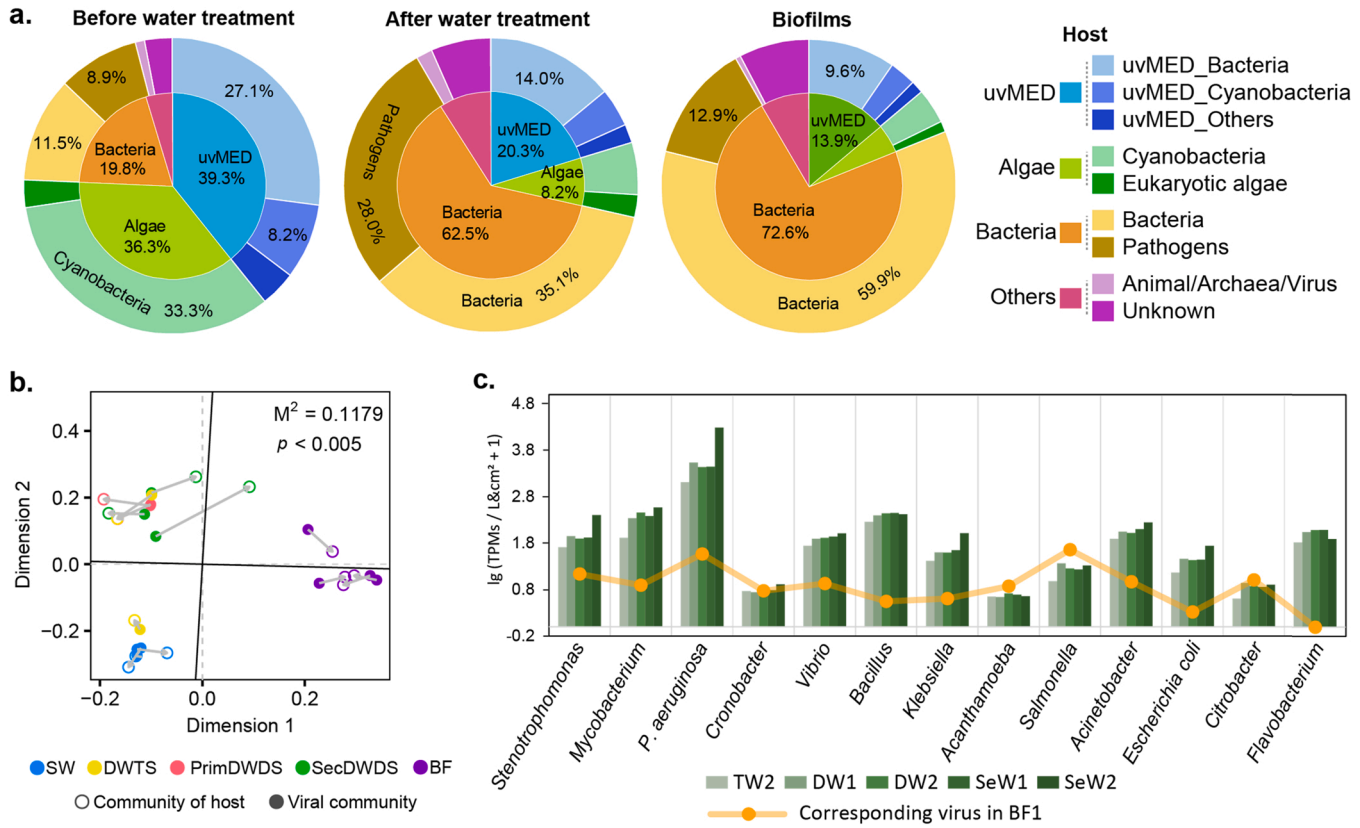

Fig. 4. . Composition of the viral hosts and their correlation with viruses in the drinking water supply system. a, Distribution of the viral host in the water before and after the water treatment and in biofilms. b, Procrustes analysis of the correlation between viral and host communities based on the Bray-Curtis dissimilarity results of TPMs of viruses and hosts (999 permutations). c, Variation in pathogenic hosts during water distribution and corresponding viruses in biofilms.

uncultured Mediterranean phage uvMED (uvMED) (Fig. 4a). As the most abundant (23.9% ± 11.9%) virus species, uvMED can infect various hosts, including *Prochlorococcus*, *Synechococcus* and *Pelagibacter*. The composition of uvMED hosts was based on previous studies (Ghai et al., 2010). *Cyanobacteria* virus (41.5%) and *Proteobacteria* virus (59.7%), which were classified by host, were the most abundant viruses before and after the water treatment, respectively.

The abundance of viruses with pathogenic hosts decrease from 10504 TPMs/L in the raw water to 208 TPMs/L in the effluent water (Tab. S13). At the same time, the abundance of *Cyanobacteria* viruses decreased from 24327 TPMs/L in the raw water to 34 TPMs/L in the effluent water (Tab. S13). The *Cyanobacteria* viruses was removed more than the viruses with pathogenic hosts by the water treatment. It should be noted that still 28.0% of the viruses infected pathogens after water treatment. Pathogenic hosts with high abundance included *P. aeruginosa* and *Mycobacterium*, which were resistant to chlorine and other disinfectants used in water treatment due to the chlorine consumption capacity of matrix and permeability barrier (Falkinham et al., 2015; Luo et al., 2021). Accordingly, the removal rate of viruses with pathogenic hosts was low. A total of 12.9% of viruses infected pathogens in pipe biofilms, such as *Salmonella* and *P. aeruginosa*. As shown in Fig. 4b, based on the NMDS analysis of the TPMs of both virus and host, the result of the Procrustes analysis indicated that there was a significant correlation between viral and host communities in different samples ($M^2 = 0.12$, $p < 0.005$). Horizontal gene transfer is more common during the replication of *Caudovirales*, the most abundant virus in DWSS (Ackermann, 1998). Therefore, the virus may carry VFs from pathogenic hosts by horizontal gene transfer.

Because of the correlation between viral and host communities, the variation in the viruses and the corresponding pathogenic hosts was explored further (Fig. S5). Student's t test showed that the TPMs of most (96.1%) of the pathogenic hosts was higher than that of the corresponding viruses (Fig. S6). Additionally, the TPMs of all the pathogenic hosts showed a positive Spearman correlation with the corresponding viruses (Fig. S6). The replication of lytic viruses begins immediately after infection and leads to phage production and lysis of the host cell (Payet and Suttle, 2013). In marine environments, 20–50% of bacterial biomass is lost daily due to viral lytic infection (Suttle, 2005). Accordingly, pathogenic virus-host interactions in the DWSS were dominated by the coexistence of lysogenic viruses and bacteria.

Because of the viruses with pathogenic hosts present in pipe biofilms, the impact of viruses in biofilms on the dissemination of pathogens in water was explored further. As shown in Fig. 4c, all pathogenic hosts except *Acanthamoeba* regrew with water stagnation (Stagnation time are shown in detail in Tab. S14). *Salmonella* virus and *P. aeruginosa* virus were the dominant viruses able to infect pathogens in biofilms, but their hosts increased by 130.7% and 1400.0% from effluent water to upper tank water, respectively (Tab. S15). Therefore, the viruses in the biofilm had no obvious effect on pathogens in water. Although specific viruses can be used for inhibiting the formation of hosts, viral infection is a stochastic process and primarily depends on the abundance of hosts (Donlan, 2009; Keshri et al., 2017). It has been reported that the bacterial population in biofilms (*P. aeruginosa*, *Acinetobacter johnsonii,* and *Bacillus subtilis*) dropped by > 3 orders of magnitude after the addition of specific bacteriophage viruses (10^6 to 10^7 PFU/mL) to the ultrafiltration membrane (Goldman et al., 2009). In wastewater, at concentrations of 400 and 4×10^7 PFU/mL, the specific viruses inhibited *P. aeruginosa* biofilm formation by 45% and 73%, respectively (Zhang and Hu, 2013). In our water system, the average concentration of heterotrophic bacteria was 2194 CFU/mL during water distribution. It can be predicted that the concentration of viruses in biofilms may be far less than 2194 CFU/mL based on the above analysis, and the viral biomass in biofilms may be extremely low for the removal of pathogens in water. Furthermore, the removal of pathogenic bacteria may not be effective due to EPS, which limits the penetration of specific viruses (Goldman et al., 2009).

3.4. Source tracking of viruses, viral virulence factors, and their hosts in bulking water

Although there was a strong relationship between the viruses and bacterial hosts, the bacterial and viral source contributions of biofilms to bulking water were different according to the source tracking by FEAST. For the bacteria in the bulking water of SeW2, the contributions were from the PrimDWDS (23.2%) and the SecDWDS (12.3%), and there was a partial contribution from the biofilm (17.3%) (Fig. 5a). In contrast, the viruses in the SeW2 major originated from the PrimDWDS (35.3%) and the SecDWDS (40.7%). The viral contribution of biofilms to bulking water of SeW2 was only 1.9%. There were minor (<0.01%) contributions of viral VFs in bulking water of SeW2 from the pipe biofilm. Total viral hosts and the pathogenic viral hosts in the DWSS had similar source contributions: the major contributions were from the PrimDWDS and the SecDWDS. Additionally, their contributions in biofilms to bulking water of SeW2 were very low, at 2.1% and 2.5%, respectively. Similarly, the FEAST results of SeW3 (Fig. 5b) showed that the contribution of bacteria, viruses, viral VFs, viral host and viral pathogenic host of biofilms to bulking water was 22.7%, 3.6%, 2.9%, 1.2% and 0.8%, respectively. As a result, both of the pipe biofilm in the PrimDWDS and the SecDWDS may have a limited effect on the dissemination of viruses and viral VFs in the DWSS.

The source tracking results revealed the low contributions of pipe biofilms to viruses, viral VFs, and viral hosts in bulking water, corresponding to the low abundance of viruses and viral VFs in the biofilm. With the decay of residual chlorine in water, microorganisms gradually regrew, and viral virulence increased slightly. However, viral virulence decreased for longer periods of stagnation (Tab. S14). More microbial cells detached from the biofilm in cooperation with stagnation. This can be proven by the increases in genes with "biofilm formation" functions (Fig. S7), which are essential for microorganisms to produce surface structures for adhesion, aggregation, and motility (Chang et al., 2014). Studies have shown that metabolic dormancy or molecular persistence programs are important traits of biofilms, which cause a high tolerance of biofilms (Koo et al., 2017). Dai's research also observed an increase in gene functions for "resistance to antibiotics and toxic compounds" in stagnated water compared with recirculating water (Dai et al., 2018). These findings may explain the low abundance of viral VFs in the upper tank water and biofilms in the distal pipeline. Furthermore, 62.3% of viral VFs were involved in the initial bacterial adherence in the DWSS (Fig. S4). The presence of EPS in biofilms was not conducive to the adherence and replication of viral VFs, which caused a low abundance of viruses and viral VFs (Amirhosein et al., 2020).

3.5. Influence of the hosts and water quality on viral virulence variation

Because there were few viral VFs in the biofilm, the transmission of viral VFs in the DWDS was more dependent on the water than the pipe biofilm. Accordingly, we further explored the key influencing factors of viral virulence variation in the DWDS. The host composition of viruses that carried VF genes was significantly different before and after DWTS. As shown in Fig. 6a, half of the viruses with VFs infected *Cyanobacteria* before DWTS, and *Synechococcus* accounted for 34.9% (Tab. S16). All the *Cyanobacteria* viruses carrying VFs decreased from 46.3% to 21.2% after the water treatment, and the dominant hosts of virulent viruses changed to *Mycobacterium* (24.4%). The proportion of *Mycobacterium* virus to all the virulent viruses increased from 1.0% in the lake to 17.8% in the effluent water and further increased to 42.7% in the SecDWDS. With increasing stagnation time, the proportion of *Mycobacterium* virus that carried VFs to all the virulent viruses gradually increased ($R^2 = 0.89$, $p < 0.05$) (Fig. 6b). Furthermore, the relationships between viral VFs and the dominant hosts of viral VFs were explored by using linear regression (Fig. S8). Both *Synechococcus* and *Mycobacterium*, the primary hosts of viral VFs, had regression coefficients of less than 0.5. Thus, the influencing factors of viral virulence variation should be explored

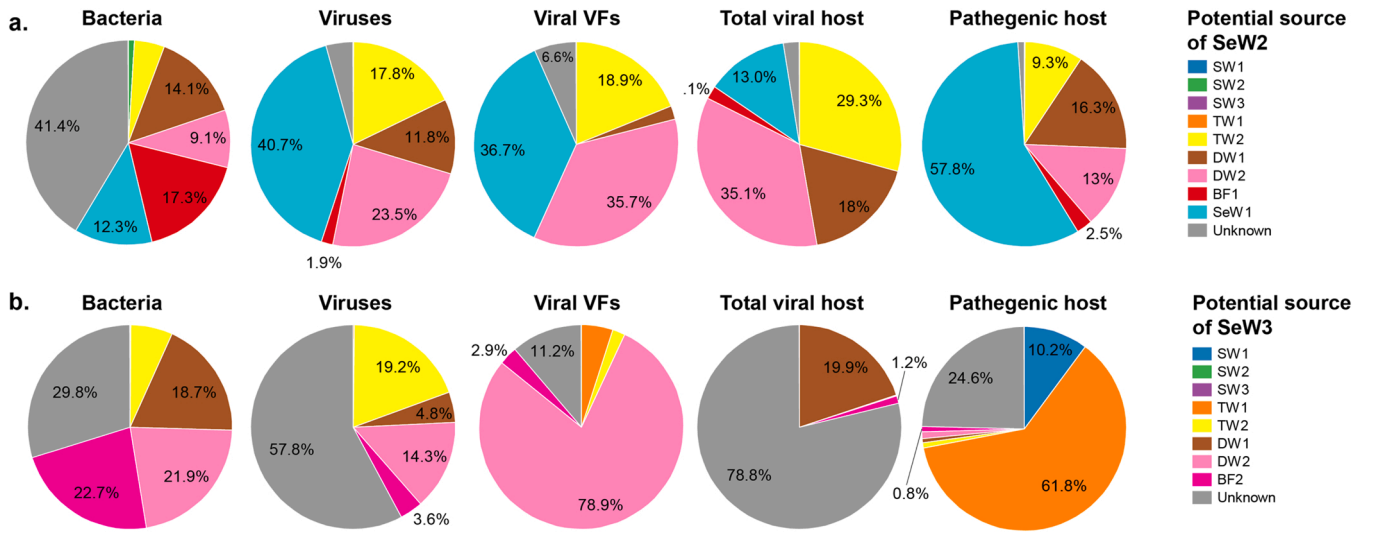

Fig. 5. . FEAST estimations of the microbial percentage contribution of each potential source to the bulking water. a, Source tracking of bacteria, viruses, viral VFs, viral host and pathogenic host in SeW2. b, Source tracking of bacteria, viruses, viral VFs, viral host and pathogenic host in SeW3.

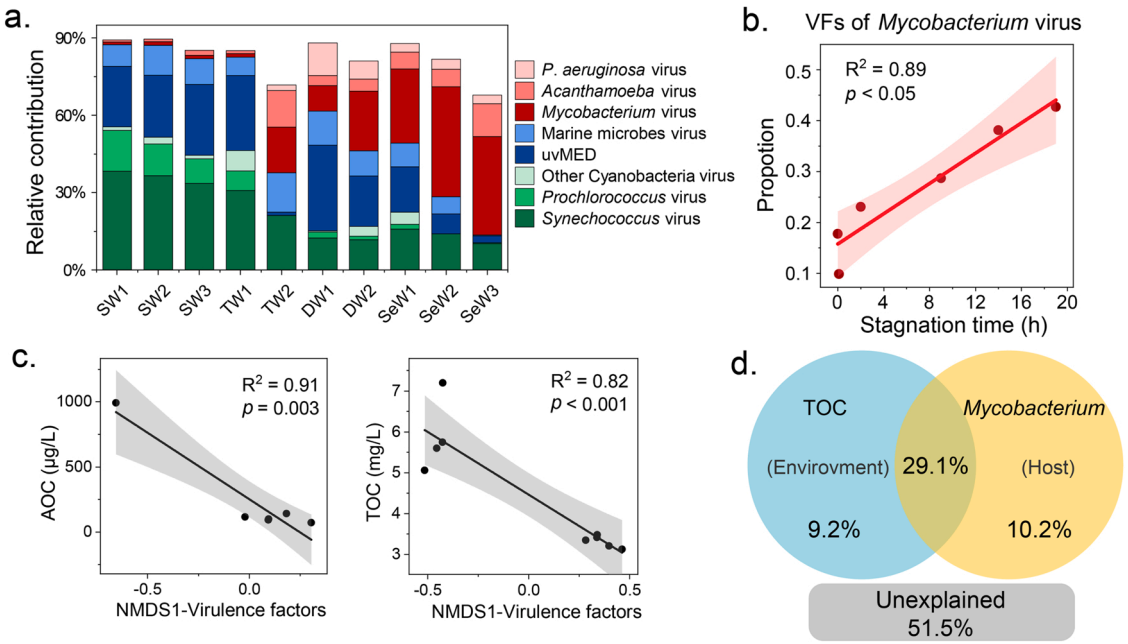

Fig. 6. Driving influences of virulence factors (VFs) of viruses. a, Relative contributions of different taxa (classified by the viral hosts) to viral VFs in different samples. b, Relationships between the proportion of *Mycobacterium* virus that carried VFs to all the virulent viruses and the stagnation time of water. c, Relationships between organic matter and the β-diversity of VF genes. d, Variance partitioning analysis (VPA) of the effects of environment and host on viral VF structure. Percentages are the variation in the viral community structure explained by the two sets of parameters.

further in combination with water quality.

By comparing the correlation between 22 water quality parameters and viral VFs, we found that both the Mantel test and Spearman's correlation analysis revealed that AOC and TOC showed significant correlations with the TPMs of viral VFs (Tab. S17). Additionally, AOC (R^2 =0.91, $p = 0.003$) and TOC (R^2 =0.82, $p < 0.001$) showed significant correlations with the composition of viral VFs (Fig. 6c). VPA was performed to quantify the relative contributions of different variables to changes in the viral VF composition. The contributions of TOC and AOC as environmental factors and of *Mycobacterium* and *Synechococcus* as hosts were compared (Fig. S9). The VPA results revealed that TOC and *Mycobacterium* had the highest contribution rates of viral virulence variation, which explained 38.3% and 39.3% of the variation, respectively, leaving 51.5% of the variation unexplained (Fig. 6d).

As an important viral host, the relative abundance of *Mycobacterium* in biofilms was only 0.07%, far less than that in water (0.20–0.47%), which corresponds to the low abundance of viruses in biofilms. The mycobacterial cell envelope is characterized by the presence of a highly impermeable second membrane, which is composed of mycolic acids intercalated with different unusual free lipids, such as LOS, the primary VFs in our DWSS (Sani et al., 2010). Thus, *Mycobacterium* was one of the key influencing factors of viral virulence in chloramine DWDS. In addition, high water age was associated with a high relative abundance of *Mycobacterium* (Haig et al., 2018). It was reported that stagnation may decrease the proportions of microbial functions involved in the cell wall and capsule while increasing the functions involved in membrane transport (Dai et al., 2018). This may explain the overall increase in the proportions of *Mycobacterium* viral VFs corresponding to stagnation

time; specifically, stagnation facilitated the transport of virulence factors and other surface molecules over the mycobacterial cell envelope. Researchers generally believe that *Mycobacterium* has a strong tolerance for disinfectants, as it is frequently detected in the water distribution system (Wang et al., 2019). Accordingly, control of *Mycobacterium*, the host of the dominant virus carrying VFs after DWTS, may be helpful for viral virulence risk reduction. The strong positive correlation of TOC and the TPMs of *Mycobacterium* suggested that reducing organic matter of drinking water may help to control of *Mycobacterium* in DWSSs (Tab. S18).

TOC (3.204–7.200 mg/L) was also one of the important factors affecting viral virulence. AOC is a part of TOC, and the concentrations of AOC were in the range of 70.88–140.66 μg/L. There are enough nutrients for the host to metabolize under high TOC, which is beneficial for the parasitism of the virus (Liu et al., 2017). The Spearman's correlation results shown in Tab. S19 revealed that TOC was positively correlated with the total viruses, viruses with pathogenic hosts, and human pathogenic viruses. The TPMs of viral VFs also showed the same results (Tab. S19). The effect of TOC on viral virulence may be due to the promotion of microbial metabolism of sufficient organic matter. Ozonation and BAC adsorption benefited from the removal of TOC. Therefore, optimizing the two treatment processes may help to reduce the risks caused by viral VFs.

4. Conclusion

This research revealed the dissemination of viruses in a DWSS and the effect of pipe biofilms on viral pathogens in bulking water. Viral pathogens, pathogenic viral hosts and viral VFs were found to disseminate from source water to tap water in the DWSS. The contribution of pipe biofilms to virus and viral VFs in bulking water was less than 4%, and viruses in the biofilm had no obvious effect on pathogens in water. Bulking water plays the main role in the dissemination of viral pathogens. The pathogenic hosts showed a positive correlation with corresponding viruses. The proportions of *Mycobacterium* virus with VFs increased with stagnation in the water distribution system. *Mycobacterium* and TOC were identified as the key factors influencing composition and abundance of viral VFs, which could explain 41.1% of the variation in viral virulence in drinking water. Host bacteria and organics may be used as the key targets to control the risk of viruses in the DWSS. This research brings new insight regarding the role of pipe biofilms in the occurrence of viruses in DWSSs. It will be beneficial for people to know more about the potential risk from some unexpected viral pathogens in raw water.

Novelty statement

This research brings new insight regarding the role of pipe biofilms in the occurrence of viruses in drinking water supply systems (DWSSs). The proportion of virus and viral VFs in the biofilm was far less than that in water. Viruses in the biofilm had no obvious effect on pathogens in water. Bulking water plays the main role in the dissemination of viral pathogens. Host bacteria and organics can be used as the key targets to control the risk of viruses in DWSSs.

CRediT author contribution statement

Xiaocao Miao: Data curation, Methodology, Visualization, Investigation, Writing – original draft. **Chenxu Liu:** Data curation. **Mingkun Liu:** Data curation. **Xue Han:** Data curation. **Lingling Zhu:** Data curation. **Xiaohui Bai:** Conceptualization, Methodology, Supervision, Writing – review & editing.

Declaration of Competing Interest

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

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2022.128694.

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