Food Environ Virol (2015) 7:173–182
DOI 10.1007/s12560-015-9190-8

ORIGINAL PAPER

Removal of Surrogate Bacteriophages and Enteric Viruses from Seeded Environmental Waters Using a Semi-technical Ultrafiltration Unit

Anne Frohnert[1] · Katja Kreißel[2] · Pia Lipp[2] · Halim Dizer[1] · Beate Hambsch[2] · Regine Szewzyk[1] · Hans-Christoph Selinka[1]

Received: 31 October 2014 / Accepted: 10 March 2015 / Published online: 19 March 2015

Abstract Experiments to determine the removal of viruses in different types of water (surface water from two reservoirs for drinking water treatment, treated groundwater and groundwater contaminated with either 5 or 30 % of wastewater) by ultrafiltration were performed with a semi-technical ultrafiltration unit. Concentrations of human adenoviruses (HAdVs), murine norovirus (MNV), and the bacteriophages MS2, ΦX174 and PRD1 were measured in the feed water and the filtrate, and log removal values were calculated. Bacteria added to the feed water were not detected in the filtrates. In contrast, in most cases viruses and bacteriophages were still present in the filtrates: log removal values were in the range of 1.4–6.3 depending on virus sizes and water qualities. Best removals were observed with bacteriophage PRD1 and HAdVs, followed by MNV and phages MS2 and ΦX174. Virus size, however, was not the only criterion for efficient removal. In diluted wastewater as compared to drinking water and uncontaminated environmental waters, virus removal was clearly higher for all viruses, most likely due to higher membrane fouling. For quality assessment purposes of membrane filtration efficiencies with regard to the elimination of human viruses the small bacteriophages MS2 and ΦX174 should be used as conservative viral indicators.

✉ Hans-Christoph Selinka
hans-christoph.selinka@uba.de

[1] Federal Environment Agency, Section II 1.4 Microbiological Risks, Corrensplatz 1, 14195 Berlin, Germany

[2] DVGW-Technologiezentrum Wasser (TZW), Karlsruher Str. 84, 76139 Karlsruhe, Germany

Keywords Ultrafiltration · Bacteriophages · Enteric viruses · Wastewater contamination

Introduction

One goal of drinking water treatment is to provide water that does not contain pathogens in numbers or concentrations that constitute a potential danger to human health. Because of their small size and their high risk for human health viruses are probably the most challenging particles to eliminate. With regard to membrane filtration technologies, only ultrafiltration membranes have pore sizes small enough to remove free viruses from water by the sieving effect without further pretreatment. Different methods, like molecular weight cut-off, fluorescent microspheres or nanoparticles can be applied to determine the pore sizes of ultrafiltration membranes (Arkhangelsky et al. 2012; Pontius et al. 2009). Other spherical particles with sizes similar to virus particles are labelled phages or silica particles coated with DNA and protein (Farkas et al. 2014; Guo and Hu 2014; Soussan et al. 2011). With regard to the use of bacteriophages, especially small icosahedral phages like MS2 and ΦX174 are often used as surrogates for human and animal viruses (Boudaud et al. 2012; ElHadidy et al. 2014; Kreißel et al. 2012; Langlet et al. 2009). However, specific virus properties as well as properties of applied materials or the composition of the water matrix can significantly influence virus removal efficiency (Boudaud et al. 2012; Kreißel et al. 2012). So far, few studies have included the use of human viruses or viruses infecting warm-blooded animals.

Human adenoviruses have been suggested to be good indicators for monitoring of contaminated waters (Haramoto et al. 2010; Hundesa et al. 2006). Adenoviruses

cause different illnesses in humans like infections of the gastrointestinal tract or the respiratory tract and conjunctivitis, respectively, and are second to rotaviruses in causing gastroenteritis in children. Up to 50 % of adenovirus gastroenteritis remains asymptomatic, but all serotypes can be shed with faeces (Mena and Gerba 2009). This may be the reason why adenoviruses are found more frequently and at higher concentrations than enteroviruses or noroviruses in sewage affected surface water and can be found throughout the year (Hamza et al. 2009; Mena and Gerba 2009). In addition to their indicator function in faecally contaminated waters, adenoviruses may also be useful to determine removal of human viruses in treatment processes.

Noroviruses can also cause severe illness in the human gastrointestinal tract. These viruses have been detected in European surface waters less frequently than adenoviruses but more frequently than enteroviruses (Hamza et al. 2009; Lodder and de Roda Husman 2005; Wyn-Jones et al. 2011). Due to their structural similarity to human noroviruses, murine norovirus (MNV) may be applied as non-pathogenic surrogate for human noroviruses.

In this study, we investigated the removal of viruses from water by ultrafiltration using various viruses and different types of waters. Up to five viruses, the bacteriophages MS2, ΦX174 and PRD1, human adenovirus (HAdV) and MNV were spiked simultaneously into different waters and filtered with a pilot-scale ultrafiltration device using a polymeric membrane with a nominal pore size of 20 nm. All viruses tested in this study were unenveloped and had capsids with icosahedral geometry. Bacteriophage ΦX174 contains its genetic information in the form of ssDNA. The capsid diameter is 25 nm with spike proteins rising 3 nm from the surface of the capsid. The isoelectric point of this phage is 6.6. Bacteriophage MS2 with a ssRNA(+) genome with an isoelectric point of 3.9 is enclosed in a capsid of 26 nm diameter (King et al. 2011; Michen and Graule 2010). Bacteriophage PRD1 contains a dsDNA genome. The capsid has a diameter of 66 nm, and 20 nm long spikes are present on the surface (King et al. 2011). The isoelectric point is in the range of 4.0–4.5 (Mesquita et al. 2010; Michen and Graule 2010). This virus was chosen due to its structural similarity to HAdVs (Belnap and Steven 2000; Benson et al. 1999; King et al. 2011). HAdVs are dsDNA viruses. Their icosahedral capsids are about 70–90 nm in diameter (depending on the strain). In addition, adenoviruses have fibres connected to the penton capsomeres varying in length (9–77.5 nm) depending on the serotype (King et al. 2011). MNVs are ssRNA viruses with icosahedral capsids of 35–40 nm in diameter (King et al. 2011). MNV was used as surrogate for human noroviruses. By comparing the log removal values (LRV) we evaluated the applicability of common surrogate viruses in the testing of ultrafiltration membranes.

Materials and Methods

Bacteriophages

Bacteriophages MS2 (DSM 13767), ΦX174 (DSM 4497) and PRD1 (DSM 19107) were quantified by double layer agar methods according to ISO 10705-1 (1995) and ISO 10705-2 (2000) using the host bacteria *Escherichia coli* K12 Hfr (NCTC 12486), *E. coli* CN (WG5; ATCC 700078) and *Salmonella* Typhimurium (WG49; NCTC 12484), respectively. Media MSA/MSB were used for ΦX174 and TYGA/TYGB for MS2 and PRD1. *Salmonella* Typhimurium can be infected by both, MS2 and PRD1. Since RNase can inhibit the infection process of the RNA phage MS2 (ISO 10705-1 1995), the semisolid agar was supplemented with RNase (40 µg/ml) in case of PRD1 quantification. Plates were incubated at (36 ± 2) °C for (18 ± 2) h. For MS2 and PRD1 plates with 10–300 plaques were counted and used for calculation/quantification. Because of their larger plaque sizes ΦX174 phages were counted only in the range of 10–150 plaques per plate. Detection limits were 1 pfu/ml for 1 ml sample volume and 0.1 pfu/ml for 10 ml volumes. Somatic and F specific phages were measured the same way as their representative strains ΦX174 and MS2 using the hosts *E. coli* CN and *Salmonella* Typhimurium.

Human Adenoviruses and Murine Norovirus

Wastewater after primary treatment containing HAdV type 41 as the most prevalent type (Hartmann et al. 2013) was used as a natural source of HAdVs. For monitoring adenovirus concentrations in diluted wastewater and in the filtrates viruses were enriched by means of glass wool filtration as described by Wyn-Jones et al. (2011). In brief, 10 l water samples were adjusted to pH 3.5 and passed through a glass wool filter. After elution with an alkaline buffer (3 % w/v beef extract, 0.05 mol/l glycine, pH 9.5), flocculation at pH 3 and subsequent centrifugation the virus containing precipitate was redissolved in phosphate buffered saline.

MNV isolate S99 (Müller et al. 2007; GenBank accession number EF531291) was propagated on RAW 264.7 cells (ATCC TIB-71) and used in spike experiments (concentrations of 3×10^5–4×10^5 copies/ml) as surrogate for human noroviruses.

Nucleic Acid Extraction and Quantitative PCR

After nucleic acid extraction using the NucliSENS® method (bioMérieux) HAdVs were measured by quantitative PCR (qPCR) and MNV by one-step reverse

transcription qPCR (RT-qPCR). In brief, 5 ml samples were mixed with lysis buffer, incubated for 10 min, 50 µl magnetic silica dispersion were added and incubated for another 10 min. Magnetic silica particles were washed and separated from the liquid. The silica particles were eluted in 100 µl buffer (for 5 min at 60 °C) to release the nucleic acids. Until use aliquots were frozen at (−20 ± 5) °C.

In all PCR assays, 10 µl of undiluted, 1/10 diluted and 1/100 diluted nucleic acid samples were tested. Dilutions of plasmid DNA of the corresponding amplified sequences were used to generate standard curves in the range of 10^1–10^6 copies per well, each in triplicates. Diethylpyrocarbonate (DEPC)-treated, deionized water served as non-template control, while three parallels of 0.5×10^3 copies of the respective PCR standards were used as positive controls. MicroAmp® optical 96-well reaction plates were run on an Applied Biosystems 7500 Real-Time PCR System with software version 1.4. Results of the three dilution steps were compared to each other to exclude inhibitory effects. If values of the dilution steps gave comparable results, the concentration in the sample was calculated from values of all dilutions. If diluted samples gave higher results, only dilutions without obvious inhibitory effects were used for calculations. Amplification efficiencies were in the range of 90–110 %.

Quantification of HAdVs was performed according to (Hernroth et al. 2002), using primers 5′-CWT ACA TGC ACA TCK CSG G-3′ and 5′-CRC GGG CRA AYT GCA CCA G-3′ (each 0.9 µM) and probe 5′-6FAM-CCG GGC TCA GGT ACT CCG AGG CGT CCT-5TAMRA-3′ (0.225 µM), labelled with 6-carboxyfluorescein (6FAM) and 5-carboxytetramethylrhodamine (5TAMRA) in 2 × Universal Mastermix (Applied Biosystems) at temperatures 50 °C (2 min), 95 °C (10 min), and 45 cycles of 95 °C (15 s), 60 °C (60 s). Quantification of MNV was performed according to (Müller et al. 2007), using, primers 5′-AGA GGA ATC TAT GCG CCT GG-3′ and 5′-GAA GGC GGC CAG AGA CCA C-3′ (each 0.25 µM) and probe 5′-6FAM-CGC CAC TCC GCA CAA ACA GCC C-Db-3′ (0.1 µM), labelled with 6-carboxyfluorescein (6FAM) and Dabcyl (Db) in 2 × Mastermix (QuantiTect™ Probe RT-PCR Master Mix, Qiagen) and 0.25 µl QuantiTect RT Mix (Qiagen). Reverse transcription to cDNA (30 min at 50 °C) and amplification (15 min at 95 °C, 15 s at 95 °C, 45 s at 55 °C, 45 cycles) took place in one tube.

Bacteria

In order to rule out that the membrane exhibits any larger damages, filtration tests with bacteria were performed. The environmental strains *E. coli* A3 and *Enterococcus faecium* Teltow11 were cultivated in liquid media for spike experiments. *E. coli* A3 was grown in a medium containing casein peptone (17 g/l), soy peptone (3 g/l), lactose (10 g/l) and NaCl (5 g/l) and *E. faecium* Teltow11 was grown in a medium containing casein peptone (15 g/l), beef extract (4,8 g/l), D(+)glucose (7,5 g/l) and NaCl (7,5 g/l). They were incubated shaking over night at (36 ± 1) °C, centrifuged at 6000 min^{-1} for 15 min and resuspended in autoclaved tap water. Both strains as well as indicator bacteria present in the wastewater were quantified using the miniaturised most probable number (MPN) methods according to ISO 7899-1 (1998) and ISO 9308-3 (1998) with fluorogenic substrates and a limit of detection of 0.15 MPN/ml.

Membrane Device

A transportable semi-technical device (width 0.8 m, length 1.2 m) was built by the DVGW-Technologiezentrum Wasser (Fig. 1). All piping was made of stainless steel. The storage tank was connected by flexible tubing to the feed pump, which delivered the water through the device. A membrane with a nominal pore size of 20 nm was chosen for these experiments. The module consisted of several capillaries composed of modified polyethersulfone (PES). Ultrafiltration was operated at a constant flux of 80 l/(m^2 h) resulting in increasing transmembrane pressures in the course of experiments. The water flow was inside-out (dead-end filtration) from bottom to top, and the total membrane area amounted to 0.2 m^2 (volume flow: 16 l/h).

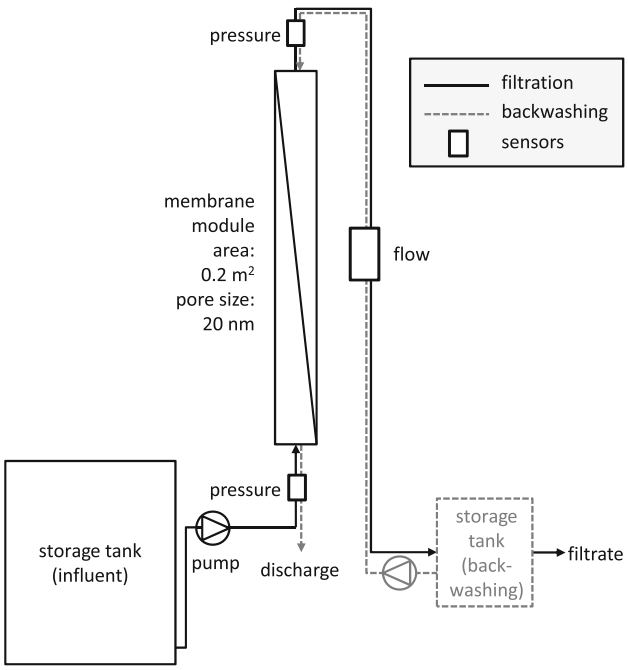

Fig. 1 Schematic diagram of the ultrafiltration unit

The control of the device was done in Labview. Temperature, flow, pressure values for feed, filtrate and the transmembrane pressure (TMP) were recorded online. Prior to each use the membrane was chemically cleaned with NaOH at pH 11 and H_2SO_4 at pH 2 (for 30 min each).

Filtration Experiments

The first set of filtration experiments was conducted using four waters from different sources. Two of them (water A and water B) were treated groundwaters with drinking water quality, the other two (water C and water D) were untreated surface waters directly taken from reservoirs used for drinking water treatment. Measured pH values of waters A, B, C and D were 7.3, 7.8, 6.8 and 6.9, respectively. Test water volumes of 50 l were spiked with bacteriophages MS2, ΦX174, PRD1 as well as MNV and mixed prior to the start of the experiment. Samples from the water tank (influent) were taken in the beginning and at the end of the experiments. Filtrate samples were taken in the first and the third filtration cycle. Each filtration cycle consisted of a filtration time of 60 min followed by 1 min backwash. The concentrations of phages in the influent were (3×10^3–2×10^5) pfu/ml for MS2, (3×10^3–4×10^4) pfu/ml for ΦX174, (3×10^3–8×10^5) pfu/ml for PRD1. MNV was spiked to achieve final concentrations of 2×10^5–4×10^5 copies/ml.

In a separate experiment bacteria *E. coli* A3 and *E. faecium* Teltow11 were spiked into the influent at concentrations of 10^6–10^7 MPN/ml. Influent and filtrates were sampled twice during the filtration cycle of 60 min.

In further filtration experiments under conditions of wastewater contamination, primary effluent of a domestic wastewater treatment plant was used as source of naturally occurring human viruses. Using water with a wastewater content of 5 %, the removal of wastewater-derived HAdVs, as well as somatic phages, F-specific phages and bacteria (*E. coli* and intestinal enterococci) were monitored over a time period of 40 h. Thereafter, cultured phages and MNV were added and mixed before the filtration was continued for another 40 h with cycles of 60 min filtration and 1 min backwashes. Since this experiment with 5 % wastewater lasted several days, the water tank (nearly 500 l) had to be refilled with fresh wastewater. This resulted in a dilution of the concentration of spiked viruses. In the beginning of the experiment concentrations in the influent were 8×10^5 pfu/ml for MS2, 5×10^4 pfu/ml for ΦX174, 2×10^6 pfu/ml for PRD1 and 7×10^5 copies/ml for MNV. Through the addition of wastewater, HAdV concentrations of only 1×10^1 copies/ml were achieved. Pairs of influent and filtrate samples were taken at eight sampling times. Additional filtrates were sampled twice and viruses were enriched by glass wool filtration.

In experiments with water containing 30 % wastewater cultured phages and MNV were spiked prior to the start of experiments. Because of the intense fouling filtration times had to be adjusted. The transmembrane pressure rose rapidly, and therefore filtration was interrupted by backwashing after about 30 min. Three influent samples and four filtrate samples were examined directly, and another two filtrate samples were enriched by glass wool filtration. The concentrations in the influent were 4×10^7 pfu/ml for MS2, 1×10^5 pfu/ml for ΦX174, 2×10^6 pfu/ml for PRD1 and 4×10^5 copies/ml for MNV. The addition of wastewater resulted in HAdV concentrations of 7×10^3 copies/ml in the influent water.

Calculations

Elimination by ultrafiltration was quantified by determination of the respective log removal value (LRV), calculated as follows:

$$\mathrm{LRV} = \log_{10}\left(\frac{c_{\mathrm{in}}}{c_{\mathrm{f}}}\right)$$

with c_{in} as the virus concentration in the influent and c_{f} as the corresponding virus concentration in the filtrate. In the short-term experiments c_{in} was calculated as the geometric mean of the influent concentrations obtained in the beginning and at the end of the experiments. To sum up, the removal of each virus during a specific experiment the arithmetic mean of the log removal values (equivalent to the geometric mean of the relation of filtrate to influent concentrations) was calculated. If virus concentrations in the filtrate were below the detection limit, LRVs were calculated based on a c_{f} value of one-half of the detection limit. Such samples were marked in the figures.

Results

Challenge tests for the removal of viruses by ultrafiltration were performed with treated groundwaters, environmental waters from drinking water reservoirs and groundwater contaminated with wastewater. All waters were spiked with viruses.

Filtration Experiments with Drinking Water and Surface Water

In the first set of experiments four clean waters of different origins, two drinking waters (water A and B) and two environmental waters from drinking water reservoirs (water C and D) were spiked with bacteriophages MS2, ΦX174 and PRD1 as well as MNV. During three cycles of filtration and backwashing, concentrations of viruses in the influents and the filtrates were measured. The time courses of these

experiments are shown in Fig. 2. During these short-term experiments, virus concentrations in the influent were constant over time. The concentrations of viruses in the filtrates were determined starting after a filtration time of 15–25 min, and at time points of 40–58, 137–148 and 153–177 min. In all experiments, viruses were significantly reduced by ultrafiltration, but still detected in the filtrates. After passing the membrane, viruses were reduced by 1.4–5.2 $\log_{10}$ units, depending on the viruses. In all four waters, time courses revealed nearly constant elimination rates for the viruses tested. For better comparison, log removal values (LRVs) were calculated and summarised in Fig. 3. By comparing the virus removals in all for waters between virus types, significant differences were found, with the exception of the pairing ΦX174 and MS2 (two sample *t*-test, $\alpha < 10^{-6}$). Lowest virus removal efficiencies were obtained with the small bacteriophages ΦX174 und MS2 (both 1.5 $\log_{10}$ units in experiments with waters B and C). However, in waters A and D the mean removal of phage MS2 exceeded the mean removal of phage ΦX174. Removals of MNV and phage PRD1 exceed those of MS2 and ΦX174. While MNV was reduced by 2.8–3.7 $\log_{10}$ units, PRD1 exhibited the highest removal of more than 5 $\log_{10}$ units. In some cases (marked by asterisks in Fig. 3) concentrations of PRD1 in the filtrate were reduced to the detection limit or below, suggesting that even higher removal could have been obtained by spiking higher concentrations into the influent. With the exception of PRD1, for which virus concentrations in the filtrate were near the detection limit, highest LRVs of all tested viruses were observed in the environmental water D, a surface water from a reservoir for drinking water treatment. Based on its absorbance at 254 nm, water D (5.4/m) is expected to have the highest content of humic substances followed by water A (3.6/m) and water B and C (both ≤1/m).

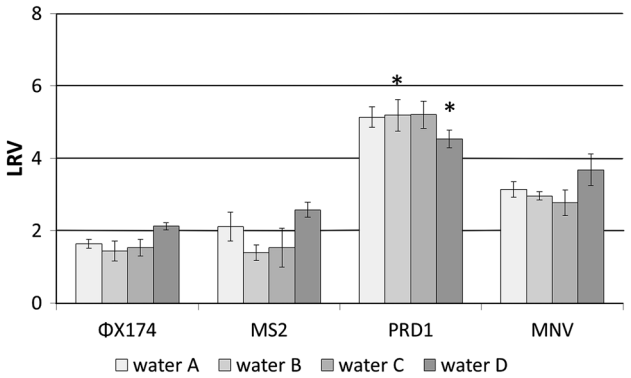

Fig. 3 Mean log removal values (LRV) of MS2, ΦX174, PRD1 and MNV in waters (*A–D*). *Error bars* represent standard deviations; *bars marked with an asterisk* denote means calculated including values at or below the detection limit

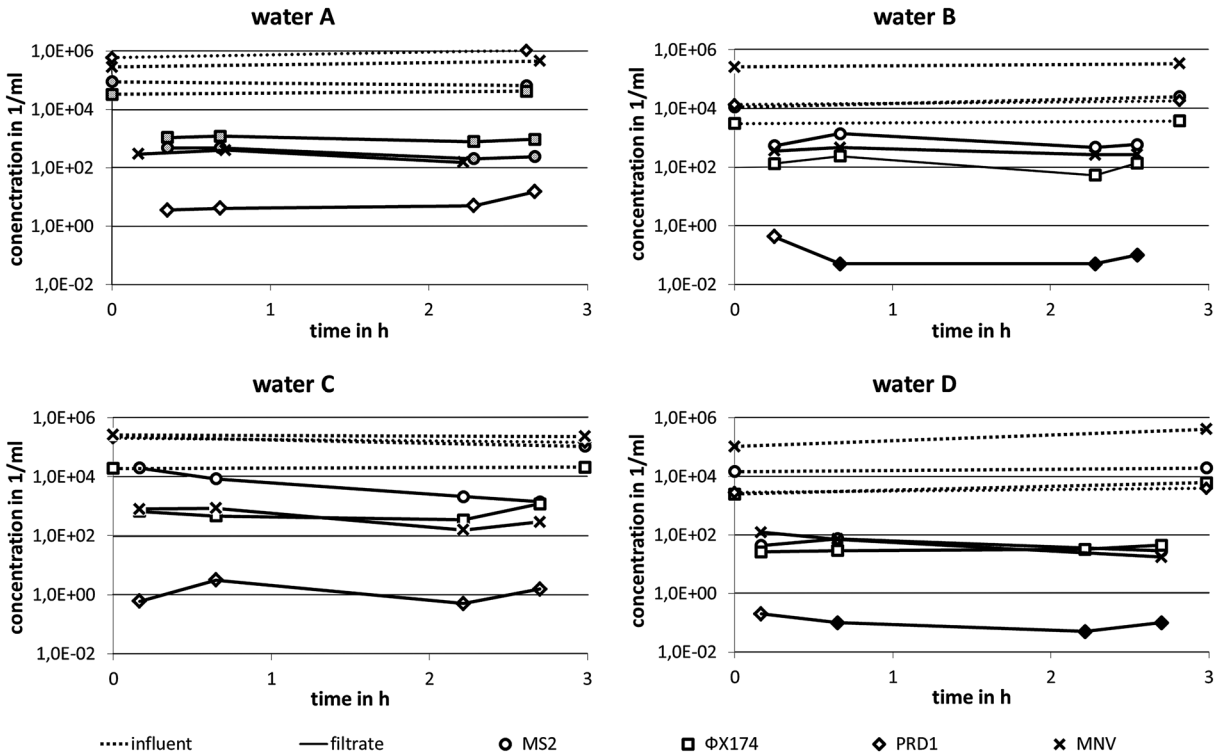

Fig. 2 Time courses of the influent and filtrate concentrations of MS2 (*circles*), ΦX174 (*squares*), PRD1 (*diamonds*) and MNV (*crosses*) spiked into four natural waters (water **A–D**); *symbols filled black* denote samples with concentrations at or below the detection limit

Mean transmembrane pressures in waters A to D were in the range of 152–174 mbar. Transmembrane pressures increased only slightly during each filtration cycle, but were reduced by backwashing to the same level as in the preceding cycles. Initial transmembrane pressures of subsequent cycles differed by no more than 5 mbar (see Fig. 4d), indicating that no significant fouling had occurred.

Since virus eliminations were not complete, we confirmed the integrity of the membrane by testing the removal of two environmental bacterial strains *E. coli* A3 and *E. faecium* Teltow11. Both strains were completely removed below the detection limit in the filtrate, resulting in LRVs higher than 7.8 and 7.2, respectively (data not shown). The corresponding transmembrane pressures were in the range of 175–185 mbar.

Filtration Experiments with Diluted Wastewater

To study virus removals in wastewater contaminated water, further ultrafiltration experiments were conducted with diluted wastewater as test water. Water A (treated groundwater) was contaminated with primary effluent to final wastewater contents of 5 and 30 %. The removal of naturally occurring viruses and indicator bacteria in the water containing 5 % wastewater was monitored for 40 h. Virus concentrations in filtrate samples were below the detection limits (data not shown). The highest concentrations observed were from indigenous somatic coliphages, resulting in log removal values >2.6. During this phase the transmembrane pressure (TMP) increased from 160 to 260 mbar. The fouling layer resulted in a mean rise of 13 mbar per filtration cycle (60 min), whereof the majority could be removed by hydraulic backwashing. The pressure increases from cycle to cycle after backwashing were about 2 mbar.

Following this period of elimination of natural contaminants, spiking experiments were performed with bacteriophages ΦX174, MS2 and PRD1 as well as MNVs to monitor the time course of removal of viruses in contaminated waters.

To allow comparisons of the ultrafiltration experiments with clean and contaminated waters, the time courses of virus elimination (LRV) and corresponding transmembrane pressures are presented in Fig. 4. As representative of clean waters, the data obtained with water D were chosen (Fig. 4a, d), revealing that the transmembrane pressure and removal of bacteriophages MS2, ΦX174, PRD1 and MNV did not depend on filtration phases and cycles. In contrast, during the course of the experiments with contaminated waters, prominent changes in the transmembrane pressures were observed (Fig. 4e, f).

As shown in Fig. 4e, the transmembrane pressure of wastewater-contaminated water (5 %) had reached 260 mbar after 40 h of filtration of naturally occurring viruses and bacteria. During the next 40 h it further increased to 850 mbar. The rise of the transmembrane pressure within one filtration cycle accounted for about 20 mbar at the 40 h time point. As in the previous phase of the experiment the majority of the fouling layer deposited in each cycle could be removed by hydraulic backwashing, resulting in an increase of the transmembrane pressure by 5 mbar per cycle (hydraulically irreversible fouling). Due to temporarily high transmembrane pressures the automatic control stopped the filtration device several times (recognisable as breaks in the transmembrane pressure curve, see

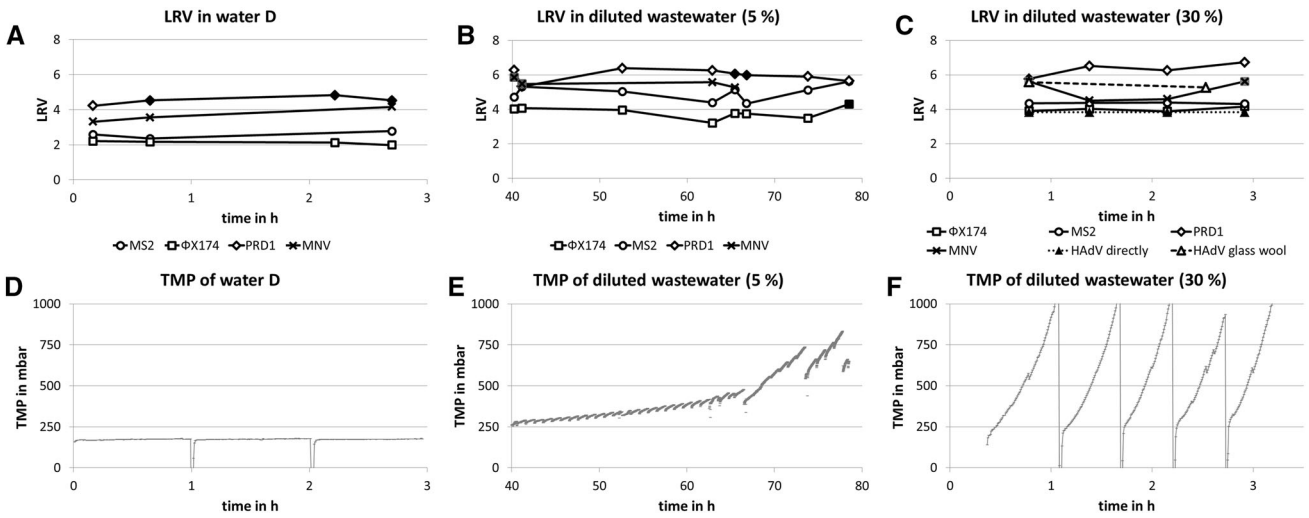

Fig. 4 Comparison of log removal values (LRV) for the viruses MS2, ΦX174, PRD1, MNV and HAdV in water D (**a**) and diluted wastewaters 5 % (**b**) and 30 % (**c**) as well as the corresponding transmembrane pressures (TMP, **d**–**f**); Please note the different time scales. *Symbols filled black* or with a *dark grey background* denote samples with concentrations at or below the detection limit

Fig. 4e at 67, 73 and 78 h). Whereas a lower transmembrane pressure developed after restart, fouling increased the TMP by up to 100 mbar during the 60 min filtration cycle in the last few hours. The hydraulically irreversible fouling also rose by 25 mbar from cycle to cycle.

The spiked bacteriophages and MNV were detected in almost all tested effluent samples and their LRVs, calculated from the data in Fig. 4b, remained quite constant at 3.8 ± 0.3 for ΦX174, 5.0 ± 0.4 for MS2, 6.0 ± 0.4 for PRD1 and 5.5 ± 0.2 for MNV (Fig. 5). The removal of HAdVs, which were present in the provided wastewater, was also tested. Even after previous enrichment of HAdVs by glass wool filtration their concentration in the filtrate was still below the detection limit. The influent concentration of HAdVs at this time (after 48 h) amounted to about 3.5×10^3/l. Therefore, the theoretical LRV of 3.5 is only the minimum removal value for HAdVs in this experiment. The real removal efficiency is expected to be significantly higher (marked by the arrow in Fig. 5).

Ultrafiltration was also performed using water A with a content of 30 % wastewater as influent water. During this experiment dramatic increases in the transmembrane pressure were observed (Fig. 4f). Fouling tendency was much higher and transmembrane pressures increased by more than 700 mbar during each filtration period. Therefore, the filtration time of each cycle had to be reduced to 30 min to meet the security levels of the membrane. The vast majority of the fouling layer was removed by backwashing (hydraulically reversible fouling). The transmembrane pressure increased by about 25 mbar from cycle to cycle (hydraulically irreversible fouling).

As in the previous experiments virus removal remained steady throughout the experiment (Fig. 4c). LRVs of 4.0 ± 0.1 for ΦX17, 4.3 ± 0.03 for MS2 and 6.3 ± 0.4 for PRD1 were obtained. MNV was below the detection limit in two samples, resulting in mean LRV of 5.1 ± 0.6. To calculate the removal of HAdVs from the contaminated water (30 % wastewater), viruses in the filtrate had to be pre-concentrated by glass wool filtration. Without prior concentration adenoviruses were not detected in the filtrate, corresponding to theoretical LRVs greater than 3.8 (identifiable as black triangles in Fig. 4c). After the glass wool concentration step adenoviruses were detected in the filtrate. For these samples a mean LRV of 5.4 ± 0.2 was determined. Concentrations of naturally occurring human noroviruses in the tested contaminated waters were too low to calculate log removals.

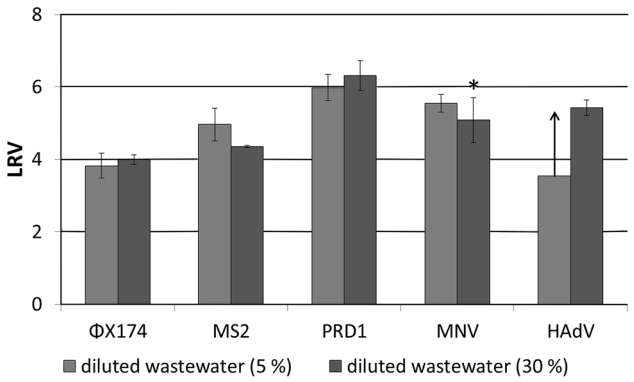

Fig. 5 Mean log removal values (LRV) of MS2, ΦX174, PRD1, MNV and HAdVs in two wastewater-contaminated waters. *Error bars* represent standard deviations; *bars marked with an asterisk* denote means calculated including values at or below the detection limit. The *bar marked with an arrow* denotes the minimum LRV since concentrations in all filtrate samples below the detection limit

Figure 5 allows a comparison of mean log removal values (LRV) of all tested viruses in waters-contaminated with 5 or 30 % wastewater. Differences in removal between all viruses (excluding HAdV) were significant (two sample *t*-test, highest $\alpha = 0.02$ for MS2 and MNV, for the rest $\alpha < 10^{-4}$). The lowest removal was observed for the small DNA bacteriophage ΦX174 with about 4 $\log_{10}$ units. Removals of the RNA phage MS2 from 5 % wastewater were by about 1 $\log_{10}$ unit higher than those of phage ΦX174, whereas in 30 % wastewater only 0.5 $\log_{10}$ units differences between MS2 and ΦX174 were observed. The larger viruses PRD1, MNV and HAdV were removed to a higher extent than phages ΦX174 and MS2 in both experiments, with phage PRD1 showing the highest removal of more than 6 $\log_{10}$ units.

Discussion

In our study on the removal of different bacteriophages and enteric animal viruses, a semi-technical ultrafiltration device with a 20 nm membrane (nominal pore size) was used. Despite the small pore size, spiked viruses were not removed completely. The experiments enabled us to calculate removal values based on measurable data. Virus removal with the tested membrane increased with virus size (all viruses had icosahedral symmetry). In waters not contaminated with wastewater a strong correlation of virus size and removal was derived (slope of about 0.1 $\log_{10}$ units/nm; $R^2 > 0.96$, with the exception of PRD1 data in water D). The small phages ΦX174 and MS2 with diameters of about 25 nm displayed the lowest removal (LRV 1.4), followed by an LRV of 3.5 for MNV, with a diameter of 35 nm, up to an LRV of about 5 for phage PRD1, with a diameter of 66 nm. In diluted wastewater, the correlation of removal and virus sizes was less pronounced (slope of about 0.04 $\log_{10}$ units/nm in 30 % wastewater; $R^2 > 0.95$) with LRVs of about 4 for phages ΦX174 and MS2, LRV of 5 for MNV, to an LRV of 6 for phage PRD1. Despite the bigger size of HAdVs (>70 nm) their LRV was

not higher than that of PRD1. These results may be explained by attachment of viruses to larger particles in the wastewater and/or by higher filtration efficiency due to a fouling layer on the membrane. A fouling layer can improve the retention of viruses (ElHadidy et al. 2014; Huang et al. 2012; Kreißel et al. 2012; Lu et al. 2014). In uncontaminated waters almost no fouling was observed during the short-term experiments and transmembrane pressures stayed constant at a low level. In contrast, the development of a fouling layer was observed in the long-term experiment with diluted wastewater. By ultrafiltration of contaminated groundwater containing 5 % wastewater the transmembrane pressure rose at first by 10 mbar and later by up to 100 mbar during 60 min filtration time. In water with 30 % wastewater fouling was significantly higher and transmembrane pressures increased by about 700 mbar during the 30 min filtration period. Compared to waters B and C, the fouling layer built up during wastewater filtration enhanced the removal of phage ΦX174 by almost 2.5 $\log_{10}$ units. For MNV the fouling layer contributed with 1–1.5 $\log_{10}$ units to the total removal in diluted wastewater. For PRD1 only a slight effect of the fouling layer of less than 0.5 $\log_{10}$ units could be deduced. Despite obvious differences in their transmembrane pressures virus removal in 5 % wastewater and 30 % wastewater was similar. In conclusion it can be said that the fouling layer, as established in experiments with diluted wastewater, led to an increase in virus removal, especially for small viruses, whereas for viruses with diameters larger than 60 nm the increase in virus removal by fouling was less pronounced.

ElHadidy et al. (2014) observed different contributions of reversible and irreversible fouling on the removal of MS2 and ΦX174 during ultrafiltration: reversible fouling lead to a minor increase in virus removal, whereas irreversible fouling leads to a continuous increase in removal of MS2 and ΦX174 by up to 2.5 $\log_{10}$ units. In our experiments virus removal was enhanced even at a low wastewater proportion with moderate reversible and irreversible fouling by about 2.5 $\log_{10}$ units for both small bacteriophages MS2 and ΦX174. It was not further enhanced at a higher wastewater proportion with more drastic fouling or longer filtration times. Intense fouling accompanied by high rising transmembrane pressures with diluted wastewater had no additional effect on virus removal.

It should be noted that the analysis for bacteriophages based on cultivation techniques detected intact infectious virus particles whereas MNV and HAdV were determined by nucleic acid-based techniques detecting genome copies. This may be a reason for the lower removal of HAdVs compared to PRD1. If non-infectious particles or free nucleic acids (without a surrounding capsid) passed through the membrane easier than full intact capsids, the LRV determined by PCR would be lower than if only complete virus particles were present. Such an effect has been observed for phages MS2 and Qβ during filtration with different membranes (Langlet et al. 2009). However, Humbert et al. (2011) report that reliable determinations of membrane performances can be achieved using both cultural and PCR-based methods with comparable results.

Many studies on the removal of viruses have used bacteriophages MS2, ΦX174 or Qβ, but most often the number of simultaneously tested viruses has been restricted to one or two. Only rare studies on membrane filtration of human viruses or viruses infecting warm-blooded animals can be found in literature (Arkhangelsky et al. 2012, Jacangelo et al. 2005, Madaeni et al. 1995). For example, Madaeni et al. (1995) have shown that poliovirus in concentrations of 10^4/ml was not detected in the filtrate of a 30 kDa membrane (LRV > 4). Arkhangelsky et al. (2012) have used phages MS2, ΦX174, PRD1, phage T4 a tailed bacteriophage of 200 nm and vaccinia virus with an elongated enveloped capsids of more than 200 nm. The bigger viruses T4, PRD1 and vaccinia virus were removed by more than 3.7 $\log_{10}$ units, while the smaller viruses MS2 and ΦX174 were removed by less than 3.7 $\log_{10}$ units using a 30 kDa PES membrane. Consistent with our results comparing the elimination of PRD1 and MS2, Jacangelo et al. (2005), using a 10 kDa membrane, observed that removal of PRD1 (LRV about 5) was higher than that of MS2, poliovirus and HAV (LRV $= 3$–4, sizes of about 25 nm).

Moreover, our results suggest that virus size is not the only criterion for efficient removal by ultrafiltration. Even though having nearly the same sizes, MS2 displayed a higher removal than ΦX174 depending on the water quality. Similar results have been obtained by Jacangelo et al. (2005): despite a similar size poliovirus has been removed to a lesser extent (LRV $= 3$) than MS2 and HAV (LRV of about 4). Boudaud et al. (2012) also showed that filtration through an ultrafiltration membrane with pore sizes of 30 nm resulted in drastically lower removal for phage GA compared to Qβ and MS2, all related phages of the same size. Therefore, the choice of the viral parameter can lead to divergent results when judging the effectiveness of ultrafiltration membranes.

It has been previously suggested that differences in the behaviour of these phages may be attributed to the fact that the surface of phage MS2 is rather hydrophobic and that of phage ΦX174 more hydrophilic. This might lead to hydrophobic interactions with the membrane material or water constituents (Duek et al. 2012; Kreißel et al. 2012). A negative surface charge for MS2 in contrast to a near neutral surface of ΦX174, especially at pH values near the isoelectric point of ΦX174, was also linked with a higher removal due to electrostatic repulsion (ElHadidy et al. 2014; Hambsch et al. 2012). In the experiments presented

here, different removal of MS2 and ΦX174 did not occur in each of the tested waters. Therefore, it is assumed that the adsorption of humic substances onto the capsid of phage MS2, as proposed by Kreißel et al. (2012) may lead to a higher hydrodynamic diameter and eventually to a higher removal. Indeed, the absorbance at 254 nm in water A and D is significantly higher than in water B and C.

These results underline that efficiency testing with ultrafiltration membranes should be conducted with viruses of different shape, size and surface characteristics, including especially the small bacteriophages MS2 and ΦX174 for reliable risk assessment. Due to their health implications, the removal of infective human virus particles putatively present in contaminated waters should exceed values of 99.99 %, equivalent to 4 $\log_{10}$ units (US EPA 1989). A 90 % virus removal is clearly not sufficient. The membrane used in our study achieved complete removal of bacteria (>7 $\log_{10}$ units). Therefore, it can be assumed that this membrane would also retain larger protozoa and their (oo)cysts, as long as the membrane is intact. Although this membrane would protect reliably from endangerments caused by bacterial and protozoan pathogens, the removal of virus particles was much lower depending on the size of the virus particles and the quality of the raw water. For larger viruses with diameters of 60 nm and more a 4 $\log_{10}$ removal could probably be achieved, but for small viruses in the range of 20–25 nm like enteroviruses removal in uncontaminated water was in the range of only 2 $\log_{10}$ units and hence remarkably lower than 4 $\log_{10}$ units in contaminated water. To estimate virus removal, information on the width of the pore size distribution and the water quality is important. Therefore, it is advisable to use viruses of different sizes and properties including the small bacteriophages ΦX174 and MS2, which are both more conservative than enteric viruses, with phage ΦX174 being the most conservative surrogate.

Acknowledgments Funding for this project was provided by the German Federal Ministry of Education and Research (BMBF), Grants 02WT0944 and 02WT0945.

References

Arkhangelsky, E., Duek, A., & Gitis, V. (2012). Maximal pore size in UF membranes. *Journal of Membrane Science, 394–395*, 89–97.

Belnap, D. M., & Steven, A. C. (2000). 'Déjà vu all over again': The similar structures of bacteriophage PRD1 and adenovirus. *Trends in Microbiology, 8*(3), 91–93.

Benson, S. D., Bamford, J. K., Bamford, D. H., & Burnett, R. M. (1999). Viral evolution revealed by bacteriophage PRD1 and human adenovirus coat protein structures. *Cell, 98*(6), 825–833.

Boudaud, N., Machinal, C., David, F., Bourdonnec, A. F.-L., Jossent, J., Bakanga, F., et al. (2012). Removal of MS2, Qβ and GA bacteriophages during drinking water treatment at pilot scale. *Water Research, 46*(8), 2651–2664.

ISO 10705-1 (1995) *Water quality—Detection and enumeration of bacteriophages—Part 1: Enumeration of F-specific RNA bacteriophages*.

ISO 10705-2 (2000) *Water quality—Detection and enumeration of bacteriophages—Part 2: Enumeration of somatic coliphages*.

ISO 7899-1 (1998) *Water quality—Detection and enumeration of intestinal enterococci in surface and waste water—Part 1: Miniaturized method (most probable number) by inoculation in liquid medium*.

ISO 9308-3 (1998) *Water quality—Detection and enumeration of Escherichia coli and coliform bacteria in surface and waste water—Part 3: Miniaturized method (most probable number) by inoculation in liquid medium*.

Duek, A., Arkhangelsky, E., Krush, R., Brenner, A., & Gitis, V. (2012). New and conventional pore size tests in virus-removing membranes. *Water Research, 46*(8), 2505–2514.

ElHadidy, A. M., Peldszus, S., & Van Dyke, M. I. (2014). Effect of hydraulically reversible and hydraulically irreversible fouling on the removal of MS2 and φX174 bacteriophage by an ultrafiltration membrane. *Water Research, 61*, 297–307.

Farkas, K., Varsani, A., & Pang, L. (2014). Adsorption of rotavirus, MS2 bacteriophage and surface-modified silica nanoparticles to hydrophobic matter. *Food and Environmental Virology,*. doi:10.1007/s12560-014-9171-3.

Guo, H., & Hu, J. Y. (2014). Fluorescent-conjugated MS2 as surrogates for viruses during drinking water treatment. *Water Science & Technology: Water Supply, 14*, 991–1000.

Hambsch, B., Bösl, M., Eberhagen, I., & Müller, U. (2012). Removal of bacteriophages with different surface charges by diverse ceramic membrane materials in pilot spiking tests. *Water Science and Technology, 66*(1), 151–157.

Hamza, I. A., Jurzik, L., Stang, A., Sure, K., Überla, K., & Wilhelm, M. (2009). Detection of human viruses in rivers of a densly-populated area in Germany using a virus adsorption elution method optimized for PCR analyses. *Water Research, 43*(10), 2657–2668.

Haramoto, E., Kitajima, M., Katayama, H., & Ohgaki, S. (2010). Real-time PCR detection of adenoviruses, polyomaviruses, and torque teno viruses in river water in Japan. *Water Research, 44*(6), 1747–1752.

Hartmann, N. M., Dartscht, M., Szewzyk, R., & Selinka, H.-C. (2013). Monitoring of adenovirus serotypes in environmental samples by combined PCR and melting point analyses. *Virology Journal, 10*, 190.

Hernroth, B. E., Conden-Hansson, A.-C., Rehnstam-Holm, A.-S., Girones, R., & Allard, A. K. (2002). Environmental factors influencing human viral pathogens and their potential indicator organisms in the blue mussel, *Mytilus edulis*: The first Scandinavian report. *Applied and Environmental Microbiology, 68*(9), 4523–4533.

Huang, H., Young, T. A., Schwab, K. J., & Jacangelo, J. G. (2012). Mechanisms of virus removal from secondary wastewater effluent by low pressure membrane filtration. *Journal of Membrane Science, 409–410*, 1–8.

Humbert, H., Machinal, C., Labaye, I., & Schrotter, J. C. (2011). Virus removal retention challenge tests performed at lab scale and pilot scale during operation of membrane units. *Water Science and Technology, 63*, 255–261.

Hundesa, A., de Motes, C. M., Bofill-Mas, S., Albinana-Gimenez, N., & Girones, R. (2006). Identification of human and animal adenoviruses and polyomaviruses for determination of sources of fecal contamination in the environment. *Applied and Environmental Microbiology, 72*, 7886–7893.

Jacangelo, J., Madec, A., Schwab, K., Huffman, D., & Mysore, C. (2005). Advances in the use of low-pressure, hollow fiber membranes for the disinfection of water. *Water Science and Technology: Water Supply, 5*(5), 109–115.

King, A. M., Adams, M. J., Carstens, E. B., & Lefkowitz, E. J. (2011). *Virus taxonomy—Classification and nomenclature of viruses: Ninth Report of the International Committee on Taxonomy of Viruses*. San Diego: Elsevier.

Kreißel, K., Bösl, M., Lipp, P., Franzreb, M., & Hambsch, B. (2012). Study on the removal efficiency of UF membranes using bacteriophages in bench-scale and semi-technical scale. *Water Science and Technology, 66*(6), 1195–1202.

Langlet, J., Ogorzaly, L., Schrotter, J.-C., Machinal, C., Gaboriaud, F., Duval, J. F., & Gantzer, C. (2009). Efficiency of MS2 phage and Qβ phage removal by membrane filtration in water treatment: Applicability of real-time RT-PCR method. *Journal of Membrane Science, 326*, 111–116.

Lodder, W. J., & de Roda Husman, A. M. (2005). Presence of noroviruses and other enteric viruses in sewage and surface waters in The Netherlands. *Applied and Environmental Microbiology, 71*(3), 1453–1461.

Lu, R., Mosiman, D., & Nguyen, T. H. (2014). Mechanisms of MS2 bacteriophage removal by fouled ultrafiltration membrane subjected to different cleaning methods. *Environmental Science and Technology, 47*, 13422–13429.

Madaeni, S., Fane, A., & Grohmann, G. (1995). Virus removal from water and wastewater using membranes. *Journal of Membrane Science, 102*, 65–75.

Mena, K. D., & Gerba, C. P. (2009). Waterborne adenovirus. *Reviews of Environmental Contamination and Toxicology, 198*, 133–167.

Mesquita, M. M. F., Stimson, J., Chae, G.-T., Tufenkji, N., Ptacek, C. J., Blowes, D. W., & Emelko, M. B. (2010). Optimal preparation and purification of PRD1-like bacteriophages for use in environmental fate and transport studies. *Water Research, 44*(4), 1114–1125.

Michen, B., & Graule, T. (2010). Isoelectric points of viruses. *Journal of Applied Microbiology, 109*(2), 388–397.

Müller, B., Klemm, U., Marques, A. M., & Schreier, E. (2007). Genetic diversity and recombination of murine noroviruses in immunocompromised mice. *Archives of Virology, 152*(9), 1709–1719.

Pontius, F. W., Amy, G. L., & Hernandez, M. T. (2009). Fluorescent microspheres as virion surrogates in low-pressure membrane studies. *Journal of Membrane Science, 335*(1–2), 43–50.

Soussan, L., Guigui, C., Alfenore, S., Mathe, S., & Cabassud, C. (2011). A new biosynthetic tracer for the inline measurement of virus retention in membrane processes: Part I—Synthesis protocol. *Analytica Chimica Acta, 690*(2), 190–198.

US EPA (1989) Drinking water; national primary drinking water regulations; filtration, disinfection; turbidity, *Giardia lamblia*, viruses, *Legionella*, and heterotrophic bacteria; final rule.

Wyn-Jones, A. P., Carducci, A., Cook, N., D'Agostino, M., Divizia, M., Fleischer, J., et al. (2011). Surveillance of adenoviruses and noroviruses in European recreational waters. *Water Research, 45*(3), 1025–1038.