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Surrogate and Pathogenic Virus Removal in Bench Scale Soil Aquifer Treatment

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Abstract

The demonstration of enteric virus removal during indirect potable reuse is necessary to ensure safe water reclamation practices. This study aimed to evaluate the efficacy of soil treatment in reducing concentrations of Pepper Mild Mottle Virus (PMMoV), Hepatitis A (HAV), and Norovirus (NoV) gene markers through bench scale soil aquifer treatment with unsaturated soil columns. Three distinct infiltration rates (4.86 mm/hr, 9.35 mm/hr, and 14.0 mm/hr) were evaluated to determine their impact on the removal of surrogate plant virus PMMoV, and enteric viruses, HAV and NoV. The concentrations of viral markers in the column influent and effluent samples were measured through RNA extraction and then RT-qPCR, and the log reduction values (LRVs) were calculated to quantify the effectiveness of removal across the columns. The LRVs for PMMoV were 2.80 ± 0.36, 2.91 ± 0.48, and 2.72 ± 0.32 for infiltration rates of 4.86 mm/hr, 9.35 mm/hr, and 14.0 mm/hr, respectively. A one-way ANOVA indicated no statistically significant differences in LRVs among the various infiltration rates (p-value = 0.329). All HAV samples fell below the detection limit both in the influent and effluent. While NoV GI and GII markers were measurable in the soil column influent, and they were below the detection limit in the effluent. The use of half the Limit of Detection (LoD) for effluent values enabled the estimation of log removals, which were calculated as 1.42 ± 0.07, 1.64 ± 0.29, and 1.74 ± 0.18 for NoV GI and 1.14 ± 0.19, 1.58 ± 0.21, and 1.87 ± 0.41 for NoV GII at infiltration rates of 4.86 mm/hr, 9.35 mm/hr, and 14.0 mm/hr. This highlights the efficacy of soil treatment in reducing virus gene marker concentrations at various infiltration rates, and that spreading basins are an effective method for reducing the presence of viral contaminants in indirect potable reuse systems.

Table of Contents

Abstract i

List of Tables iv

List of Figures v

1.0 Introduction 1

2.0 Literature Review 7

3.0 Materials and Methods 13

3.1 Column setup 13

3.2 Column operation 15

3.3 Sample collection and concentration 15

3.4 Percent recovery 16

3.5 RNA extraction and RT-qPCR 16

3.6 Log Reduction Value 19

3.7 Water quality parameters 19

3.8 Statistical analysis 20

4.0 Results and Discussion 22

4.1 Water quality parameters 22

4.2 PMMoV 32

4.3 Enteric viruses 39

5.0 Conclusions 46

6.0 References 48

7.0 Appendix 57

List of Tables

Table 1. List of Abbreviations and Acronyms vi

Table 2. Structure of viruses investigated in this study..... 3

Table 3. Average changes in DOC, TKN, and nitrate/nitrite..... 28

Table 4. Summary of ATP measurements 31

Table 5. Log removal of PMMoV, NoV GI and GII by infiltration rate 45

Table 6. Influent PMMoV gene marker, Nitrate/nitrite, TKN and DOC raw data 57

Table 7. Effluent PMMoV gene marker, Nitrate/nitrite, TKN and DOC raw data..... 58

Table 8. Influent HAV, NoV GI and NoV GII gene marker raw data..... 60

Table 9. Effluent HAV, NoV GI and NoV GII gene marker raw data 61

List of Figures

Figure 1. Schematic drawing of the three soil column setup. 14

Figure 2. Time plot of DOC removal during soil column treatment. 24

Figure 3. Time plot of nitrogen species before and after soil column treatment. 26

Figure 4. PMMoV concentration in the soil columns influent and effluent. 34

Figure 5. PMMoV LRVs after soil column treatment. 37

Figure 6. NoV GI (a) and NoV GII (b) log removal after soil column treatment..... 41

Figure 7. PMMoV, NoV GI, and NoV GII gene marker concentrations..... 43

Figure 8. PMMoV RT-qPCR standard curve using mean CT values..... 62

Figure 9. NoV GI RT-qPCR standard curve using mean CT values. 63

Figure 10. NoV GII RT-qPCR standard curve using mean CT values..... 64

Table 1. List of Abbreviations and Acronyms

A/O	Anerobic-oxic
ATP	Adenosine triphosphate
AWI	Air-water-interfaces
CT	Threshold cycle
DOC	Dissolved organic carbon
DLVO	Derjaguin–Landau–Verwey–Overbeek
DPR	Direct potable reuse
EBPR	Enhanced biological phosphorous removal
gc/L	Gene copies per liter
HAV	Hepatitis A
IPR	Indirect potable reuse
LoD	Limit of detection
LRV	Log reduction value
MAR	Managed aquifer recharge
ND	No detect
NoV	Norovirus
PMMoV	Pepper Mild Mottle Virus
SAT	Soil aquifer treatment
SWI	Solid-water-interfaces
TKN	Total Kjeldahl Nitrogen
TMWRF	Truckee Meadows Water Reclamation Facility
TN	Total nitrogen
TOC	Total organic carbon

1.0 Introduction

Water scarcity is an ongoing problem in arid climates. Water conservation strategies continue to be implemented to maintain a stable water supply when natural sources are unreliable (Garcia & Pargament, 2015). Potable water reuse is an increasingly common strategy for sustainable water management. This method uses highly purified wastewater effluent as source water for drinking water treatment. Direct potable reuse (DPR) uses wastewater effluent after advanced purification to meet drinking water regulations directly for augmentation of supply for drinking water treatment. Alternatively, indirect potable reuse (IPR) requires advanced purified water to travel through an environmental barrier before reaching the drinking water supply intake. Potable reuse has gained prominence due to its potential to address the growing concerns of freshwater resource conservation and the pressing issue of water scarcity.

An essential part of indirect potable water reuse is the intentional discharge of high-quality reclaimed water (high-quality treated wastewater effluent) into aquifers through managed aquifer recharge (MAR). MAR encompasses a range of techniques, including bank filtration, aquifer storage and recovery, and soil aquifer treatment (SAT) (Dillon et al., 2020). The selection of a MAR system depends on the geographical factors, location, the quality of reclaimed water, and the intended method of water recovery (Caldwell et al., 2021). The two primary methods for delivering reclaimed water to an aquifer are direct injection wells and spreading basins, each presenting distinct advantages and disadvantages. A key advantage of spreading basins is the filtration of water as it passes through the soil. The unsaturated soil layer known as the vadose zone, extends from the

soil surface to the groundwater table, plays a crucial role in enhancing water quality by reducing the concentration of various contaminants through adsorption and biodegradation (Bekele et al., 2011; Sharma & Kennedy, 2017).

Utilizing spreading basins to deliver water to the aquifer in IPR allows for additional attenuation of contaminants through the vadose zone. The unsaturated zone is a multi-phased flow with the potential for particle retention through straining, film straining, and adsorption at both the air-water-interfaces (AWIs) and solid-water-interfaces (SWIs) (Torkzaban et al., 2008). These mechanisms have been studied in colloid experiments. Straining refers to the retention of colloids in pore spaces too small for particle passage and is dependent on the size of the colloid relative to the pore size, and pore size is dependent on the percent saturation. Capillary forces in unsaturated systems can increase the effects of straining on particle retention. Film straining occurs when the thickness of the water film in a pore becomes smaller than the diameter of the particle, preventing further movement (Torkzaban et al., 2008). Attachment to the SWIs and AWIs depends on various factors, including Derjaguin–Landau–Verwey–Overbeek (DLVO) interactions, which include electrostatic energies, and van der Waals interactions, as well as non-DLVO interactions, such as hydrophobicity and capillary forces (Bradford & Torkzaban, 2008).

The morphology of bacteria, their size, hydrophobicity, and surface charge have been reported to affect bacterial retention in porous media (Bai et al., 2017) and are expected to similarly influence virus retention. The physical characteristics representing the structures of PMMoV, HAV, and NoV are detailed in Table 2.

Table 2. Structure of viruses investigated in this study

Virus	Size	Shape	Isoelectric Point	Enveloped	Citation
PMMoV	8 nm by 300-310 nm	Rod	3.7-3.8	No	(Symonds et al., 2018)
HAV	~30 nm	Icosahedral	2.8	No	(Michen & Graule, 2010; Stuart et al., 2019)
NoV	35-39 nm	Icosahedral	5.5-6.0	No	(Collins et al., 2006; Weaver et al., 2023)

In addition to these physical-chemical retention mechanisms, biodegradation within the vadose zone has proven to be an effective process for contaminant attenuation (Quanrud et al., 2003a). The vadose zone has proven effective in removing pharmaceuticals (He et al., 2016), emerging organic contaminants (Sopilniak et al., 2018), dissolved organic nitrogen (Gharoon & Pagilla, 2023), and viruses (Morrison et al., 2020). The focus of this study is to investigate the removal of two enteric viruses and a plant virus as a surrogate under full-scale SAT conditions in laboratory columns using actual reclaimed water from a full-scale water reclamation facility.

Indirect potable reuse (IPR) is permitted in Nevada. Strict water quality requirements are necessary to prevent groundwater contamination during recharge and to protect the public from infection. Enteric viruses primarily infect the gastrointestinal system and are transmitted through fecal-oral routes, making their removal during IPR especially important to prevent possible infection. As a result, in Nevada regulatory standards mandate a stringent 12-log reduction in enteric viruses, measured from wastewater influent to environmental extraction for drinking water treatment (https://www.leg.state.nv.us/nac/nac-445a.html#NAC445ASec27612).

Enteric viruses include NoV, HAV, hepatitis E virus, rotaviruses, and enteroviruses, and are known for their stability in the environment, which contributes to their ability to persist in water sources and cause infection at low concentrations. These viruses can maintain their infectivity over extended periods outside a host organism. Several factors contribute to their stability, including resistance to various environmental conditions such as fluctuation in temperature and pH levels (Bosch et al., 2006). They can endure

multiple treatment processes, making them challenging to remove during water treatment (Lodder et al., 2010; Wigginton & Kohn, 2012). Detecting and quantifying enteric viruses in reclaimed water can be difficult, due to seasonality, and limitations in sensitivity of the detection methods. To address these challenges, viral surrogates have emerged as an alternative approach to measuring the removal of enteric viruses in treatment systems (Symonds et al., 2018).

Surrogates such as bacteriophage MS2 and PMMoV have been used as alternatives to measuring enteric viruses' removal directly. For a surrogate to be effective, it should be present at higher concentrations than enteric viruses, show no seasonality, be removed less readily than enteric viruses, exhibit greater stability than enteric viruses, and have a similar morphology to enteric viruses (Symonds et al., 2018). PMMoV meets several of these criteria: it is present at 10^6-10^8 gene copies/L (gc/L) in treated wastewater (Symonds et al., 2018), has little seasonal fluctuation (Kitajima et al., 2018), and has been successfully used as a surrogate in similar applications (Morrison et al., 2020). It acts as a conservative tracer due to its ability to persist at higher concentrations compared to enteric viruses following treatment processes.

Previous studies used bacteriophage MS2 to measure virus attenuation in simulated SAT (Banasiak et al., 2023; Betancourt et al., 2019; Jin et al., 2000), while PMMoV was used in field studies to measure virus removal in MAR systems (Betancourt et al., 2014; Morrison et al., 2020). This study aimed to evaluate the impact of varying saturation levels on virus attenuation within a microbial active soil column by measuring surrogate

virus PMMoV and enteric viruses HAV and NoV before and after simulated SAT using laboratory unsaturated columns.

2.0 Literature Review

Soil aquifer treatment systems serve as natural barriers and filtration systems to effectively remove a wide range of contaminants. Despite their extensive use in enhancing the quality of wastewater effluents for reuse in IPR systems, a substantial research gap exists regarding the removal of indigenous viruses in SAT systems. Several studies have demonstrated virus removal through soil columns by using suspensions of surrogate viruses in homogeneous soil columns. However, only a few studies have attempted to directly measure indigenous enteric viruses present in wastewater. The limited number of studies investigating indigenous virus removal can be attributed partly to the seasonal variability of these viruses and the limitations in detection methods, which pose challenges in demonstrating their removal within simulated SAT setups. Additionally, most column studies employ uniform sand rather than the heterogeneous soil found in natural environments. This choice is due to the uniformity of sand, which allows for more accurate modeling. There is a lack of comprehensive studies utilizing natural soil mixtures in columns, combined with the measurement of indigenous viruses, to better simulate SAT and demonstrate their removal through the vadose zone.

MAR systems have been shown to effectively remove viruses. In a field-study at the Sweetwater Recharge Facility (SWRF) in Tucson, AZ, human viruses such as adenovirus and enterovirus, along with viral surrogates PMMoV and CrAssphage, were measured before and after SAT (Morrison et al., 2020). The system included infiltration basins, aquifer retention, and subsequent downstream extraction wells. The calculated LRVs for the entire SAT system ranged from 3.4 to greater than 6.2. Notably, no human viruses (adenovirus or enterovirus) were detected in the SAT water. LRVs were determined

based on the limit of detection. PMMoV exhibited the highest LRV and was consistently detected at higher concentrations in the reclaimed water before and after soil-aquifer treatment for all samples. No direct correlation was established between the LRV of PMMoV and adenovirus. Still, PMMoV was highlighted as a valuable conservative indicator for evaluating virus removal and transport in MAR. This is due to its detectability in the effluent and the ability to calculate LRVs from measured concentrations, rather than relying on limits of detection to calculate LRVs for human viruses.

Of the three sets of infiltration basins studies, it was observed that the older basins (30-year operation) compared to those in operation for 10 years, had greater virus attenuation to undetectable virus levels. Several factors, including basin maturity and infiltration rate, could have influenced the variation between infiltration basins. The study suggested that a faster infiltration rate in the younger basins led to more transport and less virus removal. The overall LRVs, ranging from 3.4 to greater than 6.2 for the system, did not allocate percentages of removal to specific steps in the treatment process to determine if soil infiltration or aquifer retention was more responsible for virus attenuation. The current study aims to measure virus attenuation through unsaturated soil and further investigate the effect of infiltration rates on virus attenuation.

The effect of soil type and infiltration rates on virus removal in simulated SAT has also been examined (Quanrud et al., 2003b). The study measured the removal of coliphage (*E. coli* phage) and poliovirus type 1 in three unsaturated soil columns. Each column was packed with a different soil type, one with loamy sand and two with sand, where one of

the sand-filled columns had sodium azide added to inhibit microbial growth. They determined that the column without microbial growth showed significantly lower coliphage removal compared to the other two columns. This finding suggested that microbial activity might play a role in removing viruses in SAT, though the specific mechanisms behind this phenomenon were not investigated in the study. Additionally, the study explored various infiltration rates in these columns. It was found that reducing the infiltration rates increased the retention time in the unsaturated columns. Notably, extending the retention time from 5 hours to 20 hours increased coliphage removal from 70% to 99% (Quanrud et al., 2003b).

Previous experiments have demonstrated greater virus removal in unsaturated column experiments compared to saturated column experiments (Banasiak et al., 2023; Powelson & Gerba, 1994). In the study by Powelson & Gerba (1994), the removal of bacteriophages MS2 and PRD1 and poliovirus type 1 was evaluated in identical soil columns, two operating in saturated flow, and two operating in unsaturated flow. The experiment assumed a first-order kinetic model for virus removal and found that the virus removal coefficient under unsaturated conditions was three times larger than the removal coefficient under saturated conditions. Similarly, Banasiak et al. (2023) measured *E. coli* and MS2 removal under varying degrees of saturation to determine the effect on removal. *E. coli* was removed more readily than MS2 for each condition evaluated. Both unsaturated conditions exhibited more removal than saturated conditions. There was 2-3 times more removal for the 10 mm/hr unsaturated condition, and 4-5 or more times removal for the 0.5 mm/hr unsaturated condition compared to the saturated conditions.

The transport mechanisms in unsaturated flow are complex and impacted by retention at both the AWI and SWI as well as through straining and film straining (Bradford & Torkzaban, 2008). Understanding and predicting the relative impact of these interfaces, especially in varying degrees of saturation, is necessary to forecast the fate of contaminants. In a study by Zhang et al. (2021), the role of both AWI and SWI in removing *E. coli* phage vB_EcoM-ep3 through an unsaturated soil columns was investigated. Different levels of saturation—80%, 50%, and 30%—were examined by measuring and modeling the breakthrough curves of the *E. coli* phage. The findings demonstrated that the removal efficiency of the *E. coli* phage increased at lower degrees of saturation. Surprisingly, the increased removal was not due to an increase in AWI interactions, but rather an increase in attachment to the SWI as the degree of saturation decreased. The soil column influent was deionized water and not reclaimed water. This approach was taken because the study also investigated the effect of ionic strength on the *E. coli* phage removal. Starting with deionized water ensured precise ionic strengths, simultaneously limiting potential microbial growth within the columns. The study did not address the potential influence of an established microbial community on the soil column's pore structure or its potential impact on the transport behavior.

NoV was selected for this study because of its high infection rate (Hall et al., 2013). The prevalence of infection increases the potential to detect NoV in the wastewater treatment plant tertiary effluent and highlights the importance of effective removal through SAT. The attention of NoV in soil column experiments has been demonstrated in several studies with LRVs ranging from 0.29 to 4 (Gamazo et al., 2020; Pang et al., 2021; Weaver et al., 2023). Gamazo et al., (2020) demonstrated the importance of the type of

media and water quality on virus removal. In the study, the NoV LRV increased from 1.7 to 4 when the conditions were changed from quartz columns with deionized spiked water, to columns packed with aquifer sand and groundwater. There was no biomass growth in the alluvial sand columns studies and it was noted that the spiking concentration may have effected NoV removal (Pang et al., 2021; Weaver et al., 2023). In field scale studies where biomass was likely present, NoV was undetectable in water after infiltration through the vadose zone (Elkayam et al., 2018). This study plans to measure the change in indigenous NoV concentration through mature biologically active soil columns to simulate SAT.

HAV is another enteric virus that was selected for this study because of its stability in the environment (Cioffi et al., 2020; Plaza-Garrido et al., 2023). Few soil column experiments have been conducted using HAV. Sobsey et al. (1995) observed over 99.9% removal of HAV in 13.3 cm long columns with different soil types. The experiment did not use RT-qPCR to analyze the HAV concentration which may have affected the results. In this study, the goal is to measure the change in indigenous HAV concentration through mature biologically active soil columns.

Previous experiments have shown that reducing saturation increases virus removal (Anders & Chrysikopoulos, 2009; Torkzaban et al., 2006). Most laboratory studies involving enteric viruses utilize stock solutions (Pang et al., 2021; Weaver et al., 2023) or fecal suspensions (Gamazo et al., 2020) to make spiked virus suspensions. Field studies have highlighted to the importance of biological degradation in virus removal (Bekele et al., 2011). This study will use mature biologically active soil columns to demonstrate the removal of NOV, HAV, and surrogate virus PMMoV. The first objective was to show

biological activity in the soil columns. Then, determine the LRV of NoV, HAV, and PMMoV using only the indigenous virus in the tertiary wastewater treatment plant effluent. This approach will closely simulate a natural infiltration basin and demonstrate the efficacy of virus removal through soil aquifer treatment.

3.0 Materials and Methods

Three identical soil columns were used to simulate soil aquifer treatment. The indigenous concentration of enteric viruses NoV and HAV, and surrogate virus PMMoV, was measured in the influent and effluent to determine the LRV through the column. Three distinct infiltration rates were used to evaluate the effect of the degree of saturation on virus removal. Water quality parameters including carbon, nitrogen, and ATP were measured in the influent and effluent to detect the presence of biomass in the columns, a feature of mature soil systems that is expected to increase contaminant attenuation. Details of the materials and methods used in this experiment are outlined below.

3.1 Column setup

Each of the three columns consisted of 120 cm long and 7 cm inner diameter acrylic glass tubing. The soil is from the vadose zone at Cold Springs Water Reclamation Facility, Washoe County, Nevada, USA, and they have a soil height of 100 cm and bulk density of 1.9 g/cm^3 (Gharoon & Pagilla, 2023). The soil was characterized in a different experiment by Gharoon & Pagilla (2023) and was found to be 86% sand, 13% silt and clay, and <1% gravel. The column porosity and pore volume were calculated to be 0.283 and 1089 cm^3 (Lewis & Sjöstrom, 2010).

The columns included a layer of glass beads at the top and bottom of the soil to evenly distribute the water on top and prevent soil washout on the bottom. There was also a layer of glass wool at the bottom under the glass beads to further prevent washout. More details are present elsewhere (Gharoon & Pagilla, 2023). A schematic diagram of the column setup is shown in Figure 1.

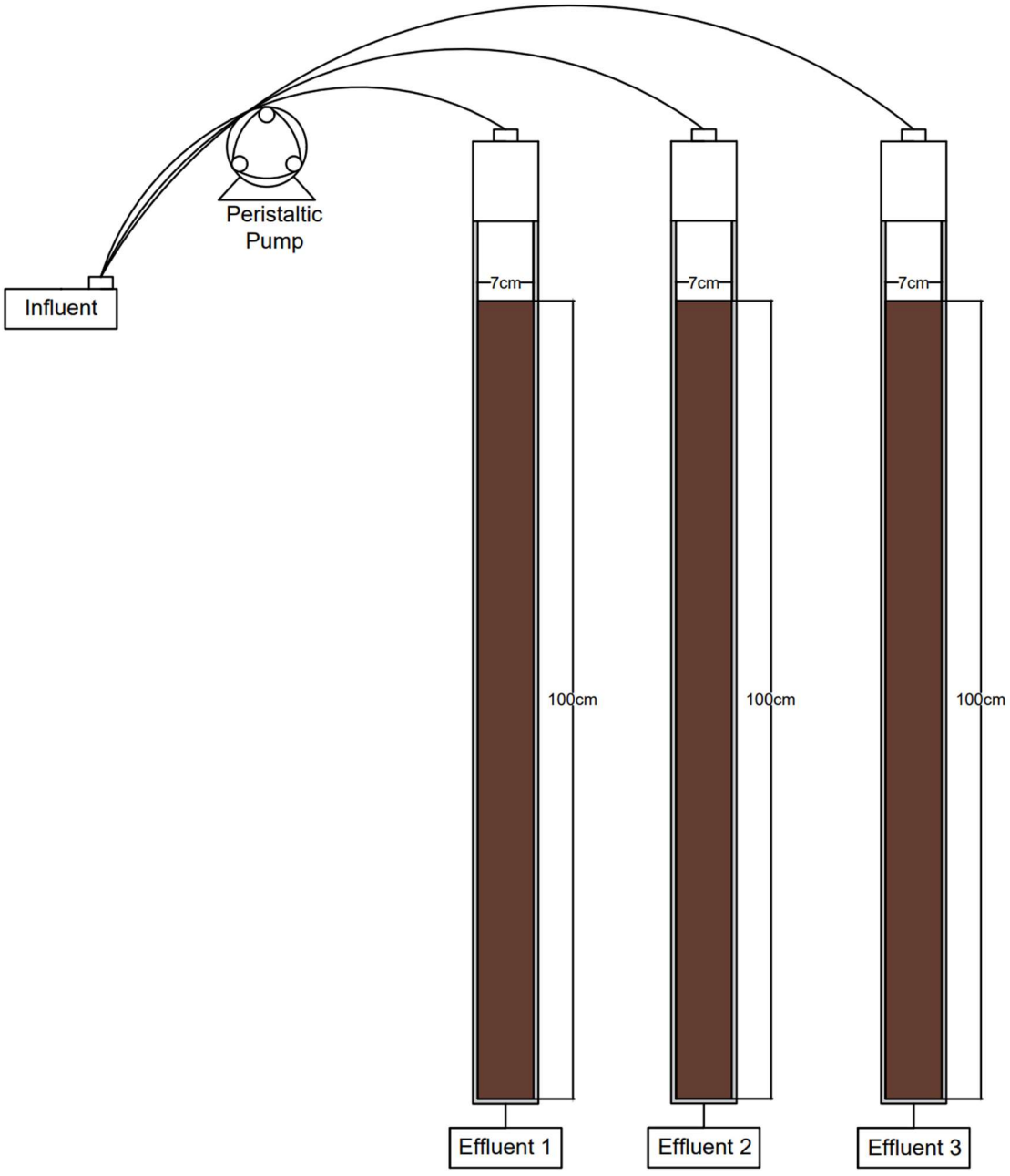

Figure 1. Schematic drawing of the three soil column setup.

3.2 Column operation

Denitrified effluent prior to disinfection from Truckee Meadows Water Reclamation Facility (TMWRF) in Sparks, NV was applied as influent to the soil columns. The water was treated with secondary treatment enhanced biological phosphorus removal (EBPR) consisting of anaerobic-oxic (A/O) basins, and tertiary nitrification and denitrification (Lacroix et al., 2020). The experimental design intentionally excluded the final effluent from the experiment to ensure the highest concentration of viral markers would be added to the soil columns and it was not disinfected.

Reclaimed water was fed to three soil columns through Masterflex L/S 13 Tygon lab tubing with a parasitic pump (Cole-Parmer Masterflex L/S® Digital Drive (Vernon Hills, Illinois, USA)). Various levels of unsaturation were evaluated using infiltration rates of 14.0 mm/hr, 9.35 mm/hr, and 4.68 mm/hr. A 2-week acclimation period was observed before sampling after adjusting the infiltration rate. The columns were operated in a temperature-controlled room ranging from 20-25°C.

3.3 Sample collection and concentration

Water samples were collected from the soil columns 1-2 times per week for viral marker analysis. Each sampling event consisted of one influent sample and three effluent samples, one from each soil column. Effluent samples were collected in sterilized 1-L amber bottles, and the same volume of influent was collected from the influent containers (500-1000 mL each). All water samples were refrigerated at 4^{o}C after collection to preserve their integrity prior to analysis.

The protocol for sample processing included concentration, RNA extraction, and RT-qPCR. Before RNA extraction, effluent samples were concentrated with 100 kDa Amicon® Ultra-15 Centrifugal Filter Units (Millipore Sigma, St. Louis, MO, USA). Effluent volumes of 60 mL were concentrated to volumes between 250 µL and 500 µL, achieving concentration factors ranging from 90 to 240 using Equation 1.

$$Concentration\ factor\ (CF) = \frac{Volume\ of\ water\ filtered}{Volume\ of\ water\ collected\ after\ filtration} \quad (1)$$

3.4 Percent recovery

Influent PMMoV concentrations were used to determine the recovery factor for the Amicon filtration. The influent samples were filtered stepwise, first with a 0.45 µm membrane filtration (MF) vacuum setup to remove suspended particles and obtain a water quality closer to the effluent water. Then, Amicon ultrafiltration (UF) was performed. The percent recovery was calculated using Equation 2 for each sampling event, and the mean percent recovery was used to adjust the measured effluent concentration to the corrected effluent concentration accounting for RNA lost during filtration.

$$\%\ recovery\ UF = \frac{PMMoV\ concentration\ after\ UF}{PMMoV\ concentration\ after\ MF} x\ 100 \quad (2)$$

3.5 RNA extraction and RT-qPCR

Total RNA was extracted from the samples using the AllPrep PowerViral DNA/RNA kit (QIAGEN, Inc., Germantown, MD, USA) following the provided user's manual. The

viral RNA samples were processed on the same-day through RT-qPCR and the remaining RNA was stored at -80°C for further downstream processing. The amount of viral gene markers was quantified with RT-qPCR on the CFX96 Touch Real-Time PCR Detection System (BioRad, Hercules, CA, USA). For PMMoV the GoTaq® Enviro PMMoV Quant Kit, Quasar® 670 (Promega, Madison, WI, USA) was used to quantify the PMMoV RNA for RT-qPCR. The reaction contained 5 µL of sample RNA, 10 µL of GoTaq(R) Enviro Master Mix, 0.4 µL of GoScript™ Enzyme Mix, 1µL of PMMoV Primer/Probe Mix, 0.2 µL of CXR, and 3.4 µL of Nuclease-Free Water for a total reaction volume of 20 µL. Reactions were performed on white 96-well qPCR plates with the following cycling program: reverse transcription at 45 °C for 15 min, reverse transcription inactivation, and GoTaq® activation at 95°C for 2 min, followed by 40 cycles of 15 s denaturation at 95 °C, and 60 s annealing/extension, and the plate read at 60 °C. The threshold cycle (CT) was determined with the default algorithm in CFX Manager Software (BioRad, Hercules, CA, USA). Nuclease-Free Water was utilized as a negative control. A calibration curve was created using six 10-fold serial dilutions of stock PMMoV RNA with a starting concentration of 4×10^6 gene copies/µL. The calibration curve was used to calculate the concentration of PMMoV viral gene markers in the RNA sample (gc/µL). The concentration of PMMoV viral gene markers in the water samples in gene copies per liter was calculated from Equation 3.

$$PMMoV\ concentration\ in\ water\ sample\ \left({}^{gc}/_{L}\right) = \frac{\left(PMMoV\ concentration\ in\ RNA\ extraction\ \left({}^{gc}/_{\mu L}\right)\right) x \frac{10^6 \mu L}{L}}{CF * \frac{Volume\ sample\ added\ to\ RNA\ extraction}{Volume\ eluted\ from\ RNA\ extraction}} \quad (3)$$

Three sampling sets from each infiltration rate with positive PMMoV detection were selected for NoV and HAV analysis. The amount of NoV genotype GI and GII, and HAV gene markers was quantified with the GoTaq® Enviro Noro/HepA RT-qPCR System (Promega, Madison, WI, USA).

The reaction contained 5 µL of sample RNA, 10µL of GoTaq(R) Enviro Master Mix, 0.4 µL of GoScript™ Enzyme Mix, 1µL of Noro GI/Noro GII/HepA Primer/Probe Mix, and 3.6 µL of Nuclease-Free Water for a total reaction volume of 20 µL. Reactions were performed on white 96-well qPCR plates with the following cycling program: reverse transcription at 45 °C for 15 min, reverse transcription inactivation, and GoTaq® activation at 95 °C for 2 min, followed by 40 cycles of 15 s denaturation at 95 °C, and 60 s annealing/extension, and the plate read at 60 °C. The CT was determined with the default algorithm in CFX Manager Software (BioRad, Hercules, CA, USA). Nuclease-Free Water was utilized as a negative control. A calibration curve was created using six 10-fold serial dilutions of stock Noro/HepA dsDNA with a starting concentration of 2×10^6 gene copies/µL. The calibration curve was used to calculate the concentration of NoV GI viral gene marker, NoV GII viral gene marker, and HAV gene marker in the RNA sample (gc/µL). The percent recovery, calculated from the PMMoV viral gene markers, was used to correct the concentrations of NoV and HAV gene markers, as the same pre-processing steps were applied to these samples. The detection limit was established using the lowest dilution of the positive control. For samples with detectable viral gene markers in the influent and viral gene markers below the detection limit in the effluent, half the detection limit divided by the concentration factor of the sample was used as the concentration (Farnham et al., 2002) to calculate the log removal.

3.6 Log Reduction Value

The log reduction value (LRV) was used to evaluate the efficacy of the soil treatment. The LRV was calculated with Equation 4 using the gene marker concentration in the influent and effluent samples collected on the same day. The mean LRV was compared for each infiltration rate to evaluate the efficiency of the treatment process.

$$LRV = log_{10}\left(\frac{Influent\ Viral\ Gene\ Marker\ Concentration\ \left({}^{gc}/_{L}\right)}{Effluent\ Viral\ Gene\ Marker\ Concentration\ \left({}^{gc}/_{L}\right)}\right) \quad (4)$$

3.7 Water quality parameters

Adenosine triphosphate (ATP), nitrogen (TKN, ammonia, nitrate/nitrite), and dissolved organic carbon (DOC) were also measured for the column influent and effluent to determine the presence or absence of microbes within the columns and to verify that the columns were at a steady state. Total ATP was measured on the soil surface from soil swabs at 15 cm from the top of the soil columns and 15 cm from the bottom of the soil columns using the Deposit & Surface Analysis (DSA) Test Kit by LuminUltra Technologies Ltd. (Fredericton, New Brunswick, Canada) based on the manufacturer's manual. Cellular ATP in the soil column influent and effluent was measured with the Quench-Gone Aqueous (QGA) test kit by LuminUltra Technologies Ltd. (Fredericton, New Brunswick, Canada) from 50 mL water samples based on the manufacturer's manual. Total nitrogen (TN), Total Kjeldahl Nitrogen (TKN-N), and nitrate/nitrite-N were measured with the TKN-N TNT 880 kit by HACH (Loveland, CO, USA). The kit measures TN and then subtracts inorganic nitrogen to determine TKN-N. Ammonia was measured with the TNT830 by HACH (Loveland, CO, USA). Dissolved organic carbon

(DOC) concentration was measured using a total organic carbon (TOC) analyzer (Shimadzu, Japan). DOC samples were filtered with a 0.45 μm syringe filter before analysis. DOC samples were acidified on the collection date with phosphoric acid to a pH below 2 and stored at less than 20°C for up to 28 days before analysis. Nitrogen measurements were completed on the day of sample collection.

3.8 Statistical analysis

Datasets for influent and effluent at each infiltration rate were analyzed for PMMoV, NoV, HAV, nitrogen, and DOC. Each dataset was assessed for normality with a Shapiro-Wilk test. The null hypothesis was that the data was normally distributed, and the alpha value was set to 0.05. The data was then categorized as parametric or nonparametric based on the p-value (Yap & Sim, 2011).

Datasets with a p-value greater than 0.05 were considered normally distributed and categorized as parametric data. Parametric data was analyzed with a one-way ANOVA test to compare the means of the different groups and assess for statistically significant differences. If the ANOVA test showed there was a statistically significant difference in means, a post-hoc Tukey HSD test was used to identify which groups had the statistically significant differences.

Datasets with p-values less than or equal to 0.05 were considered non-normally distributed and categorized as nonparametric data. Nonparametric data was analyzed with a Kruskal-Wallis test to compare the means of the different groups and assess for statistically significant differences. If the Kruskal-Wallis test showed there was a statistically significant difference in the means, post-hoc Wilcoxon rank-sum tests were

used to compare the means of paired groups and identify which groups had the statistically different means.

4.0 Results and Discussion

The biologically active soil system demonstrated over a 2 LRV in the concentration of the surrogate virus PMMoV. The simultaneous decrease in DOC, along with the reduction in TKN, and a concurrent increase in nitrate/nitrite strongly suggest the presence of biomass, likely indicating the presence of nitrifiers in the soil system. This conclusion was further supported by the presence of ATP in the effluent and on the soil surface indicating the presence of biomass. The concentration of NoV GI and GII decreased below detection limits. While, HAV remained undetectable in any of the influent samples, which is a limitation of relying on indigenous virus concentration without spiking in this study. No difference in virus attenuation was observed among the three tested infiltration rates, suggesting that other factors may have had a greater influence on their removal efficiency. Further in-depth discussion of these results is provided below.

4.1 Water quality parameters

Water quality parameters including DOC, ammonia, TKN, and nitrate/nitrite were measured in both the influent and effluent from February to July 2023. ATP was measured from the soil surface and in the influent and effluent on one sampling event. Twenty-one influent DOC samples were collected, and 61 effluent samples were collected. For ammonia, TKN, and nitrate/nitrite, 19 influent samples and 54 effluent samples were collected.

The DOC concentration decreased between the influent and effluent of the columns at each infiltration rate tested. This decrease in DOC concentration suggests the presence of

microbial activity in the columns (Shabani et al., 2020). A time plot of the DOC concentration in the influent and effluent is shown in Figure 2. The smallest change in DOC concentrations was observed for the intermediate infiltration rate (9.35 mm/hr), though the difference was only statistically significant between infiltration rates of 4.86 mm/hr and 9.35 mm/hr (p-value = 0.00053). The variation in DOC removal may be due to the differences in influent DOC concentrations, and not the removal mechanism, because the effluent DOC concentrations from infiltration rates of 14.0 mm/hr and 9.35 mm/hr were similar. The average and standard deviation of the effluent DOC concentrations for those infiltration rates were 3.2 ± 0.45 mg-C/L and 3.2 ± 0.3 mg-C/L (p-value = 0.588). This could indicate all the biologically available DOC was removed at 9.35 mm/hr and the smaller change in DOC at this infiltration rate was due to the lower influent DOC concentration.

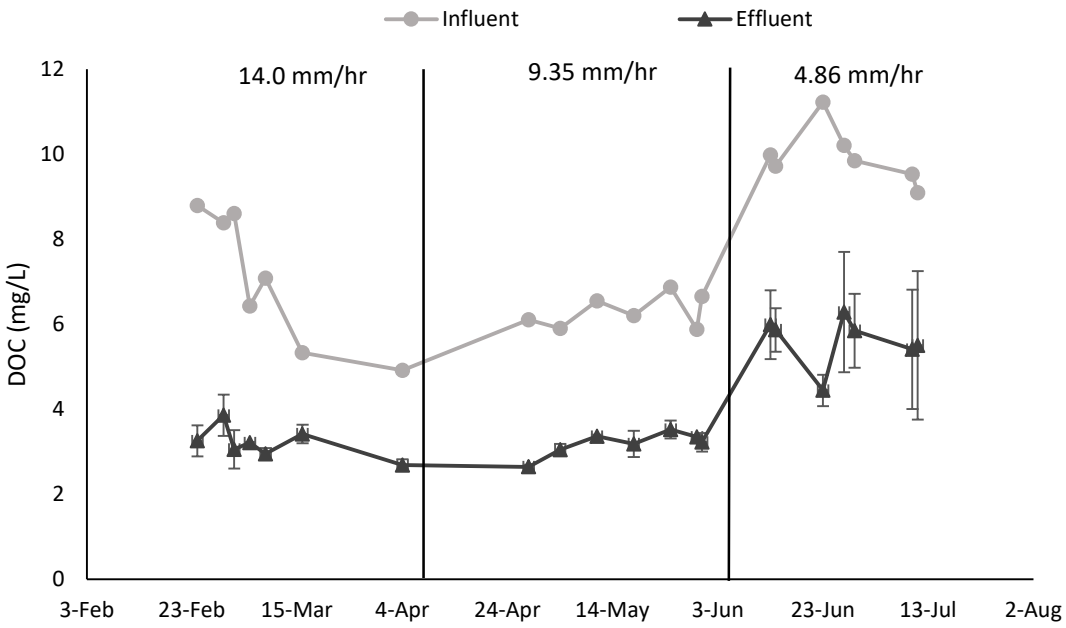

Figure 2. Time plot of DOC removal during soil column treatment.

The removal of organic carbon typically involves processes like adsorption and biodegradation. However, in this specific case, with the simultaneous conversion of nitrogen species, we expect that the DOC removal is due to heterotrophic bacteria, potentially using the DOC and/or nitrate/nitrite. In Figure 3, we observe that in the influent, the nitrogen concentration in the form of TKN is higher than the concentration in the form of nitrate/nitrite. Conversely, in the effluent, the concentration of nitrogen as nitrate/nitrite exceeds that of TKN. The effluent TKN concentrations were less than 1 mg/L for all the samples, suggesting that the organic nitrogen was either consumed by bacteria as a nutrient or converted into nitrate/nitrite.

Ammonia was also measured in the influent and effluent. The average and standard deviation of ammonia concentration in the influent was 0.78 ± 1.15 mg-N/L. Ammonia was on average less than 20% of the total nitrogen in the influent. In the effluent, ammonia was nearly completely removed, all values fell below the lower detection limit of the test kit, and were less than 0.015 mg-N/L. The removal of ammonia and TKN indicates both ammonification and nitrification are occurring as found in previous studies by Gharoon & Pagilla (2023).

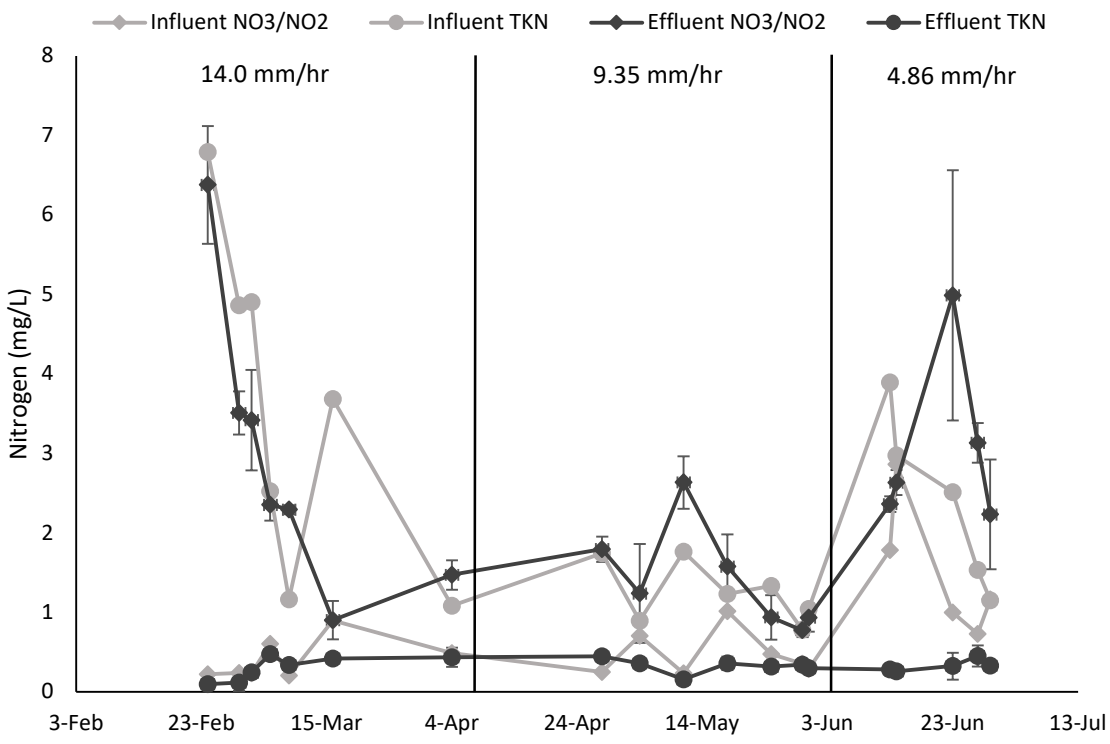

Figure 3. Time plot of nitrogen species before and after soil column treatment.

The average and standard deviations for the changes in DOC, TKN, and nitrate/nitrite through soil column treatment is summarized in Table 3. There was no statistically significant difference in the concentration of TKN-N removed and nitrate/nitrite-N produced for any of the infiltration rates tested; the p-values were 0.126, 0.359, and 0.389 for infiltration rates of 4.86 mm/hr, 9.35 mm/hr, and 14.0 mm/hr. This coupled with the low concentrations of ammonia likely indicate the conversion of TKN to nitrate/nitrite is through ammonification follow by nitrification as found in previous soil column studies (Essandoh et al., 2013; Gharoon & Pagilla, 2023).

Table 3. Average changes in DOC, TKN, and nitrate/nitrite

	Infiltration Rate		
	4.86 mm/hr	**9.35 mm/hr**	**14.0 mm/hr**
Average decrease in DOC (mg-C/L)	4.3 ± 1.4	3.1 ± 0.4	3.9 ± 1.5
Average decrease in TKN (mg-N/L)	2.1 ± 1.1	0.9 ± 0.4	3.5 ± 2.2
Average increase in nitrate/nitrite (mg-N/L)	1.6 ± 1.7	0.9 ± 0.8	2.7 ± 2.0

Nitrification is an aerobic process, and it was anticipated that changing the infiltration rate and retention times would affect nutrient loading and nitrification efficiency. This was not observed, there was no consistent pattern between infiltration rate and the change in nutrient concentrations. The lowest infiltration rate (4.86 mm/hr) did not exhibit statistically significant differences in TKN removal or nitrate/nitrite production when compared to the fastest infiltration rate (14.0 mm/hr). The intermediate infiltration rate of 9.35 mm/hr had a lower TKN removal (0.9 ± 0.4 mg-N/L) compared to infiltration rates of 4.86 mm/hr (2.1 ± 1.1 mg-N/L) and 14.0 mm/hr (3.5 ± 2.2 mg-N/L). Like with DOC removal, a statistically significant difference was only observed between 4.86 mm/hr and 9.35 mm/hr (p-value = 0.00086). The production of nitrate/nitrite was also the lowest at 9.35 mm/hr (0.9 ± 0.8 mg-N/L), in contrast to the results for the other nutrients this difference was statistically significant when compared to the nitrate/nitrite production at 14.0 mm/hr (2.7 ± 2.0 mg-N/L, p-value = 0.0013).

The variation in the nutrient concentration in the influent may have contributed to the lack of consistent patterns between the infiltration rates and the change in nutrient concentration. The influent water was reclaimed wastewater with variable concentrations of DOC, ammonia, TKN, and nitrate/nitrite which increased the variation in the change in nutrient concentration data. Additionally, the low concentrations of these water quality parameters also increased the data variability. Small fluctuations in the concentrations measured had a relatively large impact on the average concentrations and standard deviations because the fluctuations represented a large percentage of the measured concentrations. No correlation between nitrification and virus removal were observed and

the differences in TKN removal and nitrate/nitrite production between infiltration rates did not affect the PMMoV LRV.

ATP was measured on the soil surface and in the soil column influent and effluent to further examine the presence of biomass. All ATP measurements were conducted at an infiltration rate of 9.35 mm/hr. Total ATP which includes ATP from dead and alive cells was measured on the soil surface while cellular ATP was measured in influent and effluent water and only includes ATP from live cells. The ATP on the soil surface was measured at 15 cm from the top and 15 cm from the bottom of the soil columns for all three columns. The total ATP concentrations for the soil are shown in Table 4. There was a higher concentration of total ATP indicating more biomass at the bottom of the soil columns compared to at the top of the soil columns. The difference in ATP measurements between the two locations is likely because the measurement at the top of the soil column was in the transition area of the infiltration and did not receive as consistent water and nutrients for biomass growth compared to the bottom of the soil column.

The influent cellular ATP was significantly higher than the effluent cellular ATP as shown in Table 4. These results were expected because the column influent is unfiltered pre-disinfected reclaimed wastewater that has undergone biological processes including nitrification and denitrification, so it was likely that there were active microorganisms in the column influent which may have helped seed the biomass in the columns. The presence of cellular ATP in the soil column effluent further supports the observation of active microorganisms in the soil columns.

Table 4. Summary of ATP measurements

Soil tATP (pg /cm^2)		Water cATP (pg /mL)	
15 cm from top	**15 cm from bottom**	**Influent**	**Effluent**
1645.6 ± 136.3	2539.6 ± 655.5	2047.5 ± 5.2	31.2 ± 13.1

4.2 PMMoV

The surrogate virus PMMoV was successfully removed through soil treatment for each infiltration rate tested. The average LRVs of 2.80 ± 0.36 for an infiltration rate of 4.86 mm/hr, 2.91 ± 0.48 for an infiltration rate of 9.35 mm/hr, and 2.72 ± 0.32 for an infiltration rate of 0.9 mm/hr, demonstrate over 99% removal. Notably, varying the infiltration rate through the soil columns had a limited impact on the extent of PMMoV removal. This suggests that both the beginning and end of the spreading basin wetting cycle can achieve similar virus removal. The advection-dispersion equation with a first order reaction term is often used to model biocolloid transport in unsaturated porous media (Bradford et al., 2003; Šimůnek et al., 2006; Tufenkji, 2007). In this experiment changing the velocity through the soil column did not significantly affect the virus attenuation, indicating the transport was limited by either diffusion or a reaction.

The concentration of PMMoV gene markers in the soil column influent and effluent was measured from February to July 2023. Twenty-one influent samples and 61 effluent samples were collected from the three columns. The average influent PMMoV gene marker concentration was $1.25 \times 10^7 \pm 8.75 \times 10^6$ gc/L. In the effluent, the average PMMoV gene marker concentrations were $1.59 \times 10^4 \pm 1.97 \times 10^4$, $2.94 \times 10^4 \pm 3.81 \times 10^4$, and $3.53 \times 10^4 \pm 2.96 \times 10^4$ gc/L for infiltration rates of 4.86 mm/hr, 9.35 mm/hr, and 14.0 mm/hr, respectively. Standard deviation is shown for all averages with the plus/minus sign. The raw data for the influent and effluent PMMoV gene marker concentrations are in Table 6 and Table 7 of the Appendix.

Statistical analysis with the Kruskal-Wallis test demonstrated a significant difference in PMMoV concentrations between influent and effluent samples (p-value = 4.10×10^{-11}) as shown in the Figure 4 boxplot of the PMMoV concentrations. Subsequent pairwise comparisons using the Wilcoxon rank-sum test showed statistically significant differences between the mean PMMoV concentrations in the influent and effluent for all infiltration rates, yielding p-values of 3.72×10^{-12}, 3.72×10^{-12}, and 1.55×10^{-10} for infiltration rates of 4.86 mm/hr, 9.35 mm/hr and 14.0 mm/hr, respectively. These results indicate the simulated soil column treatment was effective at reducing PMMoV concentration.

Comparing the effluent PMMoV concentration at each infiltration rate, we observed a significant difference in PMMoV concentration of $3.53 \times 10^4 \pm 2.96 \times 10^4$ gc/L at 14.0 mm/hr when compared to the concentration of to $1.59 \times 10^4 \pm 1.97 \times 10^4$ gc/L at the lowest infiltration rate of 4.86 mm/hr, (p-value = 0.00203). Conversely, no statistically significant difference was found when comparing the effluent PMMoV concentrations at 4.86 mm/hr and 9.35 mm/hr (p-value = 0.102), nor at 9.35 mm/hr and 14.0 mm/hr (p-value = 0.187).

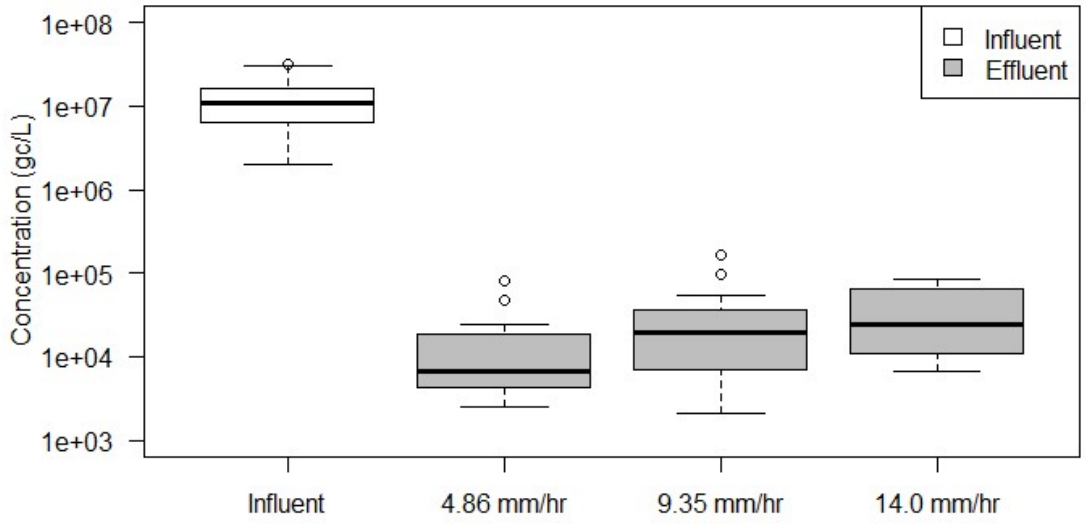

Figure 4. PMMoV concentration in the soil columns influent and effluent.

The degradation of PMMoV at the soil surface, facilitated by a combination of endogenous decay, microbial activity, and adsorption, establishes the concentration gradient that drives virus transport. It is expected that endogenous decay does not play a significant role in the attenuation of PMMoV within this specific soil treatment. Several studies have investigated the stability of viruses, including PMMoV, and have reported decay constants that vary based on the type of water tested, such as spiked freshwater, spiked saltwater, and raw sewage (Burnet et al., 2023; Greaves et al., 2020; Li et al., 2023; Roldan-Hernandez et al., 2022; Sala-Comorera et al., 2021). The decay rate of PMMoV in wastewater tertiary effluent was most accurately represented by the decay rate found in raw sewage at 20°C, which had a decay constant of k_d=0.011/day (Li et al., 2023). The amount of PMMoV removed due to endogenous decay was estimated using first-order kinetics. The average PMMoV concentration measured in the influent in this study was used as the initial concentration (C_o) in Equation 6. The residence time was estimated for each infiltration rate using the flow rate (Q) and the column pore volume (V_p) in Equation 7.

$$C_e = C_o e^{-k_d t} \quad (6)$$

$$t = \frac{V_p}{Q} \quad (7)$$

If endogenous decay were the sole mechanism responsible for PMMoV removal, the anticipated PMMoV concentration in the effluent at an infiltration rate of 4.86 mm/hr and residence time of 2.52 days, would be 1.22 x 10^7 gc/L, yielding a LRV of 0.012. Likewise, for an infiltration rate of 9.35 mm/hr and a residence time of 1.26 days, the

expected concentration would be 1.23 x 10^7 gc/L, a LRV of 0.006. Finally, at an infiltration rate of 14.0 mm/hr and a residence time of 0.84 days, the projected concentration would be 1.24 x 10^7 gc/L, resulting in a LRV of 0.004. These endogenous decay LRVs are estimations based on the pore volume and flow rates, but in an unsaturated system the entire pore volume is not filled, and the retention times would vary from these estimations. However, the estimated LRVs still demonstrate the small influence endogenous decay had on PMMoV removal. For the LRV exclusively from endogenous decay to reach 2 the retention time would need to be 418 days which given the scale of the soil columns used is very unlikely and indicates another mechanism is driving PMMoV removal.

The LRV was calculated using Equation 4 to evaluate the effectiveness of simulated soil treatment at removing surrogate virus PMMoV. Boxplots of the LRVs are shown in Figure 5. The average PMMoV LRVs were 2.80 ± 0.36 for an infiltration rate of 4.86 mm/hr, 2.91 ± 0.48 for an infiltration rate of 9.35 mm/hr, and 2.72 ± 0.32 for an infiltration rate of 14.0 mm/hr, with the highest LRV observed at the intermediate infiltration rate of 9.35 mm/hr. Notably, there was no statistically significant difference in LRVs among the different infiltration rates (p-value = 0.329).

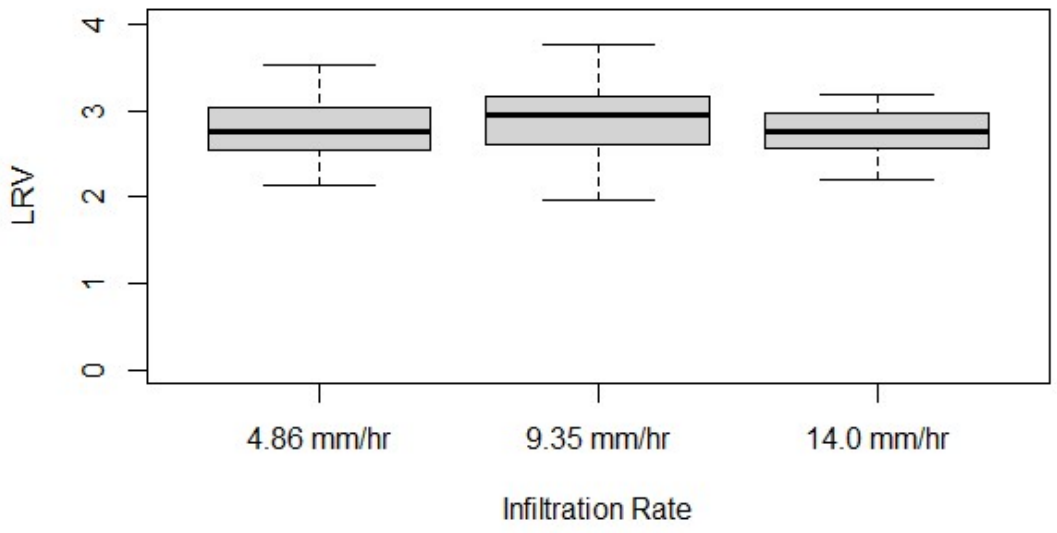

Figure 5. PMMoV LRVs after soil column treatment.

Diffusion-limited transport implies that a longer retention time would increase the virus attenuation. However, in our experiments, this was not observed for the three infiltration rates and subsequent retention times tested. We did observe a difference in effluent PMMoV concentration between the highest infiltration rate (14.0 mm/hr) and the lowest infiltration rate (4.86 mm/hr). This difference is likely due to the higher infiltration rate providing an insufficient retention time for the complete diffusion of viruses susceptible to degradation. In contrast, the slower infiltration rate (4.86 mm/hr) allows for an extended period for diffusion to the soil surface, resulting in more degradation and, consequently, lower virus concentrations. Increasing the retention time between an infiltration rate of 9.35 mm/hr and 4.86 mm/hr did not significantly alter the virus concentration in the effluent. Indicating once there is sufficient retention time for diffusion, increasing the retention time further does not change the amount of PMMoV removed. This suggests that the transport of PMMoV is reaction-limited rather than diffusion-limited because a longer retention time, which allows for more diffusion, did not increase the removal of PMMoV.

Despite the difference in effluent PMMoV concentration between 14.0 mm/hr and 4.86 mm/hr, statistically significant differences in LRVs were not observed between the two infiltration rates. This lack of significance is attributed to the requirement for order of magnitude variations in effluent concentrations to affect the LRV, which was not observed in our study.

4.3 Enteric viruses

The importance of studying PMMoV lies in its role as a surrogate for enteric viruses. In this context, comparing PMMoV concentrations with HAV and NoV concentrations in the column influent and effluent is essential. Enteric virus gene markers were measured in nine influent samples and 25 effluent samples from the three columns over the five-month sampling period. HAV was undetectable in all influent and effluent samples, likely due to the absence of an outbreak in the population during the study (Plaza-Garrido et al., 2023). NoV genotypes GI and GII were detectable in all nine influent samples at lower concentrations than PMMoV as expected (Kitajima et al., 2018), but were undetectable all measured effluent samples.

The average and standard deviations for NoV GI and GII gene marker concentrations in the influent were $1.10 \times 10^5 \pm 9.36 \times 10^4$ gc/L for NoV GI and $1.74 \times 10^5 \pm 2.61 \times 10^5$ gc/L for NoV GII. NoV gene markers were successfully reduced with soil column treatment and all effluent samples fell below the detection limit at each infiltration rate. The effluent NoV concentrations were estimated using half the LoD, as recommended by Farnham et al. (2002). This method utilized the lowest detectable concentration, which for both NoV GI and GII was the lowest dilution of the standard curve and since the same standard was used for GI and GII standard curves, the LoD for both gene markers was the same value. The average effluent concentrations of NoV gene markers were calculated as $1.38 \times 10^3 \pm 1.62 \times 10^2$ gc/L for 4.86 mm/hr, $1.98 \times 10^3 \pm 8.76 \times 10^2$ gc/L for 9.35 mm/hr, and $3.32 \times 10^3 \pm 4.08 \times 10^2$ gc/L for 14.0 mm/hr. Even though the effluent concentrations were all based on the lowest dilution of the standard curve, there was still variation within these values because of the different concentration factors of each sample which ranged

from 90 to 240. All the influent and effluent NoV samples were concentrated prior to RNA extraction therefore the below detection limit values accounted for the concentration factor in the same way the detectable values did.

Statistical analysis using the Kruskal-Wallis test showed significant differences between influent and effluent concentrations for NoV GI and GII, with p-values of 6.54×10^{-6}. The raw data for the influent and effluent HAV, NoV GI, and NoV GII gene marker concentrations are included Table 8 and Table 9 of the Appendix.

The log removal of NoV GI and GII were calculated at each infiltration rate using Equation 4. The log removal instead of LRV was used because the effluent concentrations were estimated so the calculated value was not a true LRV. The log removals of NoV GI and GII are not equivalent because the influent NoV GI and GII concentrations varied. Boxplots of the log removal of NoV GI and GII at each infiltration rate are shown in Figure 6. At an infiltration rate of 4.86 mm/hr the log removals of NoV GI and GII were 1.42 ± 0.07 and 1.14 ± 0.19, respectively. At an infiltration rate of 9.35 mm/hr the log removals were 1.64 ± 0.29 and 1.58 ± 0.21 for NoV GI and GII, respectively. For an infiltration rate of 14.0 mm/hr, the log removals were 1.74 ± 0.18 for NoV GI and 1.87 ± 0.41 for NoV GII. For NoV GI the only statistically significant difference between log removals were between 4.86 mm/hr and 14.0 mm/hr (p-value= 0.012). For NoV GII, the log removal at 4.86 mm/hr was statistically different from both of other infiltration rates, p-value = 0.000493624 between 4.86 mm/hr and 9.35 mm/hr and p-value = 0.00017 between 4.86 mm/hr and 14.0 mm/hr.

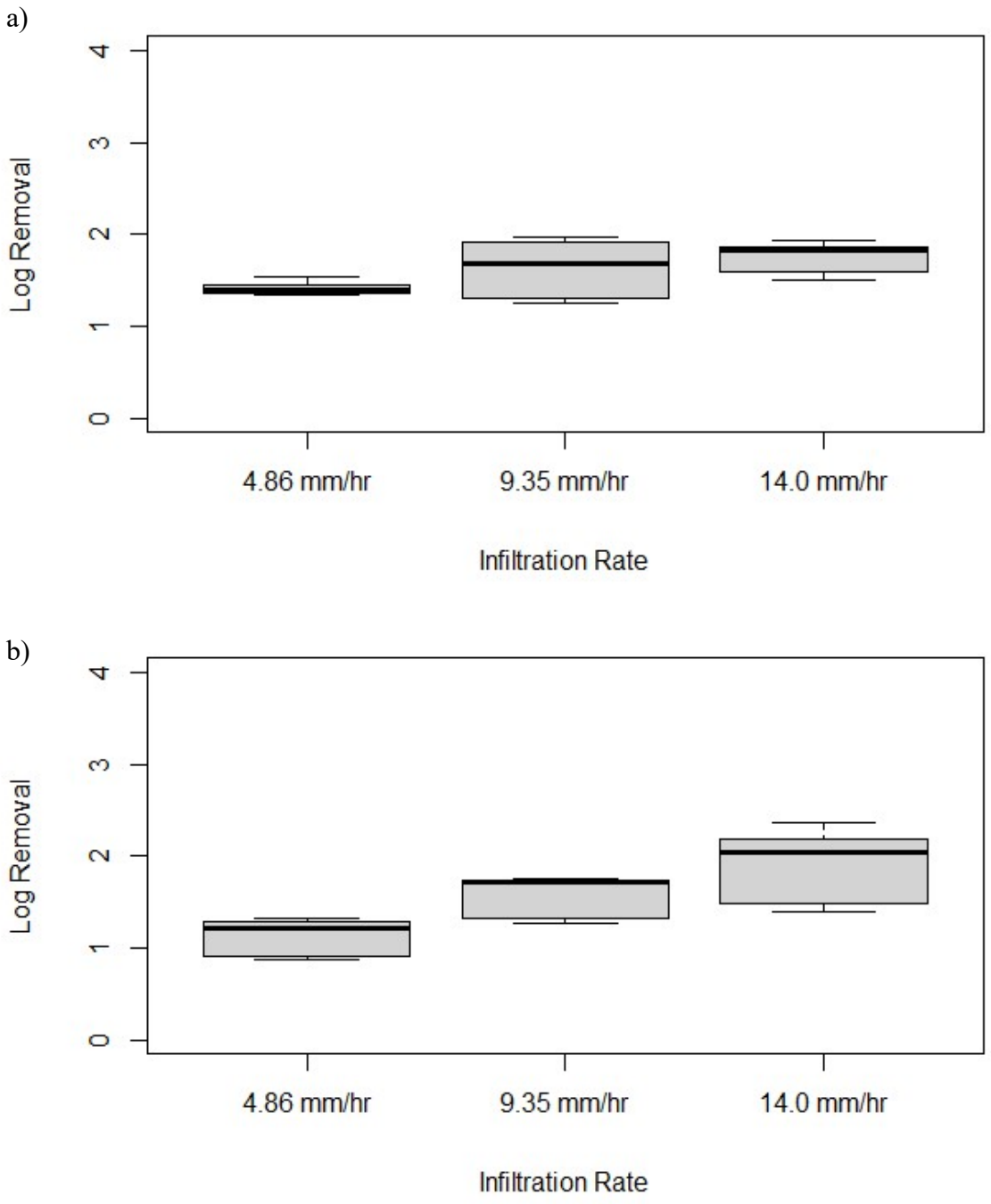

Figure 6. NoV GI (a) and NoV GII (b) log removal after soil column treatment.

Box plots of the PMMoV, NoV GI and NoV GII gene marker concentrations in the influent and effluent at infiltration rates of 4.86 mm/hr, 9.35 mm/hr, and 14.0 mm/hr are shown in Figure 7. The higher PMMoV concentration allowed the viral marker to be measured in the influent without sample concentration, unlike with NoV GI and GII. The Amicon percent recovery of 22% for the concentration process was determined by comparing the concentration of PMMoV in non-concentrated and concentrated samples using Equation 2. This process is not feasible with NoV gene markers directly since they were only detectable after concentration. Therefore, measuring PMMoV is necessary even when NoV is detectable. Additionally, both NoV GI and GII were undetectable in the soil column effluents. The concentrations shown in Figure 7 are based on half the LoD (Farnham et al., 2002). These values are approximations and could potentially over- or underestimate the removal of NoV GI and GII. Overestimating enteric virus removal could be detrimental to public health when designing a process based on estimated values. Conversely, underestimation could lead to the over-designing of systems and increased capital costs for the removal of a virus that is not actually present. Accurate LRV calculation is important for process design, and direct PMMoV measurement allows for LRVs to be directly measured.

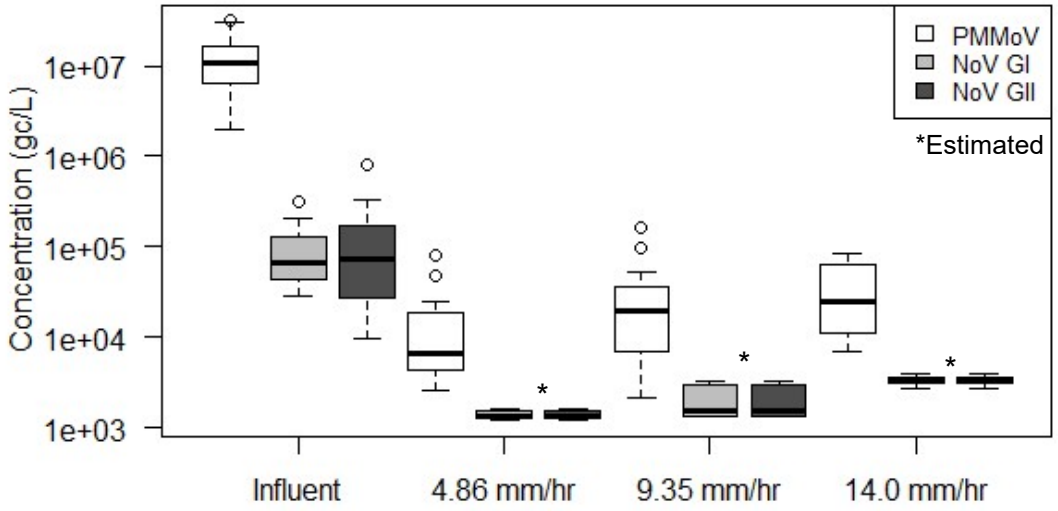

Figure 7. PMMoV, NoV GI, and NoV GII gene marker concentrations.

The ability to detect PMMoV in the effluent is essential because it allows for the calculation of LRVs to evaluate the efficiency of virus removal in the soil treatment process. Table 5 shows the log removal of PMMoV, NoV GI and NoV GII at the three infiltration rates tested. The log removal of the enteric viruses were estimated based on half the LoD for the measurement method, as the concentrations in the effluent were below the detection limit. The log removal of NoV GI and GII were consistently lower than those for PMMoV at each infiltration rate tested, with p-values of 1.718×10^{-7}, 9.406×10^{-7}, and 2.819×10^{-5} for infiltration rates of 4.86 mm/hr, 9.35 mm/hr, and 14.0 mm/hr, respectively.

This variation could be due to the physicochemical differences between the viral gene markers or because of the detection method limitations. Determining the cause for the lower log removal in enteric viruses is challenging, especially because the effluent concentrations were not detectable and may have been close to zero. If the enteric virus concentrations in the effluent were near zero, the log removal of NoV GI and GII would be larger than those estimated from half the LoD and closer to the LRVs measured for PMMoV. Even with these limitations, it is clear the concentration of enteric and surrogate viruses was reduced with soil column treatment. Conducting additional experiments to establish a correlation between NoV GI, NoV GII, and PMMoV would be valuable for assessing enteric virus removal using a surrogate.

Table 5. Log removal of PMMoV, NoV GI and GII by infiltration rate

Infiltration Rate	Virus		
	PMMoV	**NoV GI**	**NoV GII**
4.86 mm/hr	2.80 ± 0.36	> 1.41 *	> 1.14 *
9.35 mm/hr	2.91 ± 0.48	> 1.64 *	> 1.59 *
14.0 mm/hr	2.72 ± 0.32	> 1.74 *	> 1.87 *

*Effluent NoV GI and GII values based on LoD/2.

5.0 Conclusions

This study demonstrated the removal of PMMoV and enteric virus gene markers in lab-scale unsaturated soil columns and that PMMoV is an important surrogate for enteric viruses, particularly in cases where the latter are challenging to detect.

- Soil column treatment can achieve a LRV over 2 for PMMoV and non-detectable levels of enteric virus across various infiltration rates.
- The removal of DOC, ammonia, and TKN, and the production of nitrate/nitrite provide compelling evidence of microbial activity within the soil column. It is suggested that microbial mechanisms play a pivotal role in virus removal, further research is required for confirmation.
- Varying the infiltration rate had limited impact on the extent of PMMoV removal within the biologically active soil columns suggesting both the beginning and end of a spreading basin wetting cycle can achieve similar virus removal.

PMMoV exhibited larger LRVs compared to the enteric viruses which may be attributed to physicochemical variations between the viral gene markers or because the enteric virus log removals were calculated based on half the LoD because no enteric virus gene markers were detected in the soil column effluent. Further research to establish a correlation between NoV GI, NoV GII, and PMMoV LRVs would be beneficial because based on these results PMMoV removal is not guiding for determining NoV removal. It was proposed that reaction-limited transport serves as the constraining factor in gene marker transport within the soil system. Further studies on modeling the removal

mechanisms would be beneficial to gain a comprehensive understanding of how these markers are removed.

6.0 References

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7.0 Appendix

Table 6. Influent PMMoV gene marker, Nitrate/nitrite, TKN and DOC raw data

Date	PMMoV Concentration (gc/L)	Ammonia-N (mg/L)	TKN-N (mg/L)	Nitrate/Nitrite-N (mg/L)	DOC (mg/L)
24-Feb	3.02E+07	3.17	6.79	0.219	8.789
1-Mar	1.29E+07	0.674	4.86	0.234	8.381
3-Mar	1.14E+07	3.16	4.9	0.23	8.596
6-Mar	1.13E+07	1.284	2.52	0.601	6.426
9-Mar	6.25E+06	0.03	1.16	0.201	7.080
16-Mar	6.17E+06	0.082	3.68	0.893	5.321
4-Apr	1.80E+07	0.092	1.08	0.485	4.913
28-Apr	4.95E+06	0.052	1.74	0.246	6.098
4-May	7.67E+06	0.027	0.891	0.703	5.900
11-May	1.08E+07	0.02	1.76	0.229	6.547
18-May	1.26E+07	0.054	1.23	1.01	6.200
25-May	1.62E+07	0.282	1.33	0.472	6.866
30-May	3.12E+07	0.027	0.779	0.351	5.871
31-May	2.25E+07	0.027	1.04	0.283	6.646
13-Jun	6.69E+06	2.88	3.89	1.78	9.979
14-Jun	2.67E+07	2.02	2.97	2.86	9.711
23-Jun	1.08E+07	0.912	2.51	0.997	11.221
27-Jun	8.45E+06	0.088	1.53	0.726	10.203
29-Jun	3.24E+06	0.058	1.15	1.16	9.841
10-Jul	1.99E+06	*	*	*	9.526
11-Jul	2.26E+06	*	*	*	9.092

* HACH TNT 830 and TKN-N TNT 880 kit not available for July 10th and 11th samples.

Table 7. Effluent PMMoV gene marker, Nitrate/nitrite, TKN and DOC raw data

Date	Column #	Infiltration rate (mm/hr)	PMMoV Concentration (gc/L)	Ammonia -N (mg/L)	TKN-N (mg/L)	Nitrate/ Nitrite-N (mg/L)	DOC (mg/L)
24-Feb	1	14.0	8.51E+04	-0.002	0.072	5.59	3.671
24-Feb	2	14.0	2.88E+04	0.000	-0.022	7.06	2.996
24-Feb	3	14.0	8.03E+04	-0.004	0.235	6.48	3.100
1-Mar	1	14.0	7.91E+04	-0.004	0.230	3.20	4.405
1-Mar	2	14.0	2.57E+04	-0.010	0.074	3.60	3.490
1-Mar	3	14.0	8.20E+04	-0.003	0.037	3.72	3.672
3-Mar	1	14.0	7.00E+04	0.003	0.271	2.69	3.573
3-Mar	2	14.0	8.33E+03	-0.001	0.237	3.74	2.831
3-Mar	3	14.0	5.92E+04	-0.001	0.214	3.82	2.761
6-Mar	2	14.0	2.32E+04	-0.002	0.489	2.21	3.199
6-Mar	3	14.0	2.42E+04	-0.003	0.452	2.49	3.222
9-Mar	2	14.0	7.51E+03	0.004	0.289	2.33	3.042
9-Mar	3	14.0	7.20E+03	0.003	0.379	2.25	2.855
16-Mar	1	14.0	1.06E+04	0.002	**	**	3.293
16-Mar	2	14.0	6.80E+03	0.003	0.426	1.07	3.286
16-Mar	3	14.0	1.11E+04	-0.005	0.408	0.73	3.665
4-Apr	1	14.0	1.88E+04	-0.004	0.337	1.61	2.664
4-Apr	2	14.0	1.55E+04	-0.003	0.395	1.54	2.829
4-Apr	3	14.0	2.75E+04	-0.010	0.567	1.26	2.548
28-Apr	1	9.35	1.23E+04	-0.011	0.416	1.93	2.671
28-Apr	2	9.35	8.25E+03	-0.018	0.420	1.62	2.584
28-Apr	3	9.35	5.35E+04	-0.005	0.493	1.83	2.670
4-May	1	9.35	5.97E+03	-0.005	0.324	0.94	3.118
4-May	2	9.35	5.23E+03	-0.004	0.347	1.95	3.131
4-May	3	9.35	4.97E+04	-0.006	0.395	0.82	2.892
11-May	1	9.35	1.27E+04	-0.009	0.231	2.36	3.402
11-May	2	9.35	2.10E+03	-0.006	0.090	3.00	3.361
11-May	3	9.35	1.90E+04	-0.009	0.139	2.54	3.317
18-May	1	9.35	6.23E+03	-0.017	0.284	1.95	2.911
18-May	2	9.35	2.17E+03	-0.007	0.448	1.63	3.519
18-May	3	9.35	4.20E+04	-0.006	0.337	1.14	3.117
25-May	1	9.35	7.22E+03	-0.017	0.285	0.83	3.606

25-May	2	9.35	6.90E+03	-0.007	0.340	1.25	3.676
25-May	3	9.35	3.61E+04	-0.006	0.314	0.72	3.278
30-May	1	9.35	2.22E+04	-0.004	0.319	0.77	3.321
30-May	2	9.35	2.10E+04	-0.004	0.385	0.86	3.301
30-May	3	9.35	1.63E+05	0.001	0.311	0.69	3.402
31-May	1	9.35	2.15E+04	-0.004	0.253	0.98	2.976
31-May	2	9.35	2.49E+04	-0.002	0.295	1.08	3.398
31-May	3	9.35	9.63E+04	-0.008	0.339	0.74	3.290
13-Jun	1	4.86	6.12E+03	-0.011	0.213	2.28	6.296
13-Jun	2	4.86	4.36E+03	-0.010	0.359	2.47	6.601
13-Jun	3	4.86	4.82E+04	-0.008	0.261	2.33	5.073
14-Jun	1	4.86	4.76E+04	-0.010	0.278	2.56	5.981
14-Jun	2	4.86	1.85E+04	-0.009	0.299	2.81	6.307
14-Jun	3	4.86	7.93E+04	-0.001	0.189	2.52	5.304
23-Jun	1	4.86	4.59E+03	-0.009	0.268	5.10	4.149
23-Jun	2	4.86	3.20E+03	-0.007	0.186	6.50	4.859
23-Jun	3	4.86	2.39E+04	-0.002	0.512	3.36	4.312
27-Jun	1	4.86	6.66E+03	-0.010	0.406	3.08	5.609
27-Jun	2	4.86	9.93E+03	-0.010	0.598	2.91	7.908
27-Jun	3	4.86	2.42E+04	-0.010	0.343	3.40	5.330
29-Jun	1	4.86	6.16E+03	-0.008	0.298	2.19	5.131
29-Jun	2	4.86	9.44E+03	-0.009	0.371	2.94	6.814
29-Jun	3	4.86	1.27E+04	-0.002	0.310	1.56	5.594
10-Jul	1	4.86	3.65E+03	*	*	*	4.048
10-Jul	2	4.86	2.53E+03	*	*	*	6.850
10-Jul	3	4.86	1.32E+04	*	*	*	5.315
11-Jul	1	4.86	2.63E+03	*	*	*	4.019
11-Jul	2	4.86	2.97E+03	*	*	*	7.423
11-Jul	3	4.86	4.98E+03	*	*	*	5.058

* HACH TNT 830 and TKN-N TNT 880 kit not available for July 10th and 11th samples.

** Column 1 effluent error processing HACH TKN-N TNT 880 kit, unable to obtain values.

Table 8. Influent HAV, NoV GI and NoV GII gene marker raw data

Date	HAV Concentration (gc/L)	NoV GI Concentration (gc/L)	NoV GII Concentration (gc/L)
3-Mar	ND	3.08E+05	8.15E+05
16-Mar	ND	2.09E+05	3.32E+05
4-Apr	ND	1.15E+05	8.96E+04
28-Apr	ND	5.98E+04	1.69E+05
4-May	ND	1.26E+05	7.39E+04
31-May	ND	6.58E+04	2.82E+04
14-Jun	ND	3.72E+04	2.55E+04
27-Jun	ND	4.33E+04	2.65E+04
29-Jun	ND	2.80E+04	9.74E+03

ND = no detect, below detection limit

Table 9. Effluent HAV, NoV GI and NoV GII gene marker raw data

Date	Column #	Infiltration rate (mm/hr)	HAV Concentration (gc/L)	NoV GI Concentration (gc/L)***	NoV GII Concentration (gc/L)***
3-Mar	1	14.0	ND	3.57E+03	3.57E+03
3-Mar	3	14.0	ND	3.92E+03	3.92E+03
16-Mar	1	14.0	ND	3.07E+03	3.07E+03
16-Mar	3	14.0	ND	3.08E+03	3.08E+03
4-Apr	1	14.0	ND	2.75E+03	2.75E+03
4-Apr	2	14.0	ND	3.65E+03	3.65E+03
4-Apr	3	14.0	ND	3.19E+03	3.19E+03
28-Apr	1	9.35	ND	3.08E+03	3.08E+03
28-Apr	2	9.35	ND	3.32E+03	3.32E+03
28-Apr	3	9.35	ND	3.03E+03	3.03E+03
4-May	1	9.35	ND	1.35E+03	1.35E+03
4-May	2	9.35	ND	1.33E+03	1.33E+03
4-May	3	9.35	ND	1.52E+03	1.52E+03
31-May	1	9.35	ND	1.35E+03	1.35E+03
31-May	2	9.35	ND	1.33E+03	1.33E+03
31-May	3	9.35	ND	1.52E+03	1.52E+03
14-Jun	1	4.86	ND	1.60E+03	1.60E+03
14-Jun	2	4.86	ND	1.50E+03	1.50E+03
14-Jun	3	4.86	ND	1.30E+03	1.30E+03
27-Jun	1	4.86	ND	1.63E+03	1.63E+03
27-Jun	2	4.86	ND	1.26E+03	1.26E+03
27-Jun	3	4.86	ND	1.36E+03	1.36E+03
29-Jun	1	4.86	ND	1.21E+03	1.21E+03
29-Jun	2	4.86	ND	1.28E+03	1.28E+03
29-Jun	3	4.86	ND	1.23E+03	1.23E+03

ND = no detect, below detection limit

***NoV GI and GII gene marker concentrations based on half the LoD.

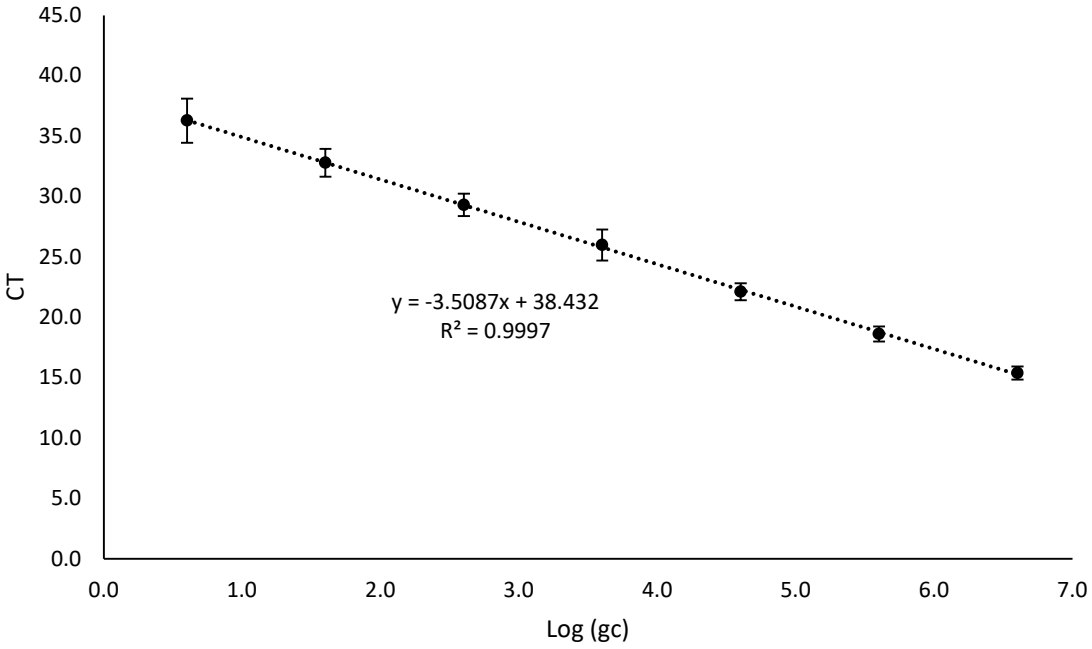

Figure 8. PMMoV RT-qPCR standard curve using mean CT values.

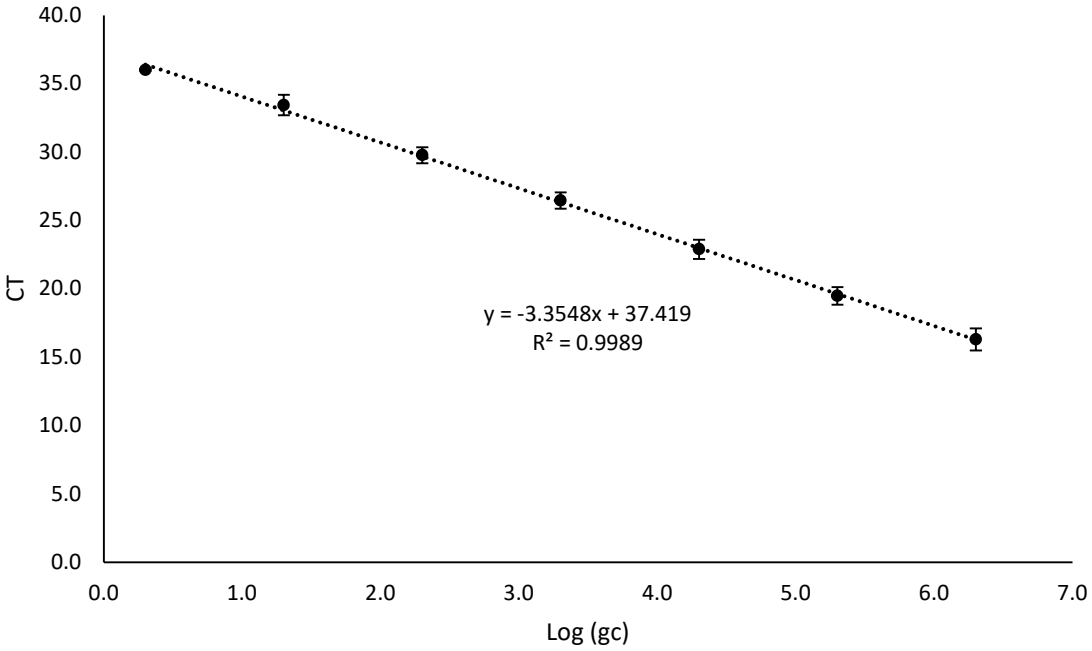

Figure 9. NoV GI RT-qPCR standard curve using mean CT values.

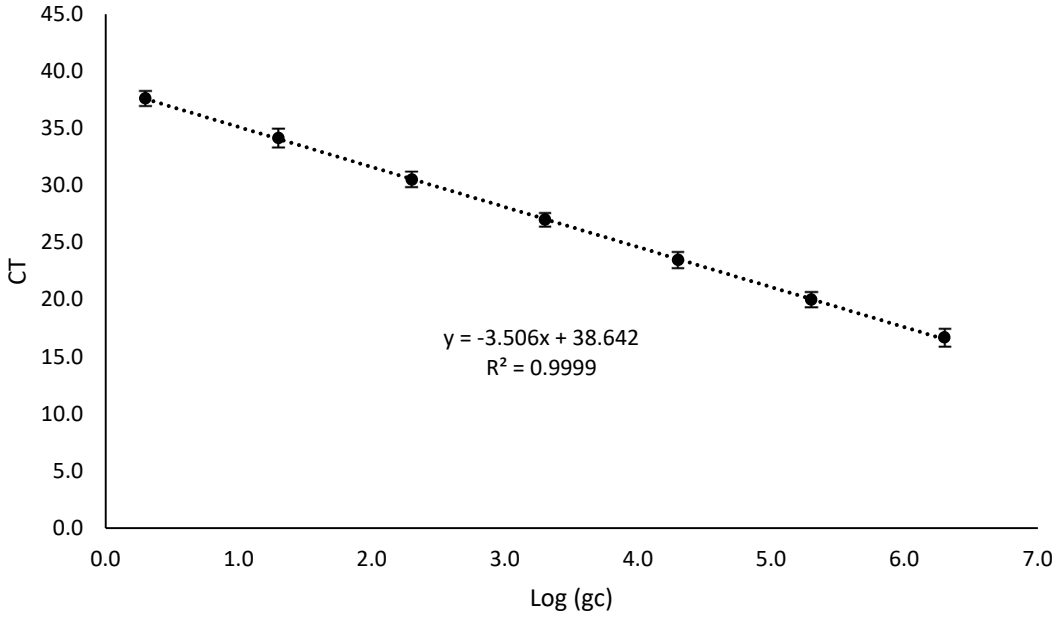

Figure 10. NoV GII RT-qPCR standard curve using mean CT values.