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Variable non-linear removal of viruses during transport through a saturated soil column

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ABSTRACT

Reduction of viral surrogates (bacteriophage MS2 and murine norovirus-1 [MNV-1]) and viruses naturally present in wastewater (enteroviruses, adenoviruses, Aichi viruses, reovirus, pepper mild mottle virus) was studied in a long-term experiment simulating soil-aquifer treatment of a non-disinfected secondary treated wastewater effluent blend using a 4.4 m deep saturated soil column (95% sand, 4% silt, 1% clay) with a hydraulic residence time of 15.4 days under predominantly anoxic redox conditions. Water samples were collected over a four-week period from the column inflow and outflow as well as from seven intermediate sampling ports at different depths. Removal of MS2 was 3.5 $\log_{10}$ over 4.4 m and removal of MNV-1 was 3 $\log_{10}$ over 0.3 m. Notably, MNV-1 was removed to below detection limit within 0.3 m of soil passage. In secondary treated wastewater effluent, MNV-1 RNA and MS2 RNA degraded at a first-order rate of 0.59 day^{-1} and 0.12 day^{-1}, respectively. In 15.4 days, the time to pass the soil column, the RNA-degradation of MS2 would amount to 0.8 $\log_{10,}$ and in one day that of MNV-1 0.3 $\log_{10}$ implying that attachment of MNV-1 and MS2 to the sandy soil took place. Among the indigenous viruses, genome copies reductions were observed for Aichi virus (4.9 $\log_{10}$) and for pepper mild mottle virus (4.4 $\log_{10}$). This study demonstrated that under saturated flow and predominantly anoxic redox conditions MS2 removal was non-linear and could be described well by a power-law relation. Pepper mild mottle virus was removed less than all of the other viruses studied, which substantiates field studies at managed aquifer recharge sites, suggesting it may be a conservative model/tracer for enteric virus transport through soil.

1. Introduction

Soil-aquifer treatment (SAT) provides a valuable soil-based natural treatment process for reduction of chemical and microbial contaminants associated with municipal wastewater effluents used as sources of water in sustainable land-based managed aquifer recharge (MAR) systems (Abel et al., 2012; Alidina et al., 2014; Amy and Drewes, 2007; Bekele et al., 2011; Hoppe-Jones et al., 2010). SAT can result in significant reductions of infectious microorganisms in the recharged source water, and thus represents an important component for indirect potable reuse applications (Asano and Cotruvo, 2004; Bekele et al., 2011; Dillon et al., 2009; Missimer et al., 2011). Enteric pathogens (i.e., viruses, protozoa, and bacteria) are generally reduced by a combination of filtration, adsorption, and inactivation/die-off as the source water moves through the soil (Yates and Gerba, 1998; Yates, 2002). Nevertheless, as viruses have great potential for long distance transport through soils and aquifers due to their small size and environmental persistence (Goyal et al., 1984, Bitton and Harvey, 1992;Yates, 2002; Xagoraraki et al., 2014; Tesson et al., 2018), understanding the factors that control virus migration and survival through subsurface formations is critical for proper design and operation of SAT facilities. Multiple factors have been shown to influence the survival and transport of viruses in the subsurface including microbial activity, soil type, soil properties (e.g., soil particle distribution, clay composition, pH, soil organic content, soil solution composition, ionic strength), flow velocity, degree of water saturation, presence of colloids, temperature, and

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virus type (Bitton and Harvey, 1992; Yates and Yates, 1998; Schijven and Hassanizadeh, 2000; Jin and Flury, 2002; Yates, 2002). Yet, virus adsorption to soil is the most important process for attenuation with most of the actual virus removal due to inactivation and irreversible attachment to soil surfaces having favorable charge characteristics (Yates et al., 1987; Schijven and Hassanizadeh, 2000).

A detailed knowledge of hydrogeological and geochemical conditions is required to properly link attenuation of viruses to dominant flow and boundary conditions and make meaningful predictions regarding their fate and transport. There have been a number of laboratory-scale studies investigating the transport behavior of viruses through aquifer material using homogeneous, repacked soil columns (Lance and Gerba, 1984; Corapcioglu et al., 1997; Jin et al., 2000; Schijven and Hassanizadeh, 2000; Walshe-Langford et al., 2010; Frohnert et al., 2014). In general, the results have been analyzed using models that consider advective-dispersive transport and first order microbial retention and release in which the pore-water velocity (v) controls the advective transport and residence time of the microbes in porous media (Bradford et al., 2017). Nevertheless, one major drawback is that most laboratory-scale studies utilized short column systems representing < 1 m of soil passage. It was shown that inactivation rate coefficients derived from such studies overestimate virus removal in the field by two to three orders of magnitude as the greatest removal of microbial contaminants usually occurs within the first meter of (unsaturated) soil passage (Pang, 2008). As a result, interpolation of inactivation rates over greater distances based on these initial values can lead to inaccurate microbial removal assessment. The non-linearity of removal with distance may have important consequences for the prediction of virus removal, thus also for the calculation of setback distances in SAT schemes to ensure adequate treatment of infiltrated source water (Schijven and Hassanizadeh, 2000). Several authors concluded that meso-scale soil column experiments with column length substantially longer than 1 m might bare a suitable compromise to better study the non-linear attenuation of viruses during travel through the vadose zone and saturated aquifer representing longer retention times (Lance and Gerba, 1984; Powelson et al., 1990, 1991; Sobsey et al., 1995; Frohnert et al., 2014; Regnery et al., 2017).

To test this research hypothesis, a meso-scale soil column system of > 4 m length was custom-built at the Colorado School of Mines to investigate the fate and transport of viruses under controlled hydrogeochemical conditions during simulated SAT with non-disinfected reclaimed water in a long-term experiment. Effluent-impacted waters applied for recharge in SAT systems usually contain high concentrations of biodegradable dissolved organic carbon (DOC), which serves as an electron donor for soil microorganisms. In a typical SAT recharge situation, a sequence of reduction processes from oxidized conditions in the initial zone of infiltration to reducing conditions in the recharged shallow aquifer will develop over time as a function of travel distance. Previous experiments simulating SAT indicated immediate depletion of dissolved oxygen in secondary treated wastewater effluent during passage through a 1.2 m long soil column operated under unsaturated flow conditions (Regnery et al., 2015). At field-scale SAT, redox conditions may change with hydraulic conditions, microbial activity, and temperature and are not constant throughout (Regnery et al., 2017) – presenting conditions difficult to simulate in the laboratory. Hence, our study focused on the fully saturated and anoxic part of the recharged shallow aquifer in a SAT scheme.

Studied viruses included the human viral surrogates bacteriophage MS2 and murine norovirus-1 (MNV-1) as well as viruses naturally present in wastewater (enterovirus [EV], adenovirus [AdV], Aichi virus [AichiV], reovirus [ReoV], pepper mild mottle virus [PMMoV]). EV has been studied the most at SAT operations, whereas AdV, AichiV, and PMMoV are among the most abundant viruses detected in wastewater (Kitajima et al., 2014). ReoV have been commonly detected in groundwater (Fout et al., 2003; Betancourt et al., 2014). Moreover, due to the ubiquitous presence of ReoV in mammals, frequent occurrence in sewage, and relative resistance to ambient conditions, this virus has been suggested as an indicator of groundwater vulnerability to fecal pollution and consequently to enteric virus contamination (Betancourt and Gerba, 2016). Bacteriophage MS2 has been used extensively to study virus transport under various soil column and field conditions (Yates and Yates, 1991; Schijven et al., 1999; Schijven and Hassanizadeh, 2000; Walshe-Langford et al., 2010; Frohnert et al., 2014; Mayotte et al., 2017). MS2 is an icosahedral phage with a diameter of 27 nm and with a low isoelectric point (pI) of 3.5. This virus has been shown to have hydrophobic properties, which may influence its transport through soil (Farkas et al., 2015). In addition, MS2 has a negative charge and is considered a relatively conservative tracer for virus transport in saturated sandy soils with low organic matter content at pH 6–8 as it shows little or no adsorption under those conditions (Jin et al., 2000; Schijven and Hassanizadeh, 2000). MNV-1 is an enteric pathogen in mice that shares many biochemical and genetic features with human noroviruses and therefore is considered a model system to study human norovirus biology (Wobus et al., 2006). MNV-1 has the size (28 to 35 nm in diameter), shape (icosahedral), and buoyant density ($1.36 \pm 0.04\ g/cm^3$) characteristic of human noroviruses. Human noroviruses in turn are considered important etiologic agents with respect to health risks associated with exposure to recycled water (Soller et al., 2017).

Although passage through a short unsaturated zone under predominantly oxic conditions was omitted in our experimental setup, it was expected that the chosen conditions (i.e., well-adapted microbial community, 4.4 m of continuous soil passage, subsurface residence time of more than two weeks) mimic SAT field conditions more closely than previous column studies investigating virus transport. To determine removal kinetics of enteric virus surrogates during simulated SAT, MNV-1 was administered as a 24-h pulse spike at the beginning of the experiment, whereas coliphage MS2 was continuously spiked to the soil column for 26 days. The fate and transport of MNV-1 and MS2 in the soil column were monitored over five days and a four-week period, respectively. Their breakthrough curves were determined and were fitted to an advection-dispersion model including a lumped term for virus attachment and inactivation using HYDRUS-1D (version 4.16.0110), a software package that simulates one-dimensional water flow and solute transport in unsaturated, partially saturated, or fully saturated porous media (Šimůnek et al., 2005). Furthermore, additional water samples were collected from the soil column system and were assayed for naturally occurring enteric viruses in the recharged treated wastewater effluent blend to assess their fate during simulated SAT.

2. Material and methods

2.1. Soil column configuration and operation

The custom-built meso-scale soil column system consisted of one continuous 4.5 m long polyvinylchloride (PVC) column with an inner diameter of 0.15 m filled with a blend of 50:50 (v/v) technical sand (i.e., quartz sand) and sandy field soil (coarse sand with approximately 6% fines sieved to grain size < 2 mm) from an active full-scale SAT infiltration basin in Colorado, United States. This allowed for adequate hydraulic properties (i.e., greater hydraulic conductivity than the applied hydraulic loading rate to maintain flow rate and meet retention time requirements) as well as biological activity in the column. The soil column had been carefully wet packed in 0.1 m incremental depths to prevent layering and achieve a uniform bulk density by stirring and tamping the soil. Soil depth in the packed column was 4.4 m. Porosity and hydraulic conductivity of the sand/soil mixture were determined to be 0.34 and 0.61×10^{-4} m/s, respectively using standard procedures at 20 °C laboratory temperature (ASTM, 2007a,b; ASTM, 2009, 2011). The proportion of sand, silt, and clay was 95%, 4%, and 1%, respectively. Grain size analysis was performed in 2012 using standard test method ASTM D422 (ASTM, 2007b). Thus, the sand/soil mixture was

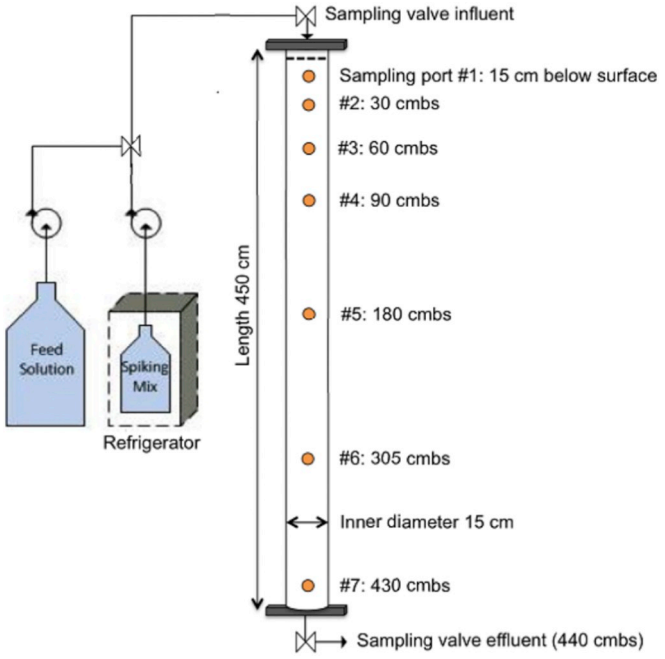

Fig. 1. Schematic of the meso-scale soil column system.

classified as sand with a soil organic carbon content of < 0.3%. The pH was 7.7 and cation exchange capacity was estimated to be 5.24 $cmol^+/kg$.

The soil column was equipped with seven intermediate sampling ports at various distances along the length of the column with the following depths: 0.15, 0.3, 0.6, 0.9, 1.8, 3.05, and 4.3 m. Customized stainless-steel sampling ports without porous cups were used to avoid sorption effects and to obtain water samples from the center of the column as previously described (Wang et al., 1980). A schematic of the soil column setup is provided in Fig. 1. The soil column was operated under saturated flow conditions in vertical direction (top to bottom) at 20 °C to simulate the saturated zone of the aquifer during SAT. The nitrogen-purged column feed solution was kept in a 50 L plastic carboy at room temperature and was delivered to the column by a peristaltic Masterflex L/S pump (Cole Parmer, Vernon Hill, IL) at a constant flow rate of 1 mL/min.

To establish a well-adapted microbial community, the soil column system received non-disinfected secondary treated effluent (conventional activated sludge system, nitrified/denitrified) from a wastewater treatment facility in Denver, CO for > 6 months during start-up. One month prior to this study (i.e., December 12, 2012), the soil column feed was switched to a blend of nanofiltration permeate and secondary treated effluent at a ratio of 50:50 (*v*/v) to prevent unforeseen clogging during virus spiking and provide more stable feed water conditions. Nanofiltration permeate was produced from tap water (Golden, CO) filtered through a 20 gpm (75.6 L per minute) membrane skid equipped with 21 Dow/Filmtec 4040 NF 270 elements (Midland, MI).

After soil column start-up, hydraulic retention time (HRT) was determined in a tracer test based on continuous conductivity readings at the column outlet as well as one intermediate sampling port using potassium bromide as a conservative tracer. CXTFIT 2.0 (Toride et al., 1995) was used to calculate the tracer's breakthrough curve and to interpolate the HRT of all intermediate sampling ports. Resulting HRT were: 0.5 days (0.15 m), 1 day (0.3 m), 2 days (0.6 m), 3 days (0.9 m), 6.1 days (1.8 m), 10.4 days (3.05 m), 15.3 days (4.3 m), and 15.4 days (4.4 m). No preferential flow paths through the column were observed during the tracer test; however, small-scale heterogeneities in the packed soil cannot be excluded.

Water samples (100 mL) for analysis of bulk organic parameters such as DOC, ultraviolet absorbance at 254 nm (UV_{254nm}), specific UV absorbance (SUVA) as well as major ions (i.e., nitrate, manganese) were collected once a week at the inflow and outflow of the soil column and analyzed subsequently according to Standard Methods (APHA, 2012). For the purpose of this study, classification of redox conditions followed the framework for identifying redox processes in aquifer systems developed by McMahon and Chapelle (2008) as detailed in Regnery et al. (2015).

2.2. Indicator virus cultures

Bacteriophage MS2 (American Type Culture Collection, ATCC 15597-B1) was propagated and assayed by the double-agar-layer method using *Escherichia coli* ATCC 15597 as the host cells (Adams, 1959). Briefly, virus propagation involved mixing 1 mL of phage stock with 100 mL of log-phase *E. coli* grown in 3% tryptic soy broth (TSB) followed by incubation at 37 °C. After incubation, the culture was centrifuged at 6000 × *g* for 15 min to remove bacterial debris and the resulting supernatant was filtered through 0.45 µm cellulose acetate membrane filters. The virus stock obtained through this procedure was aliquoted in 2 mL vials and stored at −80 °C until needed.

MNV-1 (S7-PP3 strain), kindly provided by Dr. Y. Tohya (Nihon University, Kanagawa, Japan) was propagated in a mouse leukemic monocyte macrophage cell line (RAW 264.7, American Type Culture Collection TIB-71) (Cannon et al., 2006). MNV-1 concentrations were quantified by reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) using 10-fold serial dilutions of a plasmid containing a full-length cDNA clone of MNV-1 in order to generate a standard curve and to evaluate the sensitivity of the assay (Betancourt et al., 2014). A minimum of 10 copies/reaction of the MNV-1 genome could be detected by the RT-qPCR assay. MNV-1 was also used as a sample process control to monitor the efficiency of viral nucleic acid extraction, reverse transcription, and qPCR assays (Betancourt et al., 2014).

2.3. Spiking of indicator viruses during simulated SAT

Throughout this study, a strict disinfection and spill-prevention protocol was followed to prevent contamination of the samples by the spiked or indigenous viruses present in the wastewater. MNV-1 was added to the soil column as a 24-hour pulse at the start of the experiment on January 16, 2013 at an average concentration of 10^6 genome copies (GC) per mL input while MS2 was continuously added for 26 days at an average concentration of 10^8 PFU/mL input per day. The spiking solution was kept at 4 °C in a fridge, renewed every day, and was delivered to the column by a high-precision planetary-gear driven multi-channel Ismatec IPC-8 pump (Wertheim, Germany) at a flow rate of 0.1 mL/min. Mixing of feed and spiking solutions occurred approximately 1.5 m prior to the column inlet. A sampling tap on the feed line (after the spiking, but just before entering the column) was used to collect all inflow samples (Fig. 1). All utilized tubing within this study consisted of Teflon to minimize losses of pathogens due to sorption effects. Each of the intermediate sampling ports plus column inflow and outflow were sampled once a day over a period of 15 days. After day 15 of the spiking experiment, all sampling ports as well as column inflow and outflow were sampled every second day until spiking ended on day 26. In general, 10 mL of sample were collected from each sampling location into 15 mL sterile polypropylene centrifuge tubes. A drop of fecal calf serum was immediately added to each sample to preserve the MS2 phage during subsequent freezing. The frozen samples were shipped overnight on dry ice to The University of Arizona's laboratory for analysis.

2.4. Detection of viruses naturally present in blended secondary treated effluent

After receiving blended secondary treated effluent for more than six weeks, additional water samples were collected from the soil column on January 26, 2013 and assayed for naturally occurring enteric viruses to assess their fate during simulated SAT. Samples from inflow (1 mL), seven intermediate sampling ports (1 mL each), and outflow (1 L) of the soil column were filtered through mixed cellulose esters membrane filters with a 0.45 µm pore size (EMD Millipore) to concentrate the viruses following procedures previously described (Betancourt et al., 2014).

Viral DNA (AdV) and RNA (EV, ReoV, AichiV, PMMoV) were extracted from water concentrates (400 µL) using the ZR Viral DNA/RNA kit (Zymo Research, Irvine CA), following the manufacturer's protocol. Viral RNA was reverse transcribed into complementary DNA (cDNA) using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA). Viral genome copies in the secondary effluent blend were evaluated by qPCR (Adenovirus) or RT-qPCR (EV, ReoV, PMMoV) following procedures previously described (Betancourt et al., 2014). ReoV RNA was assayed by end-point amplification of a conserved region of the viral L1 genome segment (Leary et al., 2002).

MNV-1 was used as a process control to monitor the efficiency of viral nucleic acid extraction, reverse transcription, and qPCR assay from the actual samples relative to molecular biology grade water (DNase-, RNase-, and protease-free), which was utilized as a control (i.e., no inhibition) to determine the spiked MNV-1 RNA concentration. Briefly, 2.0 µL of MNV-1 stock (4.0×10^4 copies/µL) was spiked into 200 µL of concentrated wastewater samples and molecular biology grade water. MNV-1 RNA was co-extracted with other indigenous viral nucleic acids from the water samples and the MNV-1 RNA yield was determined by RT-qPCR to calculate the extraction-RT-qPCR efficiency (E) as previously described (Betancourt et al., 2014; Schmitz et al., 2016). The MNV process control was used to identify viral nucleic acid loss during extraction, the occurrence of any RT-qPCR inhibition, or both. qPCR inhibition observed in three of the samples was resolved by 1:5, 1:10 or 1:100 dilutions The concentration of naturally occurring viruses in the wastewater samples were estimated by adjusting the concentration with the extraction –RT-qPCR efficiency (E) data for each wastewater sample. Although MNV-1 is a culturable virus, the RT-qPCR assay instead of cell culture was selected for quantification of MNV-1 genomes in order to minimize the differences in the quantification of other eukaryotic viruses naturally present in treated wastewater effluents. The persistence of AdV, EV, ReoV, AichiV, and PMMoV through SAT was evaluated by qPCR as a rapid and effective assay for detection and quantification of virus genomes in the environment. The qPCR primers and probes used in the present study for the selected viruses as well as the process control (MNV-1) are shown in Table 1. The LightCycler® 480 Real-Time PCR Instrument II (Roche Applied Science, Indianapolis, IN) was used for the qPCR assays. Reaction mixtures (25 µL) contained 12.5 µL of LightCycler® 480 Probes Master (Roche Diagnostics) primers and probes plus 2.5 µL of viral DNA or cDNA. Fluorescence data were collected after every cycle and analyzed with LightCycler® 480 Software version 1.5 (Roche Diagnostics).

Absolute quantification of viruses expressed as viral copy numbers or gene copies were derived from standard curves using tenfold serial dilutions of cDNA (EV, PMMoV, AichiV, MNV-1) or DNA (AdV) clones and the Roche system based on a second-derivative quantification cycle (*Cq*) determination and nonlinear fit algorithms. Viral copy numbers from samples (unknowns) were extrapolated from the corresponding known quantity in the standard curve. Negative (no template) controls were included to detect any false-positive results due to cross-contamination; however, no false-positive qPCR signal was observed. The qPCR reactions were performed in triplicate and considered positive when the tube fluoresced with sufficient intensity and the *Cq* was ≤40 (Bustin et al., 2009).

2.5. Inactivation of indicator viruses

The inactivation rate of MNV-1 and MS2 at room temperature in secondary treated wastewater effluent without porous media contact was assessed over 10 days based on repeated measurements of MNV-1 and MS2 concentration by qPCR. For MS2, the inactivation rate was also evaluated by the plaque assay previously described (Adams, 1959).

2.6. Data analysis

Breakthrough curves were analyzed by plotting the concentration of viral surrogates against time (in days). Total amounts of detected viral surrogates in the water samples were determined by assessing the areas under the breakthrough curves multiplied by flow rate. Fitting of the bromide tracer and virus breakthrough curves in the fully saturated porous media was conducted with HYDRUS-1D (version 4.16.0110, Šimůnek et al., 2005). As the tail of the MS2 breakthrough curve was not monitored after continuous spiking was terminated, MS2 detachment could not be evaluated. Detachment is commonly much slower than attachment (Schijven and Hassanizadeh, 2000) and was therefore neglected.

Transport of the spiked viral surrogates was analyzed using an advection dispersion equation including equilibrium sorption and first-order removal. The governing equation for an advection–dispersion model, including removal by a combination of attachment and inactivation, and neglecting detachment is as follows:

$$\frac{\partial C}{\partial t} = \frac{\partial}{\partial x}\left(\alpha_L v \frac{\partial C}{\partial x}\right) - v\frac{\partial C}{\partial x} - \lambda C \quad (1)$$

Subject to boundary conditions $C = C_0$ at $x = 0$ and $\frac{\partial C}{\partial x} = 0$ at $x = l$, where l is the transport length (cm). C is the concentration of free bacteriophages (pfu/mL); x is the travel distance (m); α_L is the dispersivity (m); v is the average interstitial water velocity (m/day); λ is the removal rate coefficient (day^{-1}), combining attachment and inactivation. The dispersivity estimate from the bromide tracer was used to characterize virus transport neglecting dispersivity.

Log_{10} removal was then calculated from:

$$Log_{10}\frac{C_t}{C_0} = -\lambda\frac{L}{v}\frac{1}{\ln 10} \quad (2)$$

Viral RNA degradation of MNV-1 and MS2 in secondary treated wastewater effluent was assumed to proceed at a first-order rate. The degradation rate coefficient was estimated by first-order regression analysis of the log-transformed concentration data versus time.

3. Results

3.1. Water quality changes during simulated SAT

Relevant organic water quality parameters in the soil column inflow and outflow and the change in nitrate and dissolved manganese after travel through 4.4 m of soil were measured and are summarized in Table 2. The nitrate reduction and dissolved manganese release, in combination with the decrease in DOC, indicated biological activity and predominantly anoxic redox conditions over the entire soil passage of 4.4 m. Levels of biodegradable DOC were in the range of 2.4 mg/L and 2.0 mg/L, respectively. On average, total ammonia concentrations in the nitrified/partial denitrified treated wastewater effluent blend were still 5.0 ± 2.0 mg N/L and revealed significant oxygen demand for further nitrification. As the feed container was purged with nitrogen gas, solely traces of oxygen could have been introduced to the soil column system via diffusion through peristaltic pump tubing and would have been utilized within the first few centimeters of the soil passage without affecting the column's predominant reducing condition. Highly reducing conditions (i.e., sulfate reduction and methanogenic conditions, respectively) were not observed in the soil column system.

Table 1
Primers and probes for viruses analyzed in the present study.

Virus	Primer or probe	Sequence (5′ → 3′)[a,b]	Reference
Aichi virus	AiV-AB-F	GTCTCCACHGACACYAAYTGGAC	Kitajima et al., 2013
	AiV-AB-R	GTTGTACATRGCAGCCCAGG	
	AiV-AB-TP	FAM-TTYTCCTTYGTGCGTGC-MGB-NFQ	
Adenovirus	AQ2	GCCCCAGTGGTCTTACATGCACATC	Heim et al., 2003
	AQ1	GCCACGGTGGGGTTTCTAAACTT	
	AP	FAM-TGCACCAGACCCGGGCTCAGGTACTCCGA-BHQ1	
PMMoV	PMMV-FP1rev	GAGTGGTTTGACCTTAACGTTTGA	Haramoto et al., 2013
	PMMV-RP1	TTGTCGGTTGCAATGCAAGT	Zhang et al., 2006
	PMMV-Probe1	FAM-CCTACCGAAGCAAATG-BHQ1	
Enterovirus	EV1F	CCCTGAATGCGGCTAAT	Gregory et al., 2006
	EV1R	TGTCACCATAAGCAGCCA	
	EV	FAM-ACGGACACCCAAAGTAGTCGGTTC-BHQ1	
MNV	MNV-S	CCGCAGGAACGCTCAGCAG	Kitajima et al., 2010
	MNV-AS	GGYTGAATGGGGACGGCCTG	
	MNV-TP	FAM-ATGAGTGATGGCGCA-MGB-NFQ	
Reovirus	L1.rv5	GCATCCATTGTAAATGACGAGTCTG	Leary et al., 2002
	L1.rv6	CTTGAGATTAGCTCTAGCATCTTCTG	
	L1.rv7	GCTAGGCCGATATCGGGAATGCAG	
	L1.rv8	GTCTCACTATTCACCTTACCAGCAG	

PMMV - Pepper mild mottle virus.
MNV - Murine norovirus.
[a] Mixed base in degenerate primer and probe is as follows: Y = C, T.
[b] The FAM (6-carboxyfluorescein) quencher is BHQ-1 (Black Hole Quencher). The FAM quencher is a minor groove binder nonfluorescent quencher (MGBNFQ).

3.2. Breakthrough curves of viral surrogates

Fig. 2 shows the observed breakthrough curves plotted with time for MS2 and best fit curves analyzed with Hydrus-1D, using the one-site kinetic sorption model and assuming first order removal (Eq. (1)). MS2 was constantly detected in the outflow of the soil column approximately 12 days after the beginning of spiking, three days faster than the chemical tracer. As described earlier, it took the conservative tracer bromide 15.4 days to pass the soil column. MNV-1 reached port #2 at 0.3 m depth within one day. The calculated breakthrough curves were investigated at a flow velocity (Darcy) of 9.6 cm/day (from 1 mL/min and the diameter of the column), a porosity of 0.34, and a low value of dispersivity of 0.87 cm^{-1}, reflecting a narrow grain size distribution with poorly graded particles. The corresponding removal rate coefficients λ (day^{-1}) of MS2 as a function of travel distance in the soil column are summarized in Table 3.

The log_{10} removal data as a function of travel distance were analyzed. Fig. 3 shows the observed removal of MS2 and MNV-1 plotted with travel distance. These results suggest that MS2 removal was nonlinear and could be described by the power-law relation indicated above the figure. As MNV-1 was removed to below detection limit (i.e., qPCR non-detects) beyond 0.3 m travel distance, its transport behavior could not be determined.

3.3. Removal of naturally occurring viruses

Table 4 summarizes the removal of naturally occurring viruses after the soil column was continuously fed with secondary treated effluent/nanofiltration permeate (50:50, *v*/v) blend for six weeks. EV and AdV were not detected in the inflow, outflow, or samples from intermediate sampling ports of the soil column. AichiV RNA was detected in the inflow and sampling port #1 corresponding to a depth of 15 cm, however after this depth there was no further detection indicating an overall reduction of > 3.1 log_{10} (99.9%). PMMoV RNA was consistently detected at high levels (10^7 GC/mL) in the inflow and the first two sampling ports at 0.15 and 0.3 m depth, respectively. After longer travel distances (i.e., ports #4 and #5 located at 0.9 and 1.8 m depth, respectively), low levels (10^1–10^2 GC/mL) of PMMoV RNA were still detected, including the outflow of the soil column, indicating an overall reduction that ranged from 1-log (90%) to 4-log (95%). These results indicate that PMMoV was transported much further through the soil column than the other viruses. As a consequence, PMMoV removal was relatively lower than removal of AichiV and MS2 coliphage. ReoV RNA was unexpectedly detected in samples collected at a depth of 4.3 m and the outflow as indicated by PCR amplification of viral complementary DNA (cDNA) templates. However, an overall reduction could not be determined since the ReoV RNA was not detected in the influent sample or samples from other intermediate sampling ports.

4. Discussion

4.1. Fate and transport of viral surrogates during SAT

Our study aimed at evaluating the removal of two human viral surrogates including a bacterial virus (bacteriophage MS2) and a mammalian virus (MNV-1) through a well-adapted 4.4 m long soil

Table 2
Bulk organic parameters of feed waters applied to the soil column and change in redox surrogates nitrate (NO^{3-}) and dissolved manganese (Mn^{2+}) after 4.4 m of subsurface soil passage.

	DOC (mg/L)		UV_{254nm} (m^{-1})		SUVA (L/mg m^{-1})		NO_3^- (mg/L)		Mn^{2+} (mg/L)	
	In	Out	In	Out	In	Out	In	Out	In	Out
SE (n = 13)	6.8 [0.7]	4.4 [0.5]	13.7 [1.9]	10.0 [0.4]	2.1 [0.2]	2.3 [0.1]	8.7 [1.6]	8.0 [2.2]	0.04 [0.02]	0.14 [0.06]
SE:NF (n = 4)	4.9 [0.6]	2.9 [0.4]	7.7 [1.2]	5.6 [2.0]	1.6 [0.9]	1.5 [0.8]	6.7 [1.4]	3.7 [1.1]	0.056 [0.02]	0.53 [0.43]

SE = Secondary treated effluent. SE:NF = Secondary treated effluent blended with nanofiltration permeate (50:50, v/v). DOC: dissolved organic carbon. UV: ultraviolet absorbance. SUVA: specific UV absorbance. Values in the table represent mean and standard deviation (in brackets). In this table, "in" denotes inflow and "out" denotes outflow.

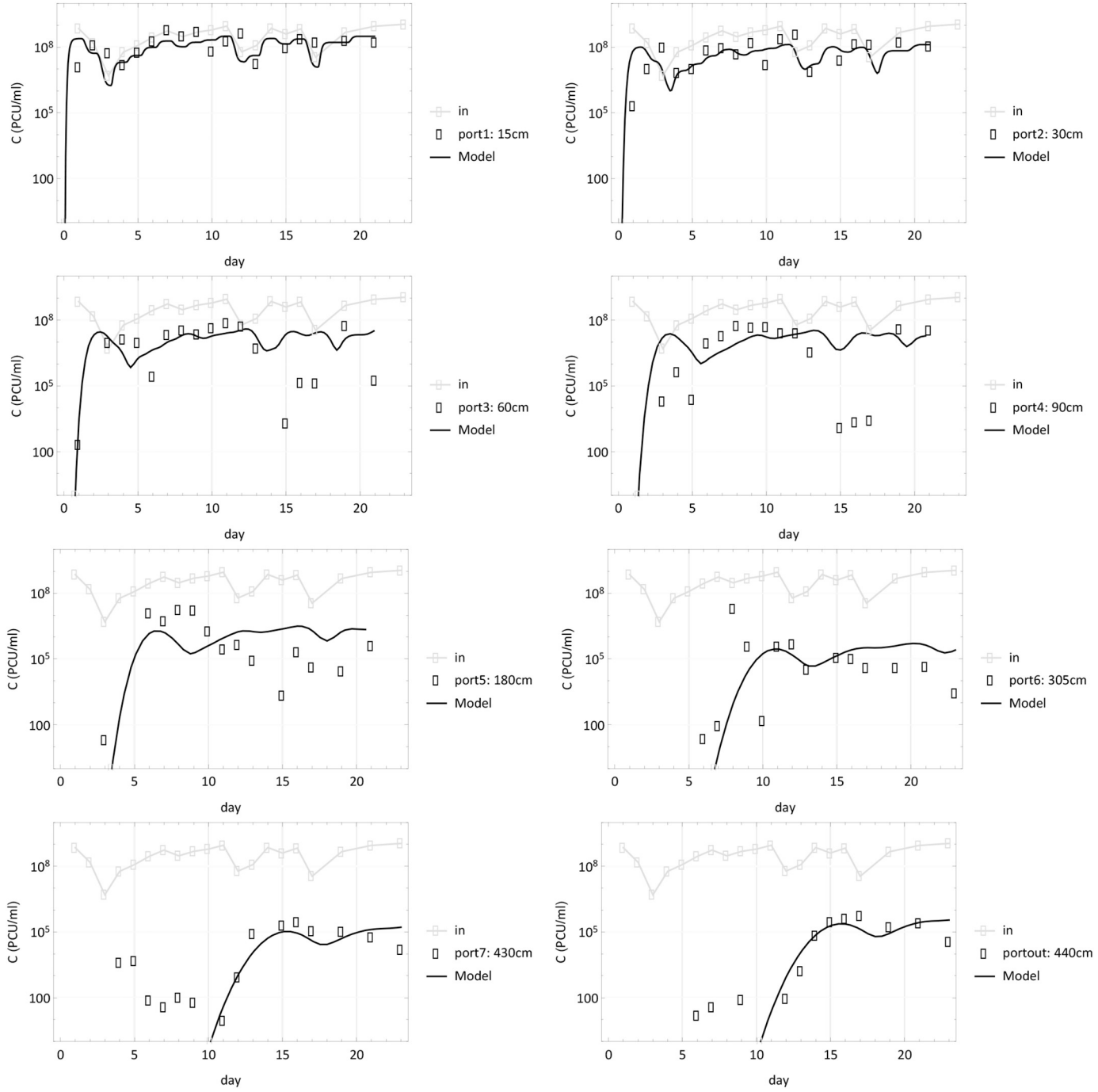

Fig. 2. Breakthrough curves of MS2 plotted with time for each sampling port of the soil column system ("in" refers to the concentrations of MS2 bacteriophages spiked onto the column).

Table 3
Estimated values of model parameters from fitting breakthrough curves of MS2.

Port #	Travel distance, L (m)	Removal rate coefficient, λ (day^{-1})	Standard error of λ (day^{-1})	R^2	$Log_{10}C/C_0$ (Eq. (2))
1	0.15	2.0	0.56	2.5%	−0.46
2	0.3	1.8	0.40	13%	−0.83
3	0.6	1.4	0.15	4%	−1.3
4	0.9	0.92	0.098	0.5%	−1.3
5	1.8	0.77	0.15	13%	−2.1
6	3.05	0.69	1.5	8.7%	−3.2
7	4.3	0.48	0.024	16%	−3.2
Outflow	4.4	0.41	0.020	26%	−2.8

column under saturated and anoxic conditions with continuous soil passage and residence time of more than two weeks for a long-term experiment. Further operating conditions included continuous dosing of MS2 over the entire runtime of the experiment (26 days) and the inclusion of MNV-1 as a 24-hour pulse at the start of the experiment to derive information about removal of a virus surrogate for norovirus in a short-term experiment. Using the advection dispersion equation including equilibrium sorption and first-order removal our results indicated that MS2 removal was non-linear and could be described by a power-law relation. MNV-1 removal, as previously mentioned, could only be observed at the first two ports, beyond port #2 MNV-1 was removed to below detection limit. The overall removal observed for MS2 was about 3.5 log_{10} over 4.4 m and almost 3 log_{10} over 0.3 m for

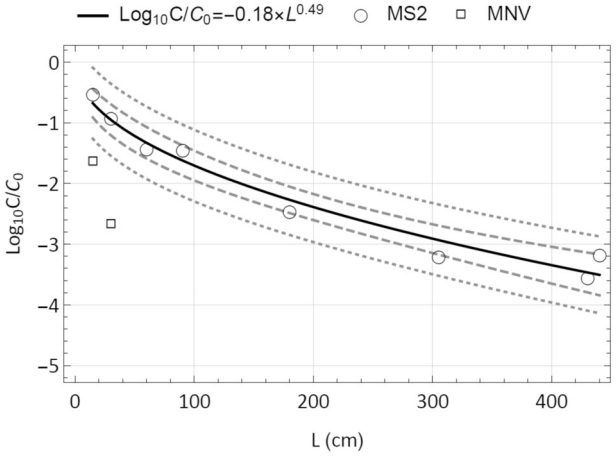

Fig. 3. Removal ($Log_{10}C/C_0$) of MS2 and MNV-1 as a function of distance during simulated SAT and continuous seeding (solid line: model, dashed line: confidence interval, dotted line: prediction interval).

Table 4
Log_{10} (%) removal of viruses naturally present in wastewater (log_{10} genomic copies) as a function of travel distance during SAT of blended secondary treated wastewater effluent.

Port #	Travel distance (m)	PMMoV	Aichi virus	ReoV	EV/AdV
1	0.15	0.2 (37%)	0.6 (75%)	–[a]	–
2	0.3	0.8 (84%)	> 3.1 (99.92%)	–	–
3	0.6	1.3 (95%)	> 3.1 (99.92%)	–	–
4	0.9	1 (90%)	> 3.1 (99.92%)	–	–
5	1.8	1.3 (95%)	> 3.1 (99.92%)	–	–
6	3.05	> 3.6 (99.97%)	> 3.1 (99.92%)	–	–
7	4.3	> 3.6 (99.97%)	> 3.1 (99.92%)	+[b]	–
Outflow	4.4	4.9* (99.9987%)	> 4.4* (99.996%)	+	–

*One liter was processed. At all other depths only 1 mL of sample could be processed. [a] indicates none detected. [b] positive by end-point PCR. PMMoV = pepper mild mottle virus. ReoV = reovirus. EV/AdV=enterovirus and adenovirus.

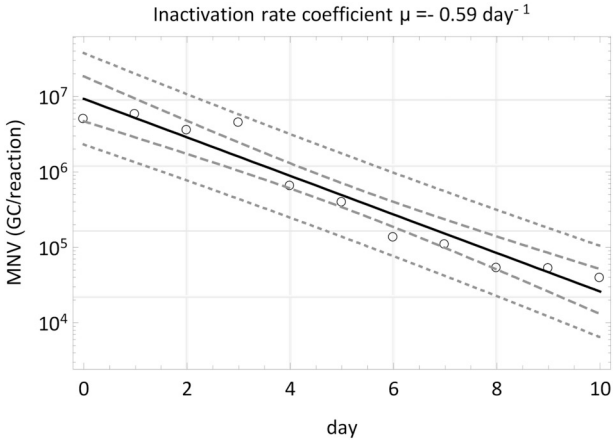

Fig. 4. Rate of degradation of MNV-1 in secondary treated effluent (solid line: model, dashed line: confidence interval, dotted line: prediction interval).

MNV-1. In secondary treated effluent, MNV-1 RNA degradation was only 0.3 log_{10} at a first-order rate of $-0.59\,day^{-1}$ (Fig. 4). Similarly, MS2 RNA degraded at a first order rate but with a much lower inactivation rate than MNV-1 corresponding to $-0.12\,day^{-1}$ that would amount to 0.8 log_{10} RNA degradation (Fig. 5). These results indicate that MNV-1 and MS2 were inactivated at different rates in secondary treated wastewater at a relatively constant temperature. One day was required for MNV-1 to travel 0.3 m indicating that most of the 3 log_{10} removal was attributable to attachment to the soil grains as observed in recent column experiments under saturated conditions (Tesson et al., 2018). Similarly, the 3.5 log_{10} removal of MS2 of over 4.4 m (15.4 days) indicates that attachment to soil grains rather than degradation was a major factor controlling the attenuation of this virus by passage through the soil column. Yates et al. (1985) reported inactivation rates of 0.075 to 0.52 [(log_{10}PFU/day^{-}(Abel et al., 2012) for MS2 in groundwater samples from five different sites at temperatures between 17 and 23 °C. No statistical difference between inactivation rate of MS2, poliovirus 1 and echovirus 1 was observed in those studies. Yates et al. concluded that the use of MS2 as a surrogate for animal virus behavior in groundwater would be justified in most cases (Yates et al., 1985). During soil passage, the attenuation of virus concentrations is largely due to the combined effect of adsorption and inactivation in addition to advection and dispersion responsible for spreading of viruses (Yates et al., 1987; Yates and Yates, 1991; Tesson et al., 2018). In our study, the apparent faster movement of MS2 in the saturated soil compared to the chemical tracer is most likely due to its transport through larger pores than the solute tracer itself (chemical tracers can diffuse into smaller pores, slowing their movement) and the greater sensitivity of the assay method for viruses (one virus can be detected in 1 mL) versus solute chemical tracers (Bales et al., 1993). Furthermore, preferential flow might have occurred within the homogeneously packed soil column. Similar phenomena have been observed in field studies (Maier et al., 2009). Viruses have been observed to travel 100 times faster than solute tracers in non-uniform substrata (Hinsby et al., 1996). Thus, chemical tracers cannot always be relied upon to reflect the transport or retention time for viruses during SAT.

The non-linear removal of MS2 with respect to travel distance observed in our study may also be attributed to heterogeneities within the uniform soil as well as within the population of transported virus particles as previously reported (Schijven and Hassanizadeh, 2000). A slow power-law decay of contaminant concentration with distance due to heterogeneities at the scale of pathogen and filtration rates (macroscale heterogeneity) was described previously by Redman et al. (2001). These studies have shown that the relatively slow rate of microbial removal predicted by power-law filtration and the uncertain spatial variability in filtration rates caused by macroscale heterogeneity place a greater burden on these other processes as the primary barriers to microbial pathogens.

The non-linearity of removal with distance may have important consequences for prediction of virus removal, thus also for the calculation of setback distances that are needed to adequately protect groundwater sources and to ensure adequate treatment of infiltrated surface water (Schijven and Hassanizadeh, 2000). Studies have indicated that virus-soil interactions, which are electrostatic and hydrophobic in nature, are determined by differences in surface characteristics between viruses and soil particles. Furthermore, surface characteristics can be altered by changes in pH, ionic strength, multivalent ions and organic matter (Goyal et al., 1984; Dowd et al., 1998; Tesson et al., 2018). As colloids, viruses appear to be partially attached to other colloidal particles in the aqueous environment, which may explain heterogeneity of the adsorptive characteristics of transported virus particles (Schijven and Hassanizadeh, 2000; Tesson et al., 2018). Studies examining the co-transport of clay colloids and viruses such as MS2 and ϕX174 at two different pore water velocities in laboratory packed columns demonstrated that the presence of clays significantly influenced virus transport. MS2 not only exhibited greater affinity than ϕX174 for clays, but also its transport was hindered with respect to ϕX174 at the lower pore water velocity (Syngouna and Chrysikopoulos, 2013). The proportion of clay in our soil column did not exceed 1%. Additional column studies in a long-term experiment under anoxic

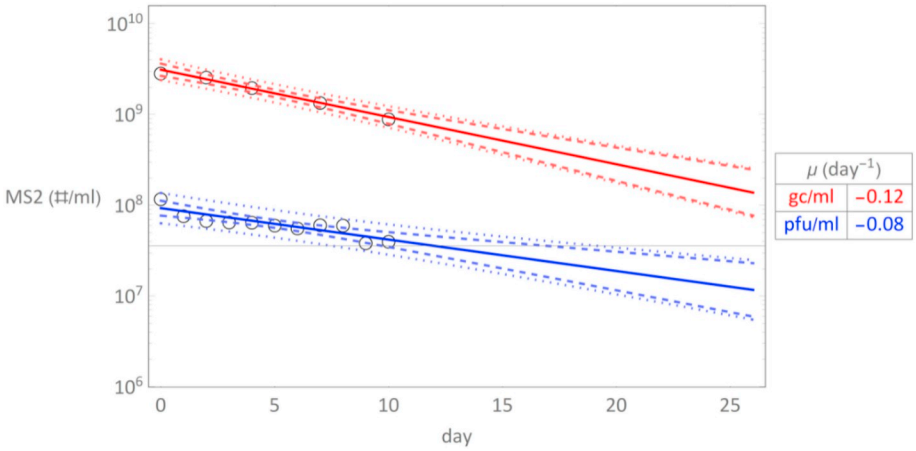

Fig. 5. Rate of degradation of MS2 in secondary treated effluent (solid line: model, dashed line: confidence interval, dotted line: prediction interval).

conditions associated low virus removal with less adsorption, less inactivation, and more detachment of MS2 and ϕX174 (Frohnert et al., 2014). Furthermore, more extensive removal of viruses and their surrogates (e.g., bacteriophages MS2 and ϕX174) was shown during unsaturated compared to saturated transport in soil column studies (Jin et al., 2000; Walshe-Langford et al., 2010; Frohnert et al., 2014). Less removal of MS2 in anoxic aquifers than in oxic aquifers was also reported in studies conducted by van der Wielen et al. (2008). The results of these studies and our research reveal practical implications about the size and impact of oxic and anoxic zones during subsurface passages and how it should be more emphasized in risk assessment studies aimed to protect drinking water resources from contaminations with human enteric viruses. It also highlights the importance of installing much larger protection zones for drinking water wells, e.g., in indirect potable reuse schemes, in order to secure against possible viral contamination (Schijven and Hassanizadeh, 2002; van der Wielen and Senden, 2008).

4.2. Behavior of naturally occurring viruses during SAT

PMMoV transport behavior in soil column systems has not been previously examined. It was selected for this study as it is consistently present in high numbers in treated wastewater and appears to persist longer in water/soil systems than human enteric viruses (Kitajima et al., 2014; Rachmadi et al., 2016; Symonds et al., 2018). This plant virus was detected more commonly than human enteric viruses at SAT field sites (Betancourt et al., 2014). PMMoV is non-enveloped rod-shaped, single stranded RNA virus with a normal length of 312 nm by 18 nm (Wetter et al., 1984), representing a relatively larger size compared to most human enteric viruses. It has a pI of 3.28 to 3.71, which is lower than the pI of most human enteric viruses (4.5–7.5). The mobility of PMMoV through the porous media column may be attributed to a large enough interstitial column velocity that facilitates the alignment of the virion long axis with the effective vertical flow field. Such a flow alignment through soil may result in preferential flow paths that enhance virus transport in the subsurface. This study and our previous study at different SAT sites (Betancourt et al., 2014) indicate that PMMoV is a conservative tracer of virus transport through aquifer material and therefore suitable for laboratory- and field-scale studies.

The present study reveals differences in removal rates for all four viruses analyzed. Under the studied conditions, removal rates during infiltration followed the order of AichiV > MNV-1 > MS2 > PMMoV. EV and AdV were never detected in our study, neither in a secondary treated effluent blend used to feed the soil column nor throughout the laboratory-scale infiltration process. The results of this investigation indicate that PMMoV removal was less than removal of the pathogenic virus (AichiV) and surrogate viruses (MS2 and MNV-1), thus quantitative data for PMMoV removal may offer a reliable and simple way to monitor virus transport in groundwater environments.

ReoV was unexpectedly detected by PCR amplification at the point of soil column outflow collection, thereby indicating that this virus could travel through the column system. No data are available in terms of ReoV removal and attenuation during laboratory-simulated SAT, thus pointing towards the need for further research. Possibly, the detection of ReoV after 4.4 m of soil passage may be due to continuous feeding of the soil column with undiluted non-disinfected secondary treated effluent for several months during the startup. ReoV is a double-stranded RNA virus with a double capsid layer that has been shown to persist longer that some single-stranded RNA virus after wastewater treatment, especially after chlorine disinfection (McDaniels et al., 1983; Brewster et al., 2005; Nieuwstad et al., 1991). Recent studies found ReoV to be the only infectious enteric virus detected after advanced wastewater treatment and ultrafiltration (Lim et al., 2015; Qiu et al., 2015).

5. Conclusions

Supporting our research hypothesis, meso-scale soil column systems clearly prove beneficial to study non-linear removal of viruses and viral surrogates such as MS2 with respect to travel distance and HRT in SAT scenarios. Furthermore, differences in removal rates among viral surrogates and viruses naturally present in treated wastewater effluents recharged during SAT were observed, which is a reflection of the differences in the physical and chemical structure of the virions. MNV-1 is undoubtedly an appropriate virus surrogate for human pathogenic viruses in laboratory studies, however additional studies may be required to obtain further information about its transport in repacked soil columns. Our study demonstrated the effect of adsorption and inactivation in the attenuation of virus concentrations where MS2 removal was more likely attributed to attachment to soil grains rather than to degradation as observed for MNV-1. PMMoV, a plant virus excreted in large numbers in human feces (10^9 virions/g dry weight fecal matter), may be selected as a conservative tracer through aquifer material in laboratory- and field-scale virus transport studies based on the results obtained in the present study. ReoV warrants further investigation to elucidate aspects of its transport and removal in the subsurface environment because of its common occurrence in disinfected wastewater.

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