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Virus Reduction during Advanced Bardenpho and Conventional Wastewater Treatment Processes

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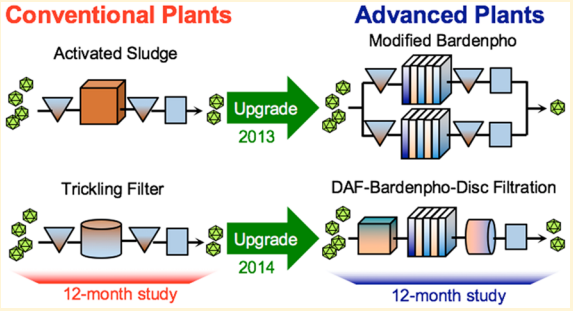
Supporting Information

ABSTRACT: The present study investigated wastewater treatment for the removal of 11 different virus types (pepper mild mottle virus; Aichi virus; genogroup I, II, and IV noroviruses; enterovirus; sapovirus; group-A rotavirus; adenovirus; and JC and BK polyomaviruses) by two wastewater treatment facilities utilizing advanced Bardenpho technology and compared the results with conventional treatment processes. To our knowledge, this is the first study comparing full-scale treatment processes that all received sewage influent from the same region. The incidence of viruses in wastewater was assessed with respect to absolute abundance, occurrence, and reduction in monthly samples collected throughout a 12 month period in southern Arizona. Samples were concentrated via an electronegative filter method and quantified using TaqMan-based quantitative polymerase chain reaction (qPCR). Results suggest that Plant D, utilizing an advanced Bardenpho process as secondary treatment, effectively reduced pathogenic viruses better than facilities using conventional processes. However, the absence of cell-culture assays did not allow an accurate assessment of infective viruses. On the basis of these data, the Aichi virus is suggested as a conservative viral marker for adequate wastewater treatment, as it most often showed the best correlation coefficients to viral pathogens, was always detected at higher concentrations, and may overestimate the potential virus risk.



1. INTRODUCTION

Increasing water consumption, limited freshwater resources, and climate change demand increased beneficial reuse of wastewater for recreational, industrial, agricultural, and potable purposes. Indirect and de facto reuse are common wastewater recycling practices worldwide. Notably, arid and water-stressed areas have escalated water management to expedite direct potable reuse,[1] most notably in Windhoek, Namibia,[2] and a few small facilities in the United States,[3] such as those located in Big Spring, Texas and Cloudcroft, New Mexico.[4] However, recycling and reclaiming municipal wastewater involves potential environmental and human health risks associated with the incidence of pathogens in these waters. Therefore, as water sustainability initiatives continue to arise, monitoring viral pathogens and utilizing the most efficient wastewater treatment technologies are necessary to minimize risks.[5−7]

Currently, there are no regulatory standards regarding the reduction of viruses during wastewater treatment;[8] however, a 12 log reduction of viruses in wastewater is required by the California Department of Public Health for reclaimed water intended for indirect potable reuse.[9] Traditional monitoring approaches and guidelines for waters intended for reuse and recreational purposes are based upon fecal indicator bacteria to detect microbial contaminants and designate waters as safe.[10−12] However, human pathogenic viruses are more resistant to treatment.[13−16] In addition, they show variable correlations with traditional indicators,[14,17] demonstrating the inadequacy of bacteria to indicate viral contamination.[16,18] Thus, the Environmental Protection Agency (EPA) recently suggested coliphages as potential indicators[11] because they are similar in structure, morphology, and assumed resistance to inactivation as human enteric viruses.[19−22] To evaluate the incidence, persistence, fate, and transport of human pathogenic viruses, a viral indicator for contamination may be appropriate. Hence, many virus types have recently been proposed as indicators for contamination and microbial-source tracking, including F-RNA phages,[16,23] pepper mild mottle virus,[24−26] polyomaviruses,[8,14,16,17,27,28] and adenoviruses.[14,16,27,28]

However, the appropriateness for an indicator can be dependent on human population dynamics, seasonal effects, and types of treatment processes.[13] Virus incidence in wastewater is dependent on human population size,[6] and removal depends on treatment efficiency. Therefore, the most-

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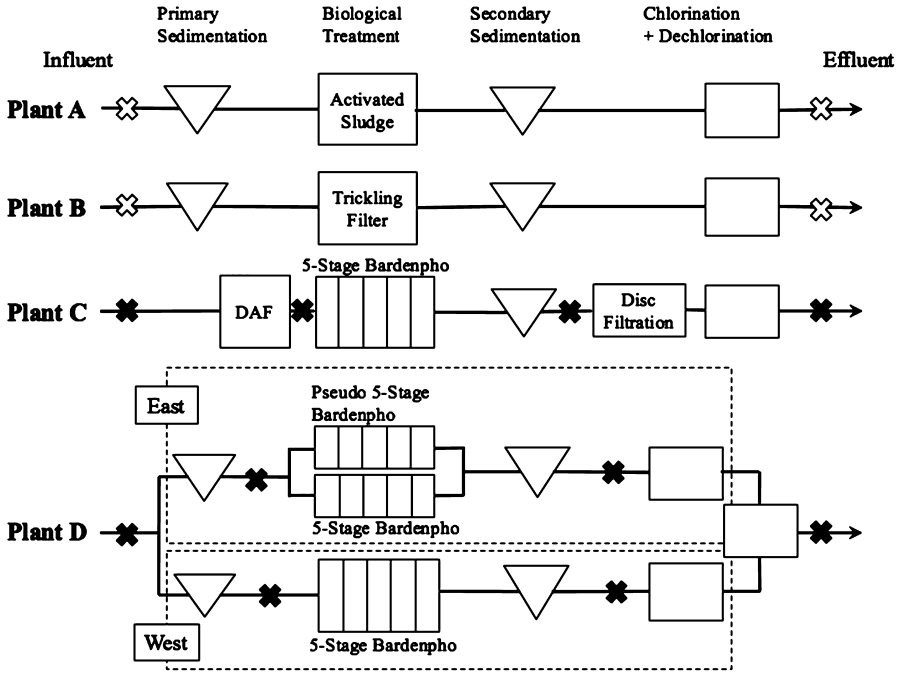

Figure 1. Schematic of each WWTP. Hollow X symbols indicate grab-sample-collection locations. Solid X symbols indicate 24 h autosampler collection locations. Plants A and B were previously reported by Kitajima et al. (2014).[25]

effective virus-removal technologies should be implemented during wastewater treatment to minimize risks associated with virus in discharged effluent waters. Previous reviews and research have evaluated virus reduction in various conventional systems such as stabilization ponds,[6] activated sludge,[7,29−31] trickling filter−biofilm systems,[6,29] and wetlands.[32] Evaluation of advanced treatment on virus removal such as membrane bioreactors (MBR) and small-pore filtration systems,[7,30,33−35] advanced oxidation processes (AOP),[36,37] reverse osmosis (RO),[9] and anaerobic−anoxic−oxic (A^2/O)[38,39] processes have also been conducted. However, comparisons between treatment processes are confounded by differences in geographical areas, wastewater origins, seasonal influences, sampling methods, concentration procedures, assays utilized, quantification methods, and detection sensitivity.[7,13,27,40] Molecular techniques, especially quantitative PCR (qPCR), have been increasingly utilized for the enumeration of the occurrence of viruses in wastewater due to its capability to detect any desired target, including emerging pathogens.[16,40,41]

The present study evaluated virus removal at WWTPs previously and recently implemented in southern Arizona. The goal was to investigate which wastewater treatment processes were most proficient at minimizing the incidence of pathogenic viruses in effluent waters intended for reclamation and recycling. Our previous study examined conventional WWTPs utilizing activated sludge and trickling filter biotowers.[25] These WWTPs were decommissioned and replaced with advanced treatment processes, providing a unique opportunity to compare virus incidence and removal before and after upgrading facilities in the same geographical region. The present study investigated advanced Bardenpho treatment processes (Plants C and D), and data were compared with results from conventional processes previously reported.[25] The conventional and advanced WWTPs were investigated for a 1 year period each within a 4 year window.

Bardenpho processes are designed with serial compartments (present study: anaerobic, anoxic, oxic, anoxic, and oxic), with the primary focus of reducing nitrogen loads via increased denitrification.[42] This evaluation is critical because little has been reported on the efficacy of the Bardenpho process with respect to pathogen removal. To our knowledge, this is the first study comparing the reduction of viruses at four different large-scale WWTPs, all receiving raw sewage from the same geographical region. These findings can have significant implications on how wastewater technologies influence restrictions and standards for water intended for reclamation and reuse.

The incidence of 11 different virus types was monitored for absolute abundance, occurrence, and reduction with the goal of identifying a conservative viral indicator of human fecal contamination, identifying a viral marker suggesting the adequate reduction of viruses during wastewater treatment, or both. Criteria for a suitable indicator were previously described by Kitajima et al. (2014).[25]

2. MATERIALS AND METHODS

2.1. Collection of Wastewater Samples. Wastewater sampling was conducted monthly at four WWTPs (conventional Plants A and B in a previous study;[25] advanced Plants C and D in the present study) located in southern Arizona throughout 12 month time periods. Between August 2011 and July 2012, a total of 48 grab samples were collected from Plant A and Plant B; the samples consisted of 12 influent (post-screening prior to primary sedimentation) and 12 final effluent (post-chlorination and dechlorination) samples from each plant.[25] Plant A utilized activated sludge for secondary treatment, while Plant B used a trickling filter (Figure 1).

Between June 2014 and May 2015, composite samples for Plants C and D were collected via 24 h autosamplers (Hach Sigma 900MAX; Loveland, CO). Plant C utilized dissolved air flotation (DAF), four parallel five-stage Bardenpho processes,

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disc filtration, and chlorination disinfection (Figure 1). A total of 48 composite samples were collected from Plant C and consisted of 12 influent (postscreening prior to primary treatment), 12 primary effluent (post-DAF), 12 secondary effluent (postbiological treatment and secondary sedimentation), and 12 final effluent (postchlorination and dechlorination) samples.

Plant D consisted of two separate treatment trains (East and West). The East train utilized a single five-stage Bardenpho process parallel to a basin that was modified into a pseudo five-stage Bardenpho process (Figure 1). The West train utilized three parallel five-stage Bardenpho processes (Figure 1). A total of 72 composite samples were collected from Plant D, consisting of 12 influent (postscreening prior to primary sedimentation), 24 primary sedimentation effluent (12 each from East and West), 24 secondary treatment effluent (12 each from East and West), and 12 final effluent (postchlorination and dechlorination) samples.

A total of 2 L of wastewater was collected for each sample in sterile 1 L Nalgene bottles and transported in a cooler containing ice to the laboratory. Virus concentration was performed within 12 h of sample collection, as described below. Excess wastewater was stored at 4 °C for a maximum of 48 h or until all analysis had been completed, whichever occurred sooner. To determine whether final effluent waters for each WWTP met microbiological criteria for recreational waters,[10] *Escherichia coli* was assayed by the Colilert method (SM 9223B; IDEXX Laboratories, Inc., Westbrook, ME) and expressed as most-probable number (MPN) per 100 mL (Figure S1).[43]

2.2. Concentration of Viruses in Wastewater Samples. The wastewater samples were concentrated using an electronegative filter method as previously described,[44] with slight modification. Briefly, 2.5 M $MgCl_2$ was added to the wastewater samples to obtain a final concentration of 25 mM. Samples were subsequently passed through an electronegative filter (0.45 μm pore size) (catalog no. HAWP-090-00; Millipore, Billerica, MA) attached to a glass filter holder (Advantec, Tokyo, Japan). A total of 100 mL of turbid samples (i.e., influent and primary effluent) and 1000 mL of samples containing low amounts of particulates (i.e., secondary effluents and final effluents) were passed through the membrane. Bacteria, protozoa, and large particulates (>0.45 μm) were removed via physical size exclusion.

Magnesium ions provided a cation bridge attaching the negatively charged viruses to the electronegative membrane and then were removed by the passage of 200 mL of 0.5 mM H_2SO_4 (pH 3.0) through the filter. The acid rinse altered environmental pH below the viruses' isoelectric point, providing virions with a net positive charge to bind directly to the filter. Then, viruses were eluted by re-establishing a net negative charge 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. Further centrifugation was performed using a Centriprep YM-50 containing a membrane with a nominal molecular weight limit (NMWL) of 50 kDa (catalog no. 4310; Merck Millipore, Billerica, MA) to obtain a final volume of approximately 650 μL. Concentrates were either processed for immediate extraction of viral nucleic acids or stored at −80 °C until further analysis.

2.3. Sample Process Control for Extraction−RT-qPCR. Murine norovirus (MNV strain S7-PP3) was kindly provided by Dr. Y. Tohya (Nihon University, Kanagwa, Japan) and propagated in RAW 264.7 cells (ATCC no. TIB-71; American Type Culture Collection, Manassas, VA). MNV was used as a sample process control to determine the efficiency of downstream assays (extraction−reverse transcription-qPCR) from actual samples relative to DI water, as previously described.[23] Briefly, 2.0 μL of MNV stock (1.8×10^4 copies/μL) was spiked into 200 μL each of concentrated wastewater samples (from Plants C and D) and molecular biology grade water (DNase-, RNase-, and protease-free), which was utilized as a control (i.e., no inhibition) to determine the spiked MNV-RNA amount. MNV-RNA spiked into the concentrated wastewater samples was co-extracted with other indigenous viral nucleic acids, and MNV-RNA yield was determined by RT-qPCR. The percent extraction−RT-qPCR efficiency (E) was calculated as follows:

$$E = C/C_o \times 100$$

where C represents the observed MNV-cDNA copy numbers per qPCR tube in a wastewater sample, and C_o represents copy numbers in the control. The MNV process control was used to identify viral nucleic acid loss during extraction, the occurrence of any RT-qPCR inhibition, or both. Concentrations of indigenous viruses in the wastewater samples were estimated by adjusting the concentration with the extraction−RT-qPCR efficiency (E) data for each wastewater sample.

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

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 a final 85 °C for 5 min to inactivate the enzyme.

2.5. 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 are provided in the Table S1. The reaction mixtures were subjected to thermal cycling, and fluorescence readings were collected and analyzed with LightCycler 480 Software version 1.5 (Roche Diagnostics, Mannheim, Germany). The genome copy numbers of each virus were determined on the basis of 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, calculated based on the plasmid DNA concentration determined by measuring the optical density at 260 nm. Negative controls were included to detect any false-positive results due to cross-contamination; however, no false-positive qPCR signal was observed. The qPCR reactions were performed in duplicate and considered positive when the tube fluoresced with sufficient

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Table 1. Characteristics of WWTPs[a]

					effluent water quality characteristics			
plant	biological treatment	capacity (m^3/day)	SRT (hours)	RAS (L/day)	BOD (mg/L)	TSS (mg/L)	ammonia (mg/L)	total N (mg/L)
A[b]	activated sludge	1.55×10^5	13–18	5.43×10^7	11–28	4–26	26–28	30–40
B[b]	trickling filter	9.46×10^4	20 min	–	8–27	9–25	26–28	30–40
C	five-stage Bardenpho	1.21×10^5	8–9	1.38×10^7	2–5.6	<2.5–4.8	0.7–5.8	5.2–11
D	five-stage Bardenpho	1.89×10^5	13–18	1.85×10^7	2–10	<2.5–5.8	1–6.5	5–9

[a]SRT, sludge and solids retention time; RAS, return activated sludge; BOD, biochemical oxygen demand; TSS, total suspended solid; MPN, most-probable number. Data were provided by the wastewater treatment facility. Data for Plants A and B were observed during the study period, August 2011–July 2012. Data for Plants C and D were observed during the study period, June 2014–May 2015; –, information not available. [b]Characteristics for Plants A and B were previously reported by Kitajima et al. (2014).[25]

Table 2. Abundance and Occurrence of Viruses and *E. coli*[a]

virus	source	Plant A (activated sludge)[b]	Plant B (trickling filter)[b]	Plant C (Bardenpho)	Plant D (Bardenpho)
PMMoV	influent	**6.5** (12/12)	**6.5** (12/12)	**7.5** (12/12)	**7.5** (12/12)
	effluent	**5.8** (12/12)	**5.7** (12/12)	**6.7** (12/12)	**4.8** (10/12)
AiV	influent	**4.9** (12/12)	**6.2** (12/12)	**5.4** (12/12)	**5.6** (12/12)
	effluent	**4.0** (12/12)	**5.4** (12/12)	**4.3** (10/12)	**3.0** (4/12)
GI NoV	influent	**4.7** (12/12)	**4.1** (12/12)	**4.6** (9/12)	**4.5** (10/12)
	effluent	**2.7** (4/12)	**2.4** (0/12)	**3.5** (8/12)	**3.1** (4/12)
GII NoV	influent	**3.8** (11/12)	**4.2** (12/12)	**4.9** (12/12)	**4.9** (11/12)
	effluent	**2.4** (2/12)	**2.5** (1/12)	**3.4** (9/12)	**2.5** (3/12)
GIV NoV	influent	**3.4** (8/12)	**3.2** (6/12)	**3.5** (5/12)	**3.9** (5/12)
	effluent	**2.8** (3/12)	**2.6** (3/12)	**2.7** (2/12)	**2.5** (1/12)
EV	influent	**5.1** (12/12)	**5.2** (12/12)	**4.8** (10/12)	**4.6** (9/12)
	effluent	**3.1** (7/12)	**3.2** (10/12)	**2.6** (2/12)	**2.4** (0/12)
SaV	influent	**5.0** (11/12)	**4.9** (12/12)	**4.4** (9/12)	**3.7** (6/12)
	effluent	**3.4** (7/12)	**2.9** (5/12)	**2.5** (1/12)	**2.4** (0/12)
ARV	influent	**3.6** (5/12)	**3.6** (7/12)	**3.4** (6/12)	**2.9** (3/12)
	effluent	**3.7** (8/12)	**3.1** (4/12)	**2.5** (1/12)	**2.6** (2/12)
AdV	influent	**4.8** (12/12)	**5.0** (12/12)	**5.7** (12/12)	**6.0** (12/12)
	effluent	**4.2** (12/12)	**4.0** (12/12)	**4.1** (11/12)	**2.7** (5/12)
JCPyV	influent	**5.5** (12/12)	**4.8** (10/12)	**4.6** (10/12)	**5.2** (12/12)
	effluent	**3.0** (6/12)	**2.6** (6/12)	**3.0** (7/12)	**2.1** (1/12)
BKPyV	influent	**4.9** (11/12)	**4.5** (10/12)	**5.0** (9/12)	**6.0** (12/12)
	effluent	**3.7** (9/12)	**3.5** (10/12)	**4.4** (10/12)	**2.6** (2/12)
E. coli	effluent	**0.9** (9/12)	**1.4** (9/12)	**0.7** (7/12)	**1.3** (1/12)

[a]Bold numbers indicate annual mean concentration values for viruses ($\log_{10}$ copies/L) and *E. coli* ($\log_{10}$ MPN/100 mL). Numerator: number of positive samples above the detection limits, 2.4 $\log_{10}$ copies/L (RNA) or 2.1 $\log_{10}$ copies/L (DNA). Denominator: total number of samples collected. [b]Data from Plant A and Plant B was previously reported by Kitajima et al. (2014).[25] However, 2.4 $\log_{10}$ copies/L (RNA) or 2.1 $\log_{10}$ copies/L (DNA) were substituted for values observed below the detection limit to keep consistency when comparisons are made with Plants C and D in the present study, as described in sections 3.3 and 3.4.

intensity and the cycle threshold (C_T) value was not more than 40, as recommended.[45]

2.6. Statistical Analysis. Nonparametric Kruskal–Wallis H tests and Wilcoxon Rank Sum posthoc tests (two-tailed) were performed with Microsoft Excel for Mac 2015 (Microsoft Corp., Redmond, WA) to determine whether $\log_{10}$ reductions at Plants A, B, C, and D were significantly different (α level of 0.05). Spearman's rank correlation coefficient tests were performed to determine the associations between potential viral markers and human enteric viruses.

3. RESULTS

3.1. Viral Nucleic Acid Extraction–RT-qPCR Efficiency. Concentrated wastewater samples were spiked with MNV as a process control to monitor RNA extraction–RT-qPCR efficiency for quantitative detection of viruses. Each sample from Plants C and D were spiked with 3.6×10^4 copies of MNV. The mean recovery efficiencies of MNV (E) for each sample are shown in Table S2. Recovery efficiency for samples from Plant A and Plant B have been previously reported.[25]

Recovery efficiencies were generally high, demonstrating minimal viral genome loss during the extraction–RT-qPCR process (Table S2). The observed viral copy numbers for each wastewater sample were adjusted according to the mean recovery efficiency (E) to estimate the actual indigenous concentrations.

3.2. Efficiency of Wastewater Treatment Plants. Data regarding each WWTP's operations was kindly provided by each facilities' management. Plants A, C, and D recycled nearly the same daily average amount of activated sludge (RAS) into secondary treatment bioreactors (Table 1). However, the sludge and solid retention time (SRT) in Plant C bioreactors was shorter than in Plants A and D (Table 1). Trickling filter biotowers in Plant B retained wastewater for only 20 min. Final effluent water quality was better after advanced treatments in Plants C and D because biological oxygen demand, total

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Table 3. Mean Reduction of Viruses at Each WWTP[a]

virus	Plant A[b] (activated sludge)	Plant B[b] (trickling filter)	Plant C (Bardenpho)	Plant D (Bardenpho)	similar reduction	significantly different
PMMoV	0.7 ± 0.5 (12)	0.8 ± 0.7 (12)	0.9 ± 0.5 (12)	> 2.7 ± 1.6 (12)[c]	A, B, C	D
AiV	0.9 ± 0.4 (12)	0.8 ± 0.1 (12)	> 1.1 ± 0.6 (12)	> 2.7 ± 0.9 (12)	A, B, C	D
GI NoV	> 2.0 ± 0.6 (12)	> 1.7 ± 0.8 (12)	> 2.0 ± 1.2 (9)	> 1.7 ± 1.4 (10)	A, B, C, D	–
GII NoV	> 1.6 ± 0.6 (11)	> 1.7 ± 0.9 (12)	> 1.6 ± 0.7 (12)	> 2.6 ± 0.6 (11)	A, B, C	D
GIV NoV	> 1.2 ± 0.7 (8)	> 1.2 ± 0.6 (6)	> 2.4 ± 1.2 (5)	> 3.6 ± 0.4 (5)	A, B, C	D
EV	> 2.1 ± 0.9 (12)	> 2.0 ± 0.7 (12)	> 2.6 ± 0.6 (10)	> 3.0 ± 0.6 (9)	A, B, C, D	–
SaV	> 1.8 ± 1.0 (11)	> 2.0 ± 0.8 (12)	> 2.7 ± 0.9 (9)	> 2.5 ± 0.5 (6)	A, B, C, D	–
ARV	> 0.3 ± 0.7 (5)	> 0.9 ± 1.3 (7)	> 1.7 ± 0.6 (5)	> 1.7 ± 1.1 (3)	A, B, C, D	–
AdV	0.6 ± 0.6 (12)	1.0 ± 1.0 (12)	> 1.7 ± 0.7 (12)	> 3.3 ± 1.2 (12)	A, B B, C	C, D
JCPyV	> 2.5 ± 1.2 (12)	> 2.8 ± 0.7 (10)	> 1.9 ± 1.0 (10)	> 3.1 ± 0.5 (12)	A, B, C, D	–
BKPyV	> 1.6 ± 1.1 (11)	> 1.4 ± 0.8 (10)	> 1.4 ± 0.8 (9)	> 3.4 ± 1.1 (12)	A, B, C	D

[a]Mean reduction of viruses and standard variation ($\log_{10}$ copies/L), as calculated as the mean difference between influent and effluent sample sets. Only sample sets that contained influent concentrations above the detection limit were considered for determining reduction values. Number in parentheses: number of sample sets used to calculate the mean annual reduction, standard deviation, and posthoc tests. Plants listed as similar are found to not have a statistically different mean reduction value (α level of 0.05), as calculated via Kruskal–Wallis H tests and Wilcoxon Rank Sum posthoc tests. Plants listed as significantly different are found to have a significantly different and greater mean reduction value than the other WWTPs. [b]Data from Plants A and B were previously reported by Kitajima et al. (2014).[25] However, mean reduction values were recalculated by substituting 2.4 $\log_{10}$ copies/L (RNA) or 2.1 $\log_{10}$ copies/L (DNA) for values observed below the detection limit to calculate the lower limit of $\log_{10}$ removals, as described in section 3.4. [c]The > symbol indicates that the actual reduction is higher than the calculated value, as at least one sample set contained effluent concentrations below the detection limit; – indicates that no WWTP showed a significantly different reduction of viruses

suspended solids, ammonia concentrations, and total nitrogen were lower than those from conventional Plants A and B (Table 1).

3.3. Incidence of Viruses in Wastewater. The incidence of 10 human enteric viruses and a plant virus commonly associated with human gut microbiota, pepper mild mottle virus (PMMoV), was detected by (RT)-qPCR.[46] Data from Plants A and B, taken between August 2011 and July 2012, have previously been reported.[25] However, values were adjusted by substituting 2.4 (RNA) or 2.1 (DNA) $\log_{10}$ copies/L for samples observed below detectable limits to maintain consistency when comparisons were made with data from advanced WWTPs in the present study. Data collected from Plants C and D between June 2014 and May 2015 are described below.

PMMoV consistently showed the highest frequency of occurrence with little seasonal variation and the highest annual mean concentrations in raw sewage influent and final effluent wastewater samples (Table 2; Figure S1A). Aichi virus (AiV) also showed a high frequency of occurrence with year-long prevalence and the second-highest annual mean concentrations (Table 2 and Figure S1B).

GI and GII noroviruses (NoV) were frequently detected in influent wastewater samples year-round, but GIV NoV, which is rarely detected in environmental samples,[25] was sporadically present (Figure S1C–E). GI and GII NoV were found at annual mean concentrations >4.5 $\log_{10}$ copies/L in raw sewage (Table 2). Enterovirus (EV) was detected at annual mean concentrations >4.6 $\log_{10}$ copies/L but either was not detected, had decreased concentrations between February to April (Table 2; Figure S1F), or both. EV was rarely detected in 2 of 24 treated wastewater samples from Plants C and D (Table 2). SaV was detected at annual mean concentrations of 4.4 and 3.7 $\log_{10}$ copies/L in sewage influent samples from Plants C and D but was only detected in 1 of 24 treated effluent samples (Table 2). ARV showed the lowest frequency of occurrence but the most specific seasonal variation, having a higher positive detection rate during the spring to early summer seasons (Table 2; Figure S1H).

Among DNA viruses, AdV showed the highest frequency of occurrence with little seasonal variation, as it was detected in 24 of 24 raw sewage influent and 16 of 24 final effluent samples from Plants C and D (Table 2, Figure S1I). JC and BK PyVs were frequently identified in 22 of 24 and in 21 of 24 sewage influent samples. (Table 2). However, the positive rate of detection and annual mean concentration of BKPyV in final effluent was higher than that of JCPyV (Table 2).

PMMoV, AiV, AdV, JCPyV, and BKPyV were analyzed as potential viral markers for human fecal contamination, adequate reduction of viruses during wastewater treatment, or both. AiV most often showed the best correlation coefficients to viral pathogens in influent and effluent waters (Table S3).

3.4. Reduction of Viruses by Wastewater Treatment Plants. The reduction of viruses by WWTPs was calculated as the difference between influent and effluent sample sets (Table 3). Data previously reported from Plant A and Plant B were used to make comparisons of virus reductions.[25] These data were adjusted by substituting 2.4 (RNA) or 2.1 (DNA) $\log_{10}$ copies/L for values observed below detectable limits to calculate the lower limit of $\log_{10}$ removals. This enabled a more-conservative determination of viral reductions rather than assuming that values observed below detectable limits provided an accurate depiction of actual concentrations. The rate of reduction to concentrations below detection limits was monitored to evaluate how often treatment resulted in no observable targets in effluent wastewaters, suggesting the efficient removal of viruses (Table S4).

Among the virus types tested, JCPyV experienced the greatest reduction values, followed by EV and SaV (Table 3). All NoV genogroups (GI, GII, and GIV), SaV, and AdV showed the greatest rates of removal to concentrations below detection limits (Table S4). PMMoV showed the greatest resistance to removal by treatment, followed by AiV, as indicated by low reduction values (Table 3) and low rates of reduction below detection limits (Table S4).

Plant D showed the greatest mean reduction values for nine virus types (Table 3) and the highest rate of removal to concentrations below the detection limit (Table S4). Statistical

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Environ. Sci. Technol. 2016, 50, 9524–9532

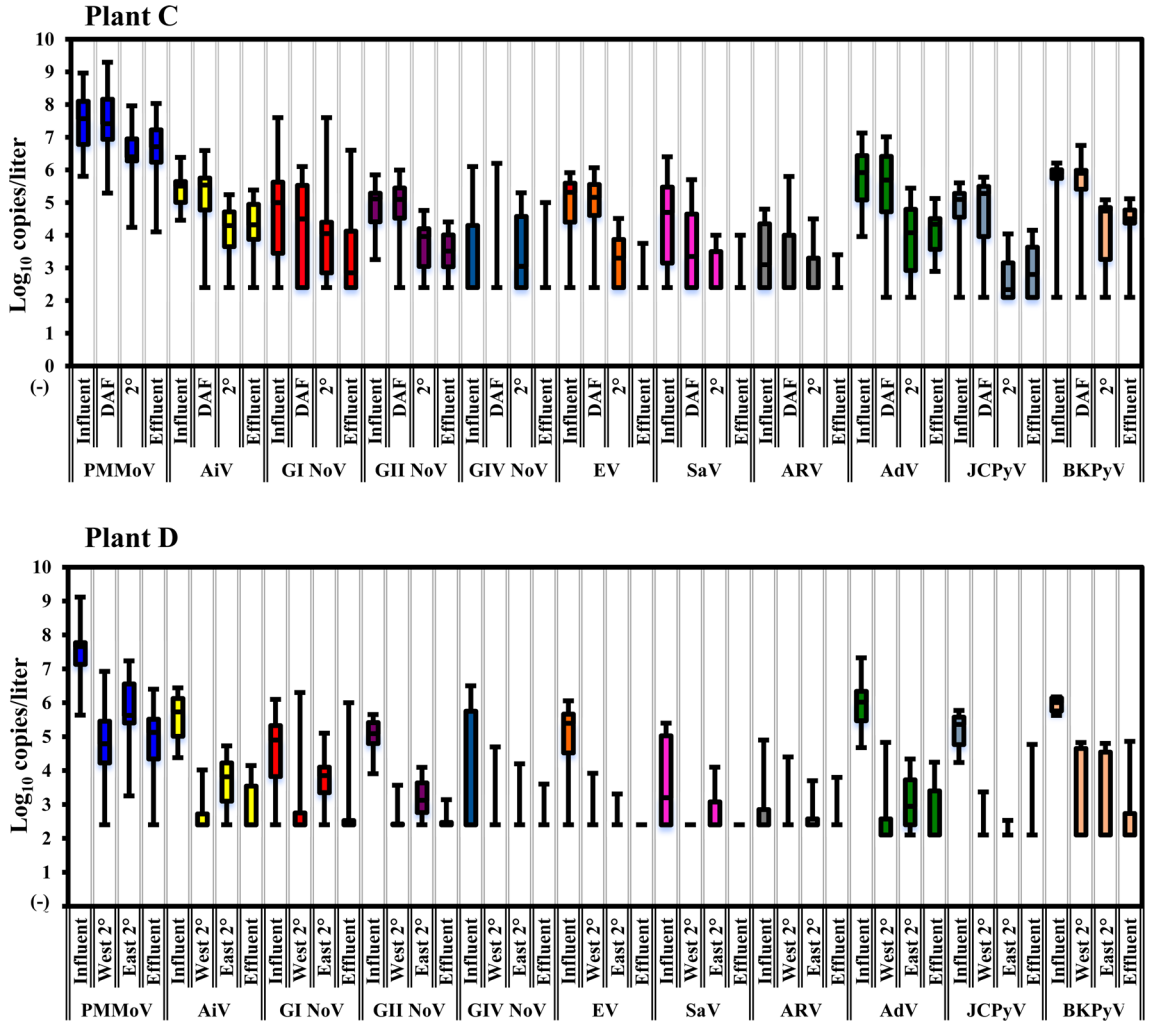

Figure 2. Concentrations of viruses in wastewater at different stages of treatment processes. Lowest possible occurrence concentrations of viruses are at the detection limits (RNA viruses: 2.4 $\log_{10}$ copies/liter; DNA viruses: 2.1 $\log_{10}$ copies/liter). Plants A and B were previously reported by Kitajima et al. (2014).[25] DAF: dissolved air flotation effluent; 2°: secondary treatment effluent; (−), not detected.

comparisons between all plants indicate that $\log_{10}$ reductions from Plant D are often significantly different (α level of 0.05) and greater than those from the other WWTPs, especially the conventional plants (Table 3).

3.5. Removal of Viruses Throughout Treatment Trains. Virus removal occurred mostly during Bardenpho secondary treatment stages (Figure 2). In Plant C, utilizing DAF did not result in a more efficient process in regards to virus removal, as concentrations following primary treatment remained similar to those in raw sewage (Figure 2). However, the mean concentration of viruses decreased 1.0−1.8 $\log_{10}$ copies/L during Bardenpho treatment (DAF effluent to 2° effluent) (Figure 2).

In Plant D, the mean concentration of viruses decreased 1.2−2.9 and 1.3−3.5 $\log_{10}$ copies/L during Bardenpho treatment in the East and West trains, respectively (Figure 2). All viruses were found at concentrations of <4.0 $\log_{10}$ copies/L following Bardenpho treatment in Plant D, except PMMoV (Figure 2). However, mean concentrations were often observed to slightly increase following disinfection, as effluent waters from both trains were mixed, causing possible contamination if either train had a higher incidence of viruses or inadequately disinfected wastewaters (Figures 1 and 2).

4. DISCUSSION

Our results suggest that Plant D, implementing an advanced Bardenpho secondary treatment process, was more proficient at minimizing the incidence of viruses in effluent wastewaters than were facilities utilizing activated sludge and trickling filter biotowers. The Bardenpho process is not intended to remove microbial contaminants but to enhance nutrient removal.[42] Yet this study demonstrated ancillary benefit of this advanced treatment, as viruses were detected less-frequently and at lower concentrations in effluent wastewaters than those treated by conventional processes. However, this study cannot differentiate whether the lack of detection is due to physical removal or damage to the nucleic acid, making it no longer detectable by qPCR.

Removal during Bardenpho treatment could be due to greater virus adsorption to suspended particles than in other treatment processes studied. Previous research suggests that viruses can adsorb to and be removed with a variety of constituents during treatment, including particulates, algae, and bacterial flocs.[53] In this scenario, viruses are not actually reduced but rather transferred to the solids portion.[13,47] In fact, viruses have been found in higher concentrations when associated with solids and lower when free in liquid

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Environ. Sci. Technol. 2016, 50, 9524−9532

supernatant.[34] This study quantified the reduction of viruses via qPCR, but further analysis with the inclusion of cell culture assays could indicate whether wastewater treatment via Bardenpho results in virus inactivation.

Plant D, implementing advanced secondary treatment, showed greater reduction, often below detection limits, than Plants A and B utilizing conventional processes. These results concur with other studies that have compared several types of advanced and conventional treatment processes for virus removal, with membrane bioreactors often showing the highest removal.[7,29,30,33,38,39] Although advanced Bardenpho treatment has not been thoroughly evaluated for virus removal, similar AO (anaerobic−oxic) and A^2/O (anaerobic−anoxic−oxic) processes in Japan have exhibited efficient virus removal, similar to that found in this study.[38,39]

However, Plant C, which also utilized advanced Bardenpho secondary treatment, showed mean virus reduction values less than Plant D and similar to those of Plants A and B (Table 3). Plant C also had the lowest rate of virus reduction to nondetectable concentrations for all virus types (Table S4). This may be explained by higher virus concentrations entering the Bardenpho secondary treatment train at Plant C due to the inefficient reduction of viruses during the DAF primary process (Figure 2). Plant C was the only WWTP to utilize DAF for primary treatment, whereas all other WWTPs utilized sedimentation tanks. Also, it should be noted that Plant C was commissioned in early 2014, and samples were collected shortly after the initial startup process. Therefore, the low efficiency of virus removal at Plant C could be associated with the initial less-than-optimal treatment processes, as reduction efficiency subsequently improved over time.

Our results indicate that Bardenpho processes were the major sources of virus removal throughout treatment trains in Plants C and D, likely due to virus sorption to solids (Figure 2). The solids retention time (SRT) at Plant D was several hours longer than Plant C, which may be the contributing factor for Plant D showing higher virus reduction values (Tables 1 and 2). Additionally, improved removal of nutrients and suspended solids during Bardenpho treatment may also contribute to enhanced virus reduction downstream due to more efficient disinfection (Table 1). These findings are significant because the removal of viruses from wastewater in facilities utilizing Bardenpho processes is relatively unknown.

This study is subject to constraints regarding sample collection and molecular techniques. Grab samples were collected during morning hours from Plant A and Plant B, whereas 24 h composite samples were collected via autosamplers in Plant C and Plant D. The grab samples of influent may not be indicative of the effluent leaving the plant because retention time was not considered. Therefore, the reduction values from Plant A and Plant B should not be considered as representative of treatment proficiency but rather of general viral contamination, as previously explained.[31] Also, qPCR results may have overestimated actual virus concentrations because the values incorporate total nucleic acids from free RNA and DNA, infectious viruses, and noninfectious viruses.[23] The removal of infectious viruses may be underestimated without infectivity analysis.[47]

This study, combined with our previous report,[25] is the first to analyze the incidence of pathogenic viruses over multiple year-long time periods in the southwest United States. EV showed high concentrations in summer and warm months, which agrees with other findings.[5,50,51] Unexpectedly, NoV did not show seasonal trends, although concentrations typically increase during colder months.[50,52,53] Group rotavirus showed the most specific seasonal variation, as it was detected most commonly during spring to early summer. ARV has also showed high occurrence in winter[38,53] or during rainy seasons,[48] with vaccinations reducing transmission.[49] Infrequent detection of ARV could be due to minimal denaturation of the double-stranded DNA during RT-qPCR. However, exposure to dimethyl sulfoxide prior to PCR could improve ARV detection, as previously described.[54] PMMoV, AiV, SaV, JC and BK PyVs, and AdV did not exhibit seasonal trends, which agrees with previous research.[8,25,26,34,49,52]

PMMoV, AiV, AdV, JCPyV, and BKPyV were analyzed as potential viral markers for human fecal contamination, adequate reduction of viruses during wastewater treatment, or both. AiV most often showed the best correlation coefficients to viral pathogens in influent and effluent waters, was always detected at higher concentrations, and may overestimate the potential virus risk (Tables 2 and S3). Therefore, AiV is suggested as a conservative viral marker for the adequate removal of viral enteric pathogens during wastewater treatment.

This study demonstrates that advanced Bardenpho wastewater treatment is more efficient than conventional processes, and AiV is a suitable marker with which to indicate the adequate removal of viral pathogens.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.6b01384.

Table S1: qPCR sequences; Table S2: recovery efficiency of MNV; Table S3: correlation coefficients between viruses; Table S4: reduction of viruses by WWTPs to below detection limit. Figure S1: concentration of viruses and *E. coli* number in influent and effluent wastewater. (PDF)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

WWTP	wastewater treatment plant
DAF	dissolved air flotation
qPCR	quantitative polymerase chain reaction

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Environ. Sci. Technol. 2016, 50, 9524−9532

RT	reverse transcription
MPN	most-probable number
PMMoV	pepper mild mottle virus
AiV	Aichi virus
NoV	norovirus
SaV	sapovirus
ARV	group-A rotavirus
PyV	polyomavirus
AdV	adenovirus
MNV	murine norovirus
GI	genogroup I
EPA	Environmental Protection Agency

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