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Enteric and indicator virus removal by surface flow wetlands

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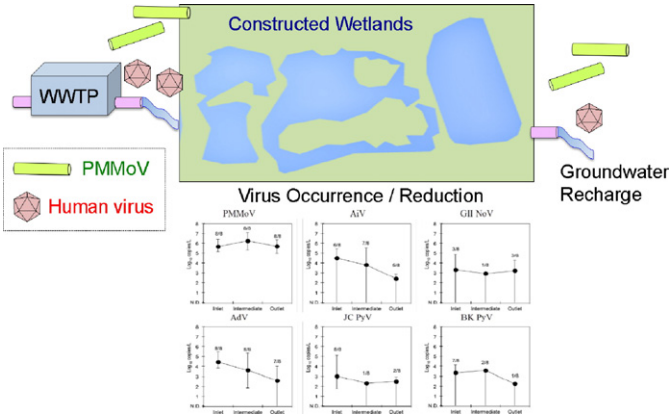
HIGHLIGHTS

- Occurrence and attenuation of viruses in surface flow wetlands were determined.
- Reduction of enteric viruses by the wetlands ranged from 1 to 3 $\log_{10}$.
- Pepper mild mottle virus showed greater persistence than human viruses in wetland.

GRAPHICAL ABSTRACT



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ABSTRACT

We investigated the occurrence and attenuation of several human enteric viruses (i.e., norovirus, adenovirus, Aichi virus 1, polyomaviruses, and enterovirus) as well as a plant virus, pepper mild mottle virus (PMMoV), at two surface flow wetlands in Arizona. The retention time in one of the wetlands was seven days, whereas in the other wetland it could not be defined. Water samples were collected at the inlet and outlet from the wetlands over nine months, and concentration of viral genomes was determined by quantitative polymerase chain reaction (qPCR). Of the human enteric viruses tested, adenovirus and Aichi virus 1 were found in the greatest prevalence in treated wastewater (i.e., inlet of the wetlands). Reduction efficiencies of enteric viruses by the wetlands ranged from 1 to 3 $\log_{10}$. Polyomaviruses were generally removed to below detection limit, indicating at least 2 to 4 $\log_{10}$ removal. PMMoV was detected in a greater concentration in the inlet of both wetlands for all the viruses tested (10^4 to 10^7 genome copies/L), but exhibited little or no removal (1 $\log_{10}$ or less). To determine the factors associated with virus genome attenuation (as determined by qPCR), the persistence of PMMoV and poliovirus type 1 (an enterovirus) was studied in autoclaved and natural wetland water, and deionized water incubated under three different temperatures for 21 days. A combination of elevated water temperature and biological activities reduced poliovirus by 1 to 4 $\log_{10}$, while PMMoV was not significantly reduced during this time period. Overall, PMMoV showed much greater persistence than human viruses in the wetland treatment.

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1. Introduction

Constructed wetlands have been receiving attention as a low-energy water treatment process and used as an additional wastewater treatment in many regions of the world (Beharrel, 2004). Studies have shown that wetlands can reduce the number of bacterial pathogens, although this process has some disadvantages: e.g., it requires a large area to build, it may harbor mosquitos, and it removes little nutrients (such as nitrogen and phosphorous) (Ramirez et al., 2005). It was reported that a small subsurface artificial wetland planted with bulrush could reduce 99% of coliforms, *Escherichia coli*, *Giardia* and *Cryptosporidium* from untreated wastewater in 10–15 days (Quiñónez-Díaz et al., 2001). Another study observed approximately 97% reduction of total coliforms from treated wastewater in a larger constructed wetland receiving secondary wastewater with 4.3 days of retention time (Wu et al., 2010). Previous studies suggested that physico-chemical and biological mechanisms and factors, such as adsorption, sedimentation, reactive oxygen species and predation by others microorganism, might be associated with fecal indicator bacteria removal in constructed wetland (Kadlec and Wallace, 2009). However, there is limited data on removal of viruses in treated wastewater by constructed wetlands (Gerba et al., 2013). Almost all of the existing data is on coliphages; in addition, data on enteric viruses is limited to enteroviruses and reovirus (Harwood et al., 2005; Lodder and Husman, 2005). Use of the polymerase chain reaction (PCR) allows for the detection of any known viruses in water, and this has been extensively used in recent years to quantify human enteric viruses in wastewater (Gerba et al., 2013). While this technology does not allow an assessment of the infectivity of viruses, it can provide information on the physical removal of virus and the loss of its genome by degradation in the environment (Kitajima et al., 2014).

Enteric viruses are always detected in raw wastewater, although the types and concentrations may vary depending upon the incidence within the community (La Rosa et al., 2010). Of the major human enteric viruses, adenovirus and Aichi virus 1 tend to be detected most commonly throughout the year (Kitajima et al., 2011, 2014). In addition to enteric viruses, other human viruses, such as polyomaviruses, can also occur in wastewater (Hewitt et al., 2013). Since polyomaviruses are specific to humans, they have been suggested as a marker to identify sources of human sewage pollution. Pepper mild mottle virus (PMMoV), a virus infecting pepper plants, has recently been proposed as a novel indicator for human fecal pollution in water environments, since it occurs in relatively high concentrations in treated sewage (Rosario et al., 2009; Hamza et al., 2011; Kitajima et al., 2014). PMMoV occurs in concentrations of up to 10^9 virions per gram of dry weight fecal matter (Zhang et al., 2006).

The goal of this study was to determine the occurrence of enteric viruses and their reduction in two surface flow constructed wetlands receiving treated (biological treatment) wastewater. In addition, the potential of PMMoV and polyomaviruses as indicators of treatment performance was assessed.

2. Materials and methods

2.1. Description of the constructed wetlands studied

Water samples were collected from two constructed wetlands located in Arizona: the Sweetwater Wetlands in Tucson and the Pinetop Wetlands in White Mountains. The Sweetwater Wetlands has been operated by Tucson Water (city of Tucson water utility) for slightly over 20 years to provide an additional treatment of the treated wastewater before infiltration into basins for soil aquifer treatment and reuse. It treats mixed media filter backwash waters (used to reduce suspended matter and protozoan parasites) as well as treated wastewater (with chlorination followed by dechlorination) from the Roger Road Water Reclamation Facility, which ceased operations in January 2014. This water reclamation facility treated the wastewater by the use of trickling filter process (biotowers) with a service population of almost 500,000 persons and a treatment capacity of 155,000 m^3 per day. The wetlands are designed to have a retention time of seven days and are approximately 0.03 km^2 in size, consisting primarily of planted bulrush and cattails (Vidales-Contreras et al., 2006).

The Pinetop wastewater treatment plant is located in Lakeside, eastern Arizona and uses conventional activated sludge process followed by chlorination. It serves approximately five thousand people and treats an average of two 7500 m^3 per day. The wastewater is discharged into the Jacques Marsh (Pinetop Wetland) consisting of 0.51 km^2. Because of high evaporation rates, little water exited in the marsh and retention time could not be defined.

2.2. Sample collection

A total of 40 samples were collected from the two wetlands in Arizona. At the Sweetwater Wetlands, three samples (inlet, intermediate, and outlet) were collected monthly from May to December 2013, after which the source of wastewater creased because of the operation of a new reclamation plant. At the Pinetop Wetland, inlet and outlet samples were collected three times in June, September, and November 2013. All samples were collected in sterile plastic bottles, stored on ice, and transported to the laboratory, where they were processed within 24 h of collection.

2.3. Analysis of physicochemical water quality and fecal indicator bacteria

Physicochemical water quality parameters such as pH, turbidity, total dissolved solid (TDS), and temperature were measured on site using a portable field sensor PCSTEst 35 (Eutech Instruments, Singapore). The most probable number (MPN) of *E. coli* per 100 mL of each water sample was determined using the Colilert® method with the Quanti-Tray®/2000 (IDEXX, Westbrook, ME) (American Public Health Association, 2005).

2.4. Virus concentration

One to two liters of water samples were collected for virus analysis and the water samples were concentrated using an electronegative filter method as described previously (Katayama et al., 2002) with slight modification. Briefly, 2.5 M $MgCl_2$ was added to the water samples to obtain a final concentration of 25 mM. The samples were subsequently passed through the electronegative filter (cat. no. HAWP-090-00; Merck Millipore, Billerica, MA) attached to a glass filter holder (Advantec, Tokyo, Japan). Magnesium ions were removed by passing 200 mL of 0.5 mM H_2SO_4 (pH 3.0) through the filter, and the viruses were eluted with 10 mL of 1.0 mM NaOH (pH 10.8). The eluate was recovered in a tube containing 50 μL of 100 mM H_2SO_4 (pH 1.0) and 100 μL of 100× Tris–EDTA buffer (pH 8.0) for neutralization. For turbid samples (more than 7 nephelometric turbidity unit [NTU]), a pre-filter treatment was applied before the virus concentration. The pre-filter treatment was conducted using the same equipment as the virus concentration but instead of using the electronegative filter, a glass fiber filter (Type A-E, 1-μm pore size and 142-mm diameter, Gellman, St Louis, MO) was used to prevent clogging of the electronegative filter by removing large debris and particles. After the pre-treatment process, the glass filter was removed and the filtrate was concentrated with the electronegative filter according to the protocol described above. The samples were further concentrated using a Centripep YM-50 (Merck Millipore) to obtain a final volume of 600–700 μL.

2.5. Sample process control for extraction-RT-qPCR

Murine norovirus (MNV, S7-PP3 strain), kindly provided by Dr. Y. Tohya (Nihon University, Kanagawa, Japan) and propagated in RAW

264.7 (ATCC TIB-71) cells (American Type Culture Collection, Manassas, VA, USA), was used as a sample process control to determine the efficiency of extraction-reverse transcription (RT)-qPCR, as previously described (Kitajima et al., 2014). Briefly, 2.0 μL of MNV stock (4.0×10^4 copies/μL) was spiked into 200 μL of concentrated wastewater samples or pure water (as a control). MNV-RNA was co-extracted with other indigenous viral nucleic acids from the water samples, and the MNV-RNA yield was determined by RT-qPCR (Kitajima et al., 2010). The % extraction-RT-qPCR efficiency (E) was calculated as follows:

$$E = C/C_0 \times 100;$$

where C represents the observed MNV-cDNA copy numbers per qPCR tube in a wastewater sample, and C_0 represents copy numbers in the control. The MNV process control was used to identify the viral nucleic acid loss during extraction and/or the occurrence of RT-qPCR inhibition, if any, but the extraction-RT-qPCR efficiency data was not used to adjust the concentration of indigenous viruses.

2.6. Extraction of viral nucleic acid and RT

Viral DNA and RNA were extracted from the concentrated water sample spiked with the MNV process control (202 μL in total) using the ZR Viral DNA/RNA Kit (Zymo Research, Irvine, CA) to obtain a final volume of 100 μL, according to the manufacturer's protocol.

The RT reaction was performed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Briefly, 10 μL of extracted RNA was added to 10 μL of RT mixture containing 2 μL of 10× reverse transcription buffer, 0.8 μL of 25× deoxynucleoside triphosphates (dNTPs), 2 μL of 10× random hexamers, 50 units of MultiScribe™ reverse transcriptase, and 20 units of RNase inhibitor. The RT reaction mixture was incubated at 25 °C for 10 min, followed by 37 °C for 120 min, and finally 85 °C for 5 min to inactivate the enzyme.

2.7. Quantification of viral genomes by qPCR

TaqMan-based qPCR assays for viruses were performed with a LightCycler® 480 Real-Time PCR Instrument II (Roche Diagnostics, Mannheim, Germany). Reaction mixtures (25 μL) consisted of 12.5 μL of LightCycler® 480 Probes Master (Roche Diagnostics), forward and reverse primers, probe(s), and 2.5 μL of (c)DNA template. The sequences of primers and probes, their final concentrations, and PCR amplification temperature profiles are shown in Table 1. The reaction mixtures were subjected to thermal cycling (initial denaturation at 95 °C for 5 min to activate DNA polymerase, followed by 50 cycles of amplification with the target-specific temperature profile described in Table 1), and fluorescence readings were collected and analyzed with LightCycler® 480 Software version 1.5 (Roche Diagnostics). The genome copy numbers of each virus were determined based on the standard curve prepared with 10-fold serial dilutions of plasmid DNA containing each virus gene to be amplified, at a concentration of 10^7 to 10^0 copies per reaction based on the plasmid DNA concentration determined by measuring the optical density at 260 nm. Negative controls were included to avoid false-positive results due to cross-contamination, and no false-positive qPCR signal was observed.

2.8. Incubation experiments

To determine the effect of temperature and microflora of the wetlands on removing/inactivating viruses, water samples were inoculated with poliovirus type 1 (Strain LSc-2ab) to obtain a final virus titer of 10^8 PFU (plaque forming units) per liter. The samples for poliovirus spiked were: sterile DI (deionized) water, non-autoclaved (i.e., untreated) wetland water collected from the Sweetwater Wetlands, and autoclaved Sweetwater wetland water. Experiments were triplicated for non-autoclaved wetland water ($n = 3$ for each virus) and no replicate experiment was conducted for the other samples ($n = 1$ for each virus). The water samples were incubated at three different temperatures (4 °C, 25 °C, 37 °C) for 21 days. Two milliliters of

Table 1
qPCR primers and probes used in the present study.

Target	Primer/probe	Name	Sequence (5′ → 3′)[a,b]	Final conc. (nM)	Amplification temperature profile	Reference
Norovirus GI	Primer	COG1F	CGYTGGATGCGNTTYCATGA	400	95 °C, 15 s → 56 °C, 60 s	Kageyama et al. (2003)
		COG1R	CTTAGACGCCATCATCATTYAC	400		
	Probe	RING1(a)-TP	FAM-AGATYGCGATCYCCTGTCCA-BHQ1	300		
		RING1(b)-TP	FAM-AGATCGCGGTCTCCTGTCCA-BHQ1	100		
Norovirus GII	Primer	COG2F	CARGARBCNATGTTYAGRTGGATGAG	400	95 °C, 15 s → 56 °C, 60 s	Kageyama et al. (2003)
		COG2R	TCGACGCCATCTTCATTCACA	400		
	Probe	RING2-TP	FAM-TGGGAGGGCGATCGCAATCT-BHQ1	300		
Enterovirus	Primer	EV1F	CCCTGAATGCGGCTAA	400	95 °C, 15 s → 60 °C, 60 s	Gregory et al. (2006)
		EV1R	TGTCACCATAAGCAGCCA	400		
	Probe	EV probe	FAM-ACGGACACCCAAAGTAGTCGGTTC-BHQ1	300		
Aichi virus 1	Primer	AiV-AB-F	GTCTCCACHGACACYAAYTGGAC	400	95 °C, 15 s → 60 °C, 60 s	Kitajima et al. (2013)
		AiV-AB-R	GTTGTACATRGCAGCCCAGG	400		
	Probe	AiV-AB-TP	FAM-TTYTCCTTYGTGCGTGC-MGB-NFQ	300		
PMMoV	Primer	PMMV-FP1-rev	GAGTGGTTTGACCTTAACGTTTGA	900	95 °C, 5 s → 60 °C, 60 s	Zhang et al. (2006), Haramoto et al. (2013)
		PMMV-RP1	TTGTCGGTTGCAATGCAAGT	900		
	Probe	PMMV-Probe1	FAM-CCTACCGAAGCAAATG-BHQ1	200		
Adenovirus	Primer	AQ2	GCCACGGTGGGGTTTCTAAACTT	500	95 °C, 3 s → 55 °C, 10 s → 65 °C, 60 s	Heim et al. (2003)
		AQ1	GCCCCAGTGGTCTTACATGCACATC	500		
	Probe	AP	FAM-TGCACCAGACCCGGGCTCAGGTACTCCGA-BHQ1	400		
JC polyomavirus	Primer	JCV-F	ATGTTTGCCAGTGATGATGAAAA	1000	95 °C, 15 s → 60 °C, 60 s	Pal et al. (2006)
		JCV-R	GGAAAGTCTTTAGGGTCTTCTACCTTT	1000		
	Probe	JCV-TP	FAM-AGGATCCCAACACTCTACCCCACCTAAAAAGA-BHQ1	600		
BK polyomavirus	Primer	BKV-F	GAAACTGAAGACTCTGGACATGGA	1000	95 °C, 15 s → 60 °C, 60 s	Pal et al. (2006)
		BKV-R	GGCTGAAGTATCTGAGACTTGGG	1000		
	Probe	BKV-TP	FAM-CAAGCACTGAATCCCAATCACAATGCTC-BHQ1	600		
MNV	Primer	MNV-S	CCGCAGGAACGCTCAGCAG	400	95 °C, 15 s → 60 °C, 60 s	Kitajima et al. (2010)
		MNV-AS	GGYTGAATGGGGACGGCCTG	400		
	Probe	MNV-TP	FAM-ATGAGTGATGGCGCA-MGB-NFQ	300		

[a] Mixed base in degenerate primer and probe is as follows: Y stands for C or T; N stands for any; R stands for A or G; B stands for not A; and H stands for not G.
[b] FAM, 6-carboxyfluorescein; TAMRA, 6-carboxytetramethylrhodamine; MGB, minor groove binder; and NFQ, non-fluorescent quencher.

each water sample was sampled in 2 mL tubes at 1, 4, 7, 10, and 21 day(s) of incubation. The samples were kept frozen at −20 °C until further analysis. The inactivation rate K_{obs} (day^{-1}) is the slope of $\ln(N_t/N_0)$ versus incubation time (day), where N_0 represents the initial virus concentration (determined by qPCR or plaque assay) and N_t represents the virus concentration after a given period of incubation time (t).

2.9. Poliovirus plaque assay

To determine the viability and inactivation of poliovirus during the incubation experiment, a plaque assay was performed using Buffalo Green Monkey kidney (BGM) cell line. The cell line was grown in minimal essential media (MEM) with 10% fetal bovine serum (FBS) and antibiotics on the six-well plate until 95% confluent cell monolayer was formed. The cell monolayer was rinsed twice with Tris-buffer (pH 7.5) before inoculated with the samples. The samples were serially diluted with FBS-free MEM. A hundred microliter of diluted samples were inoculated on the cell monolayer and then incubated in a CO_2 incubator for 30 min at 37 °C. The plates were gently rocked every 10 min, to evenly distribute the sample. The monolayer, with the sample inoculum, was covered with maintenance agar medium (MEM with 2% FBS and 1.5% of agarose) and then placed in a CO_2 incubator for 48 h at 37 °C. After incubation, the agar layer was removed and the cell monolayer was stained using 1% crystal violet solution for observation of plaques.

2.10. Statistical analysis

A Pearson correlation analysis using the Stata 20.0 was used to determine if any relationship existed between the $\log_{10}$ concentration or $\log_{10}$ removal of virus or *E. coli* and physicochemical parameters (pH, temperature, and turbidity). In the present study, correlation coefficient (R) of >0.7, 0.4 to 0.6, and <0.4 was considered a strong, moderate, and weak correlation, respectively. P value of less than 0.05 was considered statistically significant.

3. Results

3.1. Water quality parameters

We collected a total of 40 water samples from two constructed wetlands. Physicochemical and microbial water quality parameters in each water sample are summarized in Table 2. pH of the wetland water samples ranged from 7.1 to 8.5, and water temperature showed clear seasonal fluctuation especially in the Sweetwater Wetlands where water samples were collected over eight months. Turbidity in the inlet water of the Sweetwater Wetlands was much higher than that of the Pinetop Wetland; however, turbidity decline was observed after wetland treatment at the Sweetwater Wetlands. At the Pinetop Wetland, on the other hand, turbidity level always increased after wetland treatment. Reduction of *E. coli* concentration by wetland treatment was always less than 1 $\log_{10}$, except for one sampling event in September at the Pinetop Wetland where the reduction was >2 $\log_{10}$. No correlation was observed between turbidity level and $\log_{10}$ *E. coli* concentration ($R = 0.071$), suggesting that removal of turbidity does not necessarily indicate substantial removal of *E. coli* or vice versa. In addition, no clear relationship of reduction of *E. coli* numbers by the wetland treatment (0.003 to 2.553 $\log_{10}$) with the outlet water temperature was observed ($R = -0.428$).

3.2. Occurrence and reduction of viruses

Human enteric viruses and PMMoV in the wetland water samples were detected and quantified using qPCR. The most common viruses detected in the wastewater discharge into the wetlands were adenovirus and PMMoV (Table 3). Adenovirus was detected in 72.7% (8/11) of the outlet of the wetlands. PMMoV was detected in all outlet samples of both wetlands. Aichi virus 1 and polyomaviruses were also detected in most of the inlet samples. Polyomaviruses were never detected in the marsh at the Pinetop Wetland and only once in the outlet of the Sweetwater Wetlands. Aichi virus 1 was occasionally detected in the outlets of both wetlands. Enterovirus and noroviruses were only occasionally detected in the inlet samples. Enterovirus was never detected in the outlets of the wetlands. Use of MNV as a process control showed no substantial inhibition in the extraction-RT-qPCR process in any of the water samples tested in this study (recovery efficiency of greater than 10%).

3.3. Persistence of virus genomes in wetlands water

The results of incubation experiments (i.e., detection of spiked poliovirus and indigenous PMMoV) demonstrated that there was little difference in the degradation of poliovirus genome at 4 °C in all types of water (Table 4). However, at 25 °C and 37 °C, degradation of poliovirus genome was greater in non-autoclaved wetland water than that in autoclaved wetland water and deionized water (Table 4), indicating that microorganisms were playing a role in the degradation of poliovirus genome at these temperatures (Table 4). Very little decline of PMMoV genome copy numbers occurred after 21 days at any temperature, indicating that PMMoV is physically more stable and more persistent to biological degradation than poliovirus.

4. Discussion

Adenovirus and Aichi virus 1 were the most commonly detected human enteric viruses in treated wastewater in this study. The peak of adenovirus concentration at the Sweetwater Wetlands occurred in

Table 2
Physicochemical and microbial water quality parameters.

Wetland	Date (2013)	pH			Temperature (°C)			Turbidity (NTU)			*E. coli* (MPN/100 mL)		
		Inlet	Int.[a]	Outlet	Inlet	Int.	Outlet	Inlet	Int.	Outlet	Inlet	Int.	Outlet
Sweetwater	3-May	7.7	7.3	7.7	29.6	30.1	27.3	ND[b]	ND	ND	$>2.00 \times 10^2$	$>2.00 \times 10^2$	1.45×10^2
	13-Jun	7.1	8.5	7.9	29.6	30.1	27.3	8.1	5.7	6.2	1.37×10^3	8.70×10^2	6.30×10^2
	14-Jul	7.2	8.0	7.4	30.1	30.9	29.6	30.2	16.7	18.5	9.90×10^2	5.30×10^2	1.00×10^2
	27-Aug	7.7	7.9	7.7	27.5	29.0	27.7	25.4	2.7	7.0	1.06×10^3	1.21×10^3	8.40×10^2
	27-Sep	7.4	7.9	7.6	23.6	23.4	21.8	22.6	9.7	5.7	2.38×10^3	9.50×10^2	1.21×10^3
	23-Oct	7.2	7.6	7.4	22.3	19.4	18.3	99.4	1.9	2.3	1.56×10^3	9.20×10^2	1.55×10^3
	27-Nov	7.6	7.7	7.5	13.0	12.8	11.5	23.8	6.2	4.5	5.52×10^3	8.50×10^2	2.72×10^3
	23-Dec	7.8	7.9	7.7	21.9	12.1	11.7	20.3	2.8	4.2	3.36×10^3	2.41×10^3	1.99×10^3
Pinetop	27-Jun	7.5	–[c]	8.4	21.5	–	23.3	1.9	–	3.6	ND	–	6.3×10^1
	18-Sep	7.2	–	7.7	21.3	–	20.0	2.6	–	6.0	2.50×10^4	–	7.0×10^1
	18-Dec	7.5	–	7.2	13.0	–	20.3	6.4	–	6.9	1.21×10^3	–	9.5×10^2

[a] Int., intermediate.
[b] ND, no data.
[c] —, sample not collected.

Table 3
Occurrence of viruses in the constructed wetlands.

Wetland	Date (2013)	Adenovirus			Aichi virus 1			Norovirus GII			PMMoV			JC polyomavirus			BK polyomavirus		
		Inlet	Inter.	Outlet	Inlet	Inter.	Outlet	Inlet	Inter.	Outlet	Inlet	Inter.	Outlet	Inlet	Inter.	Outlet	Inlet	Inter.	Outlet
Sweetwater	3-May	8.15×10^3	7.32×10^1	6.30×10^1	4.91×10^4	2.05×10^4	6.30×10^1	ND	ND	ND	2.37×10^5	2.08×10^5	9.15×10^4	5.44×10^1	ND	ND	ND	ND	ND
	13-Jun	6.99×10^3	1.66×10^4	1.28×10^2	ND	ND	ND	ND	ND	ND	1.38×10^5	8.15×10^5	1.40×10^5	1.23×10^4	ND	1.09×10^2	9.33×10^2	4.62×10^3	1.70×10^2
	14-Jul	2.13×10^4	6.53×10^2	1.36×10^2	1.50×10^4	1.36×10^2	ND	ND	ND		8.05×10^5	1.73×10^6	3.31×10^5	2.59×10^2	ND	ND	1.48×10^3	ND	ND
	27-Aug	9.94×10^3	3.90×10^2	ND	2.56×10^5	3.25×10^5	3.90×10^2	7.18×10^4	ND	2.31×10^2	2.30×10^6	8.54×10^6	2.20×10^6	1.22×10^4	ND	ND	4.04×10^3	ND	ND
	27-Sep	2.80×10^4	2.98×10^3	9.80×10^3	ND	2.04×10^2	ND	ND	ND	ND	3.71×10^5	2.18×10^6	1.93×10^6	9.16×10^1	ND	ND	1.92×10^3	ND	ND
	23-Oct	1.64×10^4	7.12×10^3	2.83×10^3	6.97×10^3	3.21×10^3	6.77×10^2	2.84×10^2	ND	ND	3.80×10^5	9.07×10^5	2.49×10^5	2.54×10^2	ND	ND	3.21×10^2	ND	ND
	27-Nov	1.49×10^5	2.04×10^5	2.04×10^2	1.61×10^5	1.04×10^5	2.04×10^2	ND	ND	1.78×10^4	7.64×10^5	9.63×10^5	1.51×10^6	2.04×10^2	ND	8.30×10^2	3.04×10^3	ND	ND
Pinetop	23-Dec	2.92×10^5	4.07×10^4	2.25×10^2	3.07×10^3	5.54×10^3	2.78×10^2	3.75×10^2	8.29×10^2	9.56×10^2	3.10×10^5	1.17×10^7	4.83×10^5	1.20×10^5	2.02×10^2	ND	1.28×10^4	2.74×10^3	ND
	27-Jun	6.21×10^2	–	ND	6.64×10^4	–	ND	1.33×10^3	–	2.79×10^5	2.20×10^5	–	8.14×10^5	ND	–	ND	1.92×10^3	–	ND
	18-Sep	ND	–	ND	3.66×10^5	–	ND	ND	–	ND	3.42×10^5	–	1.73×10^4	ND	–	ND	ND	–	ND
	18-Dec	2.47×10^3	–	1.32×10^2	ND	–	ND	ND	–	ND	1.81×10^4	–	5.62×10^4	3.48×10^1	–	ND	ND	–	ND

November and December 2013. The incidence of adenovirus infection is more common in winter in the United States (http://www.cdc.gov/adenovirus/outbreaks.html). Dey et al. (2013) also reported that enteric adenovirus activity peaks in winter and spring (December–March) according to clinical surveillance in Japan. Other studies found that adenovirus concentration in water samples peaks in winter (Haramoto et al., 2007; Kishida et al., 2012) and March (Krikelis et al., 1985). In the present study, we observed a weak negative correlation between adenovirus $\log_{10}$ concentration and water temperature as well as pH at the Sweetwater Wetlands; on the other hand, a positive correlation was found between adenovirus concentration and turbidity. Higher persistence of adenovirus during wastewater treatment compared to other enteric viruses may be due to high stability of its double stranded DNA genome (Ogorzaly et al., 2010) as compared to RNA viruses.

At the Sweetwater Wetlands, Aichi virus 1 was detected in 51% (14/27) of samples. High abundance and persistence of Aichi virus 1 in the wetlands was probably due to their constant presence in wastewater effluent in Arizona (Kitajima et al., 2014). Noroviruses and enterovirus were only occasionally detected; this may be because removal efficiency of these viruses by wastewater treatment is relatively higher than other RNA viruses as reported previously (Kitajima et al., 2014). A previous study reported that approximately 90% reduction of enteroviruses was observed at a small pilot scale artificial subsurface flow vegetated (bulrush) bed with 5.5-day retention time (Quiñónez-Díaz et al., 2001).

We also assessed the removal of PMMoV and JC and BK polyomaviruses because they have been suggested as indicators of human sewage pollution (Rosario et al., 2009; Hewitt et al., 2013). PMMoV occurred in greater numbers in treated wastewater (inlet) and was less efficiently removed by wetland treatment than other viruses. PMMoV was detected in all wetland samples (inlet, outlet, and intermediate) ranging from 10^2 to 10^7 copies/L with less than 1 $\log_{10}$ reduction by wetland treatment. JC and BK polyomaviruses were detected at lower concentrations ($10^{2-}10^4$ copies/L) in the inlet of Sweetwater Wetlands but none were in the ponds at the Pinetop Wetlands. JC and BK polyomaviruses were detected in the Sweetwater Wetlands with 1.43 $\log_{10}$ and 0.76 $\log_{10}$ reduction on average, respectively. As a conservative indicator, PMMoV has potential due to its high abundance in treated wastewater and persistence during wetland treatment. The result of this study suggests that PMMoV is more persistent than JC and BK polyomaviruses. No correlation was observed between $\log_{10}$ PMMoV concentration and temperature ($R = -0.1492$) or turbidity ($R = -0.222$). However, we observed a weak correlation between $\log_{10}$ PMMoV concentration and pH ($R = 0.4355$, P value = 0.0334).

In general, reduction of microorganisms by wetland treatment may have been underestimated or overestimated due to generally high retention time in wetlands and limitation of the grab sampling method (i.e., the water samples were not collected from the same water body and retention time was not taken into account). This is probably why we sometimes observed virus or *E. coli* concentration being higher in a downstream point than in upstream point(s). Especially the reduction of *E. coli* by wetland treatment observed in the present study was generally low as compared to previous studies (Quiñónez-Díaz et al., 2001; Nguyen et al., 2015; Morató et al., 2014; Karimi et al., 2014). The reduction of *E. coli* can be affected by a number of environmental factors, such as water temperature, bacterial growth, sedimentation rate, vegetation, sunlight, algae presence, precipitation or stormwater inflow, inputs from droppings of waterfowl (Karimi et al., 2014; Karim et al., 2008; Morató et al., 2014; Howitt et al., 2014; VanKempen-Fryling et al., 2015; Nguyen et al., 2015; Ricca and Cooney, 1998, Palmer, 1983), but the reason of such low *E. coli* reduction in the Sweetwater Wetlands remains unclear.

Persistence of spiked poliovirus and PMMoV in wetland water under different conditions was assessed with batch experiments in the laboratory. The results of qPCR and infectivity assay for poliovirus suggested

Table 4
K_{obs} values of PMMoV and poliovirus at different temperatures.

Temperature (°C)	Water type	Spiked poliovirus		Indigenous PMMoV
		qPCR	Plaque assay	qPCR
4	Deionized water[a]	0.02	0.66	NA[c]
	Autoclaved wetland water[a]	0.02	0.33	NA
	Non-autoclaved wetland water[b]	0.37 ± 0.14	1.01 ± 0.29	0.04 ± 0.06
25	Deionized water[a]	0.38	0.45	NA
	Autoclaved wetland water[a]	0.64	0.50	NA
	Non-autoclaved wetland water[b]	1.89 ± 0.16	2.44 ± 1.01	0.05 ± 0.02
37	Deionized water[a]	1.00	3.04	NA
	Autoclaved wetland water[a]	1.24	2.45	NA
	Non-autoclaved wetland water[b]	3.71 ± 0.95	4.77 ± 1.03	0.08 ± 0.02

[a] No replicate experiment was conducted (n = 1 for each virus).
[b] Experiments were triplicated (n = 3 for each virus).
[c] NA, not applicable.

that temperature and biological activity have a significant role in virus degradation and that the biological activity itself could reduce the number of human viruses in water. In contrast, PMMoV was highly persistent (less than 0.01 $\log_{10}$ reduction per day, determined by qPCR) in non-autoclaved wetland water at all temperatures. Most previous studies suggest that wetlands with a 3- to 10-day retention time are capable of reducing removal of coliphage by coliphage numbers by ~95 to 99% (Gerba et al., 2013). One study in Spain reported 94.9% removal of coliphage by a constructed wetland (Reinoso et al., 2008). Another study reported similar results where a 3.16-$\log_{10}$ reduction of coliphages was observed at a constructed ecosystem research facility with 10 days of retention time (Karim et al., 2008). In addition, photoaction spectra and UVB irradiance used as the sunlight model have been proven to reduce >2 $\log_{10}$ of MS2 and poliovirus in wetland water (Silverman et al., 2015). PMMoV removal by biotowers or conventional activated sludge was usually less than 1 $\log_{10}$, which was lower than that observed for human viruses in wastewater (Kitajima et al., 2014). Substantial PMMoV removal can be achieved only with the advanced treatment of wastewater and its absence suggests the absence of any known human viruses (Bradley Schmitz, the University of Arizona, personal communication). Our results suggest that PMMoV is very stable and a potential conservative tracer/indicator for sewage contamination in water environment. It may also be considered too conservative, but its absence may ensure a low probability of the presence of human viruses in the treated water. It is also important to bear in mind that virus detection by qPCR does not necessarily represent the presence of infectious viruses, but failure to detect a virus by qPCR (without extraction/qPCR inhibition) does indicate the absence of the virus and may be considered a conservative performance target of a treatment system.

5. Conclusions

In the present study, we investigated the occurrence of multiple viruses at constructed wetlands, which allowed for comparative evaluation of virus removal efficiencies. The most abundant enteric viruses detected in treated wastewater (inlet of wetlands) were adenovirus and Aichi virus 1. These viruses were detected at concentrations of 10^2–10^5 copies/L in inlet of the Sweetwater Wetlands, where we observed virus removal efficiency of up to 2.5 $\log_{10}$. Other human viruses were occasionally found in outlet of the wetlands. Temperature and biological activity are likely to play a significant role in virus reduction in the wetlands. PMMoV was detected in all wetland samples with high abundance and low reduction, demonstrating the potential of PMMoV as a conservative tracer of wetland treatment performance with respect to virus occurrence and reduction.

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