COLIPHAGE REDUCTION BY THREE WASTEWATER TREATMENT TRAINS UTILIZING THE BARDENPHO PROCESS

By

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A Thesis Submitted to the Faculty of the

DEPARTMENT OF SOIL, WATER AND ENVIRONMENTAL SCIENCE

In Partial Fulfillment of the Requirements

For the Degree of

MASTER OF SCIENCE

In the Graduate College

THE UNIVERSITY OF ARIZONA

2017

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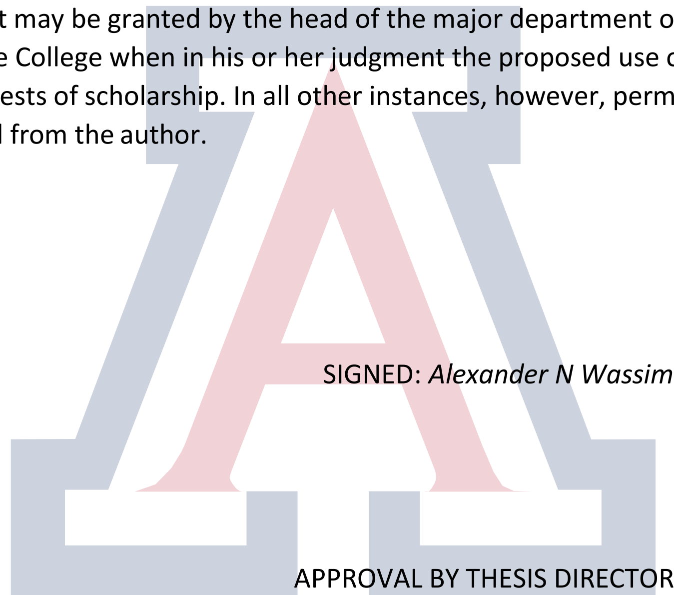

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ACKNOWLEDGMENTS

This study was supported by The National Science Foundation (NSF), Water and Environmental Technology (WET), and The University of Arizona. I would like to thank Dr. Pepper for believing in me and providing me with this amazing opportunity to conduct this research and further my academic career, without you this would not have been possible. Dr.Gerba, thank you for your abundant wealth of knowledge and humorous personality. I am honored to be a recipient of your generosity. Dr. Kelly Bright and Dr. Walter Bentacourt, thank you for your peaceful personalities and advice throughout my academic career, your advice and technical tips have helped me tremendously. A special thank you to Dr. Luisa Ikner, Dr. Bradley Schmitz, and Dr. Masaaki Kitajima, Your non-stop guidance, comfort, and support throughout school and life have made this journey unforgettable. It is people like you that have truly inspired me to follow my dreams and take advantage of every opportunity. I would like to thank David Garrett and Scott Weiler for helping set up water collections sites and answering any questions regarding the wastewater treatment plant's operations and design. Lastly, I would like to thank every single one of my Gerba and Pepper lab mates, your support and positive attitudes throughout this process have made for a great work environment and lasting friendships. I am also extremely grateful to my family and friends for their unwavering support. Thank you for always encouraging me to peruse my dreams and advancing my education.

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ABBREVIATIONS

WWTP- wastewater treatment plant

FIB- fecal indicator bacteria

USEPA- United States Environmental Protection Agency

TRWRF- Tres Rios Water Reclamation Facility

ANWRF- Agua Nueva Water Reclamation Facility

MGD- million gallons per day

DAF- dissolved air floatation

DAL- double agar layover

SAL-single agar layover

PFU- plaque forming units

CFU- colony forming units

ABSTRACT

Wastewater reuse, reclamation and recycling may provide beneficial strategies to manage limited water resources. However, insufficient treatment of municipal wastewater poses potential risk to environmental and public health regarding incidences of viral pathogens. The reduction of pathogenic microorganisms is essential to minimize human health risk associated with the reuse of wastewater. The United States Environmental Protection Agency is reviewing the use of coliphages as a potential indicator organism of fecal contamination in recreational waters. Coliphages are viruses than infect enteric coliform bacteria, and are consistently present in domestic wastewaters. They are similar in size and shape to human enteric viruses, and are more resistant to removal by disinfection than enteric bacteria. As such, they have long been proposed as indicators of fecal pollution. However, traditional bacterial indicators (i.e. *Escherichia coli*) are not reliable indicators for viral pathogens. Monitoring viral pathogens and utilizing the most sufficient wastewater treatment technologies are necessary to minimize public health risk associated with exposure. It is therefore of interest to better understand the removal of coliphages by sewage treatment processes.

PROBLEM DEFINITION

The United States Environmental Protection Agency (EPA) is considering the use of coliphage as an indicator organism for fecal contamination of recreational waters (U.S EPA, 2015). Although more efficient wastewater treatment (WWT) processes continue to be developed and implemented, the presence of fecal-associated pathogens in potable, reclaimed, and source waters continues to be a concern with respect to environmental and human health risks (Jiang et al., 2007; Sinclair et al., 2009). Traditionally fecal indicator bacteria (FIB) such as *Escherichia coli* and Enterococci have been used to detect fecal contamination in waters to protect the public from waterborne bacterial pathogens. However, it has been suggested that viral pathogens may be the leading cause of recreational waterborne illnesses (Cabelli et al., 1982; Sinclair et al., 2009). Furthermore, bacteria respond to wastewater treatment processes and environmental degradation differently than viruses. Because of this, the presence of FIB do not necessarily correlate with the human viral pathogens due to viruses being more resilient to wastewater treatment than bacterial indicators such as *E. coli* (Kitajima et al., 2014). Traditional FIB may not be useful indicators of viral pathogens associated with fecal contamination. It is therefore of interest to better understand the removal of coliphages by wastewater treatment processes due to their impact on the environment.

BACKGROUND & LITERATURE REVIEW

Introduction:

Increased population, water consumption, limited freshwater resources, and climate change have resulted in the need for beneficial reuse of reclaimed wastewater for recreation, industrial, agricultural, and potable purposes (Schmitz et al, 2016). However, there are currently no regulatory standards for virus and phage reduction during wastewater treatment (Qui et al., 2015). Gantzer et al. (1998) reported that over 140 serotypes of pathogenic viruses, bacteria, and protozoa have been found in various bodies of water known to have been contaminated with human fecal material. Of these, human pathogenic viruses are perhaps the most difficult and expensive to culture for monitoring and detection purposes. Frequent monitoring to validate the removal efficacy of human viruses on a daily operational bases is challenging due to the cumbersome and time consuming procedures necessary for virus quantification in wastewater (Amarasiri et al., 2017). In order to insure safe reuse of reclaimed waters, affordable rapid monitoring techniques are required to validate the fate and transport of viral pathogens. One approach is to utilize coliphages as a cost-effective method to monitor viral pathogens due to the well characterized nature of strains and the presence of established protocols for their quantification.

Coliphage:

In the early 1900's phages were described as a component of the human microbiome. They also exist for all known bacterial species, and a wide variety have been isolated (Pillai, 2006). Generally, the bacteriophage's entire virus particle consists of

either double-stranded (ds) or single-stranded (ss) ribonucleic acid (RNA) or deoxyribonucleic acid (DNA), and a protein capsid (Pillai, 2006). Bacteriophages use the host cell's ribosomes, protein-synthesizing machinery, amino acids, and energy generating systems to replicate.

Bacteriophages, commonly referred to as phages, are viruses that infect bacteria and therefore pose no danger to human health. Phages infect bacterial hosts in one of two ways: through receptors on the cell body (somatic phage), or through the host's F+ cell or fertility factor pili appendage (F-specific phage/ male specific phage). The USEPA has suggested that bacteriophage strains that infect and replicate in *E. coli*, known as coliphage can be used as indicators of fecal pollution or as surrogates for the fate and transport of human viral pathogens (McMinn et al., 2017). F-specific coliphages have limited replication in the environment, contain either RNA or DNA genomes. They are similar to human enteric viruses such as enteroviruses, caliciviruses, astroviruses, and hepatitis A and E in size, structure, and resistances to environmental factors (King et al., 2011). F-specific coliphages are the most extensively studied phages and are part of the multiple regulatory frameworks to monitor ground water, biosolids, water recycling, and aquaculture practices (McMinn et al., 2017). These characteristics make F-specific RNA coliphages promising candidates for indicating the presence of fecal pollution, as well as the fate and transport of human viruses in recreational waters.

Somatic coliphage, in contrast to F-specific phage, vary in size and structure, and have characteristics that are not as similar to human viruses as F-specific phage, making them less suitable as indicators of the presence of human viruses. However, some studies have demonstrated that some somatic coliphage are similar to adenovirus (King et al.,

2011). Regardless, somatic phages are excreted in higher abundance than F-specific RNA coliphage, and can replicate in the environment under certain conditions (McMinn et al., 2017). This may improve the sensitivity of detection for phages in water when pollution indices are low. Generally, coliphage will not replicate in the environment unless there is a minimum concentration of *E. coli* host cells of 10^4 colony forming units (CFU) per ml present. Thus, somatic phages may be useful as a general indicator of water quality (U.S EPA, 2015; Pillai, 2006).

Coliphages are divided into 7 major morphological families, four of which are somatic coliphages and three of which are F-specific coliphages. Somatic coliphages are found within the bacteriophage families *Myoviridae, Siphoviridae, Podoviridae,* and *Microviridae*. The somatic coliphage strain used for this study was coliphage strain Phi X174 (ATCC# 13706-B1) obtained from the American Type Culture Collection (ATCC; Manassas, VA) from the *Microviridae* family which has circular double stranded DNA. The F-specific coliphages are found within the bacteriophage families of *Inoviridae, Levivirdae,* and *Tectiviridae.* The F-specific coliphage strain used in this study, MS2 (ATCC# 15597-B1) was from the *Levivirdae* family, which has icosahedral single stranded RNA. The F-specific coliphages of the *Levivirdae* family are further categorized into genogroups GI, GII, GIII, and GIV. In general, GII and GIII F-specific RNA coliphages are found mainly in environments that are associated with human waste, while GI and GIV are mostly associated with animal waste. (Cole et al. 2003; Mesquite et al., 2010).

Indicator Criteria:

Indicators are nonpathogenic organisms that are commonly found in warm-blooded animal microflora, implicating fecal contamination and suggesting the potential presence of enteric pathogens (Gerba et al., 2015). To be a useful indicator of pathogens the following characteristics are desirable: be present whenever the target pathogen is present; be present at a density that has a direct relationship to the degree of fecal contamination; be associated with the intestinal microflora of warm-blooded animals, and have a longer survival time than the target pathogen (Gerba et al., 2015). It has also been suggested that the organisms be slightly more resistant to treatment than the pathogens of interest (Bitton, 2002). To accurately portray the incidence, persistence, fate, and transport of human viruses, an ideal indicator for human viruses is likely to be of viral origin

Previous Studies:

Several studies have been conducted to determine if both somatic and male specific coliphages are associated with fecal contamination (Mocé-Llivina et al., 2005; Love & Sobsey, 2007). However only a limited number of epidemiological studies have evaluated the use of coliphage as an indicator of fecal contamination in recreational waters. A consistent association between FIB and illness has not been reported at all beaches where epidemiological studies have been conducted (Colford et al., 2007). This is partially due to the fact that FIB in surface waters can come from multiple sources other than wastewater, such as storm and agricultural runoff.

Further studies need to be conducted comparing different wastewater treatment processes and their impact on coliphages and human enteric viruses. The appropriateness of an indicator can be dependent on multiple factors such as, human population size, seasonal effects, and types of treatment processes (Schmitz et al., 2016). Previous reviews, and projects have evaluated virus reduction in multiple conventional systems such as stabilization ponds, wetlands, activated sludge, and trickling filters (U.S EPA, 2015). Studies have also looked at the effects of advanced treatment on virus removal within membrane bioreactors, membrane filtration systems, advanced oxidation processes, and reverse osmosis systems with varying results (Schmitz et al., 2016). These findings on wastewater technologies can influence restrictions and guidelines with respect to discharge of effluent treated water intended for recreational, agricultural and potable reuse.

Wiedenmann et al. (2006) conducted a study of five freshwater bathing beaches in Germany. The study attempted to correlate the incidence of phage with gastrointestinal illness of bathers. The study concluded that there were no adverse effect levels of gastrointestinal illness symptoms such as of diarrhea, frequent bowl movements, vomiting, nausea, and stomach pains were observed with bathers in water where somatic phage levels were less than or equal to 10 PFU/100 ml, *E. coli* levels were less than or equivalent to 100 CFU/100 ml and Enterococci levels were less than or equal to 20 CFU/100 ml . However, noticeable gastrointestinal illness symptoms increased as the incidence of the organisms mentioned above increased in the freshwater sources. The study concluded that somatic coliphage would be an appropriate alternative fecal indicator that could be used to set standards for freshwater instead of *E. coli and*

Enterococci, especially in tropical climates where *E. coli* and Enterococci may be less reliable as indicators due the organisms ability to reproduce in the environment, leading to the false impression of increased fecal pollution (Solo-Gabriele et al., 2000; Yamahara et al., 2009).

Due to F-specific RNA coliphage morphological and mode of replication characteristics they have been used as surrogates for disinfection efficiencies of human enteric viruses in potable water and wastewater (McMinn et al, 2017, U.S EPA, 2015). Since monitoring data for coliphage were not used in the development of the 2012 Recreation Water Quality Criteria, there is a need to determine the feasibility of monitoring water samples for coliphage as a reliable and consistent indicator of gastrointestinal illnesses. (U.S EPA, 2015). The current study looked at the incidence and removal rates of coliphage during wastewater treatment processes, since wastewater treatment plants (WWTP) tend to be a point source of fecal contamination into recreational waters.

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APPENDIX A:

COLIPHAGE REDUCTION BY THREE WASTEWATER TREATMENT TRAINS UTILIZING THE BARDENPHO PROCESS

Alexander N. Wassimi, Charles P. Gerba, and Ian L. Pepper

Abstract:

The United States Environmental Protection Agency (USEPA) is considering the use of coliphage as indicator organisms of fecal contamination in recreational waters. Therefore, it is important to understand the removal of coliphage by wastewater treatment processes and the subsequent impact of effluent discharge on the environment. This study evaluated the incidence of coliphage within sewage at two full-scale wastewater treatment plants located in Pima County, Arizona utilizing a 5-Stage Bardenpho treatment processes. Additionally, the removal of coliphage during primary, secondary and tertiary treatment was documented. Cultural plaque assays utilized USEPA recommended *E.coli* hosts for both male specific and somatic coliphage detection. Phage incidence at each plant was consistently 10^6 PFU/100 ml for both somatic and male specific coliphage. Phage reductions of each type of phage was 4-$\log_{10}$ at one plant and 5-$\log_{10}$ at the other plant. Based on the data *E.coli* strains 15597 and 13706 were the preferred hosts for male specific and somatic phage, respectively. To our knowledge this is the first study looking at the density and $\log_{10}$ removal of male specific and somatic coliphage by culture dependent methods, at two wastewater treatment plants utilizing Bardenpho technology within the same community.

Purpose of Study:

The primary goal of the current study was to evaluate the incidences of male specific and somatic coliphages in two full scale wastewater treatment facilities in Tucson, Arizona that utilized advanced secondary Bardenpho treatment technology. An additional goal was to evaluate the removal of male specific and somatic coliphage using cultural detection USEPA methods 1601 and 1602 (U.S. EPA, 2001).

Introduction:

There are two major wastewater treatment plants (WWTP) in Tucson, Arizona, the Tres Rios Water Reclamation Facility (TRWRF) and the Agua Nueva Water Reclamation Facility (ANWRF). Tres Rios was upgraded from conventional secondary treatment processes to modern secondary treatment processes utilizing an advanced 5-stage Bardenpho technology in 2014. The 5-stage Bardenpho technology utilizes microbial processes for nutrient removal. The process consists of 5 chambers, the initial being an anaerobic tank where microbes release soluble phosphorus, followed by alternating anoxic/ aerobic tanks that promote denitrification, nitrification, and phosphorus uptake within the wastewater that is discharged into a secondary sedimentary clarifier. Overall, the Bardenpho process is designed to facilitate the removal of nitrogen and phosphorous (Sattayatewa et al., 2009). However few studies have evaluated the efficacy of virus removal by the Bardenpho process.

TRWRF consists of two separate treatment trains, designated as the East and West trains. However following primary and secondary treatment the effluent from each train is recombined prior to discharge into the environment. Overall the facility is

designed to treat a maximum of 50 million gallons per day (mgd) (Figure 1). The East train consists of a primary sedimentary clarifier, a newly designed 5-stage Bardenpho system parallel to a pseudo 5-stage Bardenpho process, and a secondary sedimentary clarifier. The pseudo 5-stage Bardenpho process was originally a Ludzack-Ettinger (activated sludge) treatment process that was modified with the attachment of an anaerobic chamber at the front of the process, and additional anoxic and aerobic chambers added to the end of the process. The West treatment train was built in 2014 utilizing a primary sedimentary clarifier, three parallel 5-stage Bardenpho processes, and a secondary sedimentary clarifier. Both trains have separate chlorine contact basins after their secondary clarifiers. The water is subjected to a 12.5% solution of sodium hypochlorite with an initial dose of 4 to12 mg/L and a step feed dose of 2 to 8 mg/ L within the first chlorine contact basin on the East and West trains. The East chlorine contact basin has an average annual flow hydraulic retention time of 20 minutes and the West chlorine contact basin has an average annual flow hydraulic retention time of 30 minutes. The East and West chlorinated waters are then combined in a final disinfection basin without additional chlorine, for an additional annual average flow hydraulic retention time of 49 minutes, intended for heterogeneous mixing of East and West train effluents. The chlorinated water overflows from the final disinfection basin in to a smaller basin where it is subsequently subjected to dechlorination with a dosage of 3-10 mg/L of a 38% solution of sodium bisulfite prior to discharge into the Santa Cruz Riverbed with total residual chlorine levels less than 1.0 mg/L (Weiler, 2017).

ANWRF is a small carbon footprint treatment facility that was commissioned in December 2013. This treatment plant was designed to treat a maximum of 32mgd.

ANWRF is unique in that it utilizes dissolved air flotation units for primary treatment, and four parallel 5-stage Bardenpho processes for secondary treatment, followed by, secondary clarifiers, disk filtration units, and two chlorine contact basins (Figure 2). The water is subjected to chlorination in both chlorine contact basins using a 12.5% solution of sodium hypochlorite, dosed at ≃3.0 mg/L. The average annual flow hydraulic retention time for one chlorine contact basins is ≃115 minutes and the second chlorine contact basin has an average annual flow hydraulic retention time of ≃231 minutes. The chlorinated water is then discharged to a service water box that goes to the City of Tucson Water Department and is subsequently distributed through a purple pipeline reclaimed water system for the irrigation of recreational and agricultural fields. ANWRF only dechlorinates a small portion of effluent that is discharged into the Santa Cruz River Bed with a 40% solution of sodium bisulfite dosed as ≃6.8 mg/L, resulting in total residual chlorine levels of less than 1.0 mg/L. Two chlorine contact basins are utilized to help with the dechlorination process (Garrett, 2017).

Methods

Preliminary One Week Pilot Study:

An initial one week long pilot study was conducted to evaluate the USEPA 1601 and 1602 cultural methodologies for the detection of coliphage. In addition, the study determined the optimum sample locations for monitoring coliphage removal along the treatment trains. One-liter grab samples of water were collected daily in sterile wide mouth Nalgene bottles from June 8th to the 12th 2015 from the influent (post screening prior to primary sedimentation), primary effluent, secondary treatment effluent, and final

effluent at both ANWRF and TRWRF. This initial study found that all four *E. coli* hosts were susceptible to phages found throughout the wastewater treatment plants (Figures 3,4 ,5,6). It was also evident that the USEPA method 1601 for double agar layer (DAL) was superior to the USEPA method 1602 for single agar layer (SAL) for optimum coliphage detection in heavily polluted waters. The DAL method allows for a maximum of 2ml of the sample to be plated while SAL allows for 10ml of the sample to be cultured. Despite this, the DAL method resulted in clearer, easier to read plaque counts throughout the treatment processes, while the SAL plates tended to build up a foggy residual layer on all influent, primary effluent and secondary effluent samples, due to high levels of pollution in the water samples making it difficult to count the plaques. The DAL method provided similar plaque forming units (PFU) values as SAL on a per ml basis, even though sample volumes assayed were different.

During the one-week pilot study, cultural methods did not show a significant difference between influent and primary effluent at both water reclamation facilities. Therefore, in the subsequent three month study, primary effluent from both treatment facilities were not collected. In addition, it was noted that there was an order of magnitude difference in coliphage PFU/100 ml when assaying 1 ml samples vs 2 ml of the sample, with the DAL method; therefore, a greater number of plates were assayed using the 2 ml sample volumes in the subsequent study. Overall, the influent, secondary effluent, and final effluent using USEPA 1601 were found to successfully demonstrate that coliphages were entering and leaving the facility, and also the overall reductions during wastewater treatment.

Three Month Study:

For the final study, at the TRWRF, two-liter samples were collected in two sterile one-liter Nalgene bottles from ten-liter carboys utilized to collect 24-hour composite wastewater samples at each location within both facilities, and immediately transported in a cooler containing ice to the laboratory. Wastewater samples were collected weekly for three months between June 2016 and August 2016 from the two WWTP. The 24-hour composites samples were collected through either flow proportional automatic sampler, which took samples every 2 million gallons at influent and effluent locations, or through a timed automatic sampler that collected samples every 2 hours at primary and secondary treatment locations within the water reclamation facilities. This resulted in a total of thirty two, 24-hour composite samples From TRWRF. These samples consisted of eight influent, sixteen post-secondary treatment, and eight final effluent. Eight of the post-secondary treatment samples were collected from the West treatment train, and the remaining eight samples were collected from the East treatment train. All final TRWRF effluent samples were post dechlorination, therefore there was no need to neutralize chlorine in the 24-hour composite final effluent wastewater carboy.

In addition, a total of twenty four, 24-hour composite samples were collected at the ANWRF. The samples consisted of 8 influent samples, 8 post-secondary treatment samples, and 8 final effluent samples. All final effluent samples were pre-dechlorination. Therefore, all final effluent samples were neutralized with 40% solution of sodium thiosulfate pentahydrate dosed at 3mg/L during the 24 hour composite water collection system.

Cultural detection of phage using EPA method 1601 was conducted within four hours of sample collection using one-liter samples from each 24-hour composite carboy. Excess water was stored at 4 C for a maximum of 48 hours until all assays and counts had been completed. The discharged wastewater was tested for *E. coli* using Colilert ® media (IDEXX laboratories, Inc. Westbrook, ME) and the most-probable number(MPN) per 100ml was determined using the Quanti-Tray ® system (IDEXX Laboratories, Inc. Westbrook, ME).

The EPA 1601 DAL method (described previously) was employed using *E. coli C* (ATCC# 13706) a bacterial host strain to detect somatic coliphages such as Phi X174. *E. coli* CN-13 (ATCC# 700609) was used as an alternate host for somatic phage. CN-13 is a Nalidixic acid resistant strain variant of *E. coli C,* which can be comparable to *E. coli C* for detection of somatic coliphages. The use of CN-13 permits the addition of Nalidixic acid to the assay media to suppress any interfering indigenous bacteria in water samples. In addition, *E.coli* C-3000 (ATCC# 15597) was employed as a bacterial host for male-specific coliphages such as MS2 phage. *E.coli* F_{amp} (ATCC# 700891) a Streptomycin and Ampicillin resistant bacterial strain was used for detection of male-specific phages that may have been hindered by indigenous bacteria in water samples. These four organisms were used as host for the detection of coliphages, to determine which host was the most effective in detecting male-specific and somatic coliphages specifically to evaluate wastewater treatment processes.

The DAL technique is a quantitative method that is based on a plaque forming assay that has been widely used, although the volumes assayed are typically low. DAL utilizes a bottom and top agar layer. The bottom agar layer is a nutrient rich media that

allows for a lawn of *E.coli* growth. The top agar media is for the mixture of the water sample containing phages and the *E. coli* host that allow for isolated plaques in the solidified agar to form due to phage infecting and lysing the specific host *E.coli*.

Media, cultural stocks, and antibiotic stocks were prepared following USEPA method 1601. The bottom agar medium consists of tryptic soy agar (TSA; Difco™, Sparks, MD) and the top agar medium was trypticase soy broth (TSB; BBL™, Sparks, MD) containing 8g/L of Bacto agar (Bacto™, Sparks, MD). Nalidixic acid and Ampicillin/ Streptomycin were aseptically added to the TSA (*E. coli* CN-13 and *E. coli* F_{amp} plates, respectively) after sterilization via autoclaving and after the TSA had cooled to approximately 50˚C to prevent degradation of the antibiotics. *E. coli* C and *E. coli* C-3000 do not require the use of antibiotics. All DAL plates, top agar media and overnight *E. coli* cultures were prepared one day prior to the collection of the samples. A more detailed procedure for DAL can be found in Appendix B .

On the day of sample analysis, tryptone top agar was prepared using USEPA method 1601. To assay each sample, 5ml of top agar medium was inoculated with 500 µl of the appropriate *E. coli* host along with 2ml or less of each water sample dilution. These were typically performed in duplicate with the exception of the 10^1 dilution (5 plates x 2 ml each). The dilutions used were dependent upon the sample being assayed (from 10^1 to 10^0 for secondary effluent and final effluent samples; from 10^{-2} to 10^{-3} for influent samples). The solution was then mixed gently and the top agar was poured over the surface of previously prepared TSA plates (bottom agar). Plates were allowed to solidify at room temperature, and were then inverted and placed in an incubator at 37°C for 16-24 hours, and examined for viral plaques (clearing in the bacterial lawn) the following day.

The number of plaques that formed on the *E. coli* lawn represented the original number of viable coliphage present in the sample and were reported as viral PFU/100 ml.

Results

The initial one-week long pilot study conducted in June 2015 evaluated the EPA 1601 and 1602 cultural methodologies designed to detect somatic and male-specific coliphage. The results of the pilot study suggested that the DAL method (1601) was superior to the SAL method (1602) for both types of coliphage. Based on this, the DAL method was subsequently used for the rest of the study. The pilot study also demonstrated that all four *E. coli* hosts were susceptible to coliphages present in the grab wastewater samples. Interestingly the initial study revealed that with in Tres Rios WWTP the East and West trains performed differently. Specifically the density of male specific and somatic coliphage were two orders of magnitude higher in the East train secondary effluent . The West secondary effluent would rarely detect coliphage present while the East secondary effluent would detect coliphage at 10^2 PFU/ml (Figures: 3-6). The study also showed that there were no also significant difference between raw sewage influent and primary effluent coliphage counts. Figures 3 through 6 show that the DAF (primary effluent) within ANWRF had relatively the same number of coliphage present in water samples as the raw sewage influent. For the Tres Rios WWTP, plaque counts in the influent and primary effluent were similar. Based on this for the subsequent three-month study, samples were not taken from the primary effluent locations with in both WWTPs.

Figure 3 shows that the effluent incidence concentration of male specific coliphage using the antibiotic resistant *E. coli* strain #700891 were 10^3 PFU/ml for both

TRWRF and ANWRF. Overall, 2 log reductions occurred from influent to final effluent. Using the non-antibiotic resistant strain #11597, male specific coliphage influent concentrations were close to 10^4 PFU/ml, with 3-4 log reductions occurring from influent to effluent (figure 4). However, the use of the antibiotic resistant *E. coli* host #700891 enabled the detection of male specific coliphage in the final effluent at TRWRF, while the use of the non-resistant *E. coli* strain resulted in very few detections. There was also larger variance in the detection of male specific phage with in ANWRF when using antibiotic resistant *E. coli* hosts (#700891) in comparison to non-resistant *E. coli* strain #15597.

Figures 5 and 6 show the effluent incidence concentration of somatic coliphage. Using either antibiotic resistant or non-resistant *E. coli* strains, incidence values were 10^4 PFU/ml at both plants. Also from influent to effluent, 3-4 log reductions were observed at both WWTPs. No significant difference in the detection of somatic coliphage was observed with respect to the use of the two *E.coli* strains.

Figures 7 and 8 show that within the three-month study, male specific coliphage were abundant in wastewater entering both treatment plants, averaging around 6-$\log_{10}$ (PFU/100ml) using either *E. coli* strains. Nonetheless, the coliphages were removed during the wastewater treatment processes at both plants. Figures 7, show that there was a greater than 4-$\log_{10}$ reduction of male-specific coliphages within both WWTP's from influent to effluent. However, at Tres Rios, differences were observed between the East and West secondary treatment trains with respect to the efficacy of coliphage removal. Within the Tres Rios West treatment train, a 4- $\log_{10}$ reduction occurred in comparison to only a 2-$\log_{10}$ reduction within the Tres Rios East treatment train. At TRWRF similar

data for male specific coliphage counts were obtained when the non-antibiotic resistant host (15597) was utilized. When 15597 was used as the host at Agua Nueva male specific coliphage were rarely detected in the final effluent samples. When comparing male specific coliphage detection using antibiotic resistant *E. coli* strain #700891 (Figure 7) and non-resistant *E. coli* strain #15597 (Figure 8) , *E. coli* host #700891 detected male specific coliphage while *E. coli* strain #15597 did not in Agua Nueva final effluent samples,. In addition, there was larger variance in PFU counts within the Tres Rios wastewater samples. These trends also occurred in the one-week pilot study. Therefore, the use of antibiotics may help in the sensitivity of detection of male specific coliphage in final effluent wastewater. Overall, the East and West secondary effluents are combined after initial chlorine disinfection within the first chlorine contact basin, and are subjected to a longer hydraulic retention time via the second contact basin. This resulted in a combined final reduction of 4-$\log_{10}$ from influent to effluent at TRWRF, and a 5-$\log_{10}$ reduction from influent to effluent at ANWRF of male specific coliphage regardless of the host strain used. The use of *E. coli* strain #15597 (Figure 8) for the detection of male specific coliphages resulted in overall less variance in phage counts in raw sewage at either plant than the antibiotic resistant *E.coli* strain #700891 (Figures 7). Based on this, *E. coli* #15597 would appear to be the more appropriate host based on the higher numbers detected, and the more precise data.

Somatic coliphage concentrations were similar regardless of whether an antibiotic resistant *E. coli* strain #700609 (Figure 9) or non-resistant *E. coli* strain #13706 (Figure 10) was utilized as the host for the detection assays. With either host strain, influent coliphage concentrations were approximately 10^6 PFUs/100 ml. Likewise, the variability

in phage concentrations in all samples appeared to be similar with final reductions of 4-log_{10} within TRWRF and a 5-log_{10} from influent to effluent of somatic coliphage at ANWRF, regardless of the host bacterial strain used. Based on these data, *E. coli* strain #13706 may be the host of choice based on the simplicity and the lower cost of the assays due to the lack of a requirement for antibiotics in the media.

Figure 11 indicates a 5-log_{10} reduction of infectious somatic and male specific coliphage from influent to effluent at Agua Nueva WWTP using either *E. coli* hosts. However, Agua Nueva's secondary treatment process indicated lower coliphage log_{10} reduction values in comparison to the West treatment train at Tres Rios. The East treatment train at Tres Rios resulted in the lowest removal rate of coliphage, with an approximate 2-log reduction. The West treatment train was the most effective at removing coliphage, with a 4-log_{10} reduction. At Agua Nueva, secondary treatment resulted in a 3-log_{10} reduction of both types of coliphages.

Figure 12 and Table 1 further illustrate the differences in the removal of coliphage with respect to each treatment train process. Table 1 indicates the average log_{10} reductions values (PFU/100ml) with standard deviations. The Agua Nueva WWTP yielded an overall 1-log_{10} greater reduction from raw sewage to final effluent than the Tres Rios WWTP . Overall, Tres Rios East treatment train was the least effective at removing infectious male specific and somatic coliphage in comparison to the Tres Rios West treatment train, and was generally less effective than the Agua Nueva secondary treatment process.

Discussion

The Tres Rios and Agua Nueva Water Reclamation Facilities were primarily designed to facilitate the removal of BOD, nitrogen and phosphorus in wastewater through the use of Bardenpho technology. Both plants have successfully reduced the levels of nitrogen and phosphorous in effluent discharged from the plants. This current study demonstrates that both plants were also effective in removing or inactivating phage from wastewater during WWT, suggesting that they may also be successful in removing human pathogenic viruses.

However, data show variable results with respect to the removal of male specific and somatic coliphage, despite the fact that they utilize the same type of secondary treatment technology. The reason for these differences may be due to the fact that these facilities have different engineering designs, and that their flow and retention times vary on a daily, monthly, and annual basis. Although both plants utilize Bardenpho technology for secondary treatment, Agua Nueva WWTP utilizes DAF as a primary treatment whereas Tres Rios utilizes conventional sedimentation as a primary treatment. In addition the final use of membrane disk filtration at ANWRF resulted in an additional final effluent reduction of coliphage by 1-log_{10} in comparison to the TRWRF final effluent. At Tres Rios, the two treatment trains resulted in different secondary treatment reduction levels of phage even though both the West and East trains utilized Bardenpho technologies. Similarly, *E. coli* levels assayed by the Colilert Quanti-Tray® method resulted in higher levels of total and fecal coliforms in the East train relative to the West train (Data not shown). These findings suggest that modifying the existing Ludzack-Ettengir technology to a pseudo Bardenpho technology in the Tres Rios East treatment

train resulted in treatment that was not as effective as the newly designed Bardenpho system on the West treatment train. Nonetheless, including the tertiary treatment, the TRWRF resulted in a 4-log_{10} reduction of both male specific and somatic coliphages. Overall, these data highlight the fact that even slightly different wastewater treatment technologies can result in variable treatment efficacy. Similarly, changing treatment operations including flow rates can likewise result in variable results.

The low incidence of phage in final disinfected effluents from both plants raises an interesting question. Were phage that entered the plants via raw sewage inactivated during treatment or did they partition into the solids portion of the treated sewage? Preliminary analysis of virus in the sewage sludge prior to digestion suggests that viruses are in fact partitioning into the solids with the sludge (Data not shown).

This study agrees with the findings of others that have looked at the incidence of coliphages in various bodies of water and WWTPs in warm climate regions (Yahya et al. 2015; Jebri et al., 2012; Agulló-Barceló et al., 2016). Multiple studies have found similar phage reduction trends during WWT utilizing different secondary and tertiary treatment processes. Overall 4-6 log reductions were typical. In this current study, concentrations of phage in effluent were still fairly high in comparison to FIB indicators such as *E. coli*. This is likely due to the enhanced resistance of coliphage to treatment, a trait also seen with a variety of enteric viruses. (McMinn et al. 2017; Purnell et al., 2016)

Overall, WWT at Tres Rios resulted in a 4-log_{10} reduction of both male specific and somatic coliphages. At Agua Nueva, treatment resulted in a 5-log_{10} reduction of male specific phage and somatic coliphages. The results of this study suggest that based on the ability to detect phage, overall cost, and simplicity of the assay, the 15597 *E. coli* strain

for male specific phage, and the 13706 *E. coli* strain for somatic phage are the preferred hosts for optimum results in the Tucson region

Figures

Figure 1: Simplified schematic of Tres Rios Water Reclamation facility. Pilot study grab samples were taken at all samples sites. Three month long study 24-hour composite samples were taken at the influent, after the secondary clarifier, and the final effluent.

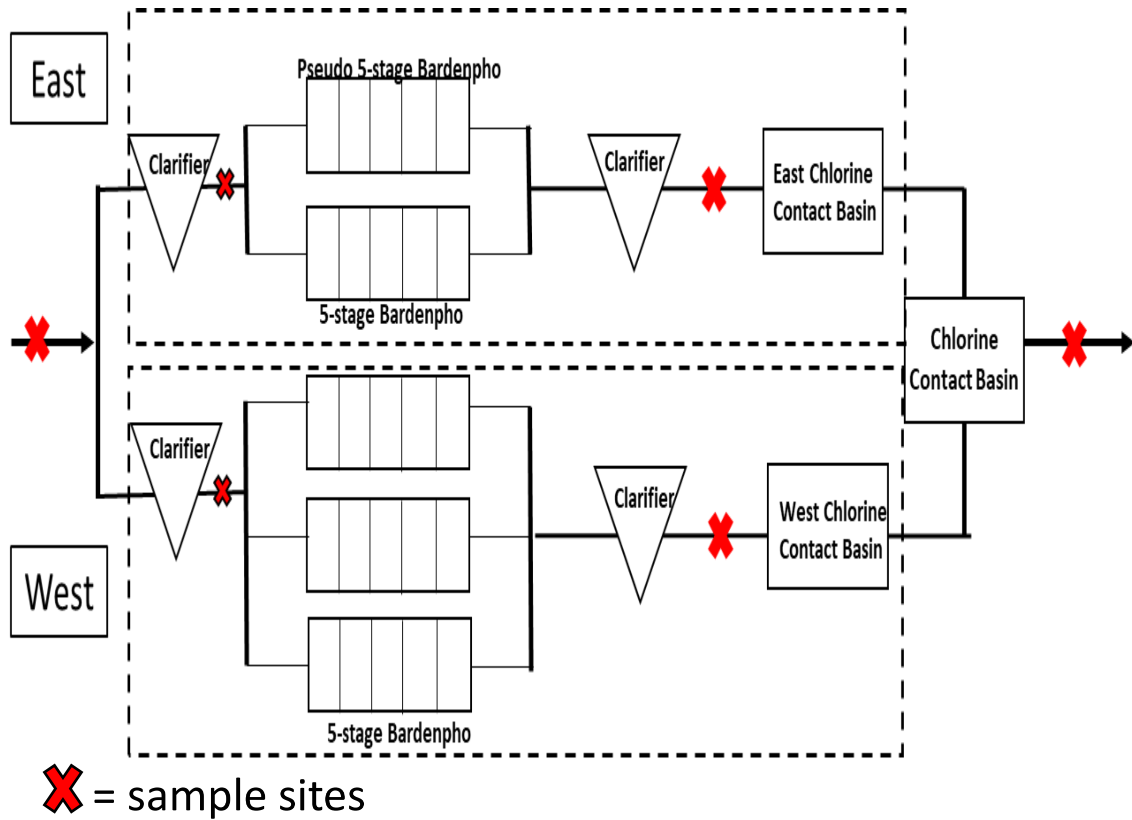


Figure 2: Simplified schematic of Agua Nueva Water Reclamation facility. Pilot study grab samples were taken at all samples sites. Three month long study 24-hour composite samples were taken at the influent, after the secondary clarifier, and the final effluent.

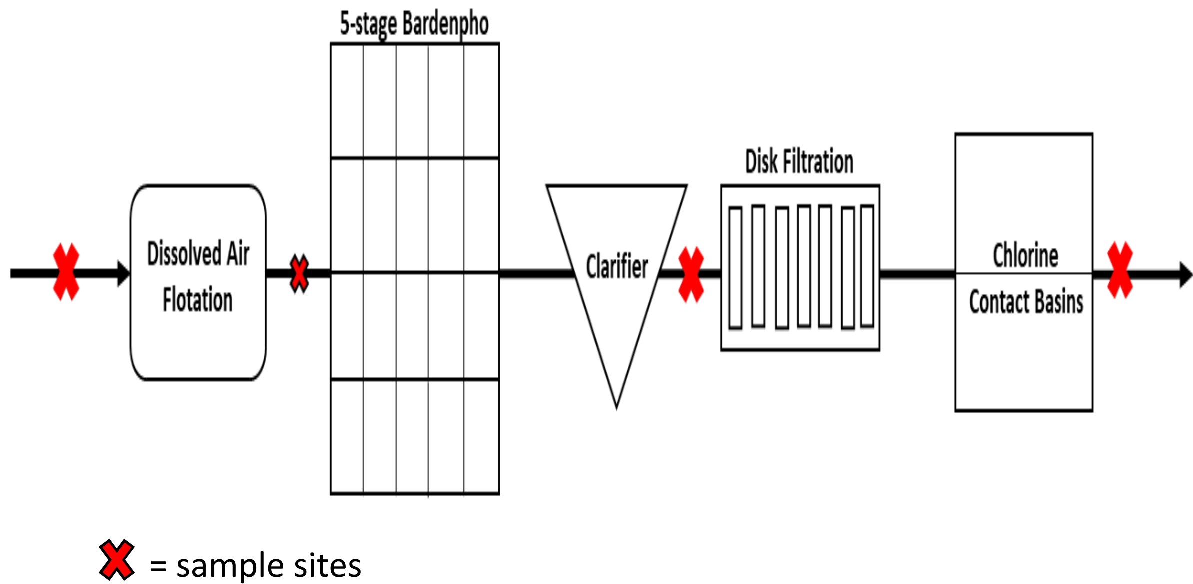


Figure 3: Detection of male-specific coliphages using antibiotic-resistant host *E. coli* strain #700891 (F_{amp}) in two WWTP in Tucson, AZ. Data represents the average of 5 grab samples collected from each location over a week period in June 8th -12th 2015.

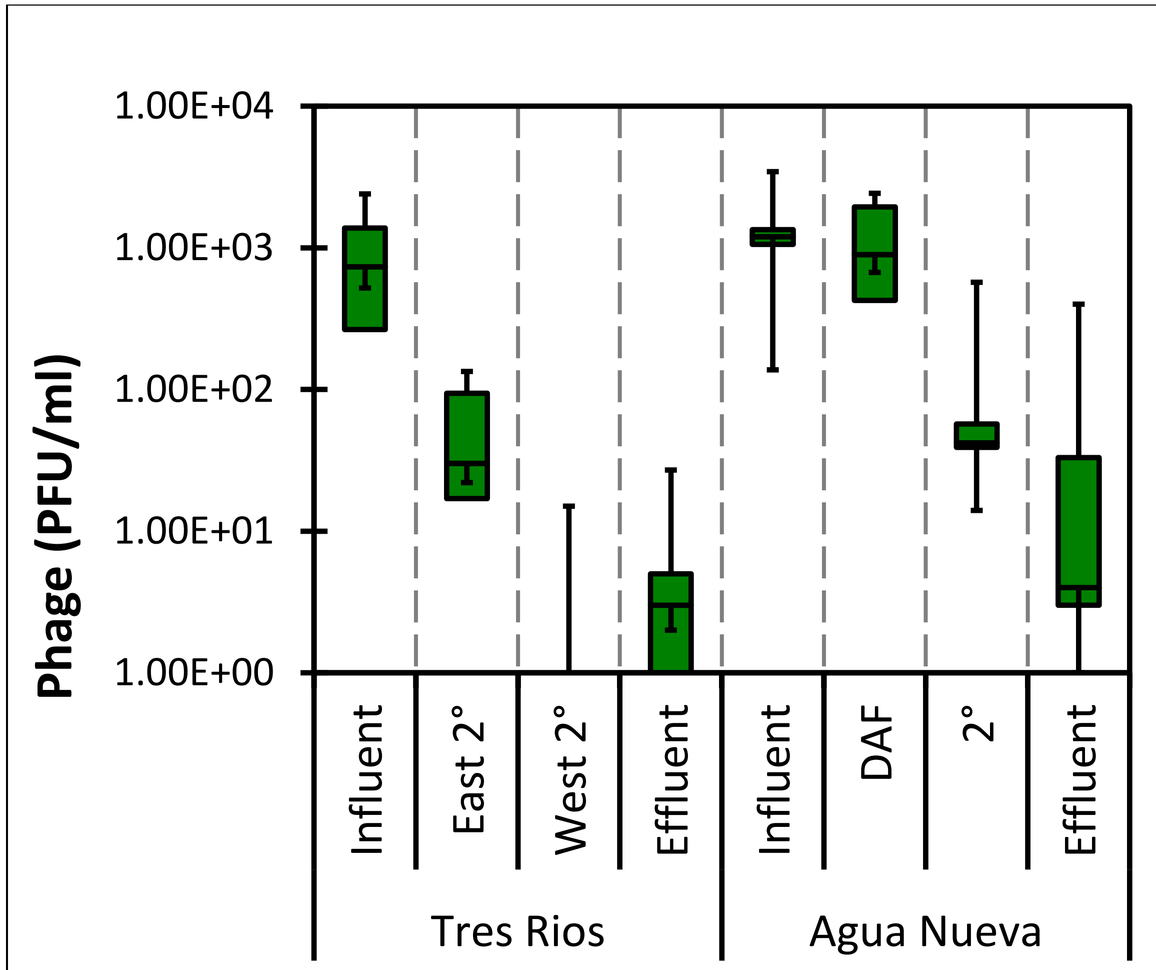


Figure 4: Detection of male-specific coliphages using host *E. coli* strain #15597 (C-3000) in two WWTP in Tucson, AZ. Data represents the average of 5 grab samples collected from each location over a week period in June 8th -12th 2015.

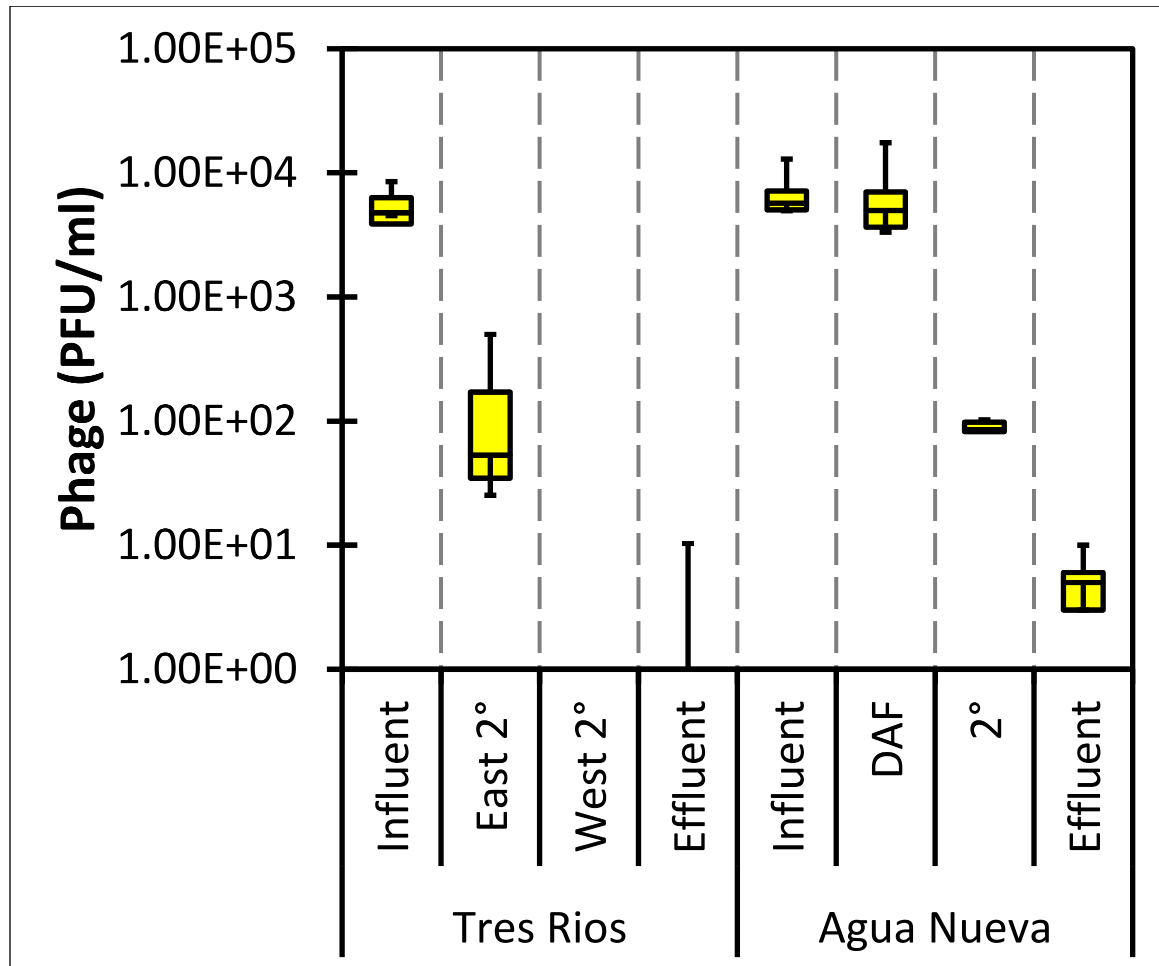


Figure 5: Detection of somatic coliphages using antibiotic-resistant host *E. coli* strain #7006009 (CN-13) in two WWTP in Tucson, AZ. Data represents the average of 5 grab samples collected from each location over a week period from June 8th -12th of 2015.

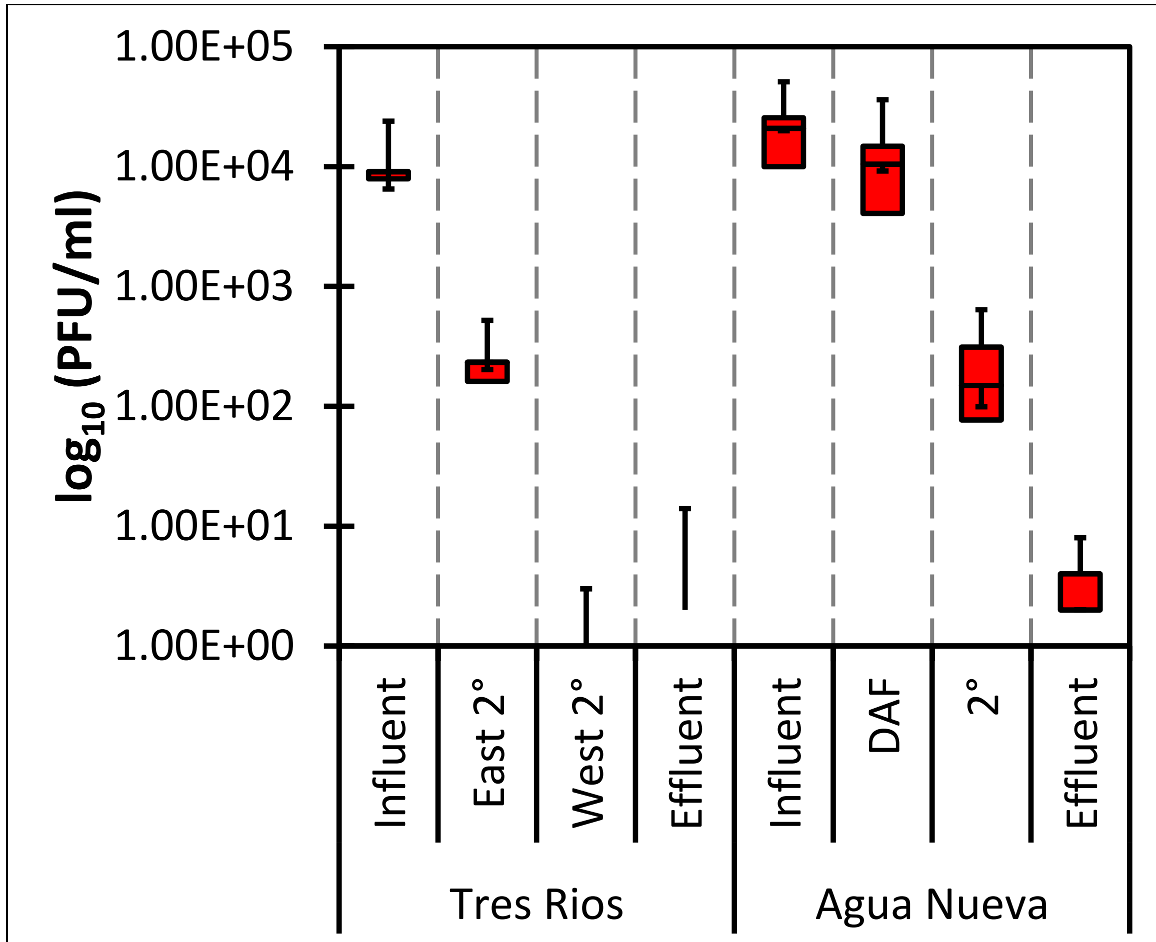


Figure 6: Detection of somatic coliphages using antibiotic-resistant host *E. coli* strain #13706 (C) in two WWTP in Tucson, AZ. Data represents the average of 5 grab samples collected from each location over a week period from June 8th -12th of 2015.

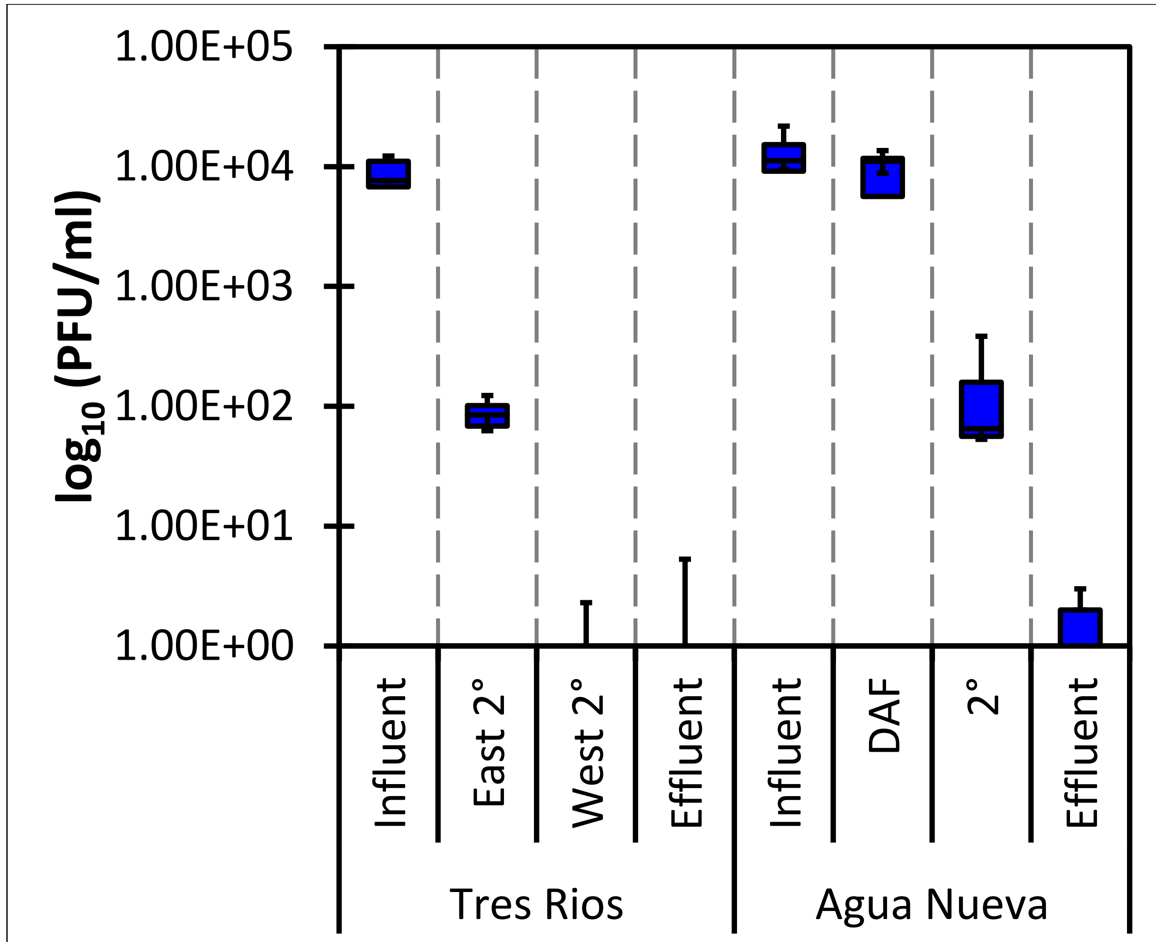
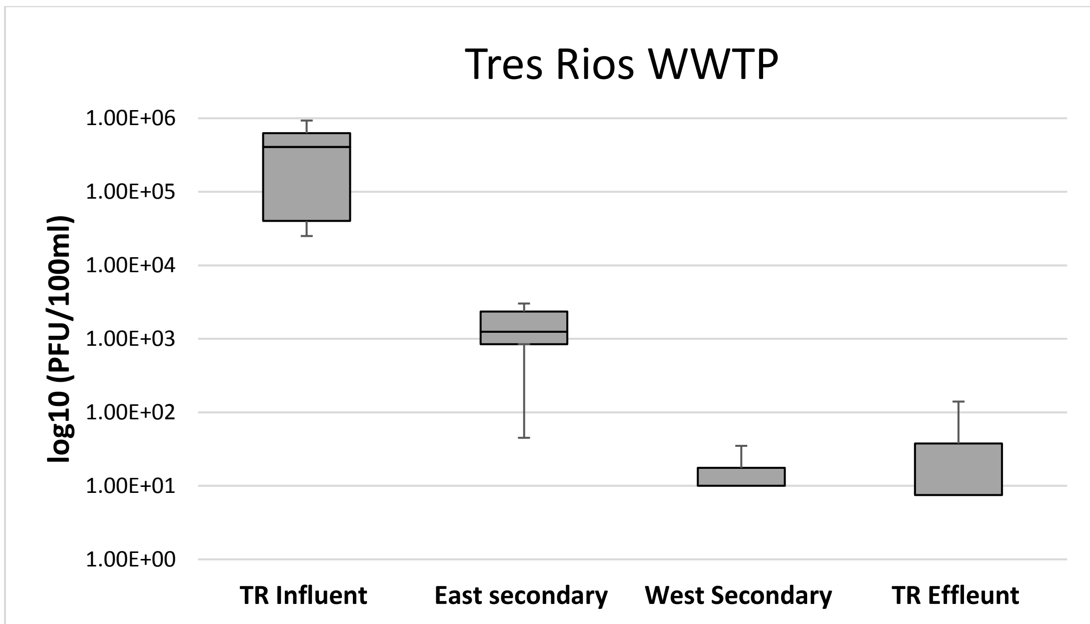


Figure 7: Detection of male-specific coliphages using antibiotic-resistant host *E. coli* strain #700891 (F_{amp}) in two WWTP in Tucson, AZ. Data represents the average of 8 samples collected from each location over a three-month period from June 2016 to August 2016.



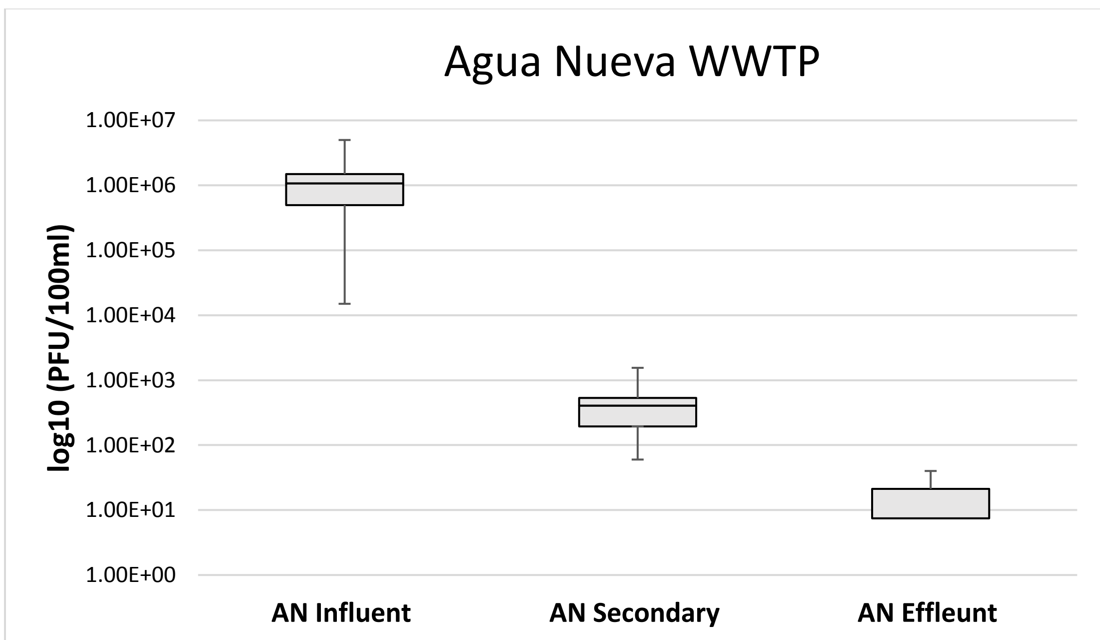
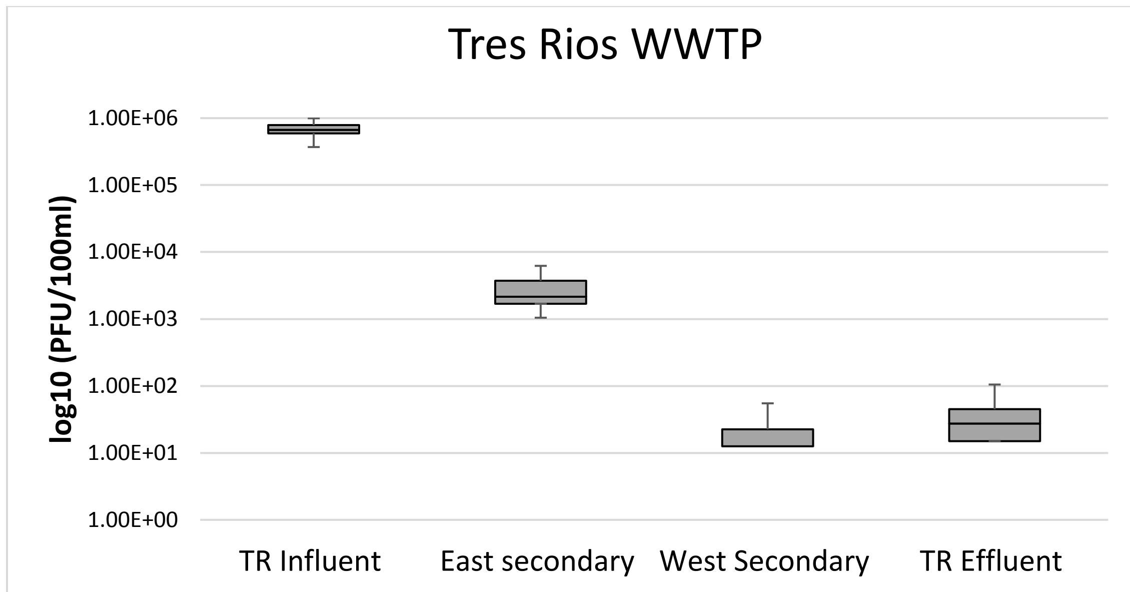


Figure 8: Detection of male-specific coliphages using host *E. coli* strain #15597 (C-3000) in two WWTP in Tucson, AZ. Data represents the average of 8 samples collected from each location over a three-month period from June 2016 to August 2016.



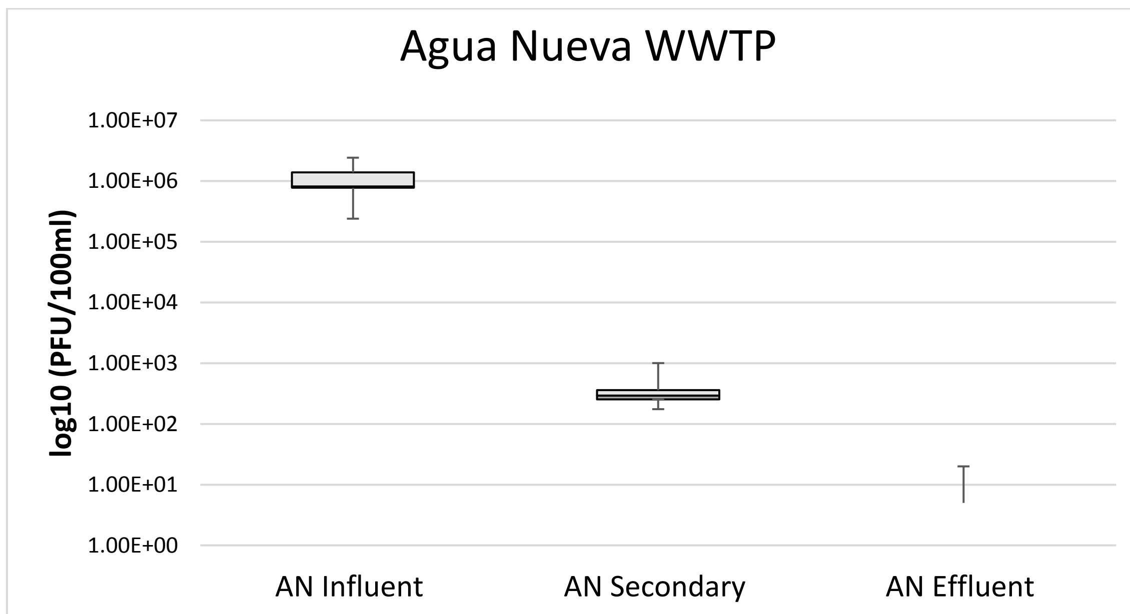
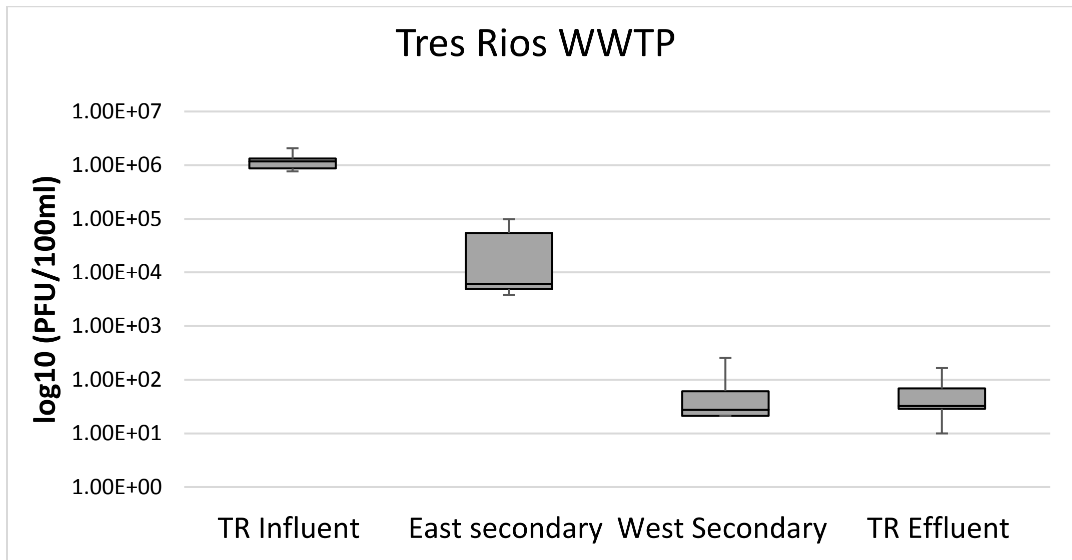


Figure 9: Detection of somatic coliphages using antibiotic-resistant host *E. coli* strain #7006009 (CN-13) in two WWTP in Tucson, AZ. Data represents the average of 8 samples collected from each location over a three-month period from June 2016 to August 2016.



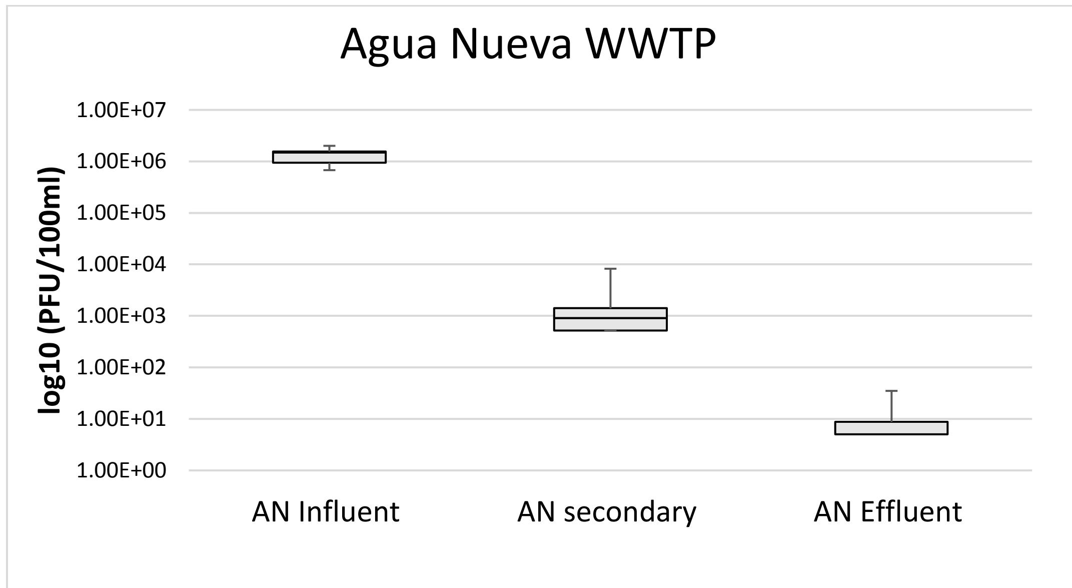
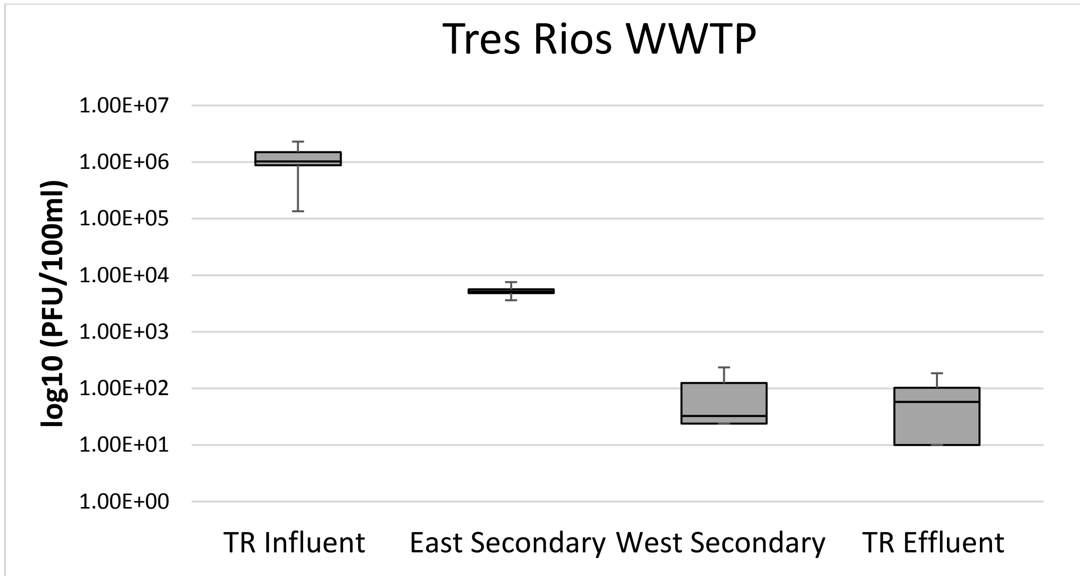


Figure 10: Detection of somatic coliphages using host *E. coli* strain #13706 (C) in two WWTP in Tucson, AZ. Data represents the average of 8 samples collected from each location over a three-month period.



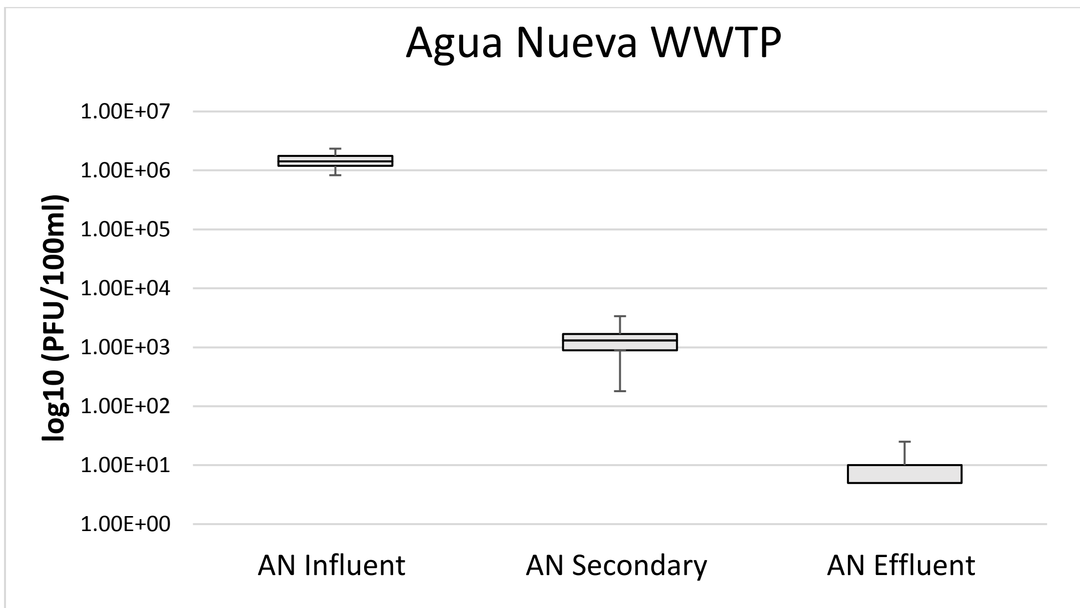


Figure 11: Coliphage reduction values at various stages of treatment processes throughout both Tres Rios (TR) and Agua Nueva (AN) WWTP. Data is an average of 56 samples over a 3-month period.

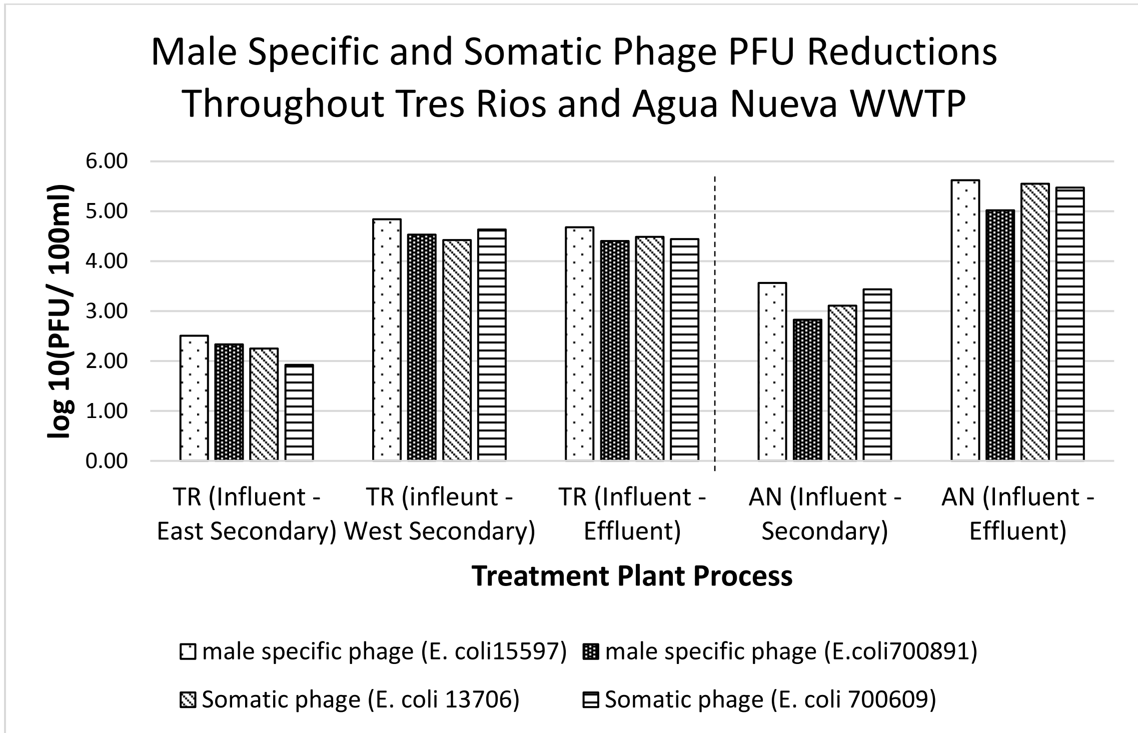


Figure 12: Concentration of Plaque Forming Units/ 100 ml of detectable male specific and somatic phage using all 4 *E.* coli hosts in Influent and final effluent samples for both Tres Rios (TR) and Agua Nueva(AN) Treatment plants. Data is an average of 56 samples over a 3-month period over summer 2016

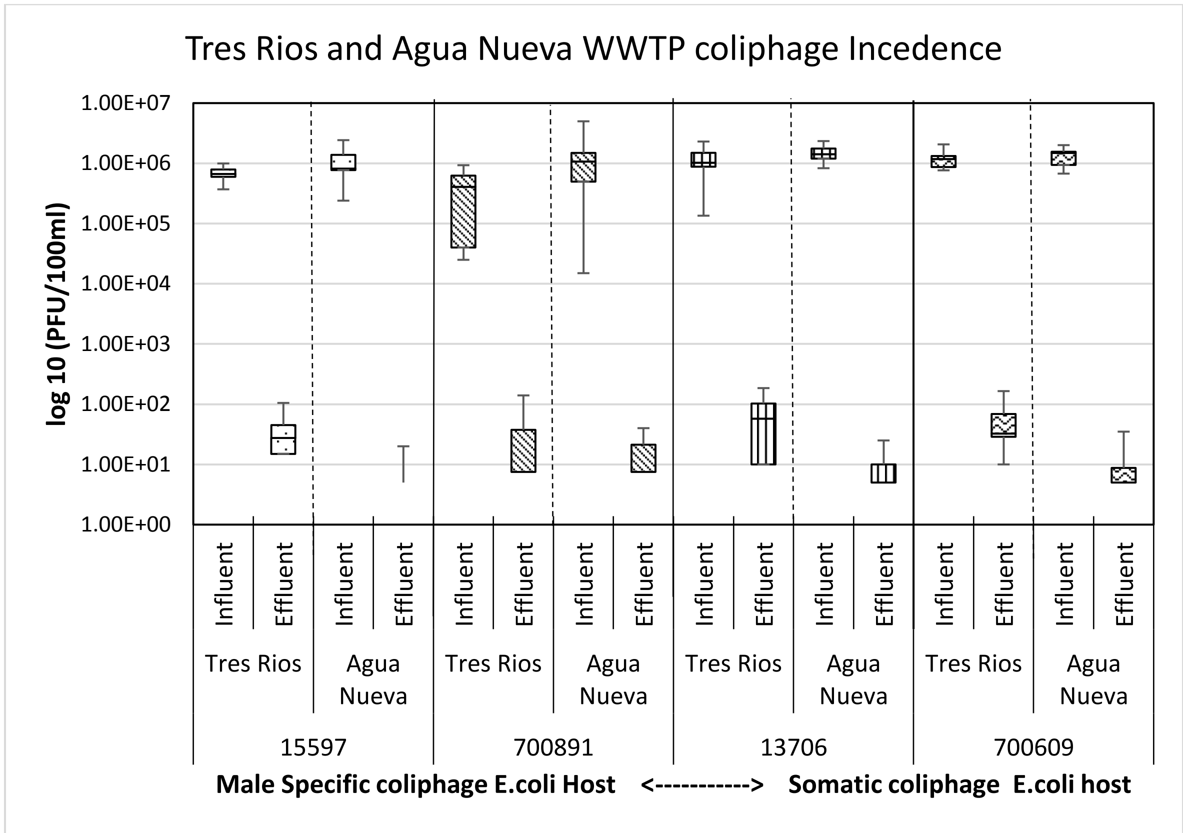


Table 1. Reduction of coliphages (average log_{10} removal ± standard deviation) between various stages of the treatment trains at the Tres Rios and Agua Nueva WWTP.

Coliphage Type (bacterial host)	WWTP (reduction between treatment steps)				
	TRES RIOS (Influent - East Secondary)	TRES RIOS (Influent - West Secondary)	TRES RIOS (Influent - Effluent)	AGUA NUEVA (Influent - Secondary)	AGUA NUEVA (Influent - Effluent)
Male-Specific (*E. coli* #15597)	2.51 ± 0.15	4.84 ± 0.72	4.68 ± 0.76	3.56 ± 0.35	5.62 ± 0.58
Male-Specific (*E. coli* #700891)	2.33 ± 0.59	4.53 ± 0.62	4.40 ± 0.68	2.83 ± 1.26	5.02 ± 0.91
Somatic (*E. coli* #13706)	2.25 ± 0.43	4.42 ± 0.49	4.49 ± 0.86	3.11 ± 0.37	5.55 ± 0.49
Somatic (*E. coli* #700609)	1.92 ± 0.50	4.63 ± 0.79	4.44 ± 0.40	3.43 ± 1.21	5.47 ± 0.60

APPENDIX B: PROCEDURES FOR DAL U.S. EPA METHOD 1601

Antibiotic stock:

Nalidixic acid: for growth of *E. coli* CN-13

1) Dissolve 1 g of Nalidixic acid sodium salt per 100ml of nanopure water
2) Stir until dissolved (may appear milky white)
3) Filter through a sterile .22 µm pore size membrane filter
4) Dispense 5 ml into 5ml freezer vials, or into a large glass container. Store in freezer at -20°C for up to one year

Ampicillin/ Streptomycin: for growth of *E. coli* Famp

1) Dissolve 0.15 g of ampicillin sodium salt and 0.15g of streptomycin sulfate per 100ml of nanopure water
2) Filter through a sterile .22 µm pore size membrane filter
3) Dispense 5 ml into 5ml freezer vials, or into a large glass container. Store in freezer at -20°C for up to one year

Procedures for DAL:

1) Bottom Agar Plates (~150 plates)
    a. Dissolve 40g TSA in 1000ml Nanopure water
        i. Create 2 flask because each 1000ml media will pour ~70 plates
    b. Place tin foil on top, Hand swirl media until dissolved
    c. Autoclave, slow exhaust
        i. Preheat water bath to 52°C
        ii. When removing media from autoclave, hand swirl to get uniformed mix
    d. Place media in water bath, wait until cool to touch prior to pouring
    e. Add 10 ml of antibiotics to 1000ml lukewarm media
        i. Add Nalidixic acid to media for CN 13 700609
        ii. Add Ampicillin/streptomycin for F_{amp} 700891
        iii. No need for antibiotics for strains 15597 and 13706
    f. Aseptically pour petri dishes
    g. Let plates solidify, invert and store in labeled petri dish sleeves inside fridge at 4°C (expires in 2 months)
2) Top Agar Tubes
    a. 100 tubes = 15g TSB, 4g Agar Agar in 500ml of Nanopure water
    b. Spin and heat on hotplate until boiling
    c. Aliquot 5 ml into disposable tubes
    d. Place blue caps on tubes lightly
    e. Autoclave, slow exhaust

   f. Cool and store at 4°C fridge, push caps down on tubes (expires in 2 months)

3) Create 24 hour culture tube
   a. Dissolve 3g of TSB in 100 ml nanopure water in Erlenmeyer flask
   b. Aliquot 10 ml TSB into screw cap tubes
   c. Autoclave, slow exhaust
   d. Label 24 hour culture tubes for each strain
   e. Aseptically add 0.2ml of antibiotics to designated culture tubes
      i. Add Naladixic acid to media for CN 13 700609
      ii. Add Ampicillin/streptomycin for PhiX 700891
      iii. No need for antibiotics for strains 15597 and 13706
   f. Obtain plate host cultures
      i. Male Specific (MS2)
         1. MS-2 (15597)
         2. F_{amp} (13706)
      ii. Somatic
         1. PhiX-174 (700891)
         2. CN-13 (700609)
   g. Sterilize loop on flame, grab a colony from plate, and place inside 24 hr TSB culture tube
   h. Place on shaker inside incubator for 24 hours at 37°C

4) Create **four** 3 hour culture flask
   a. 25 ml of nanopure water and 2g of TSB
   b. Autoclave, slow exhaust
   c. 0.5 ml of antibiotic to appropriate flasks
   d. Place 1000 µl of 24 hour into 3 hour flask
   e. Place in incubator at 37 °C for 3 hours

5) Add 3 hour bacteria growth to water sample (10ml *E. coli*/100ml sample) (100ml *E. coli*/1000ml sample)

6) Dilution Eppendorf Tubes
   a. Saline solution
      i. Dissolve 8.5g NaCl in 1000ml nanopure water inside of bottles not flasks
      ii. Autoclave saline solution along with 1.5 mL Eppendorf tubes
      iii. Fill autoclaved Eppendorf tubes aseptically with 900µL of saline solution
   b. Label dilution tubes before creating dilutions

      Dilution Example:

Clean sample

i. 10^1= 2ml of direct sample onto 5 plates, 10 ml total (10^1)
ii. 10^0= 1 mL sample directly onto 10 plates

Turbid samples

i. 10^{-2}= 100µL into 900µL saline Eppendorf tube
ii. 10^{-3}=100 µL from 10^{-2} into 900 µL saline tube

7) Plating samples (must be done quickly, time sensitive)
   a. Add appropriate dilutions based on turbidity of water samples to top agar tubes
   b. Add half an ml of 3hr culture to top agar with dilution
   c. Quickly swirl top agar tube with dilution and *E. coli* host and pour over bottom agar
   d. Swirl petri dish to make sure top agar covers bottom agar evenly
   e. Let solidify, invert, and store inside incubator at 37° C for 16-18 hours
   f. Take out of incubator after incubation period
   g. Count and calculate