Virus Removal Efficiency in Wetlands Receiving Secondary

Treated Wastewater

Abstract

Constructed wetlands are widely utilized for additional treatment of treated wastewater in Arizona. We investigated the occurrence and attenuation of several enteric viruses (i.e., norovirus, adenovirus (AdV), Aichi virus (AiV), and enterovirus) and pepper mild mottle virus (PMMoV) in wetlands. Water samples were collected monthly from three constructed wetlands in Arizona for nine months, and concentration of viral genomes was determined by quantitative PCR. AdV and AiV exhibited the highest prevalence, while norovirus was detected only during winter. Reduction of enteric viruses at the wetlands ranged from 1 to 3 $\log_{10}$. Interestingly, PMMoV was detected in all water samples with only less than 1-$\log_{10}$ reduction. To determine the environmental factors associated with virus attenuation, wetland water and sterile deionized water inoculated with poliovirus were incubated under three different temperatures and the concentration of poliovirus (inoculated) and PMMoV (indigenous) was monitored for 21 days. Water temperature and biological activities reduced 1 to 4 $\log_{10}$ of poliovirus, while PMMoV was more stable and less susceptible to these factors. Overall, PMMoV showed much higher occurrence and persistence than enteric viruses during the wetland treatment, demonstrating its usefulness as a conservative indicator of treatment efficiency and microbial water quality in water reclamation systems.

Introduction

Pathogenic viruses like Norovirus (NoV), Adeno Virus (AdV) , Aichi virus (AiV), and Enterovirus (EV) in discharged wastewater pose a potential health hazard through water use and exposure route. These viruses are well known for causing gastroenteritis and other illnesses through human and human contact, contaminated food, and environmental sources (Parshionkar 2003, Hall 2012, Pham et al. 2007, Roberts 2005). Complete removal of viruses by wastewater treatment is difficult because they are present in high concentrations in wastewater and fairly resistant to treatment processes compare it to bacterial indicators such as *E. coli*, total coliforms and fecal coliform (Gerba *et al.* 2013). These viruses have a high resistance to inactivation by heat, UV light, ozone, and salinity.

Due to extreme water scarcity/water stress especially in arid area, including Arizona as a growing population city in the desert, indirect potable reuse (IPR) is now being considered to increase supplies of drinking water. A 2012 National Research Council reported that 6% of total water use in the US was discharged to the ocean and estuary without any significant reuse. IPR refers to the use of reclaimed water via environmental buffer such as managed aquifer recharge or engineered storage tanks directly from a wastewater treatment facility to a drinking water distribution system. Consequently, scientist, water industry specialists, policy makers and community stakeholders are currently considering the IPR as a way to augment future US drinking water supplies (NRC, 2012). However, there is limited data focusing on the importance of environmental buffers such as constructed wetlands in removing viral pathogens and from the waste water.

To assess fecal contamination, the US Environmental Protection Agency (EPA) uses total coliform bacteria and generic *E. coli*. However these assays are limited in that they are not specific enough to determine whether the fecal matter is coming only from humans and correlate poorly with the removal and/or presence or absence of potentially pathogenic human viruses. (Harwood, Staley, Badgley, Borges, & Korajkic, 2014). In wetlands, warm blood animal such as ducks, birds, and other mammals are abundant. The high abundance of birds have a strong positive correlation with higher concentration of total coliform and *E. coli* in water environment (Kirschner et al., 2004), so using them as an indicator may not produce data on removal from the wastewater applied to the wetland. In addition, total coliform and *E. coli* do not always represent the human pathogenic viruses due to their diversity which depend on season, area, and the communities' hygiene (Gerba *et al.* 2013).

Recently, viruses appeared to be a better indicator of human waste contamination due to host-specificity of viruses to human, mainly for enteric viruses (Wong, Fong, Bibby, & Molina, 2012). The polymerase chain reaction base methods help the scientist to quantitate the number of viral pathogens, including the new emergent strains so data on the occurrence of viruses in environments have been rapidly accumulated (Girones et al, 2013). The reduction of human viruses in the wastewater treatment is important to be measured to know the efficacy of thr capability of wastewater treatment facility. Enteric viruses such as enteroviruses and adenoviruses are always detected in raw wastewater (Rosa, Pourshaban, Iaconelli, & Muscillo, 2010). A hundred percent and 92 % of Aichi virus genome was detected in the influent and the effluent wastewater samples from different plants in Japan, respectively (Kitajima, Haramoto, Phanuwan, & Katayama, 2011a). In addition to enteric viruses, pepper mild mottle virus (PMMoV), a dietary virus from pepper plants, has recently been proposed as a novel indicator

for human fecal pollution in water environments (Rosario, Symonds, Sinigalliano, Stewart, & Breitbart, 2009). Pepper Mild Mottle Virus (PMMoV) is a promising indicator since it is always detected idomestic sewge discharges (Hamza, Jurzik, Uberla, & Wilhelm, 2011). Furthermore, PMMoV occurs in concentrations up to 10^9 virions per gram of dry weight fecal matter (Zhang et al., 2006). Other viruses which have a good potential to be a fecal indicator are human polyoma viruses. Polyoma virus has a high prevalence (100% in the influent and 96% in the effluent of wastewater treatment in New Zealand) and only infects humans (Hewitt, Greening, Leonard, & Lewis, 2013).

Wetlands are used at several locations in Arizona for additional treatment after trickling filter treatment. A wetland is an area with saturated water and aquatic plants and is often referred to as the "kidney" of nature for its natural filtering properties. The "kidney" function in wetlands also refers to their capability for degrading organic pollutants through microbial processes.. Studies have showed that wetlands can reduce the number bacterial pathogens. A small subsurface artificial wetland (9.1 m x 3.0 m x 2.12 m) planted with bulrush in the sand can reduce 99% of coliforms, *Escherihia coli*, *Giardia* and *Cryptosporidium* from untreated wastewater in 10-15 days (Quinonez-Diaz et al., 2001). Approximately 97% of total coliforms, 55% and 30% of Biological Oxygen Demand (BOD) and nutrients were reduced from treated wastewater in a larger constructed wetland receiving secondary wastewater in 4,3 days of retention time (Wu et al., 2010).

Several mechanisms for pathogen reduction in wetlands have been proposed (Karim, Glenn, & Gerba, 2008). First of all, naturally occurring microbes reduce the amount of organic materials in saturated soils by using them as a nutrient source and converting them into reduced or oxidized forms. This process also occurs with sedimentation by aquatic plants. Rhizomes and

submerged stalks of planted reeds enhance the sedimentation and prevent microbial transport to groundwater. The second process is the nutrient competition between microbes and nematodes with the enteric bacteria and viruses. Wetlands containing aquatic plants such as bullrush, duckweed, thypa and even bare-sand wetlands have yielded promising results in reducing the number of pathogens in wetlands (Karpiscak et al., 1996). Physical conditions in wetlands such as turbidity, salinity and temperature also have role in pathogen reduction (Silva et al., 2011). Furthermore, the sunlight has capability in inactivating the viruses through UVB light radiation, absorption of photon by viruses, and reaction with reactive species which are generated by photosensitizers (Silverman, Peterson, Boehm, McNeill, & Nelson, 2013).

The goal of this study was to determine the occurrence of enteric viruses and their reduction in three wetlands receiving secondary treated wastewater. In addition, the potential of PMMoV and Polyoma JK and BK viruses as human fecal indicators in the wetland was also assessed. Furthermore, an incubation experiment was conducted to assess the role of natural microbiota and temperature in reducing the number of enteric viruses as detected by cell culture and qPCR. The Sweetwater, the Pine Top, and the Tres Rios wetlands were chosen as research sites. Tucson Water operates the Sweetwater wetlands primarily to treat mixed media filter backwash waters from its wastewater treatment facility. It is located at 2551 W Sweetwater Dr, Tucson. The wetland at times also receives wastewater directly from the Roger Road Wastewater treatment plant. The Sweetwater Wetlands is designed to provide an additional treatment of the wastewater before infiltration into basin for soil aquifer treatment and reuse. The wetlands are designed to have a retention time of five days. The wetland supports a huge variety of wildlife including dragonflies, raccoons, hawks, bobcats and dozens of other species. According to the Sweetwater wetland handbook, it contains numerous of plant species including Tamarisk,

buffelgrass, yellow starthistle, cottonwood, Gooding willow, saltbush, bulrush, mesquite, cattail, and wolfberry. The City of Phoenix uses the Tres Rios Wetland for treatment of secondarily treated wastewater. It is located at S 91st Ave, Phoenix. According to the Tres Rios website, it contains some plant species such as Freemont cottonwood, Goodings willow, cattail, bulrush, giant and alkali sacaton grasses, mesquites, desert screwbean, and saltbush. On the other hand, the White Mountain area uses their wetland to evaporate the treated waste water.

## Material and Methods

Field Sampling

A total of 40 water samples from three different wetlands in Arizona. The constructed wetlands used in this study were Sweetwater wetland in Tucson, the Tres Rios wetland in Phoenix, and Pine Top wetland in White Mountain (Image 1,2 and 3). For the Sweetwater wetland, three samples (inlet, intermediate, and outlet) were taken every month from May 2013 until January 2014.

Image 1. Sweetwater Wetland Tucson (google maps)

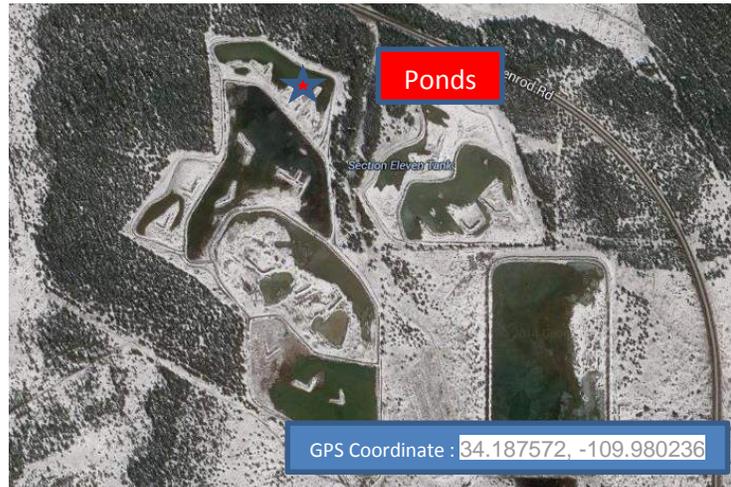Image 2. Tres Rios Wetland Phoenix (google maps)
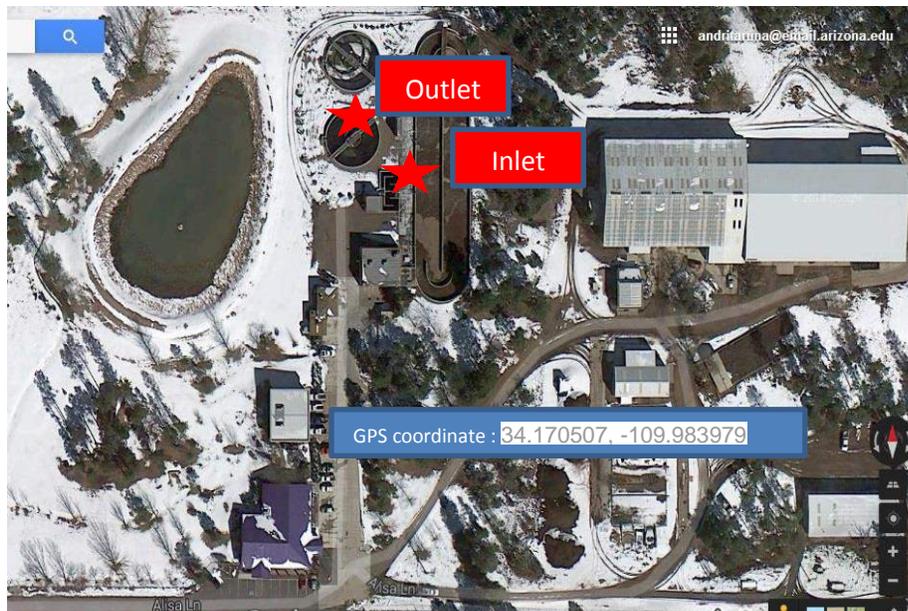
Image 3. Pine Top Wetland, White Mountain (google maps)

At the Phoenix Tres Rios wetland, three samples (inlet, intermediate, and outlet) were collected at the beginning of the summer 2013 (June 16, 2013) and winter 2013 (November 5, 2013). For Pine Top wetland, three or four samples from different stations (inlet, outlet, and pond(s) or basin) depending on the water availability were taken three times at the summer, fall, and winter 2013. Physical and chemical water quality parameters such as pH, turbidity, salinity, conductivity, total dissolved solid (TDS), and temperature were measured at the field using a portable field sensor Eutech Instruments PCSTEst 35. Duplicate one liter grab samples were taken in each station. Samples were transport to the laboratory on ice and processes within 24 hours.

Total coliforms and *E. coli*

The number of total coliforms and generic *E. coli* were measured by the most probable number; MPN using the IDEXX Colilert® method with Quanti-Trays® (IDEXX, Westbrook, ME) (American Public Health Association 2005). One to ten milliliters of sample was diluted in a sterilized DI (deionized) water 10 or 100 fold depending on the sources.

Virus Concentration

One to two liters of each wetland water sample were concentrated using HA filter (0.45 μm pore sized and 90 mm diameter). After the filtration, the membrane was rinsed with 0.5 mM of H_2SO_4 (pH 3) and eluted with 1 mM NaOH (pH 10.5) (Katayama, Shimasaki, & Ohgaki, 2002). For turbid samples (more than 7 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 HA filter, a glass fiber filter (Gellman Type A-E, 1 μm pore sized and 142 mm diameter) was applied to prevent the clogging of the HA membrane with removing all the big debris and dirt. After the pre-treatment process, the glass filter was removed and the filtered water was concentrated with HA filter according to previous protocol. After virus concentration was done, the HA filter was kept in the -20°C freezer.

The samples were further concentrated using a Centripep YM-50 (Milipore) and then centrifugation at 2,500 (1520 g) rpm for 10 minutes. Next, the liquid which passed through the ultrafiltration membrane was removed and then then the rest was centrifuged at 2,500 rpm (1520 g) for 5 minutes to obtain a final concentrate volume of 600-700 μL.

Murine norovirus process control

A volume of 2.0 μl (10^5 Plaque Forming Unit (PFU)/ml) of a stock culture of murine norovirus (MNV) was added to each of the 200 μl virus concentrates prior to extraction as a process control. Murine norovirus (MNV strain S7-PP3) which used as a process control at the DNA/RNA extraction process was obtained from Dr. Yukinobu Tohya from the Department of Veterinary Medicine, Nihon University (Kanagawa, Japan). The virus was propagated on RAW 264.7 (ATCC #TIB-71) cell line monolayers with Dulbecco's modified Eagle medium (DMEM; Mediatech Inc., Manassas, VA) containing 10% fetal bovine serum (FBS; 31 Hyclone Laboratories, Logan, UT), 0.113% sodium bicarbonate (Fisher Scientific, Fair Lawn, NJ), 10 mM HEPES buffer (Mediatech Inc.), and 1.0% antibiotic/antimycotic (Mediatech Inc.) at an incubation temperature of 37°C with 5% CO_2 as described previously (Wobus et al., 2004).

Incubation Experiment

To determine the effect of temperature and microflora of the wetlands in reducing the viruses detected by qPCR samples of water collected from the Sweetwater wetland was inoculated with laboratory grow poliovirus type 1 (Strain LSc-2ab). To compare the result, the indigenous PMMoV was determined by q-PCRin the wetland samples. One liter of the sample was spiked with one mL of 10^8 Plaque Forming Unit (PFU) per mL polio irus into 5 different water samples. The samples included sterile DI (deionized) and wetland water as a control and three of wetland water samples as the triplicate samples (Samples A,B, and C) which were incubated at three different temperatures (4 °C, 25 °C, 37 °C for 21 days). Two mL of each water samples at 1, 4, 7, 10, and 21 day(s) of incubation times were transferred and collected into 2 mL tubes. The samples were kept frozen in -21°C until further analysis.

Viral DNA/RNA extraction and RT

A 200 µL of the final concentrate was extracted to obtain the DNA/RNA using an extraction kit (Zymo Research Viral DNA/RNA Kit[TM]). The procedure was followed according to the manufacturer's protocol. For final elution of DNA/RNA, instead of adding 10 µL of DNase/RNase- free water as mentioned in the protocol, a 100 µL of the water was added.

In the case of RNA viruses, a reverse transcript (RT) PCR was used to produce a cDNA necessary before performing quantitative-PCR (q-PCR). The RT PCR was conducted using High Capacity cDNA Reverse Transcription Kits (Applied Biosystem) with the addition of an RNase inhibitor following manufacturer's protocol.

Identification of Human Pathogenic Viruses using qPCR

The q-PCR method was conducted using a Roche 480 light cycler with the established methods, primers and probes (Table 1) to detect human NoV GI and GII (Kageyama et al. 2003), Polyoma JC and BK virus (Pal et al. 2006), AiV (Kitajima et al. 2013), AdV (Heim et al. 2003), Enterovirus (Gregory, Litaker, & Noble, 2006), and PMMoV (Haramoto et al. 2013).

Table 1. Primer, probe and references used for each viruses

Viruses	Primer/probe	Name	Sequence (5' → 3')
Norovirus GI (Kageyama et al. 2003)	F primer	COG2F	CARGARBCNATGTTYAGRTGG ATGAG
	R primer	COG2R	TCGACGCCATCTTCATTCACA
	Probe	RING2-TP	FAM-TGGGAGGGCGATCGCAATCT-BHQ1
Adenovirus (Heim et al. 2003)	F Primer	AdV F	GCCACGGTGGGGTTTCTAAAC-TT
	R Primer	AdV R	GCCCCAGTGGTCTTACATGCAC AT-C
	Probe	AdV P	TGCACCAGACCCGGGCTCAGG TACTCCGA

Norovirus GI	F primer	COG1F	CGYTGGATGCGNTTYCATGA
(Kageyama et al. 2003)	R primer	COG1R	CTTAGACGCCATCATCATTYAC
	Probe	RING1(a)-TP	FAM-AGATYGCGATCYCCTGTCCA-BHQ1
		RING1(b)-TP	FAM-AGATCGCGGTCTCCTGTCCA-BHQ1
PPMoV (Zhang et al. 2006, PMMV-FP1-rev: Haramoto et al. 2013)	F primer	PMMV-FP1-rev	GAGTGGTTTGACCTTAACGTTTGA
	R primer	PMMV-RP1	TTGTCGGTTGCAATGCAAGT
	Probe	PMMV-Probe1	FAM-CCTACCGAAGCAAATG-BHQ1
Aichi Virus (Kitajima et al. 2011)	F primer	AiV-AB-F	GTCTCCACHGACACYAAYTGGAC
	R primer	AiV-AB-R	GTTGTACATRGCAGCCCAGG
	Probe	AiV-AB-TP	FAM-TTYTCCTTYGTGCGTGC-MGB-NFQ
Murine Norovirus (Kitajima et al. 2010)	F primer	MNV-S	CCGCAGGAACGCTCAGCAG
	R primer	MNV-AS	GGYTGAATGGGGACGGCCTG
	Probe	MNV-TP	FAM-ATGAGTGATGGCGCA-MGB-NFQ
Polyoma JC (Pal et al. 2006)	F primer	JCV-F	ATGTTTGCCAGTGATGATGAAAA
	R primer	JCV-R	GGAAAGTCTTTAGGGTCTTCTACCTTT
	Probe	JCV-TP	AGGATCCCAACACTCTACCCCACCTAAAAAGA
Polyoma BK (Pal et al. 2006)	F primer	BKV-F	GAAACTGAAGACTCTGGACATGGA
	R primer	BKV-R	GGCTGAAGTATCTGAGACTTGGG
	Probe	BKV-TP	CAAGCACTGAATCCCAATCACAATGCTC
Enterovirus (Gregory et al., 2006)	F Primer	EV1F	CCCTGAATGCGGCTAA
	R Primer	EV1R	TGTCACCATAAGCAGCCA
	Probe	EV probe	FAM-ACGGACACCCAAAGTAGTCGGTTC-BHQ1

Note :
*FAM is the fluorophore
 BHQ, Black hole quencher
 NFQ, Non fluorescent quencher
 MGB, Minor groove binder

Correlation Analysis

A Spearman non parametric correlation analysis using Stata 20.0 was used to describe the

relationship between pH, temperature, and turbidity related to the abundance of virus and the

$\log_{10}$ reduction during passage through the wetland. Rho value near one will be considered as a

high correlation and P value less than 0.05 is statistically significant.

<div align="center">**Results**</div>

Physical, Chemical, Total Coliform and *E. coli* Parameters in The Sweetwater wetland

In the Sweetwater wetland, the average pH, temperature, and turbidity in the inlet was 7.55, 23.3 $^{\circ}$C, and 30.3 NTU respectively (Table 2). On the other hand, in the intermediate and outlet of the Sweetwater wetland, the average pH, temperature, and tubbidity are 7.84, 7.61, 22.6°C, 21.9 $^{\circ}$C, and 7.08 NTU, 6.09 NTU respectively. The number of total coliform are 2.42 x 10^5 MPN/100 mL in the inlet and ranged from 1.58 x 10^4 – 2.42 x 10^5 MPN/100 mL in the outlet of the Sweetwater wetland (Table 2). The *E. coli* average concentration were 2.34 x 10^3 MPN/100 mL in the inlet and 1.15 x 10^3 MPN/100 mL in the outlet of the Sweetwater wetland with 0.3 Log$_{10}$ reduction after the wetland treatment.

Table 2. Physical, chemical and biological parameters in the Sweetwater Wetland

Date Collected	Site	PH	Temperature °C	Turbidity NTU	Total Coliform	*E. coli*
3-May-13	Inlet	7.73	29.6	NA	>200.5	>200.5
13-Jun-13		7.05	29.6	8.13	>2000	1.37E+03
14-Jul-13		7.2	30.1	30.2	>2000	9.90E+02
27-Aug-13		7.7	27.5	25.4	2.42E+05	1.06E+03
27-Sep-13		7.41	23.6	22.6	2.42E+05	2.38E+03
23-Oct-13		7.2	22.3	99.4	2.42E+05	1.56E+03
27-Nov-13		7.63	13	23.8	2.42E+05	5.52E+03
23-Dec-13		7.81	21.9	20.3	2.42E+05	3.36E+03
28-Jan-14		8.2	11.8	12.8	2.42E+05	2.51E+03
3-May-13	Intermediate	7.31	30.1	NA	>200.5	>200.5
13-Jun-13		8.46	30.1	5.71	>2000	8.70E+02
14-Jul-13		7.97	30.9	16.72	>2000	5.30E+02
27-Aug-13		7.91	29	2.67	2.42E+05	1.21E+03
27-Sep-13		7.88	23.4	9.74	2.42E+05	9.50E+02
23-Oct-13		7.64	19.4	1.91	19560	9.20E+02
27-Nov-13		7.68	12.8	6.18	2.91E+04	8.50E+02

23-Dec-13		7.89	12.1	2.8	8.66E+04	2.41E+03
28-Jan-14		7.85	15.78	10.9	2.42E+05	2.39E+03
3-May-13	Outlet	7.73	27.3	NA	>200.5	1.45E+02
13-Jun-13		7.9	27.3	6.15	>2000	6.30E+02
14-Jul-13		7.39	29.6	18.5	>2000	1.00E+02
27-Aug-13		7.66	27.7	6.96	2.42E+05	8.40E+02
27-Sep-13		7.6	21.8	5.67	2.42E+05	1.21E+03
23-Oct-13		7.39	18.3	2.3	1.58E+04	1.55E+03
27-Nov-13		7.48	11.5	4.49	2.25E+04	2.72E+03
23-Dec-13		7.71	11.7	4.23	1.73E+05	1.99E+03

Occurrence and Reduction of Enteric Viruses

Seasonal data and reduction of adenovirus at the Sweetwater wetland are shown at Figure 1. Of 27 samples, 77% (21/27) of them were positive for adenovirus with the highest concentration 2.92 x 10^5 copies/L in the Sweetwater wetland. In the inlet, adenovirus concentrations were stable with mean value of 5.91 x 10^4 copies/L every month in Sweetwater wetland. Of six samples collected at the Tres Rios wetland, there was one positive (4.69 x 10^3 copies/L) detected in the inlet at the beginning of winter 2013. At the Pine Top wetland, there was 6.21 x 10^2 copies/L discharged from the effluent of wastewater treatment plant into the ponds in June 2013 but there were no detectable adenoviruses at the outlet of the ponds. Only a 0.09 $\log_{10}$ reduction of adenovirus was detected at the Pine Top wetland
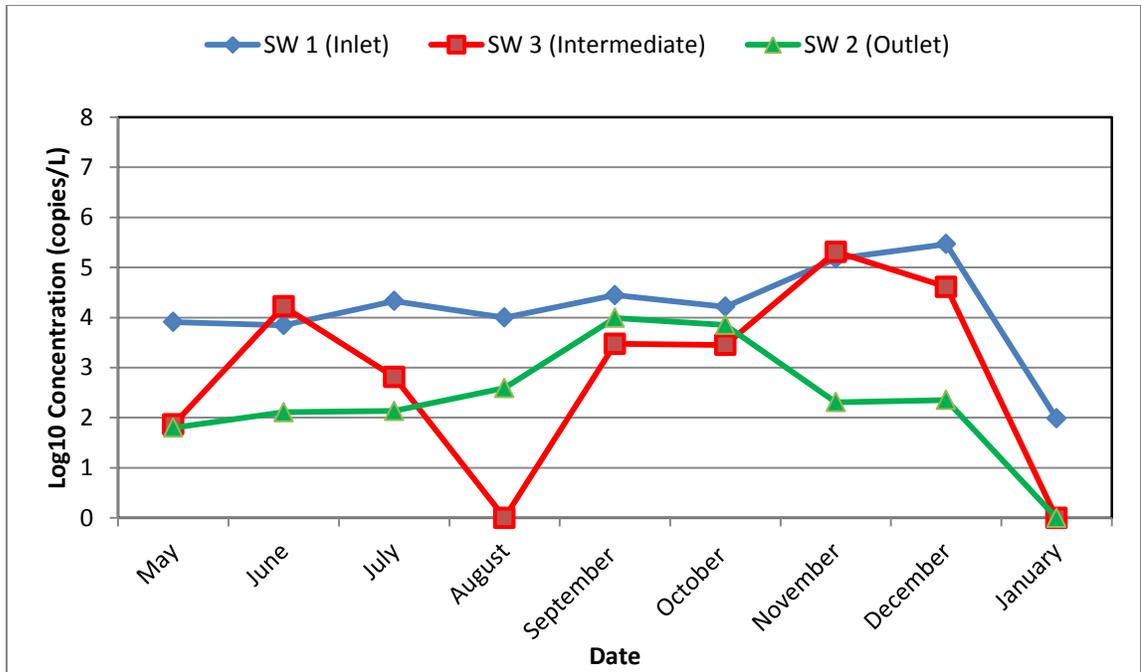
Figure 1. Seasonal occurrence of adenoviruses at the Sweetwater wetland

Greater reductions in the Sweetwater wetland were observed on May, June, November, and December ($2 - 2.5$ Log_{10} reduction) (Figure 2). On the other hand, low log_{10} reductions (approximately 1 log_{10}) were observed on July until October.
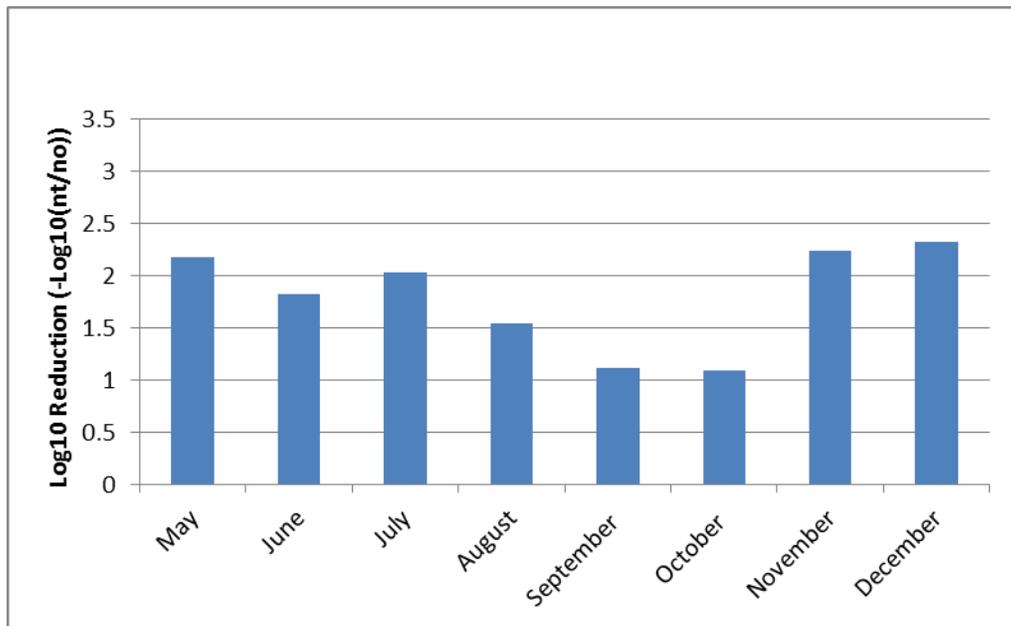

Figure 2. Log_{10} reduction of adenoviruses by the Sweetwater wetland

Enteroviruses were only detected twice at the Sweetwater wetland, and only at the intermediate

site (Figure 3). This suggests that almost all of enteroviruses was removed bythe wastewater

treatment process. Although samples were positive in the intermediate site, there was no

enteroviruses detected at the outlet. No enteroviruses were detected at the Phoenix Tres Rios
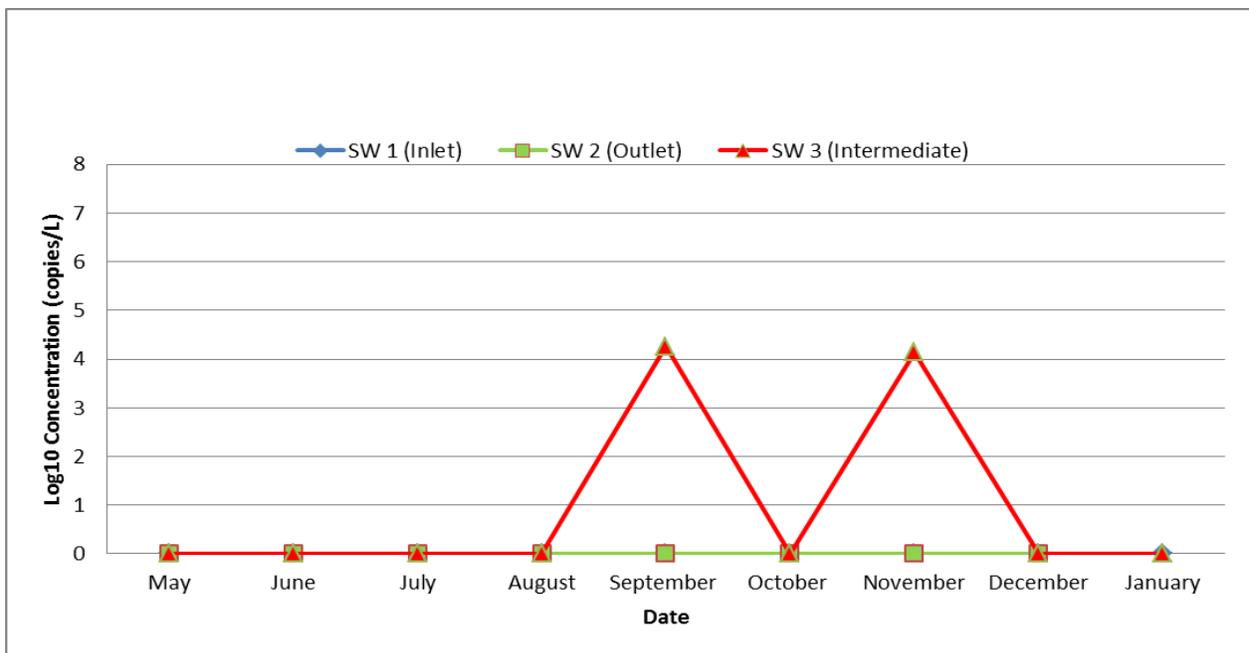
wetland

Figure 3. Seasonal occurrence of enteroviruses at the Sweetwater wetland

Norovirus (NoV) GI was only detected at the Pine Top wetland during the summer. Both

NoV GI and NoV GII were detected at 8.09 x 10^4 and 2.79 x 10^5 copies/L, respectively, at the

Pine top wetland in the influent and effluent of wastewater treatment plant and also in all the

active ponds (ponds which used in that time). On the other hand, at the Sweetwater wetland,

NoV GII, which is more infectious than NoV GI, was detected on July 2013 and November-

December 2013 (Figure 4). The NoV GII only was detected in only18% (5/27) of the samples at Sweetwater wetland.
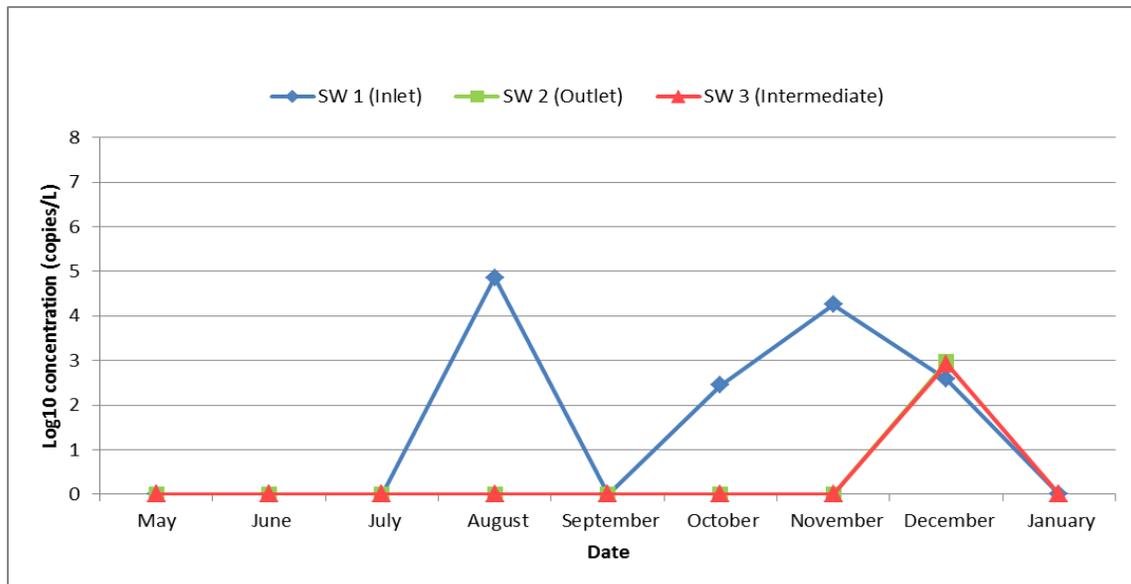
Figure 4. Seasonal occurrence of Norovirus GII at the Sweetwater wetland

There was a 2-2.5 $\log_{10}$ reduction from the inlet to the outlet of NoV GII strain by the Sweetwater wetland. No NoV GII were detected at the Tres Rios wetland, but 10^1-10^3 copies/L were detected in the inlet of Pine Top wastewater treatment plant although none were detected in the wetlands.

Aichi virus (AiV) was detected at the inlet of the Sweetwater wetland except in June and September and January (Figure 5). The highest concentration was 2.56 x 10^5 copies/L in August 2013 which was the peak of the monsoon season. In January 2014, the Sweetwater wetland was no longer supplied wastewater by the Roger Road wastewater treatment. The concentrations of AiV were high and consistent in almost all period of time except for June and September 2013.
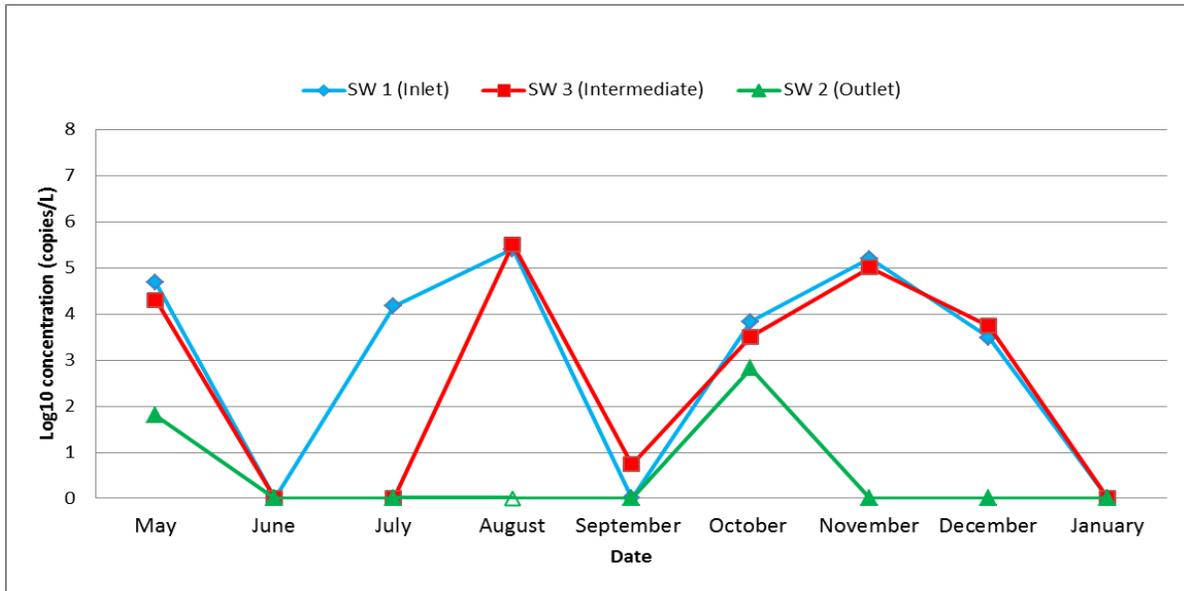
Figure 5. Seasonal occurrence of Aichi virus at the Sweetwater wetland

On the other hand, at the Tres Rios Phoenix no AiV was detected during the summer but 10^5 and 1.98×10^2 copies per liter were detected in the inlet and outlet during the winter. There was a 1.00-2.89 $\log_{10}$ reduction of AiV by the Sweetwater wetland and the highest reduction occurred in August 2013 (Figure 6).
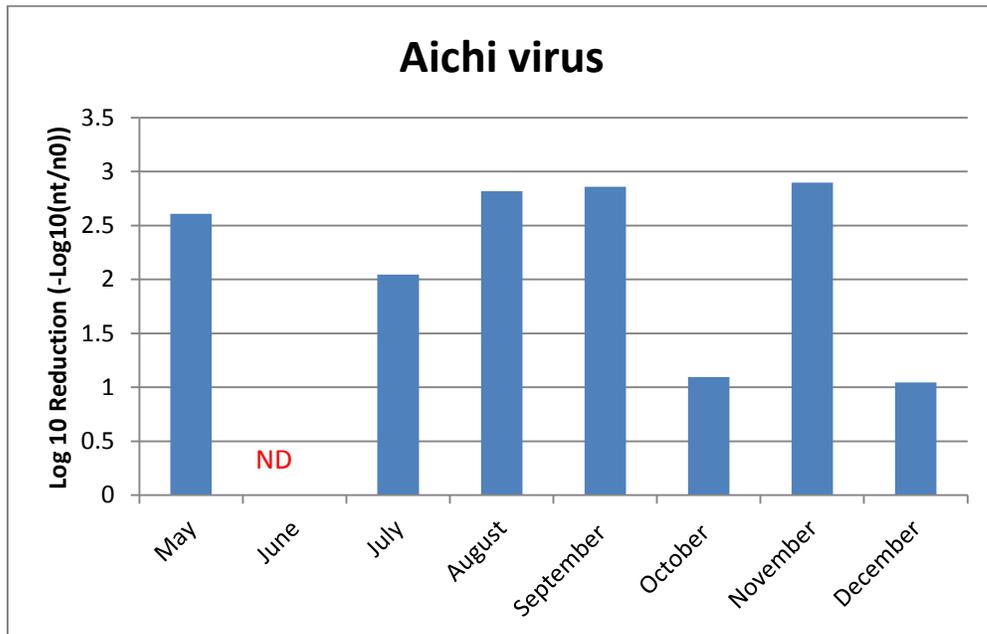
Figure 6. Log$_{10}$ reduction of Aichi virus in the Sweetwater wetland (ND = not detected)

The most persistent virus in the Sweetwater wetland was the PMMoV. PMMoV was found to be removed the least of all the viruses studied. There was less than a one log$_{10}$ reduction of PMMoV by the Sweetwater wetland. The concentration of PMMoV ranged from $10^5 - 10^7$ copies/L throughtout the study (Figure 7).
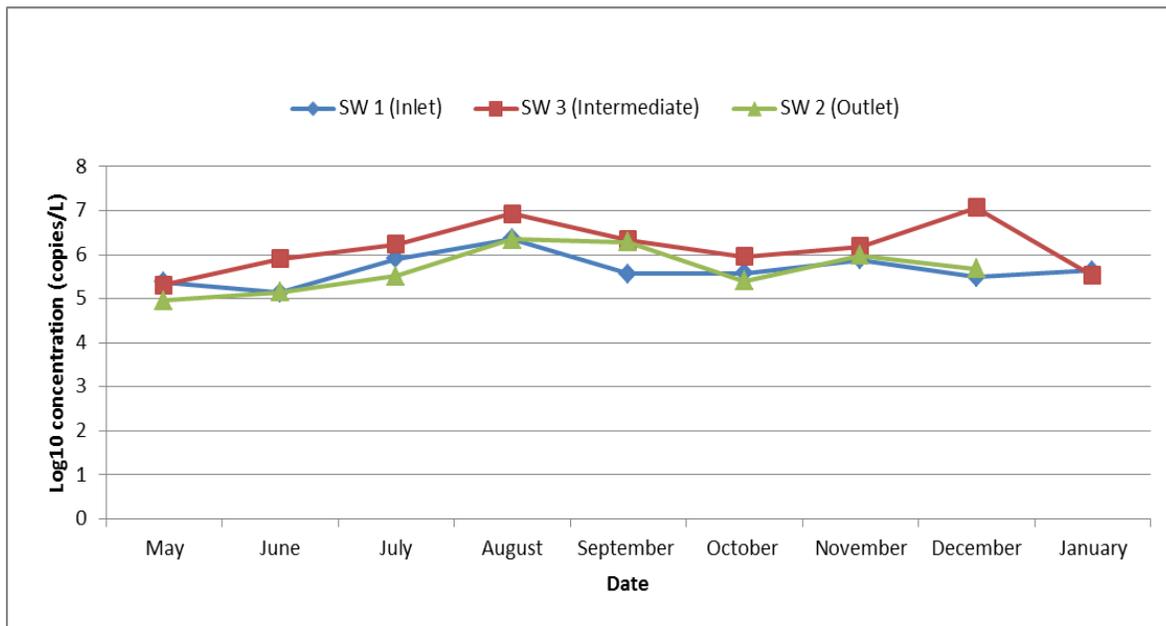

Figure 7. Seasonal occurrence of PMMoV at the Sweetwater wetland

At the Tres rios wetland, lower concentrations (10^2 -10^4 copies/L) of PMMoV were detected on June 2014 buta higher concentration was found in November (10^5 -10^6 copies/L) (Figure 8). Both HPyV BK and JC were found in the Sweetwater wetland and Pine Top wastewater treatment plant but none at the Tres Rios wetland. HPyV BK was found consistently from 10^2 -10^4 copies/L in the inlet of Sweetwater wetland (Figure 9) except in May 2013 and January 2014 with approximately 1-2 log$_{10}$ reduction (Figure 10). Higher concentrations of

HPyV JC detected (10^2 -10^5 copies/L) with a1-3 $\log_{10}$ reduction. HPyV JC was also found consistently in the inlet of the Sweetwater wetland.
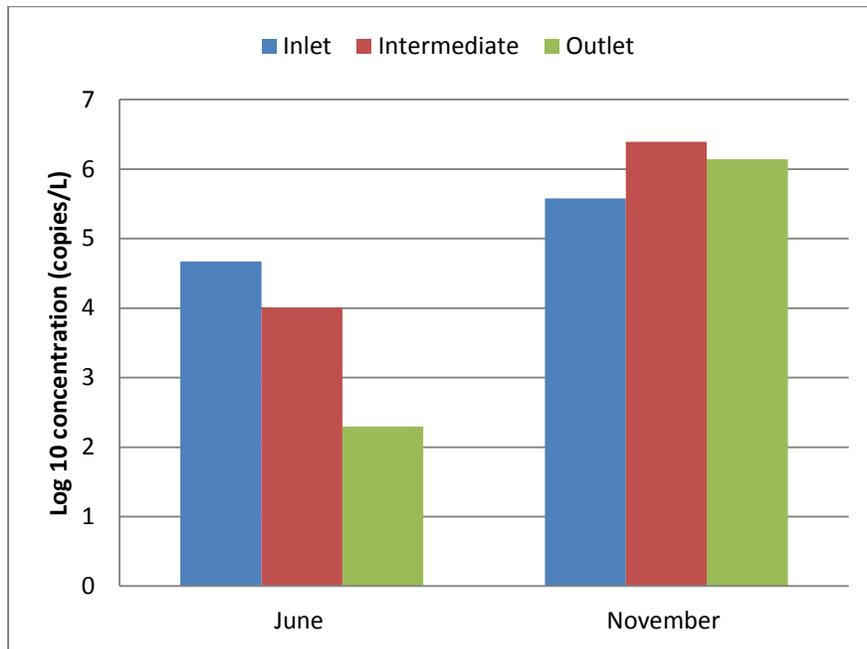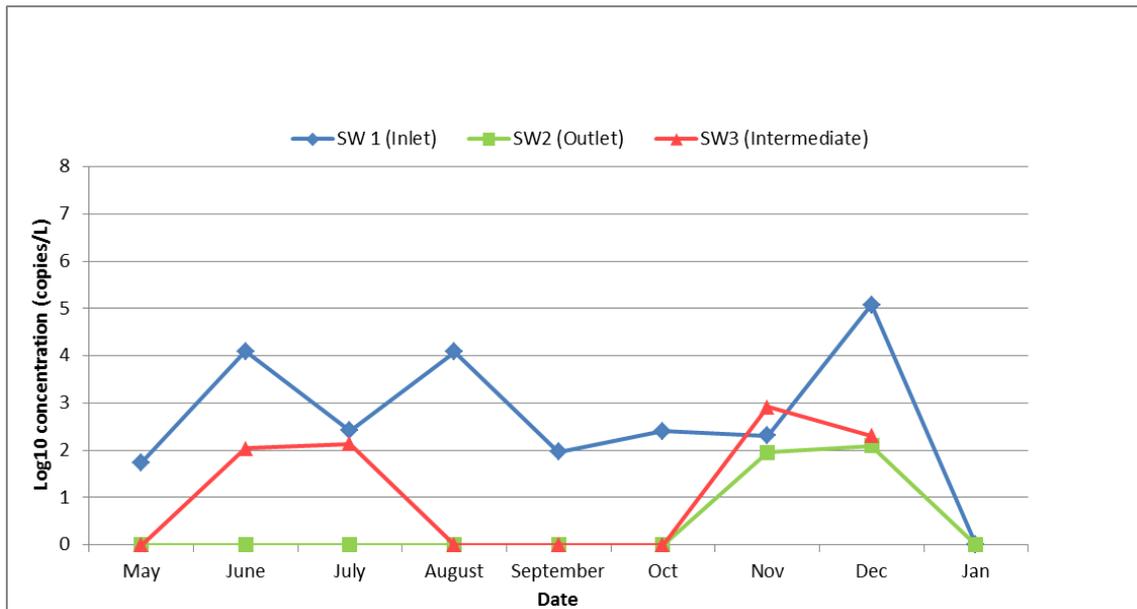

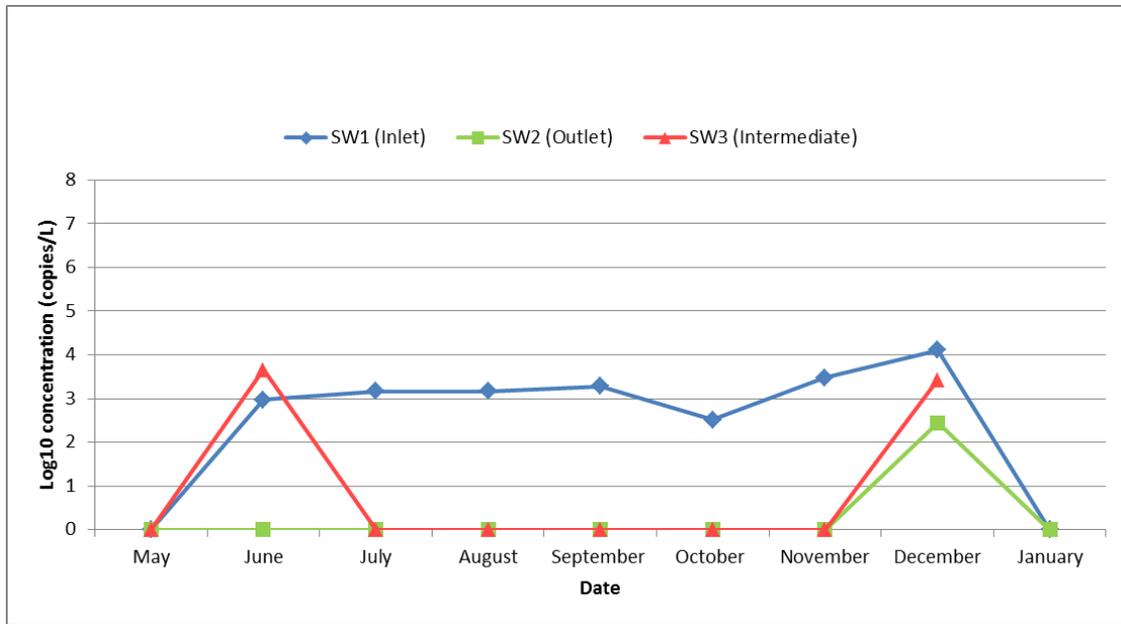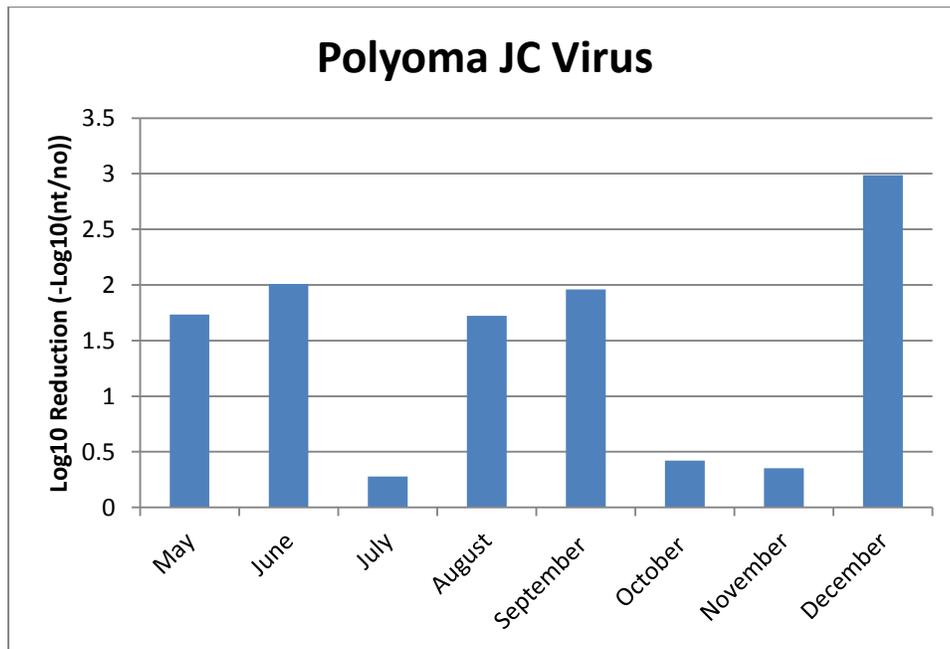Figure 8. PMMoV occurrence at Tres Rios Wetland, Phoenix

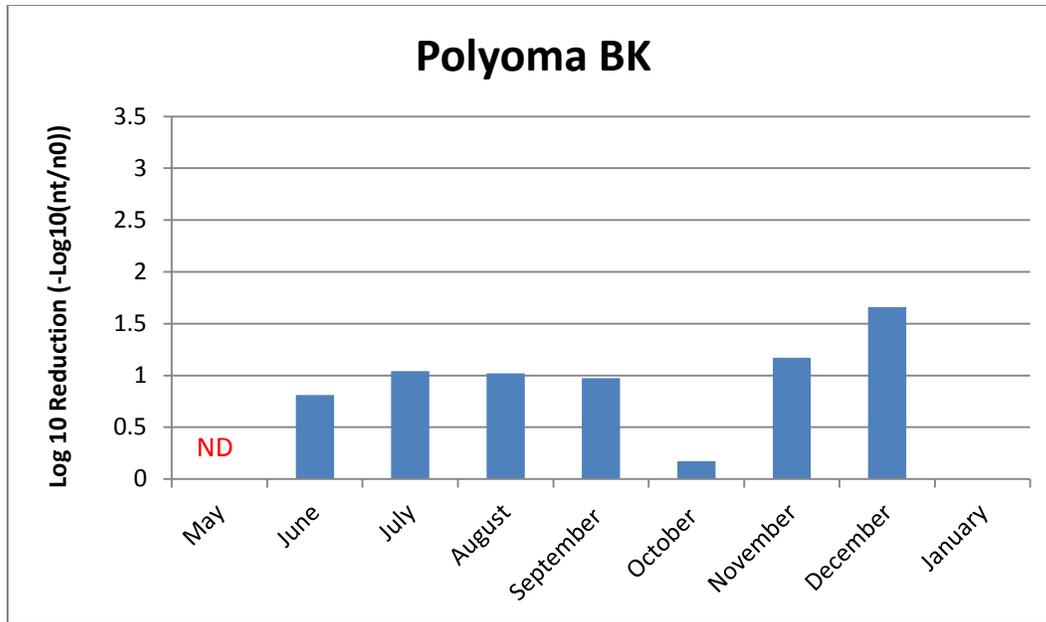Figure 9. Seasonal occurrence of Human Polyoma JC and BK at the Sweetwater wetland

Figure 10. Log$_{10}$ reduction of Human Polyoma JC and BK viruses by the Sweetwater wetland

(ND=not detected)

Correlation analysis between physical and chemical parameter with the concentration of the enteric viruses

Table 3. Rho and P value from Spearman correlation analysis

Correlation Analysis	Rho values	P values
pH and Temperature	0.0473	0.8263
pH and Turbidity	-0.3080	0.1744
Temperature and Turbidity	0.3918	0.0790
pH and adenovirus concentration	-0.1733	0.4071
pH and Aichi Virus concentration	0.1862	0.3837
pH and PMMoV concentration	-0.1846	0.0286
Temperature and adenovirus concentration	-0.1486	0.4884
Temperature and Aichi virus concentration	-0.2425	0.2537
Temperature and PMMoV concentration	-0.1015	0.6370
Turbidity and adenovirus concentration	0.3974	0.0744
Turbidity and Aichi virus concentration	0.1118	0.6295
Turbidity and PMMoV concentration	-0.2013	0.3813

Spearman analysis show there is no strong correlation between any chemical and physical parameters and enteric viruses concentration (Rho value was less than 1). There was a significant correlation between pH and PMMoV correlation based on P-value 0.02.

Incubation Experiment

An experiment was conducted to determine the persistence of poliovirus and PMMoV in wetland water at different temperatures in dark condition. This was compared to autoclaved wetland (wetland control) and reverse osmosis treated water (water control) to assess the role of microorganism on the removal of the viruses. In addition, the decline of poliovirus by infectivity assay and qPCR was determined.

In sterilized wetland water at 4°C and 25°C incubation temperature treatment, there were a slight ($1\log_{10}$) poliovirus (PV) reduction from days 1 to 4 (Figure 11). After that, it increased 1 $\log_{10}$ and remained unchanged for the next 21 days. At 37°C the concentration of PV decreased with a 2 $\log_{10}$ reduction of PV on the fourth day and then a slight increase by 1 $\log_{10}$ and then larger unchanged for the next 21 days. A similar result was found for the sterilized water control but with a lower reduction in 37 $^{\circ}$C and 21 days (Figure 11).
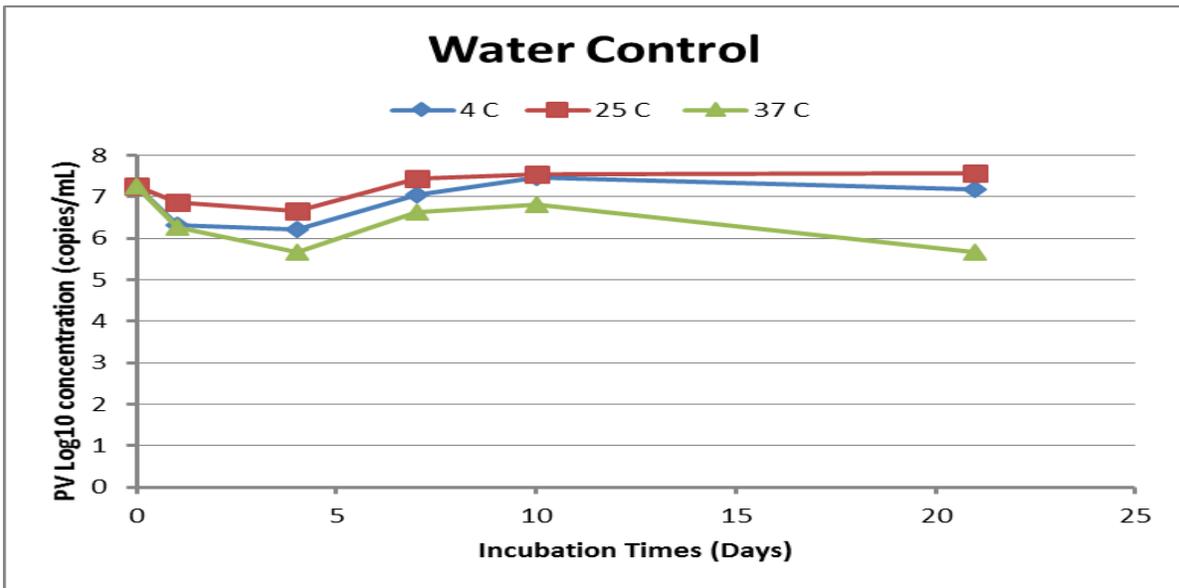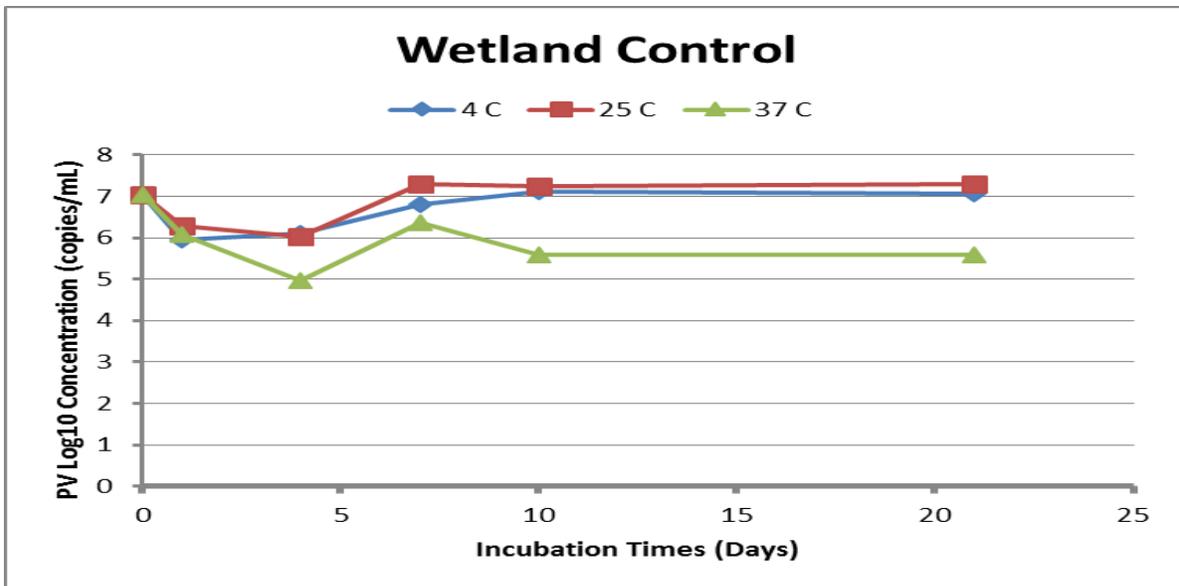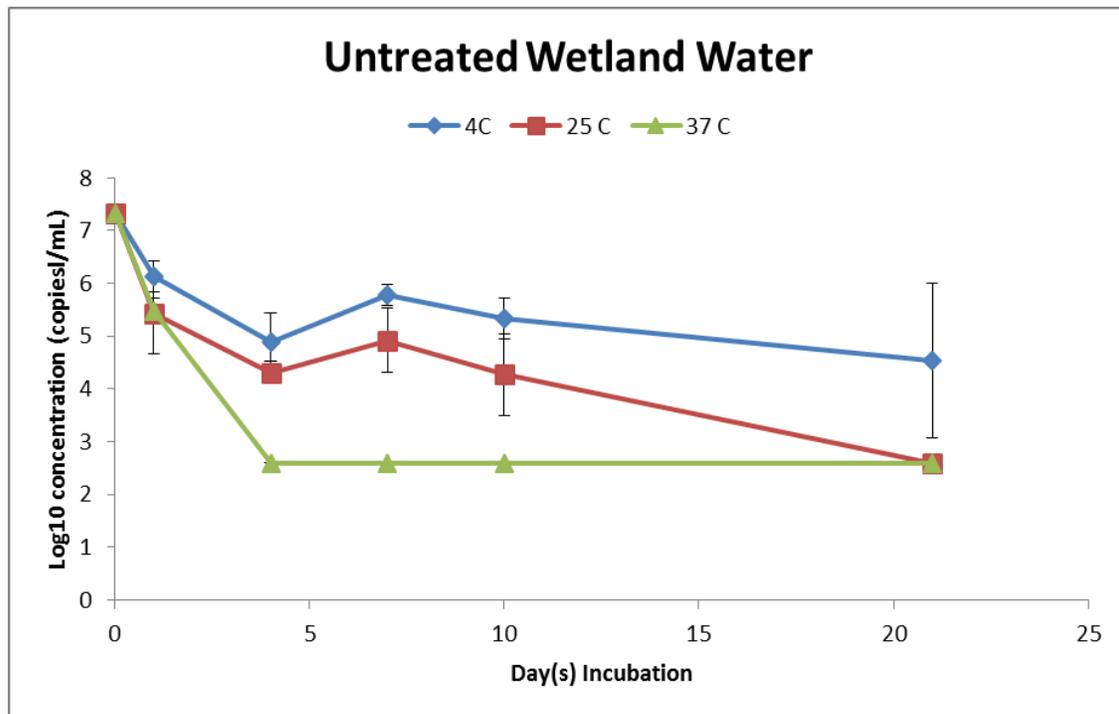
Figure 11. Log_{10} concentration of PV at the incubation experiment control's samples (water control and wetland control)

In the untreated Sweetwater wetland water, there was 4 log_{10} reduction of PV after four days at 37°C (Figure 12). After this time, no RNA was detected using q-PCR. On the other hand, a small

reduction (2-3 Log$_{10}$) was detected after 4th days at 25$^{°}$C and after 21 days of incubation there

RNA was detected (Figure 12). There was no change after 4$^{°}$C after 21 days.

Untreated Wetland Water

- Error bars mean standard deviation, limit detection was ….

Figure 12. Log$_{10}$ concentration of PV in untreated wetland water.

PMMoV shown an initial concentration in the untreated samples ranging from 2-3 x 10^{5}

copies per mL (Figue 13). During the incubation there was no significant reduction after 21 days
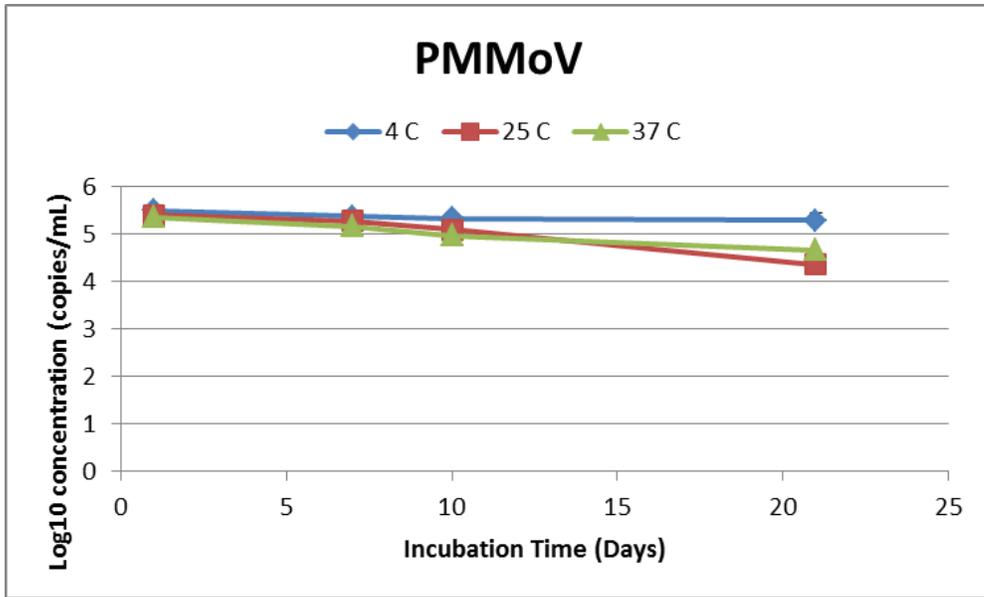
in under all experimental conditions (Figure 14).

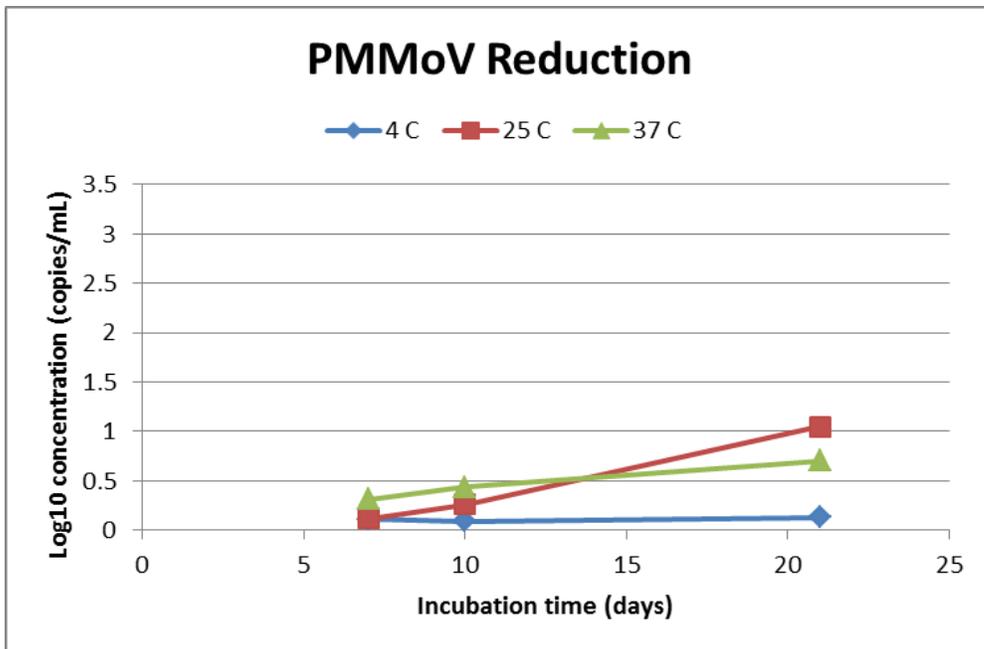Figure 13. Log$_{10}$ concentration of PMMoV in untreated wetland water

Figure 14. Log$_{10}$ reduction of PMMoV in untreated wetland water

Discussion

The human entericviruses Adenovirus and Aichi virus where the most commonly detected enteric viruses wastewater detected during this study. The peak of adenovirus concentration at the Sweetwater wetland occurred on November and December. The incidence of adenovirus infection is more common in the winter in the United States (http://www.cdc.gov/adenovirus/outbreaks.html). Dey et al 2013 also found adenovirus activity peak was in winter and spring seasons in the wastewater influent (December—March). Other studies found that the peak season for adenovirus detection in wastewater in March (Krikelis et al. 1985). Other research mentioned that adenovirus concentration peak in the river water in Japan was happened during the winter (E Haramoto, Katayama, Oguma, & Ohgaki, 2007; Kishida, Morita, Haramoto, Asami, & Akiba, 2012). A slight negative correlation was found between the concentration of AdV and pH and temperature but on the other hand, a weak positive correlation was described between Adv and the turbidity in the Sweetwater wetland (R=0.39). The persistent of adenovirus in the wastewater treatment compare it with other enteric viruses may be caused by their resistant to UV disinfection (Meng & Gerba 1996) and the higher stability of double stranded genome (Orgozally *et al.* 2010).

In the Sweetwater wetland, AiV was detected in 51% (14/27) samples. The highest concentration was observed during summer and early winter. The high abundance of AiV was also observed in 100% and 92% of AiV genomes in the influent and effluent of a wastewater treatment plant in Japan, respectively (Kitajima, Haramoto, Phanuwan, & Katayama, 2011b). There was no correlation between AiV concentration and turbidity (R = 0.118), temperature (R = -0.2425) and pH (R = 0.1862). The high abundance and persistence of Aiv in the wastewater

treatment in Arizona was also suggested due to their constant present in both of influent and effluent (Kitajima et al. 2014).

Enteroviruses and norovirus were only occasionally detected. The assumption was almost all enteroviruses were removed in the wastewater treatment before it discharged to the wetlands. There was more than 5 Log_{10} reductions (99%) of enterovirus was observed at a full-scale water reclamation facility Rose et al (1996). In addition, more than 90% reduction of enteroviruses was observed in an 9.15 m x 11 m x 2.1 m artificial subsurface flow vegetated (bulrush) bed with 5.5 days of retention times by MPN observation of cytophatic effects on cell culture media in BGM cell line (QuinoNez-Di´az et al 2001). The peak of the norovirus results were probably caused by an unreported NoV small outbreak during the summer.

The use of PMMoV and Human Polyoma JC and BK were also assessed as they have been suggested as indicators of fecal pollution. PMMoV occurred in the greatest numbers in the wastewater and wasremoved the least by the compared to the other viruses. PMMoV was detected in all wetland samples (inlet, outlet, and intermediate) ranging from 10^2 - 10^7 copies/L with less than a 1 log_{10} reduction. Polyoma JC and BK virus were detected at lower concentrations ($10^2 - 10^4$ copies/L) in the inlet of Sweetwater wetland and none were detected at the Tres Rios and PineTop wetlands. As a conservative indicator, PMMoV has a potential due to high concentratrion in raw wastewater, and the result suggested to have more resistance to wastewater treatment, temperature, and biological treatment compare it with Human Polyoma JC and BK. There was no correlation between PMMoV and temperature (R = -0.1015), and turbidity (R = -0.2013) was observed.

In general, the log_{10} reduction rate might be underestimated or overestimated because the limitation of grab sample method. The water samples not collected from the same water body,

without taking into account the retention time. The seasonal data on removal is still unclear and sometimes the concentration in the intermediate site is higher from the inlet. The higher occurrence of some of the viruses, could be explained by virus occurrence in the wastewater and polymerase inhibitors. Base on correlation analysis, there were no conclusive correlation between pH, temperature, and turbidity with the occurrence of AiV, AdV, and PMMoV due to R value which far away from 1.

An experiment was conducted to compare the decay of PMMoV loss by qPCR, and poliovirus (by both infectivity assay and qPCR) to determine how long both could be detected by qPCR in wetland water under different conditions. The results suggested that temperature and the biological process which was occurred have a significant role in viruses' removal. In autoclaved water and wetland water (water and wetland control), there were no significant reductions at 4 °C and 25 °C in the decay of the RNA viruses. In the water and wetland control where there were not any biological process happened, the PV RNA was still detected after 21 days at all temperatures by qPCR. In the untreated water samples at 37 °C no PV RNA was detected after 4 days. At 4 °C and 25 °C, 2-3 Log_{10} reductions occurred after 4 days. No PV RNA was detected at 21 days at 25 °C treatment in untreated wetland water. Similar result was shown with Coliphage where 3.16 Log_{10} reduction was observed at the constructed ecosystem research facility with 10 days of retention times (Karim et al. 2004). In contrast, there was no reduction of PMMoV occurred after 21 days at 25 °C. The result suggests PMMoV is very stable and a potential conservative tracer/indicator for human contamination in the water environment.

Conclusions

The most abundance enteric viruses detected in the secondary wastewater treatment were adenoviruses and Aichi virus. Both of the viruses are detected at concentrations of $10^2 - 10^5$ copies/L in the inlet of the Sweetwater wetland. Up to 2.5 Log_{10} reduction was detected in the field for adenovirus and Aichi virus. Temperature and biological activity likely play a significant role in the virus reduction in the wetlands. PMMoV was suggested as a potential; conservative indicator due to d abundance in the treated wastewater and persistence in the environment. This result was supported in the incubation experiment where only one log_{10} reduction its RNA after three weeks .

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